Incorporation and turnover of eicosapentaenoic and docosahexaenoic acids in human blood platelets *in vitro*

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Mass changes in the incorporation of linoleic ($C_{18:2}$), eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acids in human blood platelet phospholipids were induced by incubating the cells and these fatty acids complexed to albumin. The remodelling of [^{14}C] $C_{18:2}$, [^{14}C] $C_{20:5}$ and [^{14}C] $C_{22:6}$ in classes, subclasses and molecular species of platelet phospholipids was studied in resting and thrombin-stimulated cells. More than 85 % of the incorporation was located in phospholipids, representing 5-fold and 2.5-fold increases in the phospholipid $C_{20:5}$ and $C_{22:6}$ endogenous content respectively. Thrombin stimulation induced a 30 % degradation of 1-acyl-2- $C_{20:5}$ -glycerophosphocholine (GPC) and 1-acyl-2- $C_{22:6}$ -GPC, but did not induce significant release of $C_{18:2}$ from 1-acyl-2- $C_{18:2}$ -GPC. There was no change in the [^{14}C] fatty acid composition of 1-alkyl-2-acyl-GPC. Thrombin-dependent increases in 1-alkenyl-2- $C_{20:5}$ -glycerophosphoethanolamine (GPE) and 1-alkenyl-2- $C_{22:6}$ -GPE of 2.1-fold and 2.5-fold respectively accounted for the rise in GPE radioactivity and partly compensated for the loss of these fatty acids from 1,2-diacyl-GPC: transfer to 1-alkenyl-2-acyl-GPE was 0.4 and 1.5 nmol/ 10^9 platelets for $C_{20:5}$ and $C_{22:6}$ respectively. [^{14}C] $C_{20:5}$ and [^{14}C] $C_{22:6}$ were incorporated into six different species of 1,2-diacyl-GPC, with acylation in the major endogenous forms ($C_{18:1} + C_{16:0}$ and $C_{18:0}$ species) representing 76 % and 66 % respectively of the total radioactivity present in 1,2-diacyl-GPC. Stimulation by thrombin induced significant release of these fatty acids from the main molecular species of 1,2-diacyl-GPC, but significantly stimulated the synthesis of alkenyl forms of GPE containing $C_{18:1}/C_{22:6} + C_{16:0}/C_{22:6}$, $C_{18:0}/C_{22:6}$ and $C_{18:0}/C_{20:5}$, $C_{18:0}/C_{18:2}$, the major endogenous $C_{18:2}$ molecular species, represented only 10.5 % of the incorporation; none of the [^{14}C] $C_{18:2}$ molecular species was a substrate for transfer towards 1-alkenyl-2-acyl-GPE. It is concluded that when $C_{20:5}$ and $C_{22:6}$, but not $C_{18:2}$, are acylated in 1,2-diacyl-GPC, they participate in thrombin-dependent phospholipid remodelling, and might compete with the turnover and release of arachidonic acid from platelet phospholipids and the subsequent activation of the cells.

INTRODUCTION

Stimulation of human blood platelets by various physiological agonists such as thrombin and collagen induces the release of arachidonic acid ($C_{20:4}$) from intracellular stores [1,2] and its oxygenation through the cyclo-oxygenase and lipoxygenase pathways [3,4]. $C_{20:4}$, which is esterified in the phospholipids of platelet membranes, is present in relatively low amounts in phosphatidylcholine (PC), whereas 1,2-diacylglycerophosphoinositol (1,2-diacyl-GPI), 1,2-diacylglycerophosphoethanolamine (1,2-diacyl-GPE) and 1-alk-1-enyl-2-acyl-glycerophosphoethanolamine (1-alkenyl-2-acyl-GPE) contain large amounts of $C_{20:4}$ molecular species [5–7]. The origin of $C_{20:4}$ released from platelets on stimulation with thrombin has been widely investigated, but conflicting observations have been made [1,2]. Initially, using [3H] $C_{20:4}$ -labelled platelets, Bills *et al.* [1] hypothesized that this fatty acid was selectively released from PC and PI through the action of phospholipase A_2 . However, Colard *et al.* [8] have suggested that following stimulation by thrombin 1,2-diacyl-GPE would first be hydrolysed, generating acyl-lyso-GPE, which could in turn induce the transfer of $C_{20:4}$ from 1,2-diacylglycerophosphocholine (1,2-diacyl-GPC) to 1,2-diacyl-GPE.

The incorporation, turnover and release of other polyunsaturated fatty acids (PUFA) has been less studied. PUFA from the $n-3$ family, i.e. $C_{20:5}$ and $C_{22:6}$, were enriched in platelet phospholipids of subjects consuming fish or purified fish oil [9,10]. These structural changes were associated with a decrease in platelet aggregation and thromboxane formation [9–11]. Part of the biological activity of these PUFA might

depend upon their acylation into specific phospholipid pools or on their liberation and metabolism during platelet activation [12,13]. The incorporation of trace amounts of these labelled fatty acids into platelet lipids and determination of the subsequent fate of the radiolabel upon thrombin stimulation revealed that $C_{20:5}$ was hydrolysed from phospholipids and converted into thromboxane A_2 , whereas $C_{22:6}$ was not released by agonists [11,14–16]. Platelets have the capacity to modify their fatty acid composition when exposed to fatty acid complexed with human serum albumin [11]. We used this capacity to induce mass changes in their composition of $C_{20:5}$, $C_{22:6}$ and $C_{18:2}$. We have previously reported that the incorporation of albumin-bound $C_{20:5}$ and $C_{22:6}$ into cellular lipids decreased platelet aggregation [11,17]. However, the incorporation of linoleic acid ($C_{18:2}$), an $n-6$ fatty acid, did not affect this function [17]. In the present work, the distribution of these fatty acids among phospholipid pools and phospholipid remodelling on platelet activation have been studied. We describe the thrombin-dependent deacylation of $C_{20:5}$ and $C_{22:6}$, but not $C_{18:2}$, from 1,2-diacyl-GPC, and the transfer of these $n-3$ fatty acids into 1-alkenyl-2-acyl-GPE. Under the same conditions, $C_{18:2}$ was not metabolized.

MATERIALS AND METHODS

Chemicals

Phospholipase C (from *Bacillus cereus*) was purchased from Boehringer (Mannheim, Germany). Standard phospholipids and diacylglycerols, essential-fatty-acid-free human albumin, thrombin and 3,5-dinitrobenzoyl chloride were from Sigma (St. Louis,

Abbreviations used: PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; GPI, glycerophosphoinositol; PUFA, polyunsaturated fatty acids; PRP, platelet-rich plasma; BHT, butylated hydroxytoluene.

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Table 1. [^{14}C]Fatty acid distribution in lipid fractions of human blood platelets in resting cells and after stimulation by thrombin for 10 min

Platelets were incubated with the indicated [^{14}C]fatty acids for 2 h as described in the Materials and methods section. After washing and resuspension of the cells into Tyrode/Hepes buffer, they were further incubated with thrombin (1 unit/ml) or buffer. The distribution of radioactivity in lipid fractions was analysed on t.l.c. plates using a Berthold t.l.c. linear analyser. Results are expressed as nmol of fatty acids incorporated/ 10^9 platelets and data are means $\pm$ S.E.M. from five to six separate platelet preparations. Statistical significance (paired Student's *t* test) of thrombin-treated versus control cells is indicated by: * $P < 0.05$, ** $P < 0.01$. Abbreviations: NL, neutral lipids; SPG, sphingolipids.

	Incorporation (nmol/ 10^9 platelets)					
	[^{14}C]C $_{18:2, n-6}$		[^{14}C]C $_{20:5, n-3}$		[^{14}C]C $_{22:6, n-3}$	
	Control	Thrombin	Control	Thrombin	Control	Thrombin
PI	1.31 $\pm$ 0.29	1.25 $\pm$ 0.25	2.86 $\pm$ 0.43	2.11 $\pm$ 0.44	0.63 $\pm$ 0.13	0.60 $\pm$ 0.16
PS + SPG	2.17 $\pm$ 0.78	2.13 $\pm$ 0.32	0.85 $\pm$ 0.11	0.94 $\pm$ 0.24	1.06 $\pm$ 0.41	1.06 $\pm$ 0.43
PC	23.80 $\pm$ 2.81	22.75 $\pm$ 2.64	15.52 $\pm$ 1.51	11.04 $\pm$ 1.19**	18.98 $\pm$ 1.93	13.87 $\pm$ 1.74**
PE	2.24 $\pm$ 0.22	2.02 $\pm$ 0.32	1.89 $\pm$ 0.29	2.59 $\pm$ 0.27*	4.93 $\pm$ 0.44	6.87 $\pm$ 0.73**
NL	3.70 $\pm$ 0.79	5.13 $\pm$ 0.33	1.74 $\pm$ 0.37	6.32 $\pm$ 0.84**	3.88 $\pm$ 0.80	6.915 $\pm$ 1.13**

Table 2. Distribution of C $_{18:2, n-6}$, C $_{20:5, n-3}$ and C $_{22:6, n-3}$ in PC and PE subclasses in resting platelets and after stimulation by thrombin (1 unit/ml) for 10 min

After phospholipase C treatment of phospholipid classes, diradylglycerols were converted into their dinitrobenzoyl derivatives and quantified by t.l.c. Results are means $\pm$ S.E.M. of five experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ versus controls (Student's *t* test). For further details see the legend to Table 1.

Fatty acid	Incorporation into PC (nmol/ 10^9 platelets)			
	Control		Thrombin	
	1,2-Diacyl-GPC	1-Alkyl-2-acyl-GPC	1,2-Diacyl-GPC	1-Alkyl-2-acyl-GPC
C $_{18:2, n-6}$	24.52 $\pm$ 3.36	0.42 $\pm$ 0.08	23.54 $\pm$ 3.30	0.36 $\pm$ 0.01
C $_{20:5, n-3}$	13.86 $\pm$ 1.52	1.00 $\pm$ 0.20	10.0 $\pm$ 1.20**	0.94 $\pm$ 0.21
C $_{22:6, n-3}$	16.58 $\pm$ 1.81	1.19 $\pm$ 0.13	11.52 $\pm$ 1.60**	1.05 $\pm$ 0.11

Fatty acid	Incorporation into PE (nmol/ 10^9 platelets)			
	Control		Thrombin	
	1,2-Diacyl-GPE	1-Alkenyl-2-acyl-GPE	1,2-Diacyl-GPE	1-Alkenyl-2-acyl-GPE
C $_{18:2, n-6}$	2.17 $\pm$ 0.26	0.14 $\pm$ 0.03	2.01 $\pm$ 0.26	0.24 $\pm$ 0.09
C $_{20:5, n-3}$	1.59 $\pm$ 0.38	0.40 $\pm$ 0.08	1.60 $\pm$ 0.37	0.78 $\pm$ 0.12*
C $_{22:6, n-3}$	4.06 $\pm$ 0.38	1.06 $\pm$ 0.13	4.21 $\pm$ 0.32	2.54 $\pm$ 0.16***

MO, U.S.A.). [^{14}C]C $_{18:2}$ (55 Ci/mol) was provided by Amersham International, and [^{14}C]C $_{20:5}$ (58 Ci/mol) and [^{14}C]C $_{22:6}$ (55 Ci/mol) were from New England Nuclear (Boston, MA, U.S.A.). Unlabelled fatty acids were obtained from Sigma, and were 99% pure as checked by g.l.c. chromatography. Silica gel 60 and LK5 plates were from Merck (Darmstadt, Germany) and Whatman (Clifton, OH, U.S.A.) respectively.

Preparation and stimulation of blood platelets

Platelet suspensions were prepared as previously described [17]. Briefly, blood from healthy volunteers was collected into 1/9 vol. of anticoagulant (citric acid/citrate/dextrose, pH 4.5) and platelet-rich plasma (PRP) was prepared by centrifugation at 100 *g* for 15 min. PRP (pH 6.4) was centrifuged at 900 *g* for 10 min and the resulting pellet was gently resuspended into a Tyrode/Hepes buffer (135 mM-NaCl, 2.68 mM-KCl, 0.46 mM-NaHPO $_4$, 1 mM-MgCl $_2$, 5 mM-Hepes, 5.5 mM-glucose, pH 7.35), containing either fatty acid-albumin or albumin alone. Each [^{14}C]fatty acid (1.5 Ci/mol) complexed with essential-fatty-acid-

free human serum albumin (7 g/l) was incubated with platelets [(3–4) $\times 10^8$ cells/ml] at 37 °C for 2 h with gentle shaking. The fatty acid/albumin molar ratio was 2.0 (0.2 mM-fatty acid). The platelet suspensions were acidified to pH 6.4 with citric acid, centrifuged for 10 min at 700 *g* and finally resuspended into Tyrode/Hepes buffer. Platelets were preincubated for 2 min at 37 °C, and thrombin (1 unit/ml) or vehicle was added to the assays for 10 min at 37 °C under gentle shaking.

Extraction and fractionation of lipids

Lipids were extracted twice with ethanol/chloroform (1:2, v/v) containing 50 μM -butylated hydroxytoluene (BHT). Individual phospholipids were resolved by one-dimensional t.l.c. on silica gel LK5 plates treated with 0.8% boric acid in 50% ethanol, with chloroform/methanol/NH $_4$ OH/water (60:37.5:1:3, by vol.) as the developing solvent, according to Fine & Sprecher [18]. The radiolabelled fatty acids were distributed within various phospholipid classes which were separated into PI (R_f 0.21), phosphatidylserine (PS; R_f 0.27), PC (R_f 0.46), PE (R_f 0.56)

Table 3. Molecular species composition of 1,2-diacyl-GPC, 1,2-diacyl-GPE and 1,2-diacyl-GPI from blood platelets

Individual molecular species were separated by h.p.l.c. on an Ultrasphere ODS column and quantified by absorbance at 240 nm. Results are means $\pm$ S.D. of three to six experiments.

Molecular species	Composition (mol %)		
	1,2-Diacyl-GPC	1,2-Diacyl-GPE	1,2-Diacyl-GPI
$C_{16:1}/C_{22:6, n-3} +$	0.5 ± 0.1	—	—
$C_{18:2}/C_{22:6, n-3}$	—	—	—
$C_{16:1}/C_{20:4, n-6} +$	0.5 ± 0.3	—	—
$C_{18:2}/C_{20:4, n-6}$	—	—	—
$C_{16:0}/C_{20:5, n-3} +$	0.9 ± 0.4	0.8 ± 0.5	—
$C_{18:1}/C_{20:5, n-3}$	—	—	—
$C_{18:1}/C_{22:6, n-3} +$	1.5 ± 0.1	1.9 ± 0.5	—
$C_{16:0}/C_{22:6, n-3}$	—	—	—
$C_{18:1}/C_{20:4, n-6} +$	2.3 ± 0.3	5.0 ± 1.0	2.9 ± 0.9
$C_{16:0}/C_{22:5, n-3}$	—	—	—
$C_{16:0}/C_{20:4, n-6}$	8.7 ± 1.6	10.8 ± 0.7	6.8 ± 1.8
$C_{18:0}/C_{20:5, n-3}$	—	0.7 ± 0.2	—
$C_{18:1}/C_{18:2, n-6}$	0.6 ± 0.1	—	—
$C_{18:0}/C_{22:6, n-3}$	1.4 ± 0.2	1.8 ± 0.2	—
$C_{16:0}/C_{16:1}$	—	—	—
$C_{16:0}/C_{18:2, n-6}$	12.3 ± 0.9	5.4 ± 1.0	3.8 ± 2.1
$C_{18:1}/C_{22:4, n-6}$		0.9 ± 0.6	—
$C_{16:0}/C_{20:3, n-6}$		0.9 ± 0.01	—
$C_{16:0}/C_{22:4, n-6}$	0.5 ± 0.1	1.8 ± 0.3	—
$C_{18:0}/C_{22:5, n-3}$	0.7 ± 0.1	1.1 ± 0.2	—
$C_{18:0}/C_{20:4, n-6}$	—	49.6 ± 3.1	66.0 ± 11.0
$C_{18:0}/C_{22:5, n-6}$	9.6 ± 1.6	< 0.5	—
$C_{18:1}/C_{18:1}$	0.6 ± 0.2	2.5 ± 0.5	—
$C_{18:0}/C_{18:2, n-6}$	2.7 ± 0.4	2.2 ± 0.4	—
$C_{16:0}/C_{18:1}$	30.7 ± 4.5	4.9 ± 0.6	—
$C_{16:0}/C_{16:0}$		0.8 ± 0.2	—
$C_{16:0}/C_{20:3, n-6}$		2.5 ± 0.2	—
$C_{18:0}/C_{22:4, n-6}$	0.9 ± 0.1	< 0.5	—
Unidentified	—	1.3 ± 0.3	—
$C_{20:1}/C_{18:1}$	0.7 ± 0.2	—	—
$C_{16:0}/C_{20:1}$	0.8 ± 0.1	—	—
$C_{18:0}/C_{18:1}$	10.3 ± 1.0	1.6 ± 0.5	11.2 ± 5.3
$C_{18:0}/C_{16:0}$	2.8 ± 0.1	0.6 ± 0.1	—

and unresolved lipid species migrating above PE. This fraction included diacylglycerols, non-esterified and hydroxylated fatty acids, triacylglycerols, steryl esters and unidentified species, and was called 'neutral lipids'. For further quantification of neutral lipids, this fraction was extracted from the gel with chloroform/methanol/water (5:5:1, by vol.) and submitted to t.l.c. separation on silica gel 60G plates using hexane/diethyl ether/acetic acid (70:30:1, by vol.) as the mobile phase. Distribution of [14 C]fatty acids into lipid classes and phospholipid subclasses was quantified with a Berthold t.l.c. analyser [19].

Separation and quantification of 1,2-diacyl, 1-alkyl-2-acyl and 1-alkenyl-2-acyl phospholipid subclasses

Phospholipid classes were extracted from the silica gel with chloroform/methanol/water (5:5:1, by vol.) and converted into their respective diradylglycerols by incubation with phospholipase C according to the procedure of Takamura *et al.* [20], modified as follows. Approx. 200 μ g of phospholipids in 0.7 ml of 30 mM- H_3BO_3 /10 mM-Tris/HCl buffer (pH 7.5) containing 0.02% BHT was sonicated for 5 min before addition of 50 units of phospholipase C and 1 ml of water-saturated diethyl ether. Incubation was carried out overnight at 30 °C under vigorous shaking. Diradylglycerols were extracted with diethyl ether and converted into their dinitrobenzoyl derivatives by treatment with 3,5-dinitrobenzoyl chloride in dry pyridine for 30 min at 60 °C. After cooling, 1 ml of water was added to the

samples and the products were extracted twice with hexane. The three subclasses of diradylglyceronitrobenzoates were further resolved by t.l.c. on silica gel 60G plates using a solvent system of toluene/hexane/diethyl ether (50:45:4, by vol.). The 1-alkenyl-2-acyl, 1-alkyl-2-acyl and 1,2-diacyl subclasses were separated, having R_F values of 0.64, 0.58 and 0.38 respectively, and quantified (using a t.l.c. radioscaner) as a percentage of the amount of fatty acid incorporated into each phospholipid. They were extracted from the silica gel with diethyl ether/hexane (1:1, v/v).

Identification and quantification of molecular species in phospholipid subclasses

Separation of various molecular species within the different subclass derivatives was carried out by h.p.l.c. using a 5 μ m Ultrasphere ODS column (4.6 mm internal diam. $\times$ 250 mm) (Beckman) and an isocratic elution with acetonitrile/propan-2-ol (9:1, v/v). The products were quantified by absorbance at 240 nm and the radioactive species were counted on-line using a Berthold h.p.l.c. radioactivity monitor. Identification of molecular species was done by comparison with the retention times of standards synthesized from known phospholipids and diacylglycerols. Individual peaks were also collected and analysed for their fatty acid methyl esters or dimethylacetals by capillary g.l.c. Identification was verified by the graphical representation method of Patton [21]. The quantification of radio-

Table 4. Molecular species composition of 1-alkyl-2-acyl-GPC and 1-alkenyl-2-acyl-GPE from blood platelets

Results are expressed as means $\pm$ S.D. of five to six experiments. See Table 3 for details.

Molecular species	Composition (mol %)	
	1-Alkyl-2-acyl-GPC	1-Alkenyl-2-acyl-GPE
$C_{18:1}/C_{20:5, n-3} +$	—	0.9 ± 0.4
$C_{16:0}/C_{20:5, n-3}$	3.5 ± 0.8	3.3 ± 0.4
$C_{18:0}/C_{22:6, n-3} +$	—	—
$C_{16:0}/C_{22:6, n-3}$	1.6 ± 0.3	—
$C_{18:2}/C_{20:4, n-6}$	—	4.6 ± 1.1
$C_{18:1}/C_{20:4, n-6}$	13.1 ± 1.1	3.6 ± 0.2
$C_{16:0}/C_{22:5, n-3}$	—	23.4 ± 4.6
$C_{16:0}/C_{20:4, n-6}$	24.4 ± 1.9	1.3 ± 0.4
$C_{18:0}/C_{20:5, n-3}$	—	1.3 ± 0.3
$C_{16:0}/C_{22:5, n-6}$	—	—
$C_{18:1}/C_{18:2, n-6}$	2.4 ± 0.2	—
$C_{18:0}/C_{22:6, n-3}$	—	4.8 ± 0.5
$C_{16:0}/C_{18:2, n-6}$	6.1 ± 1.4	—
Unidentified	2.4 ± 0.3	1.0 ± 0.3
$C_{16:0}/C_{22:4, n-6}$	1.5 ± 0.3	4.9 ± 1.1
$C_{18:0}/C_{22:5, n-3}$	—	2.86 ± 0.10
$C_{18:0}/C_{20:4, n-6}$	15.2 ± 3.2	32.5 ± 2.5
$C_{18:0}/C_{22:5, n-6}$	—	0.6 ± 0.3
$C_{18:1}/C_{18:1}$	1.4 ± 0.8	0.5 ± 0.4
$C_{16:0}/C_{18:1}$	4.0 ± 1.2	0.9 ± 0.5
$C_{16:0}/C_{18:2, n-6}$	4.9 ± 0.3	0.9 ± 0.3
$C_{16:0}/C_{16:0}$	—	0.7 ± 0.2
$C_{18:0}/C_{22:4, n-6}$	—	4.5 ± 1.4
$C_{18:0}/C_{16:1}$	—	3.7 ± 1.2
$C_{18:0}/C_{18:1}$	2.9 ± 0.1	—

active molecular species was carried out by measuring the percentage of fatty acid incorporated into each species.

G.l.c. analysis

The fatty acid composition of phospholipids in control and

modified platelets was determined by g.l.c. after purification of total phospholipids by t.l.c. on silica gel 60 plates using hexane/diethyl ether/acetic acid (60:40:1, by vol.) as the mobile phase. Phospholipids were scraped off the plate and treated with 14% BF_3 in methanol [22]. The resulting fatty acid methyl esters and dimethylacetals were analysed using a Perkin-Elmer chromatograph model 5830, equipped with an SP 2380 capillary column (60 m $\times$ 0.25 mm) (Supelco, Bellefonte, PA, U.S.A.) [23].

RESULTS

Incorporation of [^{14}C]fatty acids into lipid and phospholipid classes

Platelets incorporated [^{14}C]fatty acids into cellular lipids, as measured by counting of radioactivity in aliquots of the cells after washing and resuspension into Tyrode buffer. Taking into account the initial specific radioactivity of each fatty acid, total incorporation was 33.28 ± 3.84 , 23.0 ± 2.79 and 29.97 ± 2.50 nmol/ 10^9 platelets for $C_{18:2}$, $C_{20:5}$ and $C_{22:6}$ respectively (means $\pm$ S.E.M. of five to six separate experiments). More than 85% of the fatty acids incorporated were acylated in phospholipids (Table 1). The endogenous levels of $C_{20:5}$ and $C_{22:6}$ in control platelet phospholipids were 0.43 ± 0.14 and 1.02 ± 0.19 mol % of total phospholipid fatty acids, as measured by g.l.c. This was increased to 2.20 ± 0.40 and 2.47 ± 0.73 mol % respectively after cell enrichment (means $\pm$ S.D. of three determinations). This represented a 5-fold elevation in the phospholipid $C_{20:5}$ content and a 2.5-fold increase in the $C_{22:6}$ content compared with controls.

The PC fraction accounted for 80.6, 73.0 and 74.1 % of the total phospholipid radioactivity in $C_{18:2}$, $C_{20:5}$ and $C_{22:6}$ -enriched platelets respectively. The synthesis of [^{14}C] $C_{22:6}$ -PE was significantly greater than the synthesis of [^{14}C] $C_{18:2}$ -PE ($P < 0.05$) and [^{14}C] $C_{20:5}$ -PE ($P < 0.05$).

After addition of thrombin to the platelet suspension, two major differences between the phospholipid species in control

Table 5. Redistribution of incorporated radioactive $C_{22:6, n-3}$ among molecular species of PC and PE after thrombin stimulation

Individual molecular species were separated by h.p.l.c. and quantified on-line. X represents an unidentified fatty acid. Values are means $\pm$ S.E.M.; * $P < 0.01$, ** $P < 0.001$ (Student's t test), compared with controls. ND, not determined.

Molecular species	Incorporation (nmol/ 10^9 platelets)			
	1,2-Diacyl-GPC		1-Alkyl-2-acyl-GPC	
	Control	Thrombin	Control	Thrombin
X/ $C_{22:6, n-3}$	1.66 ± 0.31	1.11 ± 0.20	ND	ND
$C_{18:2, n-6}/C_{22:6, n-3} +$	2.67 ± 0.40	$1.60 \pm 0.15^*$	ND	ND
$C_{16:1}/C_{22:6, n-3}$	1.52 ± 0.30	1.12 ± 0.15	ND	ND
$C_{14:0}/C_{22:6, n-3}$	8.39 ± 0.42	$5.90 \pm 0.30^*$	0.85 ± 0.02	0.75 ± 0.04
$C_{18:1}/C_{22:6, n-3} +$	2.60 ± 0.30	$1.80 \pm 0.21^*$	0.30 ± 0.01	0.25 ± 0.04
$C_{16:0}/C_{22:6, n-3}$				
$C_{18:0}/C_{22:6, n-3}$				
Molecular species	1,2-Diacyl-GPE		1-Alkenyl-2-acyl-GPE	
	Control	Thrombin	Control	Thrombin
	Control	Thrombin	Control	Thrombin
X/ $C_{22:6, n-3}$	2.47 ± 0.11	2.81 ± 0.23	ND	ND
$C_{18:1}/C_{22:6, n-3} +$	0.60 ± 0.03	0.35 ± 0.02	0.80 ± 0.10	$1.25 \pm 0.15^*$
$C_{16:0}/C_{22:6, n-3}$	0.61 ± 0.05	0.55 ± 0.03	0.30 ± 0.15	$1.22 \pm 0.03^{**}$
$C_{18:0}/C_{22:6, n-3}$				

Table 6. Redistribution of incorporated radioactive $C_{20:5,n-3}$ among molecular species of PC and PE subclasses after thrombin stimulation

Values are means $\pm$ S.E.M.; * $P < 0.01$, ** $P < 0.001$ (Student's t test) compared with controls. See Table 5 for details. ND, not determined.

Molecular species	Incorporation (nmol/10 ⁹ platelets)			
	1,2-Diacyl-GPC			
	Control	Thrombin	Control	Thrombin
X/ $C_{20:5,n-3}$	1.06 $\pm$ 0.56	0.75 $\pm$ 0.22		
$C_{18:2,n-6}/C_{20:5,n-3}$ + $C_{16:1}/C_{20:5,n-3}$	1.02 $\pm$ 0.37	0.44 $\pm$ 0.06		
$C_{14:0}/C_{20:5,n-3}$	1.17 $\pm$ 0.31	0.92 $\pm$ 0.65		
$C_{18:1}/C_{20:5,n-3}$ + $C_{16:0}/C_{20:5,n-3}$	6.90 $\pm$ 0.14	5.02 $\pm$ 0.10**		
$C_{18:0}/C_{20:5,n-3}$	3.71 $\pm$ 0.89	2.89 $\pm$ 0.29*		
Molecular species	1,2-Diacyl-GPE		1-Alkenyl-2-acyl-GPE	
	Control	Thrombin	Control	Thrombin
X/ $C_{20:5,n-3}$	0.53 $\pm$ 0.09	0.45 $\pm$ 0.10	ND	ND
$C_{18:1}/C_{20:5,n-3}$ + $C_{16:0}/C_{20:5,n-3}$	0.43 $\pm$ 0.06	0.47 $\pm$ 0.09	0.25 $\pm$ 0.06	0.30 $\pm$ 0.06
$C_{18:0}/C_{20:5,n-3}$	0.63 $\pm$ 0.03	0.68 $\pm$ 0.11	0.12 $\pm$ 0.03	0.49 $\pm$ 0.14**

Fig. 1. H.p.l.c. resolution of individual $[^{14}C]C_{22:6,n-3}$ molecular species of phospholipid subclasses from unstimulated platelets

Platelets were labelled with $[^{14}C]C_{22:6,n-3}$ as described in the Materials and methods section. Dinitrobenzoyl derivatives of diradylglycerols were separated by t.l.c. and submitted to h.p.l.c. analysis on an Ultrasphere ODS column. The radioactivity in individual molecular species was quantified on-line. 1, X/ $C_{22:6,n-3}$; 2, $C_{18:2}/C_{22:6,n-3}$ + $C_{16:1}/C_{22:6,n-3}$; 3, $C_{14:0}/C_{22:6,n-3}$; 4, $C_{18:1}/C_{22:6,n-3}$; 5, $C_{16:0}/C_{22:6,n-3}$; 6, $C_{18:0}/C_{22:6,n-3}$.

and stimulated samples were noted. Stimulation induced hydrolysis of 26 % of $[^{14}C]C_{20:5}$ -PC and 27 % of $[^{14}C]C_{22:6}$ -PC, but did not induce any significant release of $[^{14}C]C_{18:2}$ from this fraction. In contrast, a thrombin-dependent increase in $[^{14}C]C_{20:5}$ -PE and $[^{14}C]C_{22:6}$ -PE synthesis was observed (Table 1). In thrombin-treated platelets, the amounts of both fatty acids ($C_{20:5}$ and $C_{22:6}$) were also enhanced in the neutral lipid fraction. Only 3.07 % of the total cell-associated $[^{14}C]C_{20:5}$ was represented by its non-esterified and oxygenated forms, but this amount reached 15.4 % in stimulated cells. In $[^{14}C]C_{22:6}$ -labelled platelets the basal level of non-esterified plus oxygenated $C_{22:6}$ was 0.45 % of

the total incorporation, and this reached 1.28 % after incubation with thrombin. Most of the increase in $[^{14}C]C_{22:6}$ in the neutral lipid fraction (2.6 nmol) was located in an unidentified lipid species which was distinct from triacylglycerols and steryl esters.

Incorporation of $[^{14}C]$ fatty acids into phospholipid subclasses

The synthesis of 1-acyl-2- $C_{18:2}$ -GPC, 1-acyl-2- $C_{20:5}$ -GPC and 1-acyl-2- $C_{22:6}$ -GPC accounted for 98.2, 93.4 and 92.6 % respectively of the radioactivity incorporated into PC, whereas only 1.8, 6.4 and 7.2 % respectively of these fatty acids was acylated in 1-alkyl-2-acyl-GPC (Table 2). In PE the radioactivity

Table 7. Redistribution of incorporated radioactive $C_{18:2,n-6}$ among molecular species of 1,2-diacyl-GPC after thrombin stimulation

Y represents an unidentified fatty acid. Values are means $\pm$ S.E.M. See Table 5 and the text for details.

Molecular species	Incorporation into 1,2-diacyl-GPC (nmol/ 10^9 platelets)	
	Control	Thrombin
$C_{16:1}/C_{18:2,n-6} + C_{18:2,n-6}/C_{18:2,n-6}$	1.62 ± 1.17	0.81 ± 0.08
$C_{14:0}/C_{18:2,n-6}$	11.30 ± 2.77	10.85 ± 1.39
$C_{18:1}/C_{18:2,n-6}$	1.32 ± 0.26	2.22 ± 0.68
$C_{16:0}/C_{18:2,n-6}$	4.25 ± 0.69	4.44 ± 1.05
Y/ $C_{18:2,n-6}$	1.47 ± 0.25	1.20 ± 0.20
$C_{18:0}/C_{18:2,n-6}$	2.36 ± 0.40	2.74 ± 0.18

was distributed between 1,2-diacyl-GPE and 1-alkenyl-2-acyl-GPE (Table 2).

Stimulation by thrombin induced a 30 % degradation of 1-acyl-2- $C_{20:5}$ -GPC and 1-acyl-2- $C_{22:6}$ -GPC, but no significant release of $C_{18:2}$ from 1,2-diacyl-GPC was observed (Table 2). In contrast, there was no change in the [^{14}C]fatty acid composition of 1-alkyl-2-acyl-GPC. Thrombin-dependent increases of 2.1- and 2.5-fold in 1-alkenyl-2- $C_{20:5}$ -GPE and 1-alkenyl-2- $C_{22:6}$ -GPE respectively accounted for the rise in GPE radioactivity and partly compensated for the loss from 1,2-diacyl-GPC (Table 2). The transfers towards 1-alkenyl-2-acyl-GPE were 0.4 and 1.5 nmol/ 10^9 platelets for $C_{20:5}$ and $C_{22:6}$ respectively.

Incorporation of [^{14}C]fatty acids into molecular species of PC and PE subclasses

The endogenous distribution of molecular species within phospholipid subclasses of control platelets was studied (Tables 3 and 4). The distribution of the molecular species among the phospholipid subclasses differed markedly. 1,2-Diacyl-GPC was rich in $C_{18:1}$ - and $C_{18:2}$ -containing species, whereas 1,2-diacyl-GPE and -GPI consisted mainly of $C_{16:0}/C_{20:4}$ and $C_{18:0}/C_{20:4}$. The $C_{20:4}$ -containing species were also predominant in 1-alkenyl-2-acyl-GPE (60.5 mol % of total species) and in 1-alkyl-2-acyl-GPC (54.3 mol %), although their relative proportions were different. In contrast with what was observed in 1-alkenyl-2-acyl-GPE, higher levels of $C_{16:0}/C_{20:4}$ compared with $C_{18:0}/C_{20:4}$ were found in 1-alkyl-2-acyl-GPC. C_{22} chain fatty acids were also highly enriched in the plasmalogenic forms of PE.

[^{14}C] $C_{22:6}$ and [^{14}C] $C_{20:5}$ were incorporated into six different species of 1,2-diacyl-GPC (Tables 5 and 6 and Fig. 1), with most acylation in the predominant endogenous forms at $C_{18:1}$, $C_{16:0}$ and $C_{18:0}$ species. $C_{18:1}/C_{22:6} + C_{16:0}/C_{22:6}$ and $C_{18:1}/C_{20:5} + C_{16:0}/C_{20:5}$ represented 50.0 % and 49.8 % respectively of the total radioactivity present in 1,2-diacyl-GPC. The distribution of these two $n-3$ fatty acids in 1,2-diacyl-GPE was completely different from what was observed with 1,2-diacyl-GPC. $C_{18:1}/C_{22:6} + C_{16:0}/C_{22:6}$ and $C_{18:0}/C_{22:6}$ constituted only 14.8 % and 15.2 % respectively of the radioactivity incorporated into 1,2-diacyl-GPE, whereas 58.8 % of $C_{22:6}$ was acylated in an unidentified species which was eluted with the shortest retention time in the chromatographic system (Table 5, Fig. 1). The main $C_{22:6}$ -labelled molecular species of 1-alkyl-2-acyl-GPC were $C_{18:1}/C_{22:6} + C_{16:0}/C_{22:6}$ and $C_{18:0}/C_{22:6}$. The determination of $C_{20:5}$ acylation in this class was not attempted because of very low radioactivity incorporation. In plasmalogenic forms of PE,

most of the radioactivity was located in $C_{18:1} + C_{16:0}$ and $C_{18:0}$ species. More than 95 % of [^{14}C] $C_{18:2}$ in PC was distributed in six different molecular species of 1,2-diacyl-GPC (Table 7). Surprisingly, 46 % of the radioactivity was linked to $C_{14:0}/C_{18:2}$, a molecular species which was not detected in the endogenous composition of platelet 1,2-diacyl-GPC, whereas only 11.7 % of [^{14}C] $C_{18:2}$ was in $C_{18:0}/C_{18:2}$, the main endogenous molecular species. In [^{14}C] $C_{22:6}$ - and [^{14}C] $C_{20:5}$ -enriched platelets, thrombin stimulation induced a significant release of these fatty acids from the main molecular species of 1,2-diacyl-GPC. Concomitant with this hydrolysis, the synthesis of the two molecular species of 1-alkenyl-2-acyl-GPE was increased for both fatty acids. A 4-fold increase in the plasmalogenic form of $C_{18:0}/C_{22:6}$ indicated the preferential uptake in this species over $C_{18:1}/C_{22:6}$ and $C_{16:0}/C_{22:6}$ species. The transfer of $C_{20:5}$ occurred only towards the $C_{18:0}/C_{20:5}$ species of plasmalogens. No change in [^{14}C] $C_{18:2}$ phospholipid distribution was observed in thrombin-stimulated platelets.

DISCUSSION

Platelets incorporated fatty acids associated with albumin in both the neutral lipid fraction and phospholipids. However, more than 85 % of the cell-linked radioactivity was located in phospholipids. All of the fatty acid substrates were preferentially incorporated into 1,2-diacyl-GPC, whether they belonged to the $n-6$ fatty acid family ($C_{18:2}$) or to the $n-3$ fatty acid family ($C_{20:5}$ and $C_{22:6}$); 69.1 %, 63.2 % and 59 % of the total [^{14}C] $C_{18:2}$, [^{14}C] $C_{20:5}$ and [^{14}C] $C_{22:6}$ respectively was incorporated into this subclass, while 6.8 %, 6.6 % and 16.6 % respectively was incorporated into the PE fraction. The distributions of $C_{20:5}$ and $C_{22:6}$ within the different phospholipid pools in the enriched cells did not reflect their endogenous distribution. In accordance with previous studies [6,24], we found that $C_{22:6}$ accounted for 1.5 mol % of 1,2-diacyl-GPC and 2.2 mol % of 1,2-diacyl-GPE, while it represented 3.6 mol % of the total fatty acid in 1-alkenyl-2-acyl-GPE of human platelets (results not shown). Dietary experiments have indicated that 1-alkenyl-2-acyl-GPE represents the major form of $C_{22:6}$ - and $C_{20:5}$ -containing phospholipids in platelets from human subjects consuming fish oil [25,26], whereas our *in vitro* study showed that platelets incorporate these fatty acids preferentially into 1,2-diacyl-GPC. Using [^{14}C] $C_{20:4}$ labelling of platelet lipids, both in the short term and at equilibrium, Colard *et al.* [27] showed that $C_{20:4}$ is initially found in 1,2-diacyl-GPC and 1,2-diacyl-GPI, and rapidly undergoes transfer to 1,2-diacyl-GPE, 1-alkyl-2-acyl-GPE and -GPC, and 1-alkenyl-2-acyl-GPE during a 5 h reincubation to reach equilibrium labelling. The same time-dependent changes in the incorporation of [^{14}C] fatty acids within phospholipid pools have also been reported in neutrophils [28]. On the other hand, we found 12 % of $C_{20:5}$ acylation and 2.2 % of $C_{22:6}$ acylation in PI, which differs from the incorporation observed upon fish oil consumption, where virtually no enrichment of these fatty acids was reported [12,29]. The difference between the fatty acid uptake into phospholipid pools between the 2 h enrichment *in vitro* and studies *in vivo* may be due to the duration of incorporation. Apparently, exogenous fatty acids do not enter the cell phospholipids by synthesis *de novo*, but via a deacylation/reacylation cycle [30]. We assume that fatty acids complexed to albumin were taken up into 1,2-diacyl-GPC using the 1-acyl-2-lyso-GPC available in the resting platelet [7]. [^{14}C] $C_{20:5}$ and [^{14}C] $C_{22:6}$ were acylated heterogeneously into six different molecular species of this phospholipid class. However, 65.7 % of $C_{22:6}$ and 76.5 % of $C_{20:5}$ was esterified in the main endogenous molecular species, i.e. the $C_{16:0} + C_{18:1}$ and $C_{18:0}$ species. This is in good agreement with results obtained when platelets were labelled with trace levels of [3H] $C_{20:4}$. In

these resting cells, 80 % of the [^3H]C_{20:4} in 1,2-diacyl-GPC was equally distributed between C_{16:0}/C_{20:4} + C_{18:1}/C_{20:4} and C_{18:0}/C_{20:4}, with the remainder being in minor molecular species [7,8]. Similarly, a trace label of C_{18:2, n-6} was mostly incorporated into the main linoleoyl-containing species of platelet 1,2-diacyl-GPC [7,31]. However, when substantial amounts of [^{14}C]C_{18:2} were acylated in 1,2-diacyl-GPC, the distribution of this fatty acid within molecular species was not predominantly in the major endogenous species, but 46 % of the radioactivity was found in C_{14:0}/C_{18:2}. Moreover, unusual incorporation of [^{14}C]C_{22:6} was observed in 1,2-diacyl-GPE, the bulk of C_{22:6} being associated with an unidentified species which is different from the major endogenous C_{22:6} species. However, when platelets were incubated with only trace levels of this fatty acid, the radioactivity was equally distributed within C_{18:1} + C_{16:0} and C_{18:0} species (results not shown). This suggests that, in the presence of trace levels of fatty acid substrates in the external medium, the lysophospholipid acceptors having the highest affinity for the acyltransferases will be acylated. When large amounts of fatty acids are added, however, the lysophospholipids having lower affinities for the acyltransferases may also be substrates. This observation is in accordance with a previous report by Chilton & Murphy [32], who showed that incorporation of C_{20:4} into phospholipid molecular species of neutrophils was dependent on the concentration of C_{20:4} used in the incubation *in vitro*. They demonstrated a dose-dependent increase of 1-C_{20:4}-2-C_{20:4}-GPC in cells exposed to increasing amounts of C_{20:4}. Based on this observation and the retention times of the molecular species, we may hypothesize that, when platelets are exposed to high C_{22:6} concentrations, the major C_{22:6}-incorporating species of 1,2-diacyl-GPE would be didocosahexaenoyl-GPE.

On thrombin stimulation, [^{14}C]C_{20:5} and [^{14}C]C_{22:6} were released from most of the 1,2-diacyl-GPC molecular species and were redistributed within the two molecular species of 1-alkenyl-2-acyl-GPE, although a substantial part of C_{20:5} was released as oxygenated metabolites. This thrombin-dependent transfer of fatty acids from PC to 1-alkenyl-2-acyl-GPE has been previously reported for C_{20:4} and C_{20:5} in platelets prelabelled with trace amounts of these fatty acids [27,33–35]. In terms of mass, the transfer of C_{22:6} was, however, more than 3-fold greater than that of C_{20:5}, in agreement with our previous findings obtained under different experimental conditions [36]. Different pathways seem to be involved in the reacylation of fatty acids [37]. The acyltransferases of the CoA/ATP-dependent reacylation system reacylate the lysophospholipids using acyl-CoA formed from non-esterified fatty acids by acyl-CoA synthetases [30]. CoA- and ATP-independent transacylation prefers ether-containing lysophospholipids as acceptors [38], whereas CoA-dependent/ATP-independent transacylation shows common acceptor preference with the CoA/ATP-dependent reacylation system [39,40]. In platelets, both CoA-dependent and CoA-independent transacylation pathways have been shown to be involved in arachidonoyl transfer [37–40]. In the present study we have demonstrated that C_{20:5} and especially C_{22:6} incorporated in 1,2-diacyl-GPC participate in thrombin-dependent phospholipid remodelling, resulting in the formation of 1-alkenyl-2-C_{20:5}-GPE and 1-alkenyl-2-C_{22:6}-GPE. The fact that C_{18:2} is not a substrate for such a transfer may be due to the different incorporation of this fatty acid into the 1,2-diacyl-GPC pool as compared with *n* - 3 fatty acids, suggesting a degree of phospholipid segregation. It may also reflect the fact that the specificity of the acyltransferase is in part determined by the presence of at least four double bonds in the fatty acid substrate, as has been shown in human neutrophils [28]. Although the present data suggest that the *n* - 3 fatty acids studied might be transacylated, especially C_{22:6, n-3} which is very poorly oxygenated after its release from diacyl-

GPC, no direct evidence for such a transacylation mechanism is provided. We conclude that C_{20:5} and C_{22:6}, in participating in thrombin-dependent phospholipid remodelling, may compete with the turnover and release of C_{20:4} from platelet phospholipids and the subsequent activation of the cells.

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