Biochemical Pharmacology, Vol. 38, No. 17, pp. 2763–2772, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

STIMULATION OF 12-HETE PRODUCTION IN HUMAN PLATELETS BY AN IMMUNOMODULATOR, LF 1695

EVIDENCE FOR ACTIVATION OF ARACHIDONATE LIBERATION COUPLED TO CYCLO-OXYGENASE INHIBITION

FRANÇOISE HULLIN,* JEANNIE M. F. RAGAB-THOMAS,* CHRISTIANE SEPULCHRE,†
MARC PASCAL,† HUGUES CHAP‡ and LOUIS DOUSTE-BLAZY*

* INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, F31059 Toulouse, France; and
† Laboratoires Fournier, 50 Rue de Dijon, Daix, Fontaine les Dijon, France

(*Received* 7 *December* 1988; *accepted* 24 *February* 1989)

Abstract—Upon incubation with human platelets previously labelled with [^{14}C]arachidonic acid, a new immunomodulator, LF 1695, induced the accumulation of [^{14}C]-12-(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Although the time course of [^{14}C]HETE accumulation was identical with 60 μM LF 1695 and calcium ionophore A23187, the latter compound also promoted the formation of ^{14}C-labelled thromboxane B_2 and 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid (HHT), whereas 12-HETE was the only arachidonic acid metabolite generated under the action of LF 1695, suggesting that the drug inhibited cyclo-oxygenase. This was further confirmed by the fact that LF 1695 inhibited the second wave of platelet aggregation induced by ADP as well as arachidonic acid effects. Cell lipid analysis revealed that arachidonic acid was liberated from both triacylglycerol and phosphatidylcholine. The effect was observed in the concentration range 15–90 μM, with a half-maximal effect at 30 μM for HETE production, 15 μM for triacylglycerol hydrolysis and 45 μM for phosphatidylcholine deacylation. Incubation of platelets with [^{14}C]arachidonic acid in the presence of 60 μM LF 1695 resulted in a strong inhibition of arachidonic acid incorporation into the various cell lipids, indicating that arachidonic acid mobilization might be due to inhibition of reacylation processes. It is concluded that LF 1695 displays an original and complex effect on platelet lipid metabolism, resulting in the specific generation of lipoxygenase metabolites.

LF 1695 is a synthetic, low molecular weight compound described as an immunomodulator, able to potentiate or to stimulate lectin-induced T-cell proliferation, depending on the dose used [1]. In addition, LF 1695 also modulates interleukin production, stimulating interleukin 1 secretion by murine adherent peritoneal cells or increasing interleukin 2 production by Concanavalin A-stimulated spleen cells [2]. When considering arachidonic acid metabolism, the drug was found to inhibit prostaglandin synthesis in macrophages stimulated by calcium ionophore A23187 or lipopolysaccharide, whereas leukotriene B_4 synthesis was stimulated [2]. Since the mechanism of action of LF 1695 remains presently unknown, we decided to study its effects on human blood platelets, where arachidonate liberation from cellular lipids, as well as its subsequent metabolism, are well documented. During platelet activation, two biochemical pathways of arachidonate liberation have been described: the first one involves a phosphoinositide specific phospholipase C [3, 4], coupled to diacylglycerol/monoacylglycerol lipases [5–7], the second one, a phospholipase A_2 [8, 9]. Both pathways lead to the release of arachidonic acid, which is a prerequisite for the synthesis of cyclo-oxygenase (prostaglandin endoperoxides, thromboxane) and lipoxygenase [12-(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid, 12-HPETE,§ rapidly degraded into stable 12-(*S*)-hydroxyeicosatetraenoic acid, 12-HETE] derivatives [10].

In the present study, we used human blood platelets prelabelled with radioactive arachidonic acid in order to investigate the possible effects of LF 1695 on the pathways of arachidonate liberation and metabolism.

MATERIALS AND METHODS

Materials. LF 1695 [5-amino-2-(4-methyl-1-piperidinyl)phenyl] (4-chloro-phenyl methanone) was synthesized by the Organic Chemistry Department, Laboratories Fournier (Dijon, France). [1-^{14}C]Arachidonic acid (58 mCi/mmol) was purchased from New England Nuclear (Dreieich, F.R.G.). Human thrombin (3,000 NIH U/mg protein) and calcium ionophore A23187 were obtained from Sigma Chemical Co. (St Louis, MO). Acetylsalicyclic acid, a lysin salt, was purchased from Laboratoires Egic (Amilly, France) and 5,8,11,14-eicosatetraynoic acid (ETYA) was a generous gift from Hofmann–La Roche (Basel, Switzerland).

Preparation of radiolabelled platelet suspensions. Fresh human blood was collected from healthy volunteer donors free of any medication for at least one week, using acid/citrate/dextrose as an anticoagulant [11]. Platelet rich plasma was obtained by

‡ To whom correspondence should be addressed.

§ Abbreviations: 12-HETE, 12-(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; HHT, 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid.

Table 1. Effect of LF 1695 on thrombin-stimulated [^{14}C]arachidonic acid labelled platelets

Conditions	Total phospholipids	Triacylglycerol	Arachidonic acid and metabolites: Non-esterified arachidonic acid	Cyclooxygenase metabolites (TXB_2, HHT)	Lipoxygenase metabolites (12-HETE)	Total
Control	95.7	3.6	0.05	0.13	0.05	0.23
Thrombin 1 U/ml	88.1	3.9	0.40	4.0	1.4	5.8
Thrombin plus LF 1695 3 μM	88.1	3.6	0.30	4.1	1.9	5.3
Thrombin plus LF 1695 15 μM	89.3	2.7	0.40	2.6	3.3	6.3
Thrombin plus LF 1695 60 μM	91.3	1.5	0.20	0.4	5.3	5.9
Thrombin plus LF 1695 90 μM	92.5	2.1	0.10	0.4	3.6	4.1

Conditions of incubation were the same as in Fig. 1 and lipid extracts were processed as described under Materials and Methods. Results are expressed as percentages of total radioactivity. Since minor lipids (as diacylglycerol, monoacylglycerol) are not considered in this table, total does not come to 100% in each column.

centrifugation at 120 *g* for 20 min (20°) and was then incubated at 37° for 90 min in the presence of [^{14}C]arachidonic acid (75 nCi/ml) dissolved in ethanol (0.1%, final concentration). Radiolabelled platelets were then isolated according to Ardlie *et al.* [12]. After sedimentation at 1000 *g* for 15 min (20°), they were washed in calcium free Tyrode buffer (pH 6.5) containing 2 mM $MgCl_2$, 5.5 mM glucose, 0.35% bovine serum albumin (w/v) and 0.2 mM EGTA. After a second wash in the same buffer lacking EGTA, they were finally suspended in a Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$, 5.5 mM glucose and 1 mM $CaCl_2$, at a concentration of 5×10^8 cells/ml. The whole procedure was performed in plastic tubes or in siliconized glassware (Sigmacote®).

Incubation of [^{14}C]*arachidonic acid labelled platelets*. Platelet suspensions (2 ml) were equilibrated for 1 min at 37°, and increasing concentrations of LF 1695 (3–90 μM) were then added under a minimal volume of ethanol (0.005 ml/ml suspension), which was also included in the control tubes. All the solutions of LF 1695 were prepared immediately prior to use in glass tubes and light-sheltered in order to avoid decomposition of the drug. After 10 min at 37° (or various times for time course study), incubation was stopped by addition of 0.065 ml of ice-cold 0.2 M EDTA (pH 7.4), followed immediately by 4 ml chloroform/methanol (1/1, v/v).

In some experiments, the 10 min preincubation in the absence or in the presence of LF 1695 was followed by addition of thrombin (1 U/ml, final concentration), or of buffer (isotonic saline) in non-stimulated platelets. Cell suspensions were then further incubated for 1 min, followed by EGTA and chloroform/methanol addition as above.

In some other experiments designed to identify arachidonate metabolites formed under stimulation, 2 ml of radiolabelled platelet suspensions were pre-incubated for 20 min at 37° with 0.1 mM acetylsalicyclic acid or 0.01 mM ETYA (final concentrations), in order to inhibit cyclo-oxygenase and lipoxygenase activities, respectively. The latter one was added under a minimal volume of ethanol (0.005 ml), which was also included in controls. Then LF 1695 (60 μM, final concentration) or A23187 (1 μM, final concentration) or their respective solvents (ethanol or dimethylsulfoxide, respectively, 0.005 ml/2 ml) were added, and incubation was prolonged for 5 min with A23187 or 10 min with LF 1695, followed by addition of EDTA and chloroform/methanol as previously.

Study of [^{14}C]*arachidonic acid incorporation into platelet lipids*. In this case, platelet suspensions were incubated at 37° with 75.6 nCi/ml (1.4 μM) [^{14}C]arachidonic acid in the presence or in the absence of LF 1695. Incubations were stopped at 15 or 30 min.

Lipid analysis. Lipids were extracted according to Bligh and Dyer [13]. After recovering the first chloroformic phase, the medium was acidified with 26.5 M formic acid (0.02 ml/ml platelet suspension) and submitted to a second extraction with chloroform [14]. The two lipid extracts were pooled, evaporated to dryness under reduced pressure, dissolved in a minimal volume of chloroform/methanol (1/1, v/v), and submitted to two different chromatographic separations on thin layer plates, 0.25 mm thick (Merck, Darmstadt, F.R.G.), using the following solvents: (1) chloroform/methanol/acetic acid/water (65/43/1/3, v/v) for the separation of phospholipids [15]; (2) the upper phase of ethylacetate/acetic acid/isooctane/water (110/20/50/100, v/v), which allowed separation of phosphatidic acid, various eicosanoids, non-converted arachidonic acid, diacylglycerol and triacylglycerol from the bulk of phospholipids [16]. The various lipid spots were detected by autoradiography and then scraped directly into scintillation vials containing 10 ml Instagel (Packard). Radioactivity was determined using a Packard-Tricarb 4530 spectrometer equipped with automatic quenching correction.

Platelet aggregation. This was measured in platelet-rich plasma using the turbidimetric method of Born [17].

Statistical analysis. Statistical significance for

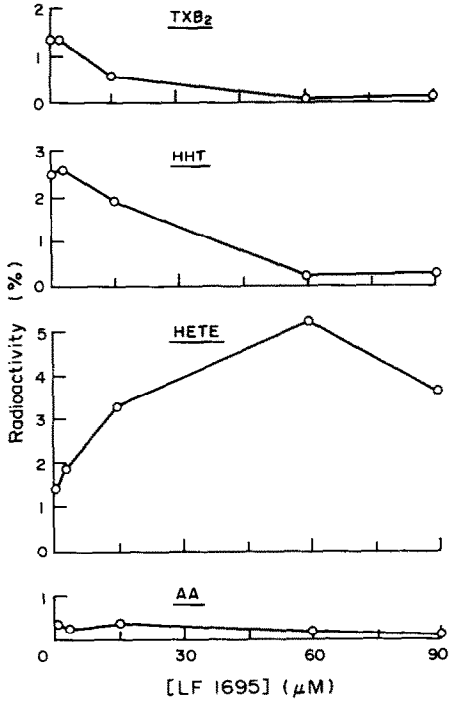

Fig. 1. Effect of LF 1695 on arachidonic acid and eicosanoid production in [^{14}C]arachidonic acid labelled platelets triggered by thrombin. Human platelet suspensions (2 ml) labelled with [1-^{14}C]arachidonic acid were incubated at 37° for 10 min with increasing concentrations of LF 1695, then were stimulated for 1 min with thrombin (1 U/ml). Lipid extracts were processed as described under Materials and Methods. Results (percentages of total radioactivity) are expressed as differences of the radioactivity present in assays compared to controls. Abbreviations: TXB_2, thromboxane B_2; HHT, 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12-(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; AA, arachidonic acid.

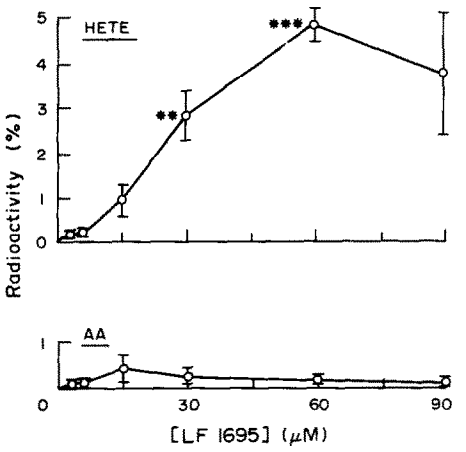

Fig. 2. Arachidonic acid and 12-HETE release in radiolabelled platelets upon LF 1695 stimulation. Two milliliters of human platelet suspensions labelled with [1-^{14}C]arachidonic acid were incubated at 37° for 10 min in the presence of increasing concentrations of LF 1695. Lipid extracts were analysed as described under Materials and Methods. Results correspond to differences of percentages of total radioactivity present in assays compared to controls and are means ± SE of 4 experiments. Statistical analyses by paired Student *t*-test are represented: ** $P < 0.02$; *** $P < 0.01$. Abbreviations: 12-HETE, 12-(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; AA, arachidonic acid.

paired data was determined by Student's *t*-test. A minimum level of significance was fixed at $P < 0.05$. All results are expressed as percentages of total radioactivity (except for arachidonic acid incorporation, which is determined as pmol/15 min/10^9 platelets) and are means ± SE of four experiments, unless otherwise stated.

RESULTS

Effect of LF 1695 *on thrombin stimulated* [^{14}C]*arachidonic acid labelled platelets*

This preliminary experiment was devised in order to see whether LF 1695 was able to modulate arachidonic acid metabolism in platelets stimulated with thrombin. For this purpose, platelets were previously incubated with [1-^{14}C]arachidonic acid under conditions allowing incorporation of 80% of it into cell lipids. Radioactivity was mainly detected in phospholipids (95% of total incorporated radioactivity), the remainder being associated with neutral lipids (diacyl- and triacylglycerol).

As shown in Table 1 and in agreement with previously reported data [10], thrombin stimulated the release of [^{14}C]arachidonate from phospholipids and its oxygenation into eicosanoids, mainly by cyclooxygenase, since thromboxane B_2, the stable metabolite of thromboxane A_2, and 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid (HHT) accounted for 1.4% and 2.6%, respectively, of total platelet radioactivity after 1 min, against 1.4% for 12-HETE. The almost twice higher radioactivity detected in HHT compared to thromboxane B_2, which are produced in equal amounts by thromboxane synthetase, probably reflects the non-enzymatic conversion of prostaglandin H_2 into HHT, in agreement with Raz *et al.* [18].

However, preincubation of platelets with LF 1695 prior to thrombin stimulation induced a dose-dependent inhibition of thromboxane B_2 and HHT production, without any accumulation of arachidonic acid (Fig. 1). Half maximal inhibition of cyclooxygenase metabolite production, as well as half maximal activation of 12-HETE production occurred at approximately 15 μM LF 1695.

This change in eicosanoid formation was not accompanied by an increased phospholipid hydrolysis, when considering the total radioactivity of eicosanoids. However, increasing doses of LF 1695 promoted a progressive decrease of phospholipid hydrolysis, which was concomitant with an increased degradation of triacylglycerol (Table 1).

These results prompted us to investigate in more detail the proper effect of the drug on [^{14}C]arachidonic acid metabolism in non-stimulated platelets.

Effect of LF 1695 *on* [^{14}C]*arachidonic acid labelled platelets*

As depicted in Fig. 2, LF 1695 added to platelet

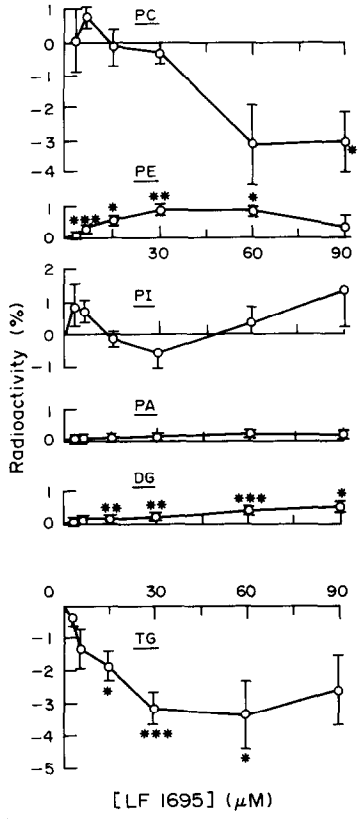

Fig. 3. [^{14}C]Arachidonate radioactivity changes in the various lipids of platelets upon LF 1695 stimulation. Human platelet suspensions (2 ml) labelled with [1-^{14}C]arachidonic acid were incubated at 37° for 10 min with increasing concentrations of LF 1695; lipids were analysed as described under Materials and Methods. Results are expressed as differences of percentages of total radioactivity present in assays compared to controls and are means ± SE of four experiments. Statistical analyses by paired Student *t*-test are represented: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanomamine; PI, phosphatidylinositol; PA, phosphatidic acid; DG, diacylglycerol; TG, triacylglycerol.

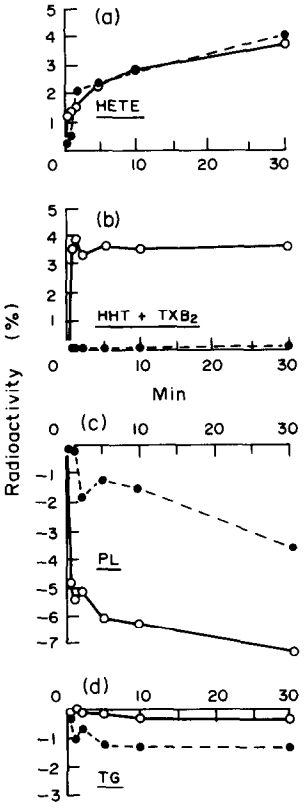

Fig. 4. Time course of the effects of LF 1695 on arachidonic acid metabolism in human platelets. Conditions of incubations are identical to those of Fig. 2, with incubation of [^{14}C]arachidonic acid labelled platelets in the presence of 60 μM LF 1695 (dotted lines, full symbols) or 1 μM A23187 (continuous lines, open symbols) for the indicated times. Data are expressed as differences of percentages of total radioactivity present in assays compared to controls, and correspond to 12-HETE (a), cyclo-oxygenase metabolites, i.e. thromboxane B_2 plus HHT (b), total phospholipids (c) and triacylglycerol (d). Abbreviations: 12-HETE, TXB_2, thromboxane B_2; HHT, 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid; PL, phospholipid; TG, Triacylglycerol.

suspensions induced a significant accumulation of 12-HETE, which was maximal at 60 μM of the drug. This occurred in the absence of any accumulation of cyclo-oxygenase metabolites (thromboxane B_2, HHT) or of non-esterified arachidonate. Up to 30 μM, HETE formation was concomitant with a dose-dependent decrease in the radioactivity of triacylglycerol, which levelled off at higher concentrations of the drug (Fig. 3). In contrast, further increase of [^{14}C]HETE occurring at 30–60 μM of LF 1695 was parallel to a fall in the radioactivity of [^{14}C]phosphatidylcholine (Fig. 3). As also shown in Fig. 3, a low but significant accumulation of [^{14}C]diacylglycerol could be already observed at the lowest concentrations of LF 1695 (difference vs controls significant at 15 μM of the drug), but this was never accompanied by an accumulation of radioactive phosphatidic acid.

Figure 4 illustrates the time course of LF 1695 effect as compared to the calcium ionophore A23187. With both agents, 12-HETE formation occurred with the same velocity, half maximal effect occurring at 2 min, followed by a progressive decline and some levelling off between 10 and 30 min (Fig. 4a). Figure 4(b) illustrates a main difference between LF 1695 and A23187, i.e. the absence of cyclo-oxygenase metabolites upon LF 1695 treatment, whereas thromboxane B_2 and HHT synthesis was completed after 30 sec of A23187 stimulation. Another difference between the two compounds concerns their effects on cell lipid radioactivity, A23187 inducing a rapid and important decrease of phospholipid radioactivity (Fig. 4c), reflecting a major stimulation of phospholipase A_2 [19], whereas LF 1695 promoted an immediate deacylation of [^{14}C]triacylglycerol (Fig. 4d) and phospholipids (Fig. 4c).

Effect of aspirin and ETYA on eicosanoid production by platelets incubated with A23187 *or LF* 1695

To substantiate these data, platelets were incubated with aspirin or ETYA prior to addition of LF

1695 or A23187. As it can be seen in Fig. 5, ETYA inhibited all eicosanoid production induced by LF 1695 or A23187. These included 12-HETE, as well as thromboxane B_2 and HHT, when A23187 was used as a stimulant, reflecting the ability of ETYA to inhibit lipoxygenase and cyclo-oxygenase, in agreement with previous literature data [20]. In both cases, this resulted in a concomitant accumulation of non-esterified [^{14}C]arachidonic acid.

In contrast, aspirin remained without effect on [^{14}C]HETE accumulation induced by LF 1695, but inhibited the synthesis of [^{14}C]thromboxane B_2 and HHT promoted by A23187. At variance with LF 1695, where a total conversion of released arachidonate to lipoxygenase had already occurred, aspirin led, in this case, to a higher 12-HETE synthesis, reflecting a shift towards the still active lipoxygenase pathway (Table 2).

Both inhibitors (aspirin and ETYA) did not modify significantly the release of arachidonate from cellular lipids (Fig. 6), despite a lower hydrolysis of phosphatidylcholine in the presence of ETYA, as previously described [10]. In addition, analysis of the various lipid classes confirmed the loss of radiolabelled arachidonate from both triacylglycerol and phosphatidylcholine, with some transfer to phosphatidylethanolamine, but without any change in the radioactivity of phosphatidylinositol, in all the LF 1695 stimulated platelet suspensions (Fig. 6). Furthermore, we still noted an obvious formation of diacylglycerol induced by LF 1695, without any effect of A23187 on this lipid (Table 2). Again, this was never accompanied by [^{14}C]phosphatidic acid formation.

Effect of LF 1695 on [^{14}C]arachidonic acid incorporation into platelet lipids

In this series of experiments, platelets were incubated with [^{14}C]arachidonic acid during 15 or 30 min in the presence of increasing concentrations of LF 1695. As shown in Fig. 7, incorporation of the fatty acids was efficiently depressed by the drug, half maximal effect occurring at 60 μM for triacylglycerol, 60 μM for phosphatidylcholine and 30 μM for phosphatidylinositol. Similar results were observed after 30 min incubation with the drug (not shown).

Effect of LF 1695 on platelet aggregation

Since, among other effects, LF 1695 appeared as a strong inhibitor of platelet cyclo-oxygenase, additional experiments were performed in order to examine whether this resulted in some modification of platelet function. As shown in Fig. 8, LF 1695 inhibited the second wave of aggregation induced by ADP as well as arachidonic acid-induced platelet aggregation. In the latter case, higher concentrations of LF 1695 (60–90 μM) were necessary to totally suppress platelet response, whereas concentrations as low as 3 μM reversed ADP-induced platelet aggregation.

DISCUSSION

The present study extends to platelets some previous observations indicating that LF 1695 stimulates the production of lipoxygenase metabolites by macrophages without any prostaglandin secretion, whose synthesis is actually inhibited [1, 2]. Altogether, our data bring clear evidence that LF 1695 promotes arachidonic acid release from both triacylglycerol and phosphatidylcholine, coupled to a total conversion of liberated arachidonic acid into 12-HETE by lipoxygenase.

The inhibition by LF 1695 of platelet cyclo-oxygenase is probably due to a direct effect of the drug on the enzyme. This is indicated by previous experiments using microsomes from sheep seminal vesicles, which revealed an inhibition of the enzyme involving the formation of a complex between LF 1695 and heme present in the enzyme (Mansuy D, personal communication). Also, an indirect effect of LF 1695 (i.e. inactivation of cyclo-oxygenase by 12-(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid or 12-HPETE, Refs 21, 22) can be excluded, since thromboxane B_2 and HHT production is not suppressed in platelets stimulated by A23187, which induces 12-HETE production with a time course very similar to that observed in the presence of LF 1695). Finally, some preliminary experiments revealed an inhibition of prostaglandin I_2 synthesis by LF 1695 in cultured human endothelial cells, which still lack lipoxygenase (Hullin F *et al.*, in preparation).

As expected, inhibition of platelet cyclo-oxygenase results in modifications of platelet aggregation, especially under those conditions where thromboxane B_2 and prostaglandin endoperoxides are required for maximal platelet activation. This is the case of ADP-induced human platelet aggregation, where LF 1695 displayed a behaviour very similar to that of aspirin or other non-steroidal anti-inflammatory drugs, i.e. a suppression of the second wave of platelet aggregation [23]. However, it still remains to be shown whether a similar effect also occurs *ex vivo* at the therapeutic doses currently used. The inhibitory effect exerted on platelet cyclo-oxygenase probably explains why phospholipid hydrolysis induced by thrombin is decreased in the presence of LF 1695 (Table 1), since thromboxane A_2 and prostaglandin endoperoxides no longer promote amplification of platelet activation. This might appear somewhat paradoxical, since LF 1695 is also shown to induce by itself deacylation of phosphatidylcholine. However, phospholipid hydrolysis promoted by the drug is only 3% of total radioactivity (Fig. 3) against 7.6% when considering the effect of thrombin (Table 1).

As to the mechanism of arachidonic acid liberation promoted by LF 1695, this differs from other stimulatory agents like thrombin or calcium ionophore A23187. For instance, thrombin activates a phospholipase C specific for inositol phospholipids, thus generating two potent intracellular messengers, inositol-1,4,5-*tris*phosphate and 1,2-diacyl-*sn*-glycerol, able to mobilize calcium and to activate protein kinase C, respectively [24–26]. The latter compound is also a substrate of diacylglycerol/monoacylglycerol lipases, representing a minor pathway of arachidonic acid liberation [5–7]. This is mainly achieved by phospholipase A_2 [8–10, 27], which seems to be activated as a consequence of an increase in cytosolic free calcium concentration [28] and possibly of lipocortin

Table 2. Effect of aspirin (ASA) and ETYA on [^{14}C]arachidonate radioactivity changes in the various lipids of human platelets stimulated with A 23187 or LF 1695

Conditions	Total phospholipids	Triacylglycerol	Diacylglycerol	Arachidonic acid and metabolites: Non-esterified arachidonic acid	Arachidonic acid and metabolites: Cyclooxygenase metabolites (TXB_2, HHT)	Arachidonic acid and metabolites: Lipoxygenase metabolites (12-HETE)
First experiment						
Control	95.6	3.6	0.1	0.06	0.14	0.10
A 23187 1 μM	86.0	4.0	0.3	0.2	5.3	3.6
A 23187 1 μM plus ASA 100 μM	87.8	3.5	0.2	0.2	0.7	6.9
A 23187 1 μM plus ETYA 10 μM	92.2	3.2	0.2	3.8	0.35	0.05
Second experiment						
Control	96.0	3.5	0.1	0.03	0.06	0.06
LF 1695 60 μM	93.3	1.7	0.6	0.2	0.2	3.4
LF 1695 60 μM plus ASA 100 μM	93.1	1.7	0.7	0.2	0.2	3.6
LF 1695 60 μM plus ETYA 10 μM	93.5	1.6	0.7	3.8	0.07	0.03

Conditions of incubation were the same as in Fig. 4 and lipid extracts were analysed as described under Materials and Methods. Results are expressed as percentages of total radioactivity.

Abbreviations: A 23187, calcium ionophore; ASA, acetylsalicylic acid (aspirin); ETYA, 5,8,11,14-eicosatetraynoic acid.

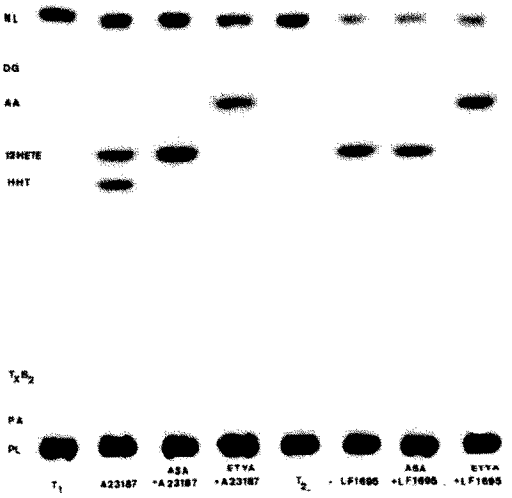

Fig. 5. Autoradiography of arachidonate metabolites produced by human platelets after A23187 or LF 1695 stimulation. Two milliliters of human platelet suspensions labelled with [1-^{14}C]arachidonic acid were preincubated for 20 min with 100 μM acetylsalicylic acid (ASA) or 10 μM 5,8,11,14-eicosatetraynoic acid (ETYA), then stimulated for 10 min with 60 μM LF 1695 or for 5 min with1 μM calcium ionophore A23187. Various eicosanoids were separated by thin-layer chromatography as described under Materials and Methods. Abbreviations: PL, phospholipids; PA, phosphatidic acid; TXB_2, thromboxane B_2; HHT, 12-(*S*)-hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12-(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; AA, arachidonic acid; DG, 1,2-diacyl-*sn*-glycerol and 1,3-diacyl-*sn*-glycerol; NL, neutral lipids (triacylglycerol).

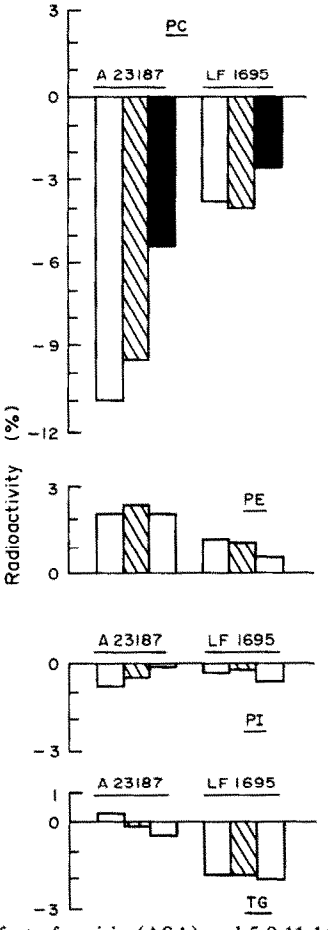

Fig. 6. Effect of aspirin (ASA) and 5,8,11,14-eicosatetraynoic acid (ETYA) on [^{14}C]arachidonate changes in phospholipids and triacylglycerol of platelets upon A23187 or LF 1695 stimulation: untreated, open bars; 100 μM ASA, dotted bars; 10 μM ETYA, full bars. Conditions of incubation were the same as in Fig. 5 and lipid extracts were analysed as described under Materials and Methods. Results are expressed as differences of percentages of total radioactivity present in assays compared to controls. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TG, triacylglycerol.

phosphorylation by protein kinase C [29]. Such a calcium-dependent activation of platelet phospholipase A_2 also occurs in response to calcium ionophores, which do not activate phospholipase C [30]. However, as is also clearly shown in the present study, neither thrombin nor A23187 are able to promote triacylglycerol hydrolysis. This is a main difference with LF 1695, which acts primarily on triacylglycerol, when considering either time course or dose dependence of LF 1695 effects, but also on phosphatidylcholine. This suggests that the cellular target for LF 1695 is probably a more general step involved in arachidonic acid turnover rather than a double stimulation of triacylglycerol lipase and phospholipase A_2. From data obtained on arachidonic acid incorporation into platelet lipids, it appears that LF 1695 greatly depresses arachidonic acid esterification into both neutral lipids and phospholipids, indicating that inhibition might well occur at the stage of arachidonic acid activation into arachidonoyl Co A or at the level of reacylation. In this respect, triacylglycerol, the lipid displaying the highest sensitivity to the action of LF 1695, is known to be in a rapid dynamic equilibrium with other lipids, this involving deacylation, reacylation and transacylation. Such an intermediary role of triacylglycerol has been well documented in other cells, like endothelial cells [31, 32]. So the apparent hydrolysis of triacylglycerol and phosphatidylcholine promoted by LF 1695 probably results from an inhibition of reacylation, as evidenced in other cells with other inhibitors of acyltransferases [33, 34].

Whatever is the mechanism of arachidonic acid liberation promoted by LF 1695, its further specific conversion into 12-HETE deserves some discussion. Some studies dealing with neutrophils [35] or endothelial cells [36] have shown that various hydroxy-eicosatetraenoic acids can be incorporated into cell lipids, mainly, but not exclusively, triacylglycerol. So it was possible that, during the labelling period, some [^{14}C]arachidonic acid was converted into 12-HETE by platelet lipoxygenase, followed by incorporation into triacylglycerol and phosphatidylcholine. If this were true, a selective hydrolysis of those lipid species containing 12-HETE could explain our present results. Indeed, phospholipase A_2 activity is higher against phosphatidylcholine con-

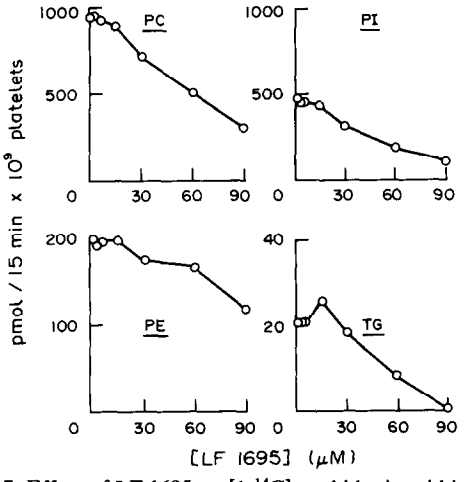

Fig. 7. Effect of LF 1695 on [1-^{14}C]arachidonic acid incorporation into platelet lipids. Platelet suspensions (5×10^8 cells/ml) were incubated in the presence of [1-^{14}C]arachidonic acid (1.4 μM, 75.6 nCi/ml) at 37° for 15 min in the absence or in the presence of increasing concentrations of LF 1695. Lipids were extracted and analysed as described under Materials and Methods. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TG, triacylglycerol.

taining HETE in the *sn*-2 position, compared to the non-oxidized phospholipid [37]. However, our experiments using ETYA allowed us to definitely exclude this hypothesis, since lipoxygenase inhibition during platelet treatment with LF 1695 completely suppressed 12-HETE production and promoted the accumulation of non-esterified [^{14}C]arachidonic acid. This unambiguously proved that 12-HETE was not pre-existing in any of the lipids undergoing net deacylation under the action of LF 1695.

Another interesting biochemical observation of the present work is the accumulation of low but significant amounts of radioactive diacylglycerol under the action of LF 1695. Such an event is well documented in platelets, where it results from inositol phospholipid hydolysis by phospholipase C upon physiological stimulation [3, 4, 26, 28, 38]. However, in this case, diacylglycerol accumulation is only transient and no longer detectable after 1 min stimulation [3, 38]. Moreover, a large part of it is immediately converted into phosphatidic acid by diacylglycerol kinase [3, 5, 26, 38–42]. This is not the case with LF 1695, where phosphatidylinositol does not display any modification of radioactivity. Another possibility might be the stimulation by LF 1695 of a phospholipase C specific for phosphatidylcholine, as recently found in various tissues or cells [43–47]. However, this was probably not the case here, since diacylglycerol accumulation actually occurred at concentrations of LF 1695 where [^{14}C]phosphatidylcholine was not modified, in contrast to [^{14}C]triacylglycerol, which was significantly decreased (see, for instance, Fig. 2). We thus suggest that [^{14}C]triacylglycerol is probably the source of [^{14}C]diacylglycerol. The lack of [^{14}C]diacylglycerol conversion into [^{14}C]phosphatidic acid in platelets treated with LF 1695 might not be due to a lack of accessibility of the substrate to the kinase, which displays an ubiquitous localization [48]. This could involve a direct inhibition of diacylglycerol kinase by LF 1695, or a specific hydrolysis of triacylglycerol at the *sn*-1 position, leading to the formation of 2,3-diacyl-*sn*-glycerol, which is not a substrate for the highly stereospecific kinase [49].

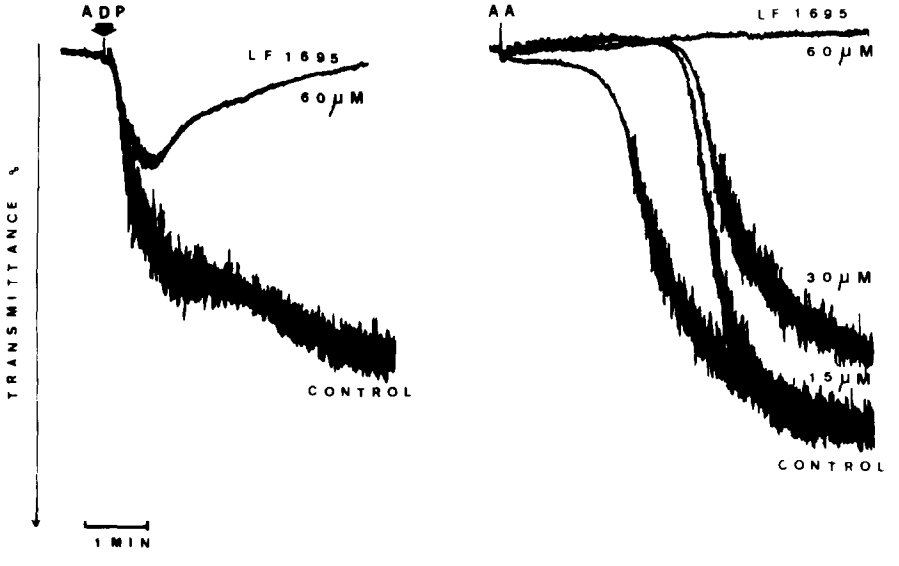

Fig. 8. Effect of LF 1695 on platelet aggregation induced by ADP or arachidonic acid. Human platelet-rich plasma was incubated at 37° for 10 min in the absence or in the presence of LF 1695, under which conditions no modifications of optical transmittance could be observed. Aggregation was triggered by further addition of ADP (2.5 μM) or arachidonic acid (AA, 1 mM) and determined according to Born [17].

In conclusion, our study reports the first observation in intact platelets of a direct stimulation of triacylglycerol deacylation subsequent to inhibition of reacylation. Although higher concentrations of LF 1695 are also able to promote phosphatidylcholine deacylation, this effect on triacylglycerol is sufficient to induce a significant and selective synthesis of 12-HETE, the stable derivative of 12-HPETE, owing to a simultaneous inhibition of cyclo-oxygenase. 12-HPETE production by platelets might participate in the immunostimulant properties of LF 1695, since platelet-derived fatty acid hydroperoxides are able to stimulate leukotriene biosynthesis in human blood leukocytes [50]. The biochemical mechanism observed here in platelets is probably the same as that leading to inhibition of prostaglandin synthesis and stimulation of leukotriene B_4 production by murine macrophages [2]. In this respect, it is interesting to note that the concentrations of LF 1695 (15–90 μM) inducing 12-HETE production by platelets are in the same order of magnitude (1–10 μg/ml) as those eliciting leucotriene B_4 synthesis, interleukin 1 secretion, inhibition of interleukin 2 synthesis and modulation of T-cell proliferation in previously studied murine cells [1, 2]. This suggests, although it does not prove, that the biochemical effect of LF 1695 described in the present study might play a central role in its immunodulatory properties. Finally, there is increasing evidence for an involvement of lipoxygenase metabolites in other fields of pathobiology, like thrombosis [51–54]. This should warrant further studies dealing with a possible effect of LF 1695 on other cells, like vascular cells, involved in thrombogenesis.

Acknowledgements—This study was supported by a grant from Ministère de l'Industrie et de la Recherche (Action Vieillissement Cardiovasculaire, contrat 82.L07557). We are indebted to Laboratoires Fournier-Dijon for supporting Françoise Hullin and to Hofmann-La Roche for the generous gift of ETYA. Thanks are also due to Dr P. Sié and Mrs M. Laroche for their help in the aggregation experiments and to Mrs Y. Jonquière for correcting the English manuscript.

REFERENCES

1. Othmane O, Touraine JL, Sanhadji K and Pascal M, A new immunomodulator, LF 1695. *In vitro* effect on the T lymphocytes lineage in man. *Int J Immunopharmac* **7**: 543–548, 1985.
2. Pascal M and Derrepas P, Increased interleukin-1 and modulation of interleukin-2 production by murine macrophages and lymphocytes treated with LF 1695. *Int J Immunopharmac* **8**: 499–508, 1986.
3. Rittenhouse-Simmons S, Production of diglycerides from phosphatidylinositol in activated human platelets. *J Clin Invest* **63**: 580–587, 1979.
4. Mauco G, Chap H and Douste-Blazy L, Characterization and properties of a phosphatidylinositol phosphodiesterase (phospholipase C) from platelet cytosol. *FEBS Lett* **100**: 367–370, 1979.
5. Mauco G, Chap H, Simon MF and Douste-Blazy L, Phosphatidic and lysophosphatidic acid production in phospholipase C and thrombin-treated platelets. Possible involvement of a platelet lipase. *Biochimie* **60**: 653–661, 1978.
6. Bell RL, Kennerly DA, Stanford N and Majerus PW, Diglyceride-lipase: a pathway for arachidonate release from human platelets. *Proc Natl Acad Sci USA* **76**: 3238–3241, 1979.
7. Mauco G, Fauvel J, Chap H and Douste-Blazy L, Studies on enzymes related to diacylglycerol production in activated platelets. II. Subcellular distribution, enzymatic properties and positional specificity of diacylglycerol- and monoacylglycerol-lipases. *Biochim Biophys Acta* **796**: 169–177, 1984.
8. Kannagi R and Koizumi K, Effect of different physical states of phospholipid substrates on partially purified platelet phospholipase A_2 activity. *Biochim Biophys Acta* **556**: 423–433, 1979.
9. Apitz-Castro RJ, Mas MA, Cruz MR and Jain MR, Isolation of homogeneous phospholipase A_2 from human platelets. *Biochem Biophys Res Commun* **91**: 63–71, 1979.
10. Bills TK, Smith JB and Silver MJ, Metabolism of ^{14}C-arachidonic acid by human platelets. *Biochim Biophys Acta* **424**: 304–314, 1976.
11. Aster RH and Jandl JH, Platelet sequestration in man. I. Methods in rat platelets. *J Clin Invest* **43**: 843–855, 1964.
12. Ardlie NG, Packham MA and Mustard JF, Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. *Br J Haematol* **19**: 7–17, 1970.
13. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification, *Can J Biochem Physiol* **37**: 911–918, 1959.
14. Chap H, Mauco G, Simon MF, Benveniste J and Douste-Blazy L, Biosynthetic labelling of platelet activating factor from radioactive acetate by stimulated platelets. *Nature (Lond)* **289**: 312–314, 1981.
15. Owens K, A two dimensional thin layer chromatographic procedure for the estimation of plasmalogens. *Biochem J* **100**: 354–361, 1966.
16. Sun FF, Chapman JP and McGuire JC, Metabolism of prostaglandin endoperoxides in animal tissues. *Prostaglandins* **14**: 1055–1075, 1977.
17. Born GVR, Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond)* **194**: 927–929, 1962.
18. Raz A, Aharony D and Kenig-Wakshal R, Biosynthesis of thromboxane A_2 and 12-L-hydroxy-5,8,10-heptadecatrienoic acid in human platelets. Evidence for a common enzymatic pathway. *Eur J Biochem* **86**: 447–454, 1978.
19. Rittenhouse-Simmons and Deykin D, The activation by Ca^{2+} of platelet phospholipase A_2. Effects of dibutyryl cyclic adenosine monophosphate and 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate. *Biochim Biophys Acta* **543**: 409–422, 1978.
20. Sun FF, McGuire DR, Morton DR, Pike JE, Sprecher H and Kunau WH, Inhibition of platelet arachidonic acid 12-lipoxygenase by acetylenic acid compounds. *Prostaglandins* **21**: 333–343, 1981.
21. Hammarström S and Falardeau P, Resolution of prostaglandin endoperoxide synthetase and thromboxane synthetase in human platelets. *Proc Natl Acad Sci USA* **74**: 3691–3695, 1977.
22. Siegel MI, McConnell RT, Abrahams SL, Porter NA and Cuatrecasas P, Regulation of arachidonate metabolism via lipoxygenase and cyclo-oxygenase by 12-HPETE, the product of human platelet lipoxygenase. *Biochem Biophys Res Commun* **89**: 1273–1280.
23. Mustard JF, Perry DW, Kinlough-Rathbone RL and Packham MA, Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* **228**: 1757–1765, 1975.
24. Berridge MJ, Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* **220**: 345–360, 1984.
25. Nishizuka Y, The role of protein kinase C in cell surface

signal transduction and tumor promotion. *Nature (Lond)* **308**: 693–698, 1984.
26. Majerus PW, Wilson DB, Connolly TM, Bross TE and Neufeld EJ, Phosphoinositide turnover provides a link in stimulus–response coupling. *Trends Biochem Sci* **10**: 168–171, 1985.
27. McKean ML, Smith JB and Silver MJ, Formation of lysophosphatidylcholine by human platelets in response to thrombin. Support for the phospholipase A_2 pathway for the liberation of arachidonic acid. *J Biol Chem* **256**: 1522–1524, 1981.
28. Simon MF, Chap H and Douste-Blazy L, Selective inhibition of human platelet phospholipase A_2 by buffering cytoplasmic calcium with the fluorescent indicator quin 2. Evidence for different calcium sensitivities of phospholipases C and A_2. *Biochim Biophys Acta* **875**: 157–164, 1986.
29. Touqui L, Rothhut B, Shaw AM, Fradin BB, Vargaftig BB and Russo-Marie F, Platelet activation, a role for a 40 K antiphospholipase A_2 protein indistinguishable from lipocortin. *Nature (Lond)* **321**: 177–180, 1986.
30. Rittenhouse SE, Activation of human platelet phospholipase C by ionophore A23187 is totally independent upon cyclooxygenase products and ADP. *Biochem J* **222**: 103–110, 1984.
31. Denning GM, Figard PH, Kaduce TL and Spector AA, Role of triglycerides in endothelial cell arachidonic acid metabolism. *J Lipid Res* **24**: 993–1001, 1983.
32. Sivarajan M, Hall ER, Wu KK, Rafelson ME and Manner C, Regulation of intracellular arachidonate in normal and stressed endothelial cells. *Biochim Biophys Acta* **795**: 271–276, 1984.
33. Kröner EE, Peskar BA, Fisher H and Ferber E, Control of arachidonic acid accumulation in bone marrow-derived macrophages by acyltransferases. *J Biol Chem* **256**: 3690–3697, 1981.
34. Körner CF, Hausmann G, Gemsa D and Resch K, Rate of prostaglandin synthesis is not controlled by phospholipase A activity but by reincorporation of released fatty acids into phospholipids. *Agents Actions* **15**: 28–29, 1984.
35. Bonser R, Siegel MI, Chung SM, McConnell RT and Cuatrecasas P, Esterification of endogenously synthesized lipoxygenase products into granulocyte cellular lipids. *Biochemistry* **20**: 5297–5301, 1981.
36. Richards CF, Johnson AR and Campbell WB, Specific incorporation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid into phosphatidylcholine in human endothelial cells. *Biochim Biophys Acta* **875**: 569–581, 1986.
37. van Kuijk FJGM, Sevanian A, Handelman WB and Dratz EA, A new role for phospholipase A_2: protection of membranes from lipid peroxidation damage. *Trends Biochem Sci* **12**: 31–34, 1987.
38. Simon MF, Chap H and Douste-Blazy L, Activation of phospholipase C in thrombin-stimulated platelets does not depend on cytoplasmic free calcium concentration. *FEBS Lett* **170**: 43–48, 1984.
39. Lloyd JV and Mustard JF, Changes in ^{32}P-content of phosphatidic acid and the phosphoinositides of rabbit platelets during aggregation induced by collagen or thrombin. *Br J Haematol* **26**: 243–253, 1974.
40. Lapetina EG and Cuatrecasas P, Stimulation of phosphatidic acid production in platelets precedes the formation of arachidonate and parallels the release of serotonin. *Biochim Biophys Acta* **573**: 394–402, 1979.
41. Broekman MJ, Ward JW and Marcus AJ, Fatty acid composition of phosphatidylinositol and phosphatidic acid in stimulated platelets. Persistence of arachidonyl–stearyl structure. *J Biol Chem* **256**: 8271–8274, 1981.
42. Mauco G, Dangelmaier CA and Smith JB, Inositol lipids, phosphatidate and diacylglycerol share stearoylarachidonoylglycerol as a common backbone in thrombin-stimulated platelets. *Biochem J* **224**: 933–940, 1984.
43. Wolf RA and Gross RW, Identification of neutral active phospholipase C which hydrolyzes choline glycerophospholipids and plasmalogen selective phospholipase A_2 in canine myocardium. *J Biol Chem* **260**: 7295–7303, 1985.
44. Daniel LW, Waite M and Wykle RL, A novel mechanism of diglyceride formation. 12-*O*-Tetradecanoylphorbol-13-acetate stimulates the cyclic breakdown of phosphatidylcholine. *J Biol Chem* **261**: 9128–9132, 1986.
45. Besterman JM, Duronio V and Cuatrecasas P, Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for the generation of a second messenger. *Proc Natl Acad Sci USA* **83**: 6785–6789, 1986.
46. Martin TW, Wysolmerski RB and Lagunoff D, Phosphatidylcholine metabolism in endothelial cells: evidence for phospholipase A and a novel Ca^{2+}-independent phospholipase C. *Biochim Biophys Acta* **917**: 296–307, 1987.
47. Ragab-Thomas JMF, Hullin F, Chap H and Douste-Blazy L, Pathways of arachidonic acid liberation in thrombin and calcium ionophore A23187-stimulated human endothelial cells: respective roles of phospholipids and triacylglycerol and evidence for diacylglycerol generation from phosphatidylcholine. *Biochim Biophys Acta* **917**: 388–397, 1987.
48. Call FL and Rubert M, Diglyceride kinase in human platelets. *J Lipid Res* **14**: 466–474, 1973.
49. Lands WEM, Pieringer RA, Slakey SPM and Zschocke A, A micromethod for the stereospecific determination of triglyceride structure. *Lipids* **1**: 444–448, 1966.
50. Maclouf J, Fruteau de Laclos B and Borgeat P, Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet derived 12-hydroperoxy-eicosatetraenoic acid. *Proc Natl Acad Sci USA* **79**: 6042–6046, 1982.
51. Duthil CE, Haddeman E, Don JA and Ten Hoor F, The role of arachidonic acid lipoxygenase and fatty acids during irreversible blood platelet aggregation *in vitro*. *Prostaglandins* **6**: 111–126, 1981.
52. Aharony D, Smith JB and Silver MJ, Regulation of arachidonate-induced platelet aggregation by the lipoxygenase product, 12-hydroperoxyeicosatetraenoic acid. *Biochim Biophys Acta* **718**: 193–200, 1982.
53. Croset M and Lagarde M, Stereospecific inhibition of PGH_2-induced platelet aggregation by lipoxygenase products of arachidonic acid. *Biochem Biophys Res Commun* **112**: 878–883, 1983.
54. Buchanan MR, Haas TA, Lagarde M and Guichardant M, 13-Hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. *J Biol Chem* **260**: 16056–16059, 1985.