Subcellular Fractionation of Bone Marrow-Derived Macrophages: Localization of Phospholipase A_1 and A_2 and Acyl-CoA:1-Acylglycero-3-phosphorylcholine-0-acyltransferase

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Analysis of the subcellular distribution of lipid-metabolizing enzymes was carried out in bone marrow-derived macrophages with special respect to a comparison of the subcellular localisation of phospholipase A_1 and A_2 and to acyl-CoA:1-acylglycero-3-phosphorylcholine-0-acyltransferase.

After cell disruption differential centrifugation was followed by additional sucrose gradient purification of three main fractions.

Satisfactory enrichment factors were obtained by this method for the following marker enzymes. The plasma-membrane enzyme alkaline phosphodiesterase I was enriched up to 25-fold and the acyl-CoA:1-acylglycero-3-phosphorylcholine-0-acyltransferase was enriched up to 30-fold. The marker enzyme for the endoplasmic reticulum, NADPH-cytochrome c reductase showed a similar enrichment and distribution as the acyltransferase. Therefore it was concluded that the acyl-CoA:1-acylglycero-3-phosphorylcholine-0-acyltransferase of bone marrow-derived macrophages is mainly located in the endoplasmic reticulum. Phospholipase A_1 and A_2 occurred in a high proportion together with the lysosomal marker enzyme N-acetyl-β-glucosaminidase in the soluble supernatant and in the gradient fractions. In the endoplasmic reticulum phospholipase A_2 occurred only in trace activities whereas phospholipase A_1 was maximally enriched in this subcellular fraction. No subcellular fraction could be obtained where phospholipase A_2 was enriched exclusively. However, it can be concluded that the two enzymes which are responsible for the balance of fatty acid liberation and re-acylation are located in two different cellular compartments. Furthermore it can be claimed that in the cell there has to exist an exchange of substrates and products between these compartments to achieve a complete metabolic cycle of the de- and re-acylation reaction of phospholipids in bone marrow-derived macrophages.

Introduction

In a previous study [1] we observed that the turnover of arachidonic acid of phospholipids in bone marrow-derived macrophages is mainly controlled by the activities of phospholipase A_2 and the acyl-CoA:lysophosphatide acyltransferase. Since these studies were only carried out in intact cells no information could be obtained whether this turnover also is influenced by the distribution of these enzymes in different subcellular compartments.

Analysis of the subcellular distribution of enzymes was carried out in great detail on peritoneal macrophages by Canonico *et al.* [2], however, the activities of phospholipases and of acyltransferases were not determined. To our knowledge there exists no study where both enzymes are compared directly. Only a rough indication for the subcellular localization of these enzymes was obtained by Wang *et al.* [3] and Franson *et al.* [4]. Moreover, there exists no fractionation study on bone marrow macrophages. Macrophages of this origin now can be prepared in large quantities and high purity (95%). Therefore we chose this cell type and report here on the distribution of lipid-metabolizing enzymes in characterized subcellular fractions.

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Materials and Methods

Materials

C_{57}Bl/10 mice, male and female, 6 – 12 weeks old were purchased from our own breeding colony and used throughout the experiments. Unlabelled 1α-phosphatidyl choline was purchased from Sigma, St. Louis, Mo. USA. N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid = HEPES was purchased from Roth, Karlsruhe, FRG. Eagle's medium (Dulbecco's modification) horse serum and fetal calf serum were obtained from G.I.B.C.O., Grant Is-

land, N.Y., USA; sera were inactivated by heating at 56 °C for 30 min.

1-[1-^{14}C]palmitoyl-2-acylglycero-3-phosphorylcholine was prepared by enzymatic acylation of unlabelled 2-acylglycero-3-phosphorylcholine with [1-^{14}C]-palmitic acid according to Ferber and Resch [5]. Similarly 1-acyl-2-[1-^{14}C]oleoylglycero-3-phosphorylcholine was prepared using 1-acylglycero-3-phosphorylcholine and [1-^{14}C]oleic acid as substrates for the acyl-CoA:lysophosphatide acyltransferase from rat liver microsomes. All other substances and reagents were of analytical purity.

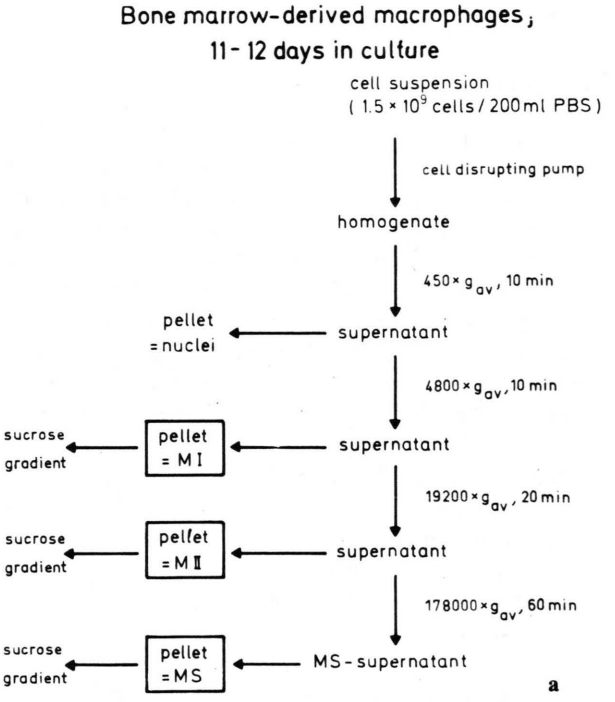
Methods

Cell cultivation

Bone marrow-derived macrophages were cultivated from bone marrow of mice according to Meerpohl *et al.* [6]. The culture medium contained 30% conditioned medium [7, 8] which shifted the cell proliferation towards the production of a 95% pure population of macrophages (Meerpohl *et al.* [6]). 5×10^7 cells were grown in 250 ml bags of polymeric fluorocarbon according to Munder *et al.* [9]. The cells were harvested from the culture bags at day 11–12 by gently shaking the culture containers at room temperature for 60 min. Then the cells could be collected by centrifugation at $300 \times g_{av}$ and 4 °C for 10 min. Cell viability was always over 90% as checked by trypan blue exclusion.

Cell disruption and preparation of subcellular fractions

All procedures were carried out at 4 °C. Harvested cells were washed once to remove serum and resuspended in phosphate buffered saline (pH 7.4) at a concentration of 1.5×10^9 cells/200 ml. Then the cells were disrupted by the method of Crumpton and Snary [10] in a Stansted cell-disrupting pump (Stansted Fluid Power Ltd., Stansted, Essex, UK), using a backward pressure of 20 psi. After passage through the pump the suspension was squirted twice through a syringe (No. 1 needle) to free nuclei from adherent material and to avoid clumping. Under these conditions cell disruption exceeded 90% as checked by phase contrast microscopy. The nuclei, debris and unbroken cells were spun down at $450 \times g_{av}$. The pellet was resuspended in 0.03 M HEPES-buffer (pH 6.0, containing 0.13 M KCl and 0.005 M $MgCl_2$). The supernatant was centrifuged at $4800 \times g_{av}$ for 10 min. The pellet containing the large granules (M I) was resuspended in 10 mM HEPES-buffer (pH 7.2) after washing once in the same buffer. The supernatant was centrifuged at $19\,200 \times g_{av}$ for 20 min. The pellet containing the



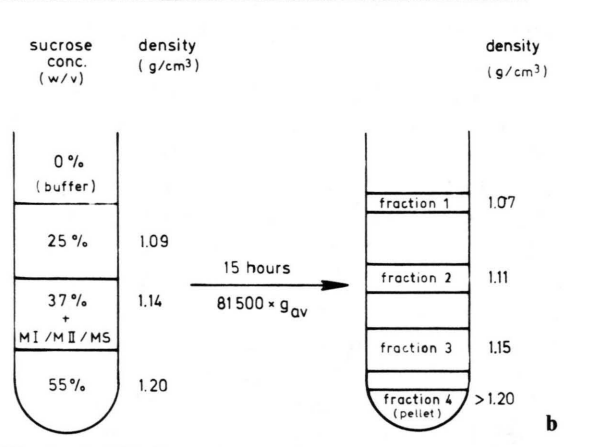


Fig. 1. a) Cell disruption of bone marrow-derived macrophages and differential centrifugation. Three main fractions were obtained: M I, M II, MS. b) Sucrose gradient separation of the three main fractions. The fractions M I or M II or MS were suspended in sucrose which was adjusted to 37% (w/v). After centrifugation, 4 fractions (subfraction 1–4) were obtained.

second granular fraction (M II) was resuspended in 10 mM HEPES-buffer (pH 7.2) after washing once in the same buffer. The supernatant was centrifuged at 178000 × g_{av} for 60 min. The cell free microsomal supernatant was saved and the pellet containing the microsomal fraction (MS) was washed once and resuspended in 10 mM HEPES-buffer (pH 7.2).

Gradient centrifugation

The particle containing fractions M I, M II and MS were submitted to further purification by sucrose gradient centrifugation. In preceeding experiments linear sucrose gradients had been carried out to establish an optimal step gradient. The sucrose concentration covered a range from 20% to 60% sucrose (w/v) in 10 mM HEPES-buffer (pH 7.2) *i.e.* a density range from 1.07 g/cm³ to 1.22 g/cm³. Under these conditions the plasma marker enzyme alkaline phosphodiesterase I for example peaked at a sucrose concentration of about 30% (w/v, *i.e.* density 1.11 g/cm³). The lysosomal marker enzyme N-acetyl-β-glucosaminidase peaked at a sucrose concentration of about 45% (w/v, *i.e.* density 1.17 g/cm³) and the mitochondrial inner membrane marker enzyme succinate dehydrogenase peaked at about 50% sucrose concentration (w/v, *i.e.* density 1.18 g/cm³). The enrichment of the different marker enzymes at these various sucrose concentrations led to the display of a step gradient of equal volumes of the following sucrose concentrations (w/v). 55% sucrose (density 1.20 g/cm³) was overlaid with 37% sucrose (density 1.14 g/cm³) containing one of the three fractions (M I, M II, MS) to be purified. This gradient step was overlaid with 25% sucrose (density 1.09 g/cm³). 10 mM HEPES-buffer (pH 7.2) was laid on top to tare the tubes. After centrifugation for 15 h in a swing out rotor (SW 27, Beckman Corp.) at 81500 × g_{av} without brake all three main fractions resulted in three bands and a pellet of the following sucrose density: first band (fraction 1) 1.07 g/cm³; second band (fraction 2) 1.11 g/cm³; third band (fraction 3) 1.15 g/cm³ and pellet (fraction 4) > 120 g/cm³. All three main fractions (M I, M II, MS) showed this distribution pattern in the step gradient. The four subfractions were washed with 10 mM HEPES-buffer (pH 7.2) and sedimented at 178000 × g_{av} for 120 min before finally resuspending in the same buffer. After this differential centrifugation and purification by step gradient the fractions were submitted to protein and enzyme determinations. In case of storage at − 20 °C the different fractions were resuspended in 8% sucrose (w/v, final concentration) in 10 mM HEPES-buffer (pH 7.2).

Enzyme assays

The activities of alkaline phosphatase (EC 3.1.3.1), alkaline phosphodiesterase I (EC 3.1.4.1), γ-glutamyl transferase (EC 2.3.2.2) Mg^{2+}-ATPase (EC 3.6.1.3), succinate dehydrogenase (EC 1.3.99.1), β-N-acetyl-glucosaminidase (EC 3.2.1.30), lactate dehydrogenase (EC 1.1.1.27) and acyl-CoA: 1-acylglycero-3-phosphorylcholine-0-acyltransferase (EC 2.3.1.23) were determined as described previously [11]. The activity of 5′-nucleotidase (EC 3.1.3.5) was determined using the same technique as described for the ATPase with the exception that [^{32}P]AMP was used as substrate. The activity of the NADH- and NADPH-cytochrome c reductase (EC 1.6.2.4) was carried out according to Crane and Löw [12].

Determination of the activity of phospholipase A_1 and A_2

Standard assays were carried out with sodium cholate as detergent which effectively inhibited the lysophospholipase. Samples contained either 0.05 μCi 1-[1-^{14}C]palmitoyl-2-acylglycero-3-phosphorylcholine or 1-acyl-2-[1-^{14}C]oleoylglycero-3-phosphorylcholine, 50 nmol non-labelled phosphatidylcholine in 0.5 ml 0.1 M TRIS-buffer, pH 7.6 with 4 mM $CaCl_2$ and 0.35% sodium cholate. After incubation for 60 min at 37 °C the reaction was terminated by the addition of 5 ml methanol and the lipids were processed and separated by thin-layer chromatography as described previouly [5].

From the amount of generated lysolecithin and free fatty acids the positional specificity and the activities were determined.

pH optima depended on the type of detergent used. Without detergent only low activities were measured at pH 4 − 5. Using cholate or deoxycholate phospholipase A_1 and A_2 activities were highest at pH 9 − 10. Non-ionic detergents as Triton X-100 could not be used, because no inhibiton of the lysophospholipase was achieved.

Lipid extraction and analysis was carried out as described previously [11].

Results

The procedure of subcellular fractionation of bone marrow-derived macrophages by differential centrifugation and gradient separation is given in Fig. 1 a, b.

Table I shows the specific and total activities of the main fractions obtained by differential centrifugation. These crude fractions already show for some enzymes a notable enrichment *e.g.* the plasma membrane marker enzyme alkaline phosphodiesterase I is enriched 9-fold and the acyl-CoA: lysophos-

Table I. Enzyme activities in the main subcellular fractions of bone marrow-derived macrophages. Specific activities are expressed as nmol × mg^{-1} × min^{-1}. The enrichment factors are given in brackets. Total activities are given as percentage of the total activity of the homogenate and specific contents of cholesterol and phospholipid as nmol/mg protein.

Enzymes	Homogenate	Nuclei	M I	M II	MS supernatant	MS
Acyl CoA: lysolecithinacyltransferase						
specific activity	6.859 (1)	12.533 (1.83	28.736 (4.19	54.820 (7.99)	0.036 (0.005)	50.945 (7.43)
total activity %	100	20.54	26.41	18.24	0.15	19.07
Phospholipase A_1						
specific activity	0.220 (1)	0.420 (1.91)	0.386 (1.75)	0.073 (0.33)	0.278 (1.26)	0.114 (0.50)
total activity %	100	27.20	13.79	1.02	51.08	1.66
Phospholipase A_2						
specific acitivity	0.360 (1)	0.622 (1.73)	0.627 (1.74)	0.130 (0.36)	0.306 (0.85)	0.141 (0.39)
total activity %	100	24.57	13.68	1.16	34.39	1.25
Alkaline phosphodiesterase I						
specific activity	8.637 (1)	10.304 (1.20)	32.723 (3.79)	86.000 (9.95)	1.314 (0.15)	59.284 (6.86)
total activity %	100	13.44	23.88	22.73	4.49	17.62
Mg^{2+} ATPase						
specific activity	58.700 (1)	39.200 (0.67)	235.800 (4.02)	676.400 (11.52)	0.657 (0.011)	335.200 (5.71)
total activity %	100	7.52	25.32	26.30	0.33	14.66
5'Nucleotidase						
specific activity	2.900 (1)	4.940 (1.70)	14.650 (5.03)	8.790 (3.03	0.820 (0.28)	1.370 (0.47)
total activity %	100	19.85	35.12	11.62	9.05	0.28
Succinate dehydrogenase						
specific activity	4.778 (1)	4.268 (0.89)	41.698 (8.72)	15.600 (3.26)	0.201 (0.04)	2.077 (0.43)
total activity %	100	10.06	55.00	7.45	1.24	1.12
N-Acetyl-β-glucosaminidase						
specific activity	310.335 (1)	733.028 (2.36)	843.614 (2.72)	325.942 (1.05)	312.095 (1.01)	763.919 (2.46)
total activity %	100	26.61	17.14	2.39	29.72	6.32
Acid phosphatase						
specific activity	21.131 (1)	23.292 (1.34)	40.492 (2.06)	40.247 (1.90)	10.951 (0.80)	19.837 (0.94)
total activity %	100	22.97	56.84	6.11	23.56	1.82
NADPH cytochrome c reductase						
specific activity	17.363 (1)	34.613 (1.99)	53.683 (3.08)	127.478 (7.36)	3.399 (0.19)	123.736 (7.11)
total activity %	100	22.46	19.48	16.76	5.78	18.30
NADH cytochrome c reductase						
specific activity	42.192 (1)	36.298 (0.86)	198.107 (4.72)	156.279 (3.71)	0.658 (0.01)	99.146 (2.36)
total activity %	100	10.03	32.64	14.20	0.50	1.41
Cholesterol						
specific content	49.743 (1)	45.120 (0.91)	146.677 (2.95)	236.292 (4.75)	5.916 (0.12)	121.646 (2.44)
total activity %	100	12.03	27.71	22.34	5.20	6.19
Phospholipid						
specific content	149.45 (1)	142.444 (0.95)	415.182 (2.78)	618.289 (4.14)	8.454 (0.06)	372.728 (2.49)
total activity %	100	12.64	26.11	19.46	2.47	6.32

phatide acyltransferase 8-fold. In contrast to lymphocytes bone marrow macrophages exhibit only trace activities of the plasma membrane marker enzymes alkaline phosphatase and γ-glutamyl transferase *. High percentages of the total activities of the following enzymes were recovered in the soluble supernatant: N-acetyl-β-glucosaminidase (30%), acid phosphatase (23%), phospholipase A_1 (51%) and phospholipase A_2 (34%). The total activities of most other enzymes were exceedingly low in the soluble supernatant. With the exception of the crude nuclear fraction the highest proportions of protein and most enzymes were found in the large granules (M I, M II) and not in the microsomal fraction. On the other hand the microsomal fraction contained appreciable amounts of ATPase, acyl-CoA: lysophosphatide acyltransferase and NADPH-cytochrome c reductase. In the three main fractions (M I, M II, MS) no clear separation of lysosomes, mitochondria and endoplasmic reticulum was achieved. Therefore we subjected these fractions to a further purification by step gradient centrifugation. In preliminary experiments continuous gradients were used to establsih optimal sucrose concentrations.

As can be seen from Fig. 2 the two plasma membrane marker enzymes alkaline phosphodiesterase I and Mg^{2+}-ATPase were enriched up to 26-fold compared to the homogenate in gradient fraction 1 and 2. Also the majority of the total activities of these two enzymes were recovered in subfraction 1 and 2. The cholesterol distribution followed a similar pattern.

The lysosomal marker enzyme N-acetyl-β-glucosaminidase was found mainly in gradient fraction 3 of all main fractions (M I, M II, MS) however, with clear preference for M I-3. A similar distribution of

* The alkaline phosphatase activity in homogenates of bone marrow macrophages did not exceed 0.6 nmol × mg^{-1} × min^{-1} and γ-glutamyl transferase did not exceed 0.06 nmol × mg^{-1} × min^{-1}.

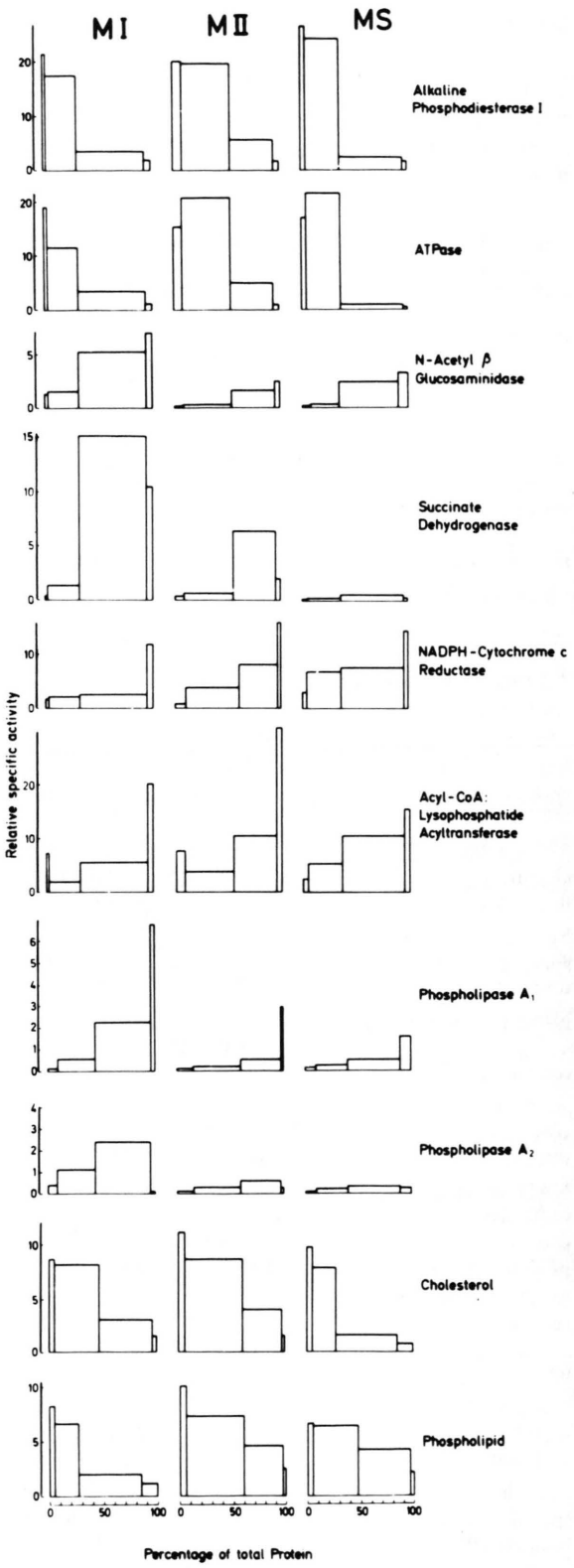


Fig. 2. Enzyme activities and composition of subfractions after separation on sucrose gradients. Abscissa: the percentage of total protein (homogenate = 100%) is given. Ordinate: the relative specific activities are plotted as enrichment factors (homogenate = 1.0). Thus the area of each column is proportional to the total activity recovered in the corresponding fraction. Each graph consists of 4 columns representing (from left to right) subfraction 1–4 of the gradient of the main fractions M I, M II, MS.

the total activities was observed for phospholipase A_1 and A_2 and for succinate dehydrogenase.

The marker enzyme for endoplasmic reticulum NADPH-cytochrome c reductase and the acyl-CoA: lysophosphatide acyltransferase were also found mainly in subfraction 3 but with clear preference for MS-3. A further indication for the localization of these two enzymes in the same intracellular particles comes from the fact that their enrichment factors were found to be highest in subfraction 4 of all gradients. Interestingly, subfraction 4 (M I, M II, MS) exhibits only trace activities of phospholipase A_2 whereas phospholipase A_1 was enriched maximally in subfraction 4.

Discussion

In a previous study [1] we reported on the turnover of arachidonic acid in intact bone marrow macrophages. We had evidence that the incorporation of arachidonic acid was due to the reacylation of lysophosphatides. The main aim of this study was to establish the subcellular localization of the related lipid-metabolizing enzymes *i. e.* phospholipase A_1 and A_2 and the acyl-CoA: lysophosphatide acyltransferase.

We developed a method for the separation of subcellular fractions which was highly reproducible and yielded fractions with high enrichment of subcellular components as shown by the high specific activities of marker enzymes.

The highest purity was achieved for the separation of plasma membranes. Thus the alkaline phosphodiesterase I was enriched in gradient fraction 2 about 25-fold. Moreover, the recovery of this membrane-bound enzyme was highest in this fraction. None of the other enzymes was enriched to a similar extent in this plasma membrane fraction.

Even higher enrichment factors of about 30-fold were achieved for the acyl-CoA: lysophosphatide acyltransferase in gradient fraction 4. However, in this fraction only a minor proportion of the total activity was recovered compared to subfraction 3 where the bulk activity was found.

Since the marker enzyme for endoplasmic reticulum the NADPH-cytochrome c reductase showed a very similar enrichment and distribution of total activities within subfraction 3 and 4 it seems very likely that the acyl-CoA: lysophosphatide acyltransferase of bone-marrow macrophages is located in the endoplasmic reticulum.

This is in accordance with findings of Wang *et al.* [3] who analyzed subcellular components of rabbit alveolar macrophages.

The distribution of phospholipase A_1 and A_2 was found to be much more complex. Both enzymes occured mainly in the soluble fraction and in fraction M I. The lysosomal marker enzymes acid phosphatase and N-acetyl-β-glucosaminidase were also recovered mainly in these two fractions. This indicates that a major proportion of phospholipases A_1 and A_2 is associated with lysosomes.

Phospholipases A_1 and A_2 showed a striking difference in their occurence in the endoplasmic reticulum. In this fraction phospholipase A_2 occurred only in trace activities whereas phospholipase A_1 was maximally enriched in subfraction 4 of all major fractions. This distribution is further supported by the fact that phospholipase A_1 in contrast to phospholipase A_2 was found in appreciable quantities in the microsomal fraction which also contains high quantities of endoplasmic reticulum. The localization of phospholipase A_1 thus is in accordance with findings in many other cell types [13, 14]. On the other hand we could not obtain a fraction which contains phospholipase A_2 exclusively or with high preference as was reported for mitochondria [13, 14].

There are two possible explanations for this finding, either that mitochondria of macrophages contain both phospholipases or more likely, that the separation of lysosomes from mitochondria in subfraction M I-3 was not optimal. Thus the high activities of phospholipase A_1 found in this fraction is probably caused by contamination with lysosomes.

The combination of differential centrifugation followed by gradient purification of all three main fractions (M I, M II, MS) was advantageous especially for the purification of the endoplasmic reticulum and the correlation of the acyl-CoA: lysophosphatide acyltransferase with this fraction.

With respect to the turnover of arachidonic acid one has to consider that the corresponding enzymes phospholipase A_2 and acyl-CoA: lysophosphatide acyltransferase are located in different subcellular compartments. Therefore in order to achieve a complete metabolic cycle of de- and re-acylation of

phospholipids, substrates and products have to be exchanged between these compartments. It has to be elucidated whether a similar separation exists for the phospholipase A_1 and the corresponding acyltransferase which transfers preferentially saturated fatty acids.

Acknowledgements

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