Formation of diacyl- and alkylacylphosphatidylcholine by the membranes of human platelets

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Received 30 October 1985

We have investigated the distribution and fatty acid preference of two acyl-CoA transferase activities in a human platelet mixed membrane fraction and in well-characterised surface and intracellular membrane subfractions prepared from it by high-voltage free-flow electrophoresis. One transferase inserts long-chain unsaturated fatty acids into 1-acyllysophosphatidylcholine (1-acyl-LPC) and the other into lyso-platelet-activating factor (LPAF). Both transferase activities were approx. 4-fold enriched in the intracellular membranes with respect to their specific activities in the mixed membranes. The surface membrane activities were correspondingly depleted. Using 1-acyl-LPC as the acceptor, all the intracellular membrane preparations showed transferase preference for the CoA ester of 8,11,14-eicosatrienoic acid. In contrast when LPAF was the acceptor the CoA esters of linoleic and arachidonic acid were the preferred donors.

(Human platelet membrane) *Acyltransferase* *Enzyme activity* *Diacylphosphatidylcholine* *Alkylacylphosphatidylcholine*

1. INTRODUCTION

The choline-containing phosphoglycerides of platelets are one source of the free arachidonic acid (AA) used for the biosynthesis of prostaglandin endoperoxides and thromboxanes [1–2]. Diacylphosphatidylcholine (diacyl-PC) is presumed to be the predominant PC species from which AA can be liberated and the total amount of this fatty acid esterified in diacyl-PC may influence the size of the free AA pool that stimulated platelets can produce. It has been recently demonstrated that alkylacyl-PC is a minor but significant component of the total choline-containing phosphoglycerides in cell membranes [3,4] and since it is enriched in AA [4] it may also contribute as a precursor for prostanoid synthesis. Alkylacyl-PC is, however, both a precursor and a metabolite of platelet-activating factor (PAF) since not only can it be converted to PAF via a deacylation-acetylation reaction [4–6], but both alkylacyl-PC and lyso-PAF (LPAF) are formed when PAF is incubated with human platelets [7].

The fatty-acyl-CoA transferases are known to insert fatty acids into lysophospholipids of other cells via the remodelling of Land's pathway [8] and this pathway is probably a major route for the incorporation of highly unsaturated fatty acids into platelet phospholipids [9]. Moreover, this process, in combination with the known selectivity for AA of the fatty-acyl-CoA synthetase [10], the enzyme(s) which produces the fatty acyl donors for the transferases, could be important in regulating the distribution of AA in platelet phospholipids. In previous reports, using a crude mixed membrane fraction prepared from sonicated platelets, two fatty-acyl-CoA transferase activities were identified [9,11]. These enzyme activities insert unsaturated fatty acids into 1-acyl-LPC or LPAF giving respectively diacyl-PC and alkylacyl-PC.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

Here, we have used highly purified human platelet surface and intracellular membranes to investigate the distribution and activities of the fatty-acyl-CoA transferases which produce diacyl-PC and alkylacyl-PC from 1-acyl-LPC and LPAF, respectively. Additionally, some evidence is presented for the predominant localisation of alkylacyl-PC in the intracellular membranes and this is in general agreement with the subcellular distribution of its fatty-acyl-CoA transferase activity reported here.

2. EXPERIMENTAL

Unsaturated fatty acyl chlorides were obtained from NuChek Prep (Elysian, MN). Avanti Biochemicals (Birmingham, AL) supplied semisynthetic LPAF (prepared from beef heart PC) and 1-acyl-LPC (prepared from egg PC). This commercial LPAF has previously been shown to be free of any detectable contamination with 1-acyl-LPC [11]. Both LPAF and 1-acyl-LPC gave a single spot on TLC (chloroform:methanol:acetic acid:water, 100:65:14:6, by vol.). Coenzyme A, lithium salt (Chromatopure), was obtained from P-L Biochemicals (Milwaukee, WI) and DTNB was supplied by Sigma. The [*choline-methyl*-^{3}H]dipalmitoyl-PC and [1,2-*alkyl*-^{3}H]PAF were purchased from New England Nuclear (Boston, MA).

2.1. *Preparation of platelet membrane fractions*

The procedures for preparation of the mixed membrane fraction and for the isolation of human platelet surface and intracellular membranes were essentially as reported in [12] with only minor modifications. Briefly, human platelets were isolated by differential centrifugation from fresh whole blood or buffy coat residues supplied by the Blood Transfusion Service Laboratories at Tooting, London. Generally, processing of the buffy coat in the laboratory began within 2–3 h of donation. After washing, the platelets were exposed to neuraminidase (Sigma grade X, 0.05 U/ml) for 20 min to remove sialic acid and lower the surface membrane electronegativity. They were then washed, resuspended in buffer (10 mM Hepes, 0.34 M sorbitol, pH 7.2) and sonicated. After a low-speed centrifugation to remove unbroken cells and large debris, the sonicate was applied to the surface of a 1.0–3.5 M sorbitol density gradient and centrifuged for 90 min at 42000 × *g*. The mixed membrane fraction, which was well separated from the higher density granular components, was removed from the low-density region of the gradient and washed by centrifugation (90 min at 100000 × *g*). This membrane fraction, which contained both surface and intracellular elements, was applied to the chamber of a VAP-5 free-flow electrophoresis apparatus (Bender-Hobein, Munich) operating at 140 mA, 100 V/cm and 6°C. The mixed membrane fraction separated into 2 discrete subfractions consisting of surface and intracellular membrane vesicles with the former represented by the least electronegative peak. After profiling this separation by protein assay, these 2 major membrane subfractions were pooled across the peaks, centrifuged and resuspended to wash and concentrate for analytical and enzyme determinations.

2.2. *Fatty-acyl-CoA transferase assay*

The unsaturated fatty acyl-CoA esters were prepared and characterized as in [9]. They were stored in 10 mM citrate-phosphate buffer, pH 5.9, containing sodium ascorbate, 2.5–5.0 mM final concentration, to prevent oxidation as described [9].

The acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase and acyl-CoA:1-alkyl-*sn*-glycero-3-phosphocholine acyltransferase activities of the membrane fractions were measured at room temperature using the spectrophotometric assay described by Lands and Hart [13]. All substrates were used at saturating concentrations. The assay mixtures consisted of 0.8 ml of 85 mM phosphate buffer, pH 7.0, in the case of 1-acyl-LPC or pH 7.5 in the case of LPAF; 0.1 ml of 3.3 mM DTNB; 0.1 ml of 1.0 mM 1-acyl-LPC or 1.5 mM LPAF; and 1–10 μl of the fatty acyl-CoA solution of final concentration 63.6 ± 0.63 and 20.4 ± 0.55 μM for 1-acyl-LPC and LPAF, respectively. Reference cuvettes were exactly the same except that 0.1 ml water was substituted for the lysophospholipid solutions. The membrane preparation (6–30 μl, containing 23.6–133.5 μg membrane protein) was added simultaneously to the test and reference cuvettes to initiate the reaction. Initial rates were calculated from a continuous recording of the enzyme activity.

3. RESULTS

All enzyme activities have been expressed as initial rates determined from continuous recordings of activity measured under conditions of substrate saturation (predetermined for each donor/acceptor system). Table 1 shows the activities (mean ± SD for 4 preparations) for the enzyme which transfers fatty acyl groups to 1-acyl-LPC in the mixed membrane fractions and in the surface and intracellular membrane subfractions derived from them by the free-flow electrophoresis procedure. It can be seen that for all the fatty acyl groups studied the specific activities of the transferase, associated with the intracellular membranes were ~4-fold higher than those in the mixed membrane fraction from which the intracellular membrane was derived. In contrast, the surface membrane activities all showed depletion with respect to the corresponding mixed membrane activity. When the specific activities for the different donor CoA esters are compared, it can be seen that for this lysophospholipid acceptor, 1-acyl-LPC, the CoA ester of 8,11,14-eicosatrienoic acid was the pre-

Table 1

Acyl-CoA-transferase activities in human platelet membrane fractions: acceptor – 1-acyllysophosphatidylcholine

Fatty acyl-CoA	Acyl-CoA transferase activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)			
	Mixed membranes (MM)	Surface membranes (SM)	Intracellular membranes (IM)	Activity ratio IM/MM
18:1 (9)	28.4 ± 6.0	9.5 ± 2.8	113.6 ± 33.5	4.06
18:2 (9,12)	29.0 ± 5.3	9.8 ± 2.6	114.9 ± 22.6	3.96
20:3 (8,11,14)	56.2 ± 10.1	18.3 ± 4.3	221.6 ± 43.0	3.94
20:4 (5,8,11,14)	28.4 ± 5.4	11.6 ± 2.5	115.5 ± 22.6	4.07
			mean activity ratio	4.00

The spectrophotometric assay is described in section 2. All activities are means ± SD for 4 different platelet preparations. The double bond positions for the CoA ester fatty acids are in parentheses

Table 2

Acyl-CoA-transferase activities in human platelet membrane fractions: acceptor – lyso-platelet-activating factor

Fatty acyl-CoA	Acyl-CoA transferase activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)			
	Mixed membranes (MM)	Surface membranes (SM)	Intracellular membranes (IM)	Activity ratio IM/MM
18:1 (9)	0.5 ± 0.8	1.0 ± 1.1	2.2 ± 1.3	4.40
18:2 (9,12)	5.2 ± 1.2	2.7 ± 2.1	20.6 ± 4.1	3.96
20:3 (8,11,14)	2.6 ± 0.9	1.6 ± 1.0	10.9 ± 2.8	4.19
20:4 (5,8,11,14)	4.8 ± 0.5	2.3 ± 0.9	21.6 ± 6.0	4.50
			mean activity ratio	4.26

The spectrophotometric assay is described in section 2. All activities are means ± SD for 4 different platelet preparations. The double bond positions for the CoA ester fatty acids are in parentheses

ferred donor in all membrane fractions, with oleate, linoleate and arachidonate, all being transferred at significantly lower rates.

Table 2 shows the activities for LPAF as the acceptor and again the intracellular membrane transferase activities for all donor CoA esters were over 4-fold enriched with respect to activities in the mixed membrane fractions. The surface membrane activities showed depletion with respect to the mixed membranes. However, with LPAF the CoA esters of linoleic and arachidonic acids were the preferred fatty acyl group donors in all 3 membrane fractions from all the platelet preparations studied.

The preliminary determinations of endogenous alkylacyl-PC in the membrane fractions were made. These were carried out by extraction of the phospholipids, isolation of the choline-containing phosphoglycerides, conversion of any alkylacyl-PC present into PAF by alkaline hydrolysis followed by acetylation and assay by aggregometry exactly as described earlier [11]. The intracellular membrane fractions, in which the transferase enzymes were predominantly localised, contained 6.2 and 7.1 $nmol \cdot mg^{-1}$ protein ($n = 2$) of alkylacyl-PC, whereas its concentration in the surface membrane fraction was only 1.9 and 3.5 $nmol \cdot mg^{-1}$ protein ($n = 2$).

4. DISCUSSION

Here, we have demonstrated that 2 fatty-acyl-CoA transferase activities, previously characterized in a mixed population of human platelet membranes as acyl-CoA: 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase [9] and acyl-CoA: 1-alkyl-*sn*-glycero-3-phosphocholine acyltransferase [11] are substantially enriched in a highly purified intracellular membrane fraction. Additionally, the rank order of fatty acyl group selectivity appears to be maintained for each lysophospholipid acceptor in each membrane fraction regardless of the differences in specific activity between the different membrane fractions. In the case of 1-acyl-LPC, there is no clear preference observed in the rate of transfer of oleate, linoleate, or arachidonate, but a significant preference was shown for the CoA ester of 8,11,14-eicosatrienoate. However, when LPAF is the acceptor, the CoA esters of arachidonic and linoleic acids were the preferred donors.

The intracellular membrane fraction has been earlier shown to contain a higher percentage of choline-containing phosphoglycerides than the surface membrane fraction [15]. Included in this group of phospholipids, diacyl-PC is believed to be the most important source of the AA liberated by phospholipase A_2 in stimulated platelets. Our preliminary data show that another choline-containing phosphoglyceride, alkylacyl-PC, the product of the acylation of LPAF with unsaturated fatty acids, is also enriched in the intracellular membrane fraction. Alkylacyl-PC is the probable precursor of PAF as well as a metabolite of PAF in platelets [7,14] and the selectivity of the fatty-acyl-CoA transferase for arachidonate suggests that this alkylacyl-PC could be enriched in AA relative to other unsaturated fatty acids. This would be in agreement with the recent findings that over 40% of the fatty acid at *sn*-2 of alkylacyl-PC is arachidonate [4,14].

The selectivity of the fatty-acyl-CoA transferases for unsaturated fatty acyl groups, when 1-acyl-LPC and LPAF are acceptors, strengthens the concept that the remodelling pathway is an important one for the incorporation of unsaturated fatty acids into platelet phospholipids [9]. Supporting evidence for this has been provided by Neufeld and Majerus [16] who estimated the rate of turnover of AA in 'resting' platelets and suggested that this may be associated with the remodelling of platelet phospholipids. It is now accepted that the fatty acid composition of membrane phospholipids is not stable, but responds dynamically to changes in the environment and the remodelling pathway is probably the major route by which phospholipids of platelets are labelled when radioactive AA is used [17]. This remodelling probably also explains the finding that radioactivity from 2-[1-^{14}C]arachidonyl-PC, which is apparently taken up as an intact phospholipid into platelets, eventually appears in phospholipid classes other than PC [17]. The participating enzymes phospholipase A_2 and the fatty-acyl-CoA transferase, are both enriched in the intracellular membranes and in earlier studies the intracellular membrane phospholipase A_2 activity showed a preference for 2-[1-^{14}C]arachidonyl-PC over other 2-acylphosphatidylcholines [18,19]. From our earlier analytical studies of these membrane subfractions prepared by free-flow electrophoresis it

was shown that PC and phosphatidylinositol (another source of free AA) were enriched in the intracellular membranes and moreover the percentage of AA in PC was higher in the intracellular membranes than in the PC of the surface membranes [15].

There is now a strong body of evidence suggesting that considerable metabolism of AA by human platelets can proceed at intracellular sites [20,21] and from the present findings we can now include acyltransferases in this list. The key role of the metabolites of AA in thrombosis [22] suggests that a successful pharmacological approach to this disease may require agents which can penetrate the surface membrane rapidly and allow selective interference with intracellular membrane processes.

ACKNOWLEDGEMENTS

The authors are grateful for the skilled technical assistance of Mrs Joan M. Caracappa, and we wish to thank Mrs Faith S. Williams and Miss Heather D. Watson for secretarial assistance. This work was supported in part by the British Heart Foundation and by a Hemostasis and Thrombosis Training Grant HL-07371, Grants HL-14890 and HL-29668 of the National Institutes of Health. It was also sponsored by a Grant-in-Aid from the American Heart Association with funds contributed in part by the Southeastern Pennsylvania Chapter.

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