CoA-DEPENDENT CLEAVAGE OF ARACHIDONIC ACID FROM PHOSPHATIDYLCHOLINE AND TRANSFER TO PHOSPHATIDYLETHANOLAMINE IN HOMOGENATES OF MURINE THYMOCYTES

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1. Introduction

Increased turnover of the fatty acyl moieties of membrane phospholipids and in particular the metabolism of arachidonic acid play a central role in the early phase of ligand–receptor-mediated activation in many cell types.

Alterations in the membrane lipid phase are thought to initiate changes in the membrane permeability to ions and the activation of membrane-bound enzymes [1]. The synthesis of arachidonic acid metabolites by the cyclo-oxygenase and lipoxygenase systems is also considered to be controlled by the availability of free arachidonic acid; this in turn is determined by the release of arachidonic acid from membrane-bound lipids [2–4].

In mitogen-induced activation of T lymphocytes, one of the early changes reported in activated cells is an increased incorporation of long-chain fatty acids into phosphatidylcholine and phosphatidylethanolamine [5]. In mitogen-activated human T cells an elevated release of arachidonic acid from membrane phospholipids has been reported [6,7], coupled with a synthesis of arachidonic acid derivatives [8].

There is good evidence that phospholipid synthesising enzymes are activated in stimulated T cells [9] but we have been unable to detect significant phospholipase A_2 activity in thymocytes using exogeneous substrate [10]. The rapid incorporation of fatty acids into membrane phospholipids appears to be due to a turnover cycle of the fatty acyl chains rather than de novo synthesis of phospholipids. This fatty acyl chain turnover necessitates the existence of degradative mechanisms. In spite of the finding that thymocyte homogenates lack significant phospholipase A_2 activity it has been reported that cells prelabelled with arachidonic acid liberated free fatty acids from phospholipids when incubated at 37°C [6,7]. One possible pathway which could lead to a phospholipase A_2-independent generation of free fatty acids is the breakdown of phosphatidylinositol by a specific phospholipase C followed by the degradation of the resulting diglyceride by diglyceride lipase [8,11,12].

Here we demonstrate that thymocytes exhibit a CoA-dependent breakdown of phosphatidylcholine catalysed by the acyl-CoA:lysophosphatide acyltransferase. This pathway was first demonstrated in rat liver microsomes in [13].

2. Experimental

2.1. *Preparation and labelling of thymocytes*

Thymuses from C3H or C57bl mice were carefully removed, freed of connective tissue and finely minced with scissors. Cell suspensions were prepared as in [14]. The first two cell supernatant fractions obtained by dissociation of the thymi fragments were used; these contain the cells from the dissociation step in cold phosphate-buffered saline and the cells obtained after the first trypsin treatment.

The cells were washed twice with Hepes-buffered Eagle's medium and adjusted to 2×10^7 cells/ml in the above medium containing 0.05% fatty acid free

Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine

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bovine serum albumin (Sigma, München) and 0.1 μCi/ml (1.8 nmol) 1 [^{14}C]arachidonic acid. They were then incubated for 60 min at 37°C. After this incubation period the cells were washed once with cold Hepes-buffered Eagle's medium containing 0.5% fatty-acid-free albumin to remove unincorporated free arachidonic acid.

2.2. *Preparation of homogenates*

The cells were resuspended in cold phosphate-buffered saline and disrupted on ice using a Branson sonifier (setting 3 for 3 min). To remove water-soluble cytoplasmic constitutents such as ATP and CoA, the homogenate was centrifuged at 177 000 × g_{av} for 30 min (50 000 rev./min, rotor 60 Ti Beckman) at 4°C. The supernatant was discarded and the pellet was resuspended by sonication in cold phosphate-buffered saline. Assays were set up and incubated for 60 min at 37°C as table 1.

2.3. *Lipid substrates, extraction, chromatography and measurement of labelled lipids*

Lysophosphatidylethanolamine was purchased from P-L Biochemicals, Milwaukee and lysophosphatidylcholine was obtained from Sigma Chemicals, St Louis. Lysophosphatidylinositol was prepared by degradation of phosphatidylinositol (Sigma), with phospholipase A_2 (from pancreas, Boehringer, Mannheim) in an incubation mixture containing 7 ml diethyl ether, 25 mg phosphatidylinositol, 70 μl 0.1 M $CaCl_2$, 70 μl 0.1 M Tris buffer (pH 7.4) and 3 mg phospholipase A_2. After overnight incubation at 37°C on a shaker the mixture was applied to silica plates (Kieselgel H, Merck, Darmstadt) and separated with a solvent system chloroform:methanol:water 65:40:10 (by vol.). This system gives a good separation of lysophosphatidylinositol from phosphatidylinositol, leaving phospholipase A_2 at the origin. After staining of reference lanes with rhodamine 6GO the area of gel containing the lysophosphatidylinositol was scraped off the plate and the lipid eluted with the above solvent mixture.

All lysocompounds were shown to be free of phospholipase A_2 contamination by assay as in [10]. Further proof of the identity of the lyso-compounds and their ability to act as acceptors for fatty acyl-CoAs was obtained by acylation to their corresponding diacylphospholipid using the acyl-CoA:lysophosphatide acyltransferase as in [9]. Before use lysocompounds were taken to dryness by evaporation and dissolved in phosphate-buffered saline and briefly sonicated.

Labelled lipids were extracted, separated by thin-layer chromatography and quantified as in [9]. In addition, separation of phosphatidylinositol and phosphatidylserine from phosphatidylcholine was achieved with the system chloroform/methanol/acetic acid/water (100:55:16:8, by vol.) at 4°C.

Phospholipids were quantified by phosphorus determination as in [9].

3. Results and discussion

After 60 min incubation of crude membrane preparations of prelabelled thymocytes only a small degradation of phosphatidylcholine (3%) and a concomitant increase in free fatty acid was detected. This could be due to trace phospholipase A activity, being detected more easily in this system using endogeneously labelled cells as compared to exogeneous substrate [10]. No significant change was observed in the phosphatidylethanolamine and neutral lipid fractions. Addition of sodium cholate (0.35%), which enhances phospholipase action, did not alter this distribution pattern.

Provided that a suitable lysoacceptor (lysophosphatidylethanolamine) is present in the incubation mixture the addition of CoA caused an impressive degradation of phosphatidylcholine (17%) and a corresponding increase in the phosphatidylethanolamine fraction. Addition of lysophosphatidylethanolamine or CoA alone induced only a very slight change in the level of phosphatidylcholine and phosphatidylethanolamine.

As a control experiment the capacity of such thymocyte homogenates to acylate lysophosphatidylcholine with free arachidonic acid was examined. The results shown in table 2 rule out the possibility that the CoA-dependent phosphatidylcholine breakdown is caused by the combined action of a phospholipase A_2 and an ATP-dependent re-acylation of an available lyso-compound. From table 2 it can be seen that the homogenate pellet contains insignificant levels of ATP or CoA as the addition of either ATP or CoA to the pellet did not induce incorporation of exogeneously added free arachidonic acid. Only the simultaneous addition of CoA and ATP resulted in acylation of lysophosphatidylcholine.

These results favour the hypothesis that the CoA-dependent transfer of arachidonic acid from phospha-

Table 1
Transfer of arachidonic acid from phosphatidylcholine to phosphatidylethanolamine in homogenates of mouse thymocytes (% radioactivity)

Additions	Incubn.	LPC	PC	PE	FFA	NL
None	0	0.55 ± 0.09 (0.0)	77.59 ± 0.11 (0.0)	15.78 ± 0.06 (0.0)	0.77 ± 0.12 (0.0)	4.86 ± 0.01 (0.0)
None	1 h	0.68 ± 0.04 (+0.13)	74.35 ± 0.47 (−3.24)	15.48 ± 0.23 (−0.30)	2.73 ± 0.03 (+1.96)	5.04 ± 0.19 (+0.18)
CoA	1 h	0.74 ± 0.06 (+0.19)	74.28 ± 0.36 (−3.31)	16.40 ± 0.05 (+0.62)	2.84 ± 0.12 (+2.07)	5.00 ± 0.05 (+0.14)
LPE	1 h	0.78 ± 0.02 (+0.23)	73.20 ± 0.20 (−4.39)	18.08 ± 0.21 (+2.30)	2.51 ± 0.06 (+1.74)	4.93 ± 0.06 (+0.07)
CoA + LPE	1 h	0.93 ± 0.08 (+0.38)	59.71 ± 0.07 (−17.38)	30.82 ± 0.35 (+15.04)	2.73 ± 0.18 (+1.96)	5.17 ± 0.14 (+0.31)
CoA + LPE + cholate	1 h	0.77 ± 0.04 (+0.22)	75.26 ± 0.22 (−2.33)	17.21 ± 0.24 (+1.43)	1.05 ± 0.07 (+0.28)	5.18 ± 0.08 (+0.32)

Thymocytes were isolated and prelabelled with 1-[^{14}C]arachidonic acid as in section 2. Aliquots of homogenate equivalent to 2×10^7 cells were used per assay. Assays were performed in 1.0 ml phosphate-buffered saline with different additions: CoA, 65 nmol; lysophosphatidylethanolamine (LPE), 25 nmol; sodium cholate, 3.5 mg. Values are given as means ± SE ($n = 3$) of the percentage of total radioactivity in cellular lipids. Figures in parentheses represent the differences between 1 h and time 0 incubations. Lysophosphatidylethanolamine was purchased from P-L Biochemicals (Milwaukee WI) and found by assay [10] to be free of phospholipase A contamination. 100% incorporation was in the range of 56 000 cpm

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine, FFA, frre fatty acids; NL, neutral lipids

Table 2
The capacity of thymocyte homogenates to acylate lysophosphatidylcholine with free arachidonic acid

Additions	Incorporation into phosphatidylcholine
None	0.6 ± 0.09%
CoA	0.7 ± 0.06%
ATP	0.8 ± 0.02%
CoA + ATP	20.0 ± 0.50%
CoA + ATP + cholate	0.9 ± 0.04%

Unlabelled thymocytes were isolated, homogenised and freed of cytoplasmic constitutents as in section 2. Aliquots of homogenate equivalent to 2×10^7 cells (90 μg protein) were used per assay. Assays were performed in 1.0 ml phosphate-buffered saline containing as substrates 10 nmol 1-[^{14}C]-arachidonic acid (0.05 μCi) and 25 nmol lysophosphatidylcholine. Amounts per assay of different additions as indicated were: CoA, 65 nmol; ATP 5.0 μmol; sodium cholate 3.5 mg. Values are given as means ± SE (n = 3) of the percentage of total radioactivity in cellular lipids

tidylcholine to phosphatidylethanolamine is catalysed by the acyl-CoA:lysophosphatide acyltransferase operating in reverse (eq. (1)) as suggested in [13].

$$\text{PC} + \text{CoA-SH} \rightarrow \text{Lyso-PC} + \text{Acyl-CoA} \quad (1)$$

$$\text{Acyl-CoA} + \text{LPE} \rightarrow \text{PE} + \text{CoA-SH} \quad (2)$$

Reactions catalysed by the acyl-CoA:lysophosphatide acyltransferase

The results given in table 2 emphasise that high activities of this enzyme are present in homogenate pellets of thymocytes. Consistent with this interpretation is the observation that cholate, which is known to inhibit the acyltransferase (see table 2) also completely eliminates the CoA-dependent breakdown of phosphatidylcholine and the transfer of fatty acid to lysophosphatidylethanolamine.

Control experiments in which unlabelled free arachidonic acid (10 nmol/ml) was added showed no inhibition of the CoA-dependent transfer of labelled fatty acid. This is further proof that the CoA-dependent transfer of fatty acyl moieties from phosphatidylcholine to phosphatidylethanolamine does not involve liberation of free fatty acids as an intermediate stage.

Experiments in which thymocytes were pre-labelled with free oleic acid and further incubated in the same manner as cells pre-labelled with arachidonic acid (table 1) revealed no CoA-mediated transfer of oleic acid from phosphatidylcholine to phosphatidylethanolamine (not shown). The action of phospholipase A_2 would not account for this fatty acid specificity of the transfer; on the other hand, it is known that the acyl-CoA:lysophosphatide acyltransferase exhibits a marked specificity for poly-unsaturated fatty acids [15].

In contrast to the results obtained using liver microsomes and exogeneous radiolabelled phospholipids as donor substrates [13], in our system using endogeneously labelled membrane phospholipids of thymocytes, only lysophosphatidylethanolamine functioned as a good acceptor for a CoA-mediated transfer of fatty acids from a phospholipid donor.

The ability of lysophosphatidylinositol and lysophosphatidylcholine to act as acceptor substrates was tested in experiments where phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine were separated from each other. The addition of lysophosphatidylinositol or lysophosphatidylcholine, in conjunction with CoA, did not alter the distribution of ^{14}C radiolabel in the different lipid fractions. In similar experiments with complete separation of phospholipids, the addition of lysophosphatidylethanolamine (plus CoA) did not cause any decrease in the phosphatidylinositol fraction.

Thus these results demonstrate that in our system phosphatidylcholine is the only phospholipid donor and lysophosphatidylethanolamine the only acceptor substrate for the CoA-mediated transfer of arachidonoyl moieties.

4. Conclusions

We have reported that in bone marrow-derived macrophages, which contain high activities of phospholipase A_2, the accumulation of free unsaturated fatty acids, such as arachidonic acid, is regulated by the reacylation of lysophosphatides catalysed by the acyl-CoA:lysophosphatide acyltransferase [4].

Our finding in thymocytes of a CoA-dependent transfer of fatty acyl chains between phospholipids catalysed by the acyl-CoA:lysophosphatide acyltransferase provides a means of achieving turnover of phospholipid acyl chains without the participation of a phospholipase A_2. Although this pathway does not lead directly to the accumulation of free fatty acids, fatty acyl-CoA is generated as an intermediate and in intact cells this compound could be

further hydrolyzed by acyl-CoA hydrolases. The acyl-CoA:lysophosphatide acyltransferase has a known specificity for polyenoic acids [15]. In thymocyte homogenates we have demonstrated the reverse action of this enzyme which consequently leads to a specific cleavage of arachidonic acid from phosphatidylcholine. This mechanism could account for the preferential release of arachidonic acid from phospholipids observed in intact lymphocytes [6,7].

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