Thrombin induces a biphasic 1,2-diacylglycerol production in human platelets

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The 1,2-diacylglycerol (DAG) mass content was measured in thrombin-stimulated human platelets. Thrombin stimulates a biphasic accumulation of DAG, with an early phase reaching a peak at 10 s and a later phase reaching a peak at 2–3 min. The time course of first-phase DAG production corresponded well to that of $\text{Ins}(1,4,5)\text{P}_3$ formation, which was rapid and transient. The second phase of DAG accumulation occurred after the level of $\text{Ins}(1,4,5)\text{P}_3$ returned to nearly basal. Thrombin stimulated the decrease in PtdIns and phosphatidylcholine contents. The source of second-phase DAG was examined in platelets prelabelled with three radioactive fatty acids, i.e. arachidonic, palmitic and myristic. Thrombin stimulated the increase in radioactivity of DAG with decline of PtdIns in platelets labelled with [^3H]arachidonic acid or [^3H]palmitic acid, in which PtdIns was considerably labelled. In contrast, significant accumulation of [^3H]DAG was not observed in [^3H]myristic acid-labelled platelets, in which PtdIns was poorly labelled. In platelets prelabelled with [^3H]inositol, an increase in InsP in response to thrombin was seen for more than 5 min. In contrast, upon stimulation, significant increases in [^3H]phosphocholine and [^3H]choline were not observed in [*methyl*- ^3H]choline-labelled platelets. Thrombin induced a small production of phosphatidylethanol, when ethanol was present during stimulation. However, the formation of DAG and phosphatidic acid was not significantly affected by ethanol. These results suggest that thrombin stimulates a biphasic accumulation of DAG, initially from PtdInsP_2 and later from PtdIns in human platelets.

INTRODUCTION

1,2-Diacylglycerol (DAG) functions as an intracellular second messenger which activates protein kinase C (PKC) [1]. Initially PtdIns was considered to be the source of agonist-induced DAG formation [2]. However, in response to the agonist, hydrolysis of polyphosphoinositides has been shown to precede the decline in PtdIns in many types of cells. On the basis of these observations, the decrease in PtdIns upon cell stimulation was assumed to be the consequence of its consumption during the resynthesis of PtdInsP_2 [3]. The concept is widely accepted that hydrolysis of PtdInsP_2 by phospholipase C (PLC) upon stimulation of Ca^{2+} -mobilizing receptors leads to the formation of DAG and $\text{Ins}(1,4,5)\text{P}_3$ [4]. However, quantitative and temporal discrepancies between DAG and $\text{Ins}(1,4,5)\text{P}_3$ formation in agonist-stimulated cells have been demonstrated [5,6]. More recently, agonist-induced DAG formation from phosphatidylcholine (PtdCho) has also been demonstrated in many types of cells (see [7]). PtdCho contains 1-alkyl-2-acyl molecular species in addition to the diacyl form. The formation of 1-alkyl-2-acyl-glycerol upon cell stimulation was also demonstrated in MDCK cells [8] and neutrophils [9]. It is suggested that the activation profiles of PKC isoenzymes would be different among the three types of diacylglycerols [10–12].

The formation of DAG has often been investigated in radio-labelled cells. However, in these studies the changes in DAG mass content could not be determined. A simple and sensitive method has been developed for measurement of DAG mass content by using *Escherichia coli* DAG kinase [13]. Several studies have demonstrated the biphasic accumulation of DAG in agonist-stimulated cells [14–21]. In most cases, the first phase of DAG increase is rapid and transient, which is concurrent with $\text{Ins}(1,4,5)\text{P}_3$ production. On the other hand, the second sustained DAG accumulation was reported to occur in the absence of

$\text{Ins}(1,4,5)\text{P}_3$ elevation, suggesting that DAG comes from other sources than PtdInsP_2 .

PKC plays an important role during stimulus-induced platelet responses. At least two types of PKC were reported to exist in platelets [22,23]. The formation of DAG during agonist stimulation has been demonstrated. However, the details of DAG formation, e.g. phospholipid source(s) and pathway, are not fully understood in platelets. In the present study, the thrombin-induced DAG accumulation was quantitatively analysed, and the possible phospholipid sources and pathway are discussed.

EXPERIMENTAL

Preparation of platelets

Human platelets were obtained from healthy volunteers [24], and isolated at room temperature. Platelet-rich plasma was obtained by centrifugation at 164 g for 10 min, and was then mixed with prostaglandin I_2 (0.1 $\mu\text{g}/\text{ml}$) and potato apyrase (0.5 unit/ml) and gently layered on 40% (w/v) BSA. Tubes were centrifuged for 15 min at 1000 g. Plasma and BSA were carefully removed, and platelets were suspended in Tyrode–Hepes buffer (134 mM-NaCl, 12 mM- NaHCO_3 , 2.9 mM-KCl, 0.36 mM- NaH_2PO_4 , 1 mM- MgCl_2 , 5.6 mM-dextrose, 0.1% BSA, 10 mM-Hepes, pH 7.40). Platelets were then filtered through a Sepharose 2B column equilibrated with Tyrode–Hepes buffer. For the studies of fatty-acid-labelled lipid metabolism, the platelets were labelled with [^3H]arachidonic, [^3H]palmitic or [^3H]myristic acid (0.2 $\mu\text{Ci}/\text{ml}$) for 120 min in platelet-rich plasma. For [^3H]choline labelling, platelets were incubated with [*methyl*- ^3H]choline chloride (10 $\mu\text{Ci}/\text{ml}$ of platelet-rich plasma) for 180 min. For [^3H]inositol labelling, gel-filtered platelets ($2 \times 10^9/\text{ml}$) were incubated with [^3H]inositol (10 $\mu\text{Ci}/\text{ml}$) for 150 min in low-glucose (0.56 mM) buffer containing 2 mM- MnCl_2 instead of MgCl_2 [25].

Abbreviations used: DAG, 1,2-diacylglycerol; PtdOH , phosphatidic acid; PtdCho , phosphatidylcholine; PtdEtn , phosphatidylethanolamine; PtdSer , phosphatidylserine; PtdEtOH , phosphatidylethanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

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[^3H]Inositol-labelled platelets were finally suspended in Tyrode-Hepes buffer containing 10 mM-LiCl and left for 20 min before use.

Platelet incubation and lipid extraction

Platelets ($1 \times 10^9/250 \mu\text{l}$) were equilibrated at 37 °C for 5 min and then activated with thrombin. Just before incubation, 1 mM- Ca^{2+} or 1 mM-EGTA was added. At appropriate times, incubations were terminated by addition of 2 ml of chloroform/methanol (1:2, v/v). For polyphosphoinositide analysis, the reaction was terminated by adding 2 ml of chloroform/methanol/HCl (20:40:1, by vol.). The lipids were extracted by the method of Bligh & Dyer [26] with slight modification [24]. The extracted lipids were analysed within 24 h. If necessary, the samples were dissolved in hexane/benzene (1:1, v/v) and were stored at -20 °C to minimize acyl-group migration.

Assay of DAG mass content

DAG mass content in crude lipid fractions was measured by the conversion of DAG into [^{32}P]phosphatidic acid (PtdOH) by *E. coli* DAG kinase in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP, by the method of Preiss *et al.* [13]. Incubations were carried out for 60 min at 30 °C. To quantify the content of alkylacyl-glycerol, 500 units of *Rhizopus arrhizus* lipase [27], which possesses phospholipase A_1 activity, was added after 60 min of incubation, and then incubation continued for an additional 60 min. Under these conditions, standard diacyl-PtdOH (from 1 nmol of DAG) was completely hydrolysed. After extraction, ^{32}P -labelled lipids were separated on silica-gel 60 plates in the solvent system chloroform/methanol/acetic acid/water (30:15:4:2, by vol.). Radioactive spots were identified by autoradiography and were scraped into scintillation vials, to which 5 ml of scintillation cocktail was added. The radioactivity was counted in a Packard scintillation counter (Tricarb 4000). The amount of DAG present in the samples were calculated from the amount of [^{32}P]PtdOH produced [13].

Measurement of inositol phosphates

The aqueous phase of chloroform/methanol/HCl extract was neutralized with 1 M-Tris and diluted to 4 ml with water. Labelled inositol-phosphates were then separated by chromatography on a 1.0 ml Bio-Rad AG 1X8 anion-exchange column as described by Berridge *et al.* [28]. Individual fractions (4 ml) were mixed with Aquasol-2 and the radioactivity was counted. The mass of $\text{Ins}(1,4,5)\text{P}_3$ was measured as described previously [29].

Analysis of water-soluble choline metabolites

The aqueous phase of the chloroform/methanol extract was dried and resuspended in 50 % (v/v) ethanol. A portion of each sample was spotted on a Whatman LK6D plate, which was then developed with 0.5 % NaCl/methanol/conc. NH_3 (50:50:1, by vol.) [30]. Spots corresponding to choline, phosphocholine and glycerophosphocholine standards were scraped into vials and counted for radioactivity.

Analysis of radiolabelled lipids

Radiolabelled phospholipids were separated by two-dimensional t.l.c. on silica-gel 60 plates impregnated with 2.5 % (w/v) magnesium acetate, by using chloroform/methanol/13.5 M- NH_3 (65:35:6, by vol.) in the first direction, and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) in the second direction [31]. Neutral lipids were separated on silica-gel 60 plates impregnated with 0.4 M-boric acid in the solvent system chloroform/acetone (24:1, v/v). For the separation of phosphoinositides, lipids were applied to a LK6D plate impregnated with 1 % potassium oxalate containing 2 mM-

EDTA. Plate was developed in chloroform/methanol/4 M- NH_3 (9:7:3, by vol.). Individual lipids were observed by exposure of the plates to iodine vapour and identified by co-migration with authentic standards. The areas corresponding to individual lipid fractions were scraped into vials, and radioactivity was determined in a liquid-scintillation counter as described above.

Measurement of phosphorus content of phospholipids

Phospholipids were separated by two-dimensional t.l.c. as described above. Fractionated phospholipids were digested with 70 % (w/v) HClO_4 at 180 °C for 30 min, and inorganic phosphorus content was determined [32,33].

Materials

[^3H]Arachidonic acid (207 Ci/mmol), [^3H]palmitic acid (180 Ci/mmol), [^3H]inositol (72 Ci/mmol), DAG assay system and $\text{Ins}(1,4,5)\text{P}_3$ assay system were obtained from Amersham. [^3H]Myristic acid (42 Ci/mmol) and Aquasol-2 were from New England Nuclear. [*methyl*- ^3H]Choline chloride (85 Ci/mmol) was from American Radiolabeled Chemicals. Silica-gel 60 and LK6D plates were purchased from Merck and Whatman respectively. *Rhizopus* lipase was from Boehringer Mannheim. Other chemicals were of reagent grade.

RESULTS

DAG formation

Platelets were stimulated with 1 unit of thrombin/ml for the indicated periods of time. After lipid extraction, the DAG mass contents were determined. Control resting platelets contain 98 ± 36 pmol of DAG/ 10^9 cells ($n = 8$). Stimulation with thrombin induced a biphasic accumulation of DAG, as shown in Fig. 1. The early phase showed a sharp rapid peak at 10 s after stimulation. On the other hand, the second-phase DAG generation is rather slow and sustained, reaching a maximum at 2 min. When extracellular Ca^{2+} was chelated by EGTA, the thrombin-induced increase in DAG was monophasic, reached its peak at 10 s and returned to the control level by 1 min. The second phase, observed in the presence of extracellular Ca^{2+} , disappeared.

Recently the accumulation of alkylacylglycerol from alkylacyl-PtdCho has been demonstrated, when human neutrophils were stimulated with *N*-formylmethionyl-leucyl-phenylalanine [9,27]. Platelets also contain alkylacyl-PtdCho, which is about 10 % of total diacyl-PtdCho [34]. Next, the formation of alkylacylglycerol was investigated in thrombin-stimulated platelets. *E. coli* DAG kinase converts alkylacylglycerol to alkylacyl-[^{32}P]PtdOH. However, the latter could not be resolved on the t.l.c. plate, because it co-migrated with diacyl-[^{32}P]PtdOH. Therefore *Rhizopus arrhizus* phospholipase A_1 treatment was used for quantitative measurement of these two PtdOH types, in which diacyl-[^{32}P]PtdOH was hydrolysed to lyso-[^{32}P]PtdOH, whereas alkylacyl-[^{32}P]PtdOH was not. Thus cell extracts subjected to *E. coli* DAG kinase were assayed for total amounts of both DAG and alkylacylglycerol. Thrombin did not stimulate the accumulation of alkylacylglycerol in platelets throughout 5 min of incubation (results not shown), indicating that only the DAG type was produced.

$\text{Ins}(1,4,5)\text{P}_3$ formation

In contrast with DAG, $\text{Ins}(1,4,5)\text{P}_3$ production in response to thrombin was monophasic (Fig. 2). Upon stimulation, the mass content of $\text{Ins}(1,4,5)\text{P}_3$ increased rapidly and reached a peak within 2 s, the earliest time technically measurable. Control resting platelets contain about 1 pmol of $\text{Ins}(1,4,5)\text{P}_3/10^9$ platelets. The maximal $\text{Ins}(1,4,5)\text{P}_3$ content at 2 s after thrombin

Fig. 1. Time course of DAG mass change in thrombin-stimulated human platelets

Human platelets were incubated without (○) or with (●, ▲) 1 unit of thrombin/ml for the times indicated. Lipids were extracted and the DAG content was determined as described in the Experimental section. Each point is the mean of triplicate determinations. Similar results were obtained in seven additional experiments.

Fig. 2. Time course of $InsP_3$ mass change in thrombin-stimulated human platelets

Human platelets were incubated without (○) or with (●) 1 unit of thrombin/ml for the times indicated. $InsP_3$ mass content was determined as described in the Experimental section. Data are means $\pm$ range of duplicate determinations of a representative experiment. Five other experiments gave similar results.

stimulation was 10.5 pmol/ 10^9 platelets. Within 30 s $Ins(1,4,5)P_3$ returned nearly to the basal level, and no increase was observed thereafter. The $Ins(1,4,5)P_3$ response in platelets was very prompt. In platelet-activating-factor-stimulated human neutrophils, $Ins(1,4,5)P_3$ reached a peak at 10 s and was increased by about 7-fold (from 3.8 to 26.3 pmol/ 10^7 cells).

Mass changes of phospholipids

The time courses of DAG and $Ins(1,4,5)P_3$ formation suggest that the second phase of DAG is produced from other source(s) than $PtdInsP_2$. To explore DAG sources, the alterations in phospholipids upon thrombin addition were quantitatively measured. Mass contents of PtdCho, phosphatidylethanolamine

Table 1. Thrombin-induced phospholipid changes in human platelets

Human gel-filtered platelets were stimulated with 1 unit of thrombin/ml for 2 and 5 min. Phospholipid contents were determined as described in the Experimental section. The content of unstimulated control was designated as 100 %. The 100 % values for PtdCho, PtdEtn, PtdSer and PtdIns correspond to 151.4, 108.2, 34.8 and 16.5 nmol/ 10^9 platelets respectively. The results are the means $\pm$ s.d. of five experiments each performed in duplicate: ^a $P < 0.05$, ^b $P < 0.01$ compared with control.

Phospholipid	Incubation time...	Content (%)	
		2 min	5 min
PtdCho		96.3 $\pm$ 2.8 ^a	94.5 $\pm$ 3.6 ^a
PtdEtn		98.9 $\pm$ 3.5	98.7 $\pm$ 4.8
PtdSer		98.8 $\pm$ 3.5	99.1 $\pm$ 4.8
PtdIns		59.4 $\pm$ 8.7 ^b	53.3 $\pm$ 7.6 ^b

Fig. 3. Time courses of thrombin-induced radioactivity changes of PtdIns and DAG in $[^3H]$ myristic acid-labelled human platelets

Human platelets prelabelled with $[^3H]$ myristic acid were incubated without (○) or with (●) 1 unit of thrombin/ml for the times indicated. Lipids were extracted and radioactivity was determined as described in the Experimental section. Each point is the mean of triplicate determinations. Bars indicate s.d. Two other experiments gave similar results.

(PtdEtn), phosphatidylserine (PtdSer) and PtdIns were 148.5 $\pm$ 15.2, 110.8 $\pm$ 12.4, 37.2 $\pm$ 5.3 and 15.8 $\pm$ 4.7 nmol/ 10^9 platelets ($n = 5$) respectively. At 2 and 5 min after thrombin stimulation, the contents of PtdCho and PtdIns decreased (Table 1). However, significant changes were not seen in PtdEtn and PtdSer. These trends were observed in every experiment. These results indicate that PtdIns and/or PtdCho could function as DAG sources in thrombin-stimulated platelets.

Analysis of DAG sources in platelets labelled with radioactive fatty acids

When platelets were incubated with $[^3H]$ myristic acid, it was preferentially incorporated into PtdCho, being 90 % of the total radioactivity. On the other hand, the radioactivity in PtdIns did not exceed 2 %. In $[^3H]$ myristic acid-labelled platelets thrombin did not give a significant decrease in $[^3H]PtdIns$. Although thrombin caused a decrease in $[^3H]PtdCho$, $[^3H]DAG$ accumulation was hardly observed (Fig. 3). $[^3H]$ Palmitic acid was incorporated into PtdCho up to 75 % of total radioactivity, and into PtdIns as much as 8 %. Thrombin stimulation caused a decrease in $[^3H]PtdIns$ and a biphasic $[^3H]DAG$ formation (Fig.

Fig. 4. Time courses of thrombin-induced radioactivity changes of PtdIns and DAG in [^3H]palmitic acid-labelled human platelets

Human platelets prelabelled with [^3H]palmitic acid were incubated without (○) or with (●) thrombin. Other experimental conditions are described in Fig. 3. Each point is the mean of triplicate determinations. Bars indicate S.D. Two other experiments gave similar results.

Fig. 5. Accumulation of InsP in thrombin-stimulated human platelets

[^3H]Inositol-labelled human platelets were incubated without (○) or with (●) 1 unit of thrombin/ml. InsP radioactivity was determined as described in the Experimental section; that (999 d.p.m.) in unstimulated control was designated as 100%. Results are means $\pm$ range of duplicate determinations of a representative experiment. Three other experiments gave similar results.

4). Similar results were observed in [^3H]arachidonic acid-labelled platelets (results not shown). However, in [^3H]palmitic acid- or [^3H]arachidonic acid-labelled platelets, a decrease in [^3H]PtdCho was also observed. When platelets were prelabelled with radioactive fatty acids, the decrease in radioactivity in phospholipids could be explained by their hydrolysis by an A-type phospholipase. Thus the DAG source was further examined in [^3H]inositol- or [^3H]choline-labelled platelets.

Inositol phosphate formation

[^3H]Inositol was time-dependently incorporated into phosphoinositides up to 300 min. After 150 min incubation, the distribution of ^3H radioactivity in PtdIns (86%), PtdInsP (7%) and PtdInsP₂ (6%) became similar to the mass ratio of phosphoinositides [33,35]. To prevent the decrease in platelet responsiveness after long incubation, platelets were labelled for 150 min. Thrombin stimulated the increase in the radioactivity of inositol phosphates. [^3H]InsP₃ and [^3H]InsP₂ rapidly increased within

Fig. 6. Time courses of thrombin-induced radioactivity changes of phosphocholine (a) and choline (b) in [^3H]choline-labelled human platelets

Human platelets prelabelled with [^3H]choline chloride were incubated without (○) or with (●) 1 unit of thrombin/ml for the times indicated. Water-soluble choline metabolites were extracted and separated on t.l.c. plates. Details are described in the Experimental section. The radioactivity (d.p.m.) in phosphocholine (90845) and choline (9417) in unstimulated control was designated as 100%. Each point is the mean of three experiments performed in duplicate. Bars indicate S.D.

30 s, and maintained their levels for 3–5 min (results not shown). Similar trends have previously been reported [36,37]. However, the InsP₃ accumulated after 30 s appeared to be Ins(1,3,4)P₃, and Ins(1,4,5)P₃ was not detectable [38], in accordance with Fig. 2, suggesting that PtdInsP₂ hydrolysis was transient. The radioactivity of [^3H]InsP gradually increased throughout 5 min incubation (Fig. 5). The accumulation of InsP without PtdInsP₂ breakdown may reflect the hydrolysis of PtdIns by PLC.

[^3H]Choline metabolites

When platelets were incubated for 3 h with [^3H]choline, about 40% of incorporated radioactivity was found in PtdCho. The major water-soluble metabolites in resting platelets are phosphocholine and choline, which comprise approx. 45% and 5% of cell-associated radioactivity respectively. The radioactivity in [^3H]PtdCho decreased slightly but time-dependently (by about 5% at 5 min) upon thrombin stimulation. However, [^3H]phosphocholine and [^3H]choline were hardly increased (Fig. 6). The increase in radioactivity was found in lyso-PtdCho and glycerophosphocholine fractions (Table 2), suggesting PtdCho hydrolysis by an A-type phospholipase. The decrease in [^3H]PtdCho was largely (over 80% at 2 min and over 70% at 5 min) compensated by increases in [^3H]lyso-PtdCho and [^3H]glycerophosphocholine.

Phosphatidylethanol (PtdEtOH) formation

The agonist-induced DAG formation from PtdCho via the phospholipase D (PLD)/PtdOH phosphohydrolase pathway has been reported in human neutrophils [9]. In the presence of ethanol, activation of PLD results in the formation of PtdEtOH, owing to a PLD-catalysed transphosphatidylation [39]. The thrombin-induced accumulation of PtdEtOH in platelets has previously been reported by Rubin [40]. PtdEtOH formation was examined in [^3H]arachidonic acid-labelled platelets in the presence of 1% ethanol. The addition of ethanol did not affect the basal levels of [^3H]DAG and [^3H]PtdOH. We could detect some increase in the radioactivity in the PtdEtOH fraction upon thrombin stimulation (Table 3). However, at 2 min after throm-

Table 2. Thrombin-induced radioactivity changes of PtdCho, lyso-PtdCho and glycerophosphocholine in [methyl-³H]choline-labelled human platelets

Human platelets prelabelled with [methyl-³H]choline chloride were stimulated with 1 unit of thrombin/ml. Radioactivity was determined as described in the Experimental section. Results are means $\pm$ range of duplicate determinations. Two other experiments gave similar results.

Phospholipid	Incubation time (s)...	Radioactivity (d.p.m.)			
		0	60	120	300
PtdCho		81 208 $\pm$ 1201	79 341 $\pm$ 1304	77 618 $\pm$ 1012	76 264 $\pm$ 1524
Lyso-PtdCho		340 $\pm$ 38	2105 $\pm$ 94	1543 $\pm$ 82	1269 $\pm$ 97
Glycerophosphocholine		4480 $\pm$ 187	5216 $\pm$ 220	6253 $\pm$ 211	7156 $\pm$ 384

Table 3. Thrombin-induced increase in radioactivity of DAG, PtdOH and PtdEtOH in [³H]arachidonic acid-labelled platelets

[³H]Arachidonic acid-labelled human platelets were stimulated with 1 unit of thrombin/ml for 2 min in the absence or presence of 1% ethanol. Lipid extraction and determination of radioactivity were carried out as described in the Experimental section. Results are expressed in d.p.m., as (radioactivity in thrombin-stimulated platelets) – (radioactivity in control platelets). The radioactivity of DAG and PtdOH in the unstimulated control was 150 and 227 d.p.m. respectively. Ethanol had no effect on basal DAG and PtdOH levels. Data are means $\pm$ S.D. of triplicate determinations. Two other experiments gave similar results.

Ethanol	Lipid...	Radioactivity (d.p.m.)		
		DAG	PtdOH	PtdEtOH
None		487 $\pm$ 24	4314 $\pm$ 56	0
1%		478 $\pm$ 32	4292 $\pm$ 72	198 $\pm$ 18

bin stimulation, the increase in radioactivity of the PtdEtOH fraction was less than 5% of that of PtdOH. The formation of [³H]DAG and [³H]PtdOH in response to thrombin was not significantly affected by 1% ethanol.

DISCUSSION

The present results indicate that thrombin induces a biphasic accumulation of DAG in human platelets, which has previously been reported in many types of cells [14–21]. The first DAG peak was rapid and transient after stimulation. The time course of the first DAG peak well corresponded to that of a transient Ins(1,4,5) P_3 accumulation (Figs. 1 and 2). The coincident formation of Ins(1,4,5) P_3 and DAG suggest that the DAG source is PtdIns P_2 . The content of PtdIns P_2 has been shown to be about 1 nmol/10⁹ platelets [41,42]. By mass analysis Broekman [41] demonstrated that 30–40% of PtdIns P_2 was hydrolysed within 10 s upon thrombin stimulation. Stimulation with thrombin produced an increase of approx. 300 pmol of DAG/10⁹ platelets at 10 s (Fig. 1), which could be explained by hydrolysis of 30% of the PtdIns P_2 . However, the net increase in the first phase of DAG exceeds that of Ins P_3 , as often observed in other types of cells [20,21]. This may indicate a very rapid turnover of Ins(1,4,5) P_3 ; phosphorylation to Ins P_4 by Ins(1,4,5) P_3 3-kinase, or degradation to Ins P_2 by Ins(1,4,5) P_3 5-phosphatase. Alternatively, it is also likely that the first phase of DAG is produced from phospholipid(s) other than PtdIns P_2 . The molecular-species analysis of DAG and phospholipids provides more direct information about the DAG source.

The second sustained phase of DAG accumulation was observed after Ins(1,4,5) P_3 returned nearly to the basal level,

suggesting that DAG came from other phospholipid(s) than PtdIns P_2 . The phospholipid mass analysis showed the decreases in PtdIns and PtdCho upon thrombin stimulation. The direct hydrolysis of PtdIns by PLC has been demonstrated in some types of cells [14,43]. However, accumulating evidence suggests that agonist-induced PtdCho hydrolysis generates DAG. In [³H]palmitic acid- and [³H]arachidonic acid-labelled platelets, in which PtdIns was considerably radiolabelled, thrombin-induced DAG formation was detectable, with a concomitant decrease in PtdIns. In contrast, the increase in radioactivity of DAG was not observed in [³H]myristic acid-labelled platelets, in which PtdIns was poorly labelled. In platelets prelabelled with [methyl-³H]choline, thrombin did not significantly change the levels of [³H]phosphocholine and [³H]choline. In platelets prelabelled with radioactive fatty acids or [methyl-³H]choline, the decrease in radioactivity of PtdCho was observed. The data in Table 2 suggest that this may be due to its hydrolysis by an A-type phospholipase. Our assumption was supported by the literature, which reported quantitative measurements of thrombin-induced formation of lyso-PtdCho [31,44] and loss of arachidonic acid content from PtdCho [45,46].

The decrease in PtdIns was accompanied with accumulation of [³H]InsP (Table 1 and Fig. 5) and may reflect generation of the second phase of DAG from PtdIns. Although InsP is formed through degradation of inositol polyphosphates, the accumulation of [³H]InsP far exceeded the levels of [³H]Ins P_2 and [³H]Ins P_3 , which decreased very slowly (results not shown). InsP formed through inositol polyphosphate hydrolysis appeared to be mainly Ins(4) P [47]. However, Siess & Binder [37] observed the accumulation of Ins(1) P and Ins(2) P (probably derived from inositol 1,2-cyclic monophosphate). Our assumption is also in good agreement with previous reports. Wilson *et al.* [42] have described that during thrombin stimulation, PtdIns, in addition to PtdIns P_2 , is hydrolysed directly by PLC. Holmsen's group [48] argued against their hypothesis to draw this conclusion, but subscribed to the proposal that PtdIns is hydrolysed by PLC. Broekman *et al.* [49] observed that in thrombin-stimulated platelets the fatty acid composition of PtdOH was similar to that of PtdIns, mostly stearyl-arachidonoyl molecular species. As PtdOH is generated from DAG by DAG kinase, it is reasonable to consider that a principal part of DAG is derived from PtdIns. At 2 min after thrombin stimulation, the loss of PtdIns (6.7 nmol/10⁹ platelets) far exceeded the gain of DAG (0.3 nmol/10⁹ platelets). On the basis of the findings that the loss of PtdIns was almost compensated by the sum of increases in PtdOH, PtdInsP and PtdIns P_2 , Tysnes *et al.* [33] suggested the rapid conversion of DAG into PtdOH. The second phase of DAG formation was extracellular-Ca²⁺-dependent. Several types of PLCs in platelets have been purified [50–53]. Most of them hydrolyse PtdIns in a Ca²⁺-dependent manner, and require higher concentrations of Ca²⁺ than for PtdIns P_2 hydrolysis. Taken together, it may be possible to assume that the sustained

increase in intracellular Ca^{2+} by Ca^{2+} influx triggers PtdIns hydrolysis by PLC.

It has recently been proposed that DAG was mainly derived from the PtdCho-PLD/PtdOH phosphohydrolase pathway in *N*-formylmethionyl-leucyl-phenylalanine-stimulated human neutrophils [9]. In the presence of ethanol PLD catalyses a transphosphatidyl transfer reaction to form PtdEtOH. The formation of PtdEtOH is a sensitive and useful indicator of PLD activity [39]. However, stimulatory or inhibitory effects of ethanol on platelet phospholipid turnover have been demonstrated. Rand *et al.* [54] have shown that ethanol has inhibitory effects on platelet functions induced by very low concentrations (< 0.01 unit/ml) of thrombin by attenuating the production of InsP_3 . The responses to ADP and higher concentrations of thrombin were not affected by ethanol. In contrast, Rubin & Hoek [55] have reported the opposite results, that ethanol itself stimulates PLC by facilitating its interaction with a GTP-binding protein, resulting in the formation of InsP_3 . However, in the present study ethanol did not change the levels of [^3H]DAG and [^3H]PtdOH in [^3H]arachidonic acid-labelled platelets. The production of DAG and PtdOH in response to 1 unit of thrombin/ml was not significantly affected by ethanol. The formation of PtdEtOH was observed in thrombin-stimulated platelets, indicating the presence of PLD in human platelets, as previously described [40]. However, the increase in PtdEtOH was much less compared with PtdOH production. These results suggest that PLD plays a minor role, if any, in platelets. Accordingly, Randall *et al.* [56] reported that the PLD activity of human platelets is one-fiftieth of that of human neutrophils.

Accumulating evidence has suggested that the biphasic DAG formation, especially the second sustained phase, is closely coupled with mitogenic signal transduction [16,57,58]. We have shown here that thrombin stimulates a biphasic DAG accumulation in the platelet, anucleated and non-mitogenic cell. The first phase of DAG accumulation and $\text{Ins}(1,4,5)\text{P}_3$ production was independent of extracellular Ca^{2+} . 5-Hydroxytryptamine secretion in response to thrombin occurred regardless of extracellular Ca^{2+} . Thus $\text{Ins}(1,4,5)\text{P}_3$ and first-phase DAG accumulation may be closely related to granule secretion. In contrast, second-phase DAG formation was largely prevented in the absence of extracellular Ca^{2+} . Platelet aggregation was dependent on extracellular Ca^{2+} . Taken together, it is tempting to speculate that the second phase of DAG causes PKC activation, which may be one of the signals leading to platelet aggregation.

In summary, from the present results, we propose that thrombin stimulates a biphasic DAG accumulation, and that the first phase of DAG is derived from PtdIns $_2$ and the second phase is mainly from PtdIns. We know that data based on prelabelling experiments do not always indicate mass changes. Each prelabelling experiment individually provides insufficient evidence to conclude that the second phase of DAG comes from PtdIns. However, all experiments showed consistent results. Based on the combined weight of the present data, the above proposal seems to be reasonable. Further studies are necessary to clarify the physiological significance and the regulatory mechanism of DAG formation in platelets.

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