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Inositol lipids, phosphatidate and diacylglycerol share stearoylarachidonoylglycerol as a common backbone in thrombin-stimulated human platelets

Gerard MAUCO,* Carol A. DANGELMAIER and J. Bryan SMITH
Center for Thrombosis Research, Temple University, Health Science Center, 3400 North Broad Street, Philadelphia, PA 19140, U.S.A.

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Gel-filtered human platelets were stimulated with 5 i.u. of thrombin/ml for times up to 1 min. The fatty acid composition of inositol-containing phospholipids, phosphatidic acid and diacylglycerol was determined by g.l.c. in control and thrombin-stimulated platelet suspensions. Inositol phospholipids were found to have similar proportions of stearic and arachidonic acids, the sum of these representing 86.6% of the total fatty acids in phosphatidylinositol (PtdIns), 76.9% in phosphatidylinositol 4-phosphate (PtdIns4*P*) and 85.4% in phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. However, arachidonic and stearic acids were less abundant in phosphatidic acid (PtdA) and diacylglycerols in non-stimulated platelets. A transient decrease in the mass of PtdIns(4,5)P_2 was observed after 5–10s of thrombin stimulation, followed by an increase after 30s. The amounts of PtdIns4*P* and PtdIns decreased throughout the experiment. A transient accumulation of stearoyl-arachidonoylglycerol was observed at 5s, whereas stearoylarachidonoylglycerol 3-phosphate (PtdA) was produced in increasing amounts throughout the experiment. The decrease in inositol-containing phospholipids was not fully compensated for by the production of diacylglycerol or PtdA [or PtdIns(4,5)P_2] at 1 min. All the changes in inositol phospholipids, as well as those observed in diacylglycerols and PtdA, were due to a parallel reduction or increase in the contents of stearic and arachidonic acids, with a stoichiometry equal to 1. Taken together, this suggests an interconversion of all these lipids with the utilization of a common backbone, stearoylarachidonoylglycerol. The deacylation of this diacylglycerol could account for up to 4–5 nmol of arachidonate/10^9 platelets after 1 min stimulation by thrombin.

Activation of the turnover of PtdIns and its phosphorylated derivatives PtdIns4*P* and PtdIns(4,5)P_2 is a common phenomenon accompanying receptor activation of cells (Michell, 1975; Berridge, 1980, 1984; Downes & Michell, 1982; Fisher *et al.*, 1984). Platelets display the same kind of response upon stimulation by thrombin (Lloyd & Mustard, 1974; Billah & Lapetina, 1982), ADP (Lloyd *et al.*, 1973; Vickers *et al.*, 1982) or platelet activating factor (MacIntyre & Pollock, 1983; Mauco *et al.*, 1983). This activated PtdIns metabolism is believed to be primarily due to the cleavage of inositol phospholipids by a phospholipase C active on PtdIns (Rittenhouse-Simmons, 1979; Mauco *et al.*, 1979*a*; Siess & Lapetina, 1983; Lenstra *et al.*, 1984) and/or on PtdIns(4,5)P_2 (Agranoff *et al.*, 1983; Low & Weglicki, 1983; Rittenhouse, 1983). Both pathways produce diacylglycerol, which can be metabolized further either by (1) degradation by a lipase which releases arachidonic acid (Mauco *et al.*, 1978, 1979*b*; Bell *et al.*, 1979) for transformation into thromboxane A_2, or (2) phosphorylation into PtdA (Lapetina & Cuatrecasas, 1979; Mauco *et al.*, 1978) and sub-

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdA, phosphatidic acid; lysoPtdIns, lysophosphatidylinositol; lysoPtdA, lysophosphatidic acid; CMP-PtdA: cytidine monophosphate phosphatidic acid.

*On leave of absence from Universite P. Sabatier, Faculté de Médecine Purpan, Toulouse, France. Present address: INSERM Unité 101, Hôpital Purpan, 31059 Toulouse-Cedex, France.

sequent transformation into CMP-PtdA (Lucas *et al.*, 1970), leading to the resynthesis of PtdIns, giving the so-called PtdIns cycle (Michell, 1975).

To date, most of the studies on platelets have been carried out by measuring changes in radioactivity in ^{32}P- or arachidonate-prelabelled cells and specific radioactivities have not been determined. Thus, only partial data are available regarding the relative mass changes during inositol phospholipid metabolism. Furthermore, available information is fragmentary, since there is no study describing the transformations of all the lipids involved in the inositol phospholipid responses. Broekman *et al.* (1980, 1981) measured the amounts of PtdIns and PtdA and Perret *et al.* (1983) have described an increase in the mass of PtdIns4*P* and PtdIns(4,5)P_2 2min after thrombin stimulation. No information is available on their mass at earlier times, when a decrease of [^{32}P]-PtdIns(4,5)P_2 is observed concomitantly with the appearance of [^{32}P]inositol-1,4,5-trisphosphate (Agranoff *et al.*, 1983). Moreover, the fatty acid composition of PtdIns4*P* and PtdIns(4,5)P_2 is unknown and the decrease in PtdIns is not exactly compensated by an increase of PtdA after thrombin stimulation (Broekman *et al.*, 1980, 1981).

Thus it appeared of interest to measure the amounts of all the lipids involved in the inositol phospholipid response in the same platelet preparation in order to discriminate between the different metabolic pathways briefly described above.

Experimental

Platelet preparation and labelling

Human blood was obtained from informed healthy volunteers, who had not taken any drug during the previous week. Blood was treated with citric acid/citrate/dextrose as anticoagulant according to Aster & Jandl (1964). The platelet-rich plasma obtained by centrifugation at 180*g* for 15min at room temperature was acidified to pH6.5 with the anticoagulant mixture and then recentrifuged (1500*g*, 15min, room temperature). The platelet pellet was resuspended in 10ml of autologous platelet-poor plasma and incubated with $^{32}P_i$ (0.3mCi/ml; ICN, Irvine, CA, U.S.A.) for 1h at 37°C. The radioactive platelets were gel-filtered on Sepharose 2B (Pharmacia, Uppsala, Sweden) using a Ca^{2+}-free Tyrode's buffer without phosphate and containing 0.2% serum albumin and 5mM-glucose (Lages *et al.*, 1975).

Platelet stimulation and lipid extraction

Aliquots [$(2.0–2.5)\times10^9$ cells] of the labelled, gel-filtered platelets were warmed at 37°C for 3min and either extracted directly or stimulated with 5i.u. of thrombin/ml for times up to 1min, without stirring. The extraction was performed at 4°C as previously described (Schacht, 1981; Mauco *et al.*, 1983). Briefly, to 1vol. of platelet suspension was added sodium EDTA (20mM final concentration) plus 3.75 vol. of ice-cold chloroform/methanol (1:2, v/v). The extract was partitioned into two phases by addition of 1.25 vol. of chloroform and 1.25 vol. of 2.4M-HCl. The lower phase was removed and the upper phase was washed with 2.5 vol. of chloroform. The pooled organic extracts were evaporated under N_2 at 37°C and then stored in chloroform/methanol (2:1, v/v) at −18°C. All the above procedure was carried out in presence of butylated hydroxytoluene (50μg/ml) as an antioxidant.

Separation of the lipid classes

This was achieved by t.l.c. on 0.25mm silica gel plates (Merck, Darmstadt, Germany) using one of the following solvents: (*a*) chloroform/methanol/acetic acid/water (81:10:45:1, by vol.) for the resolution of PtdIns (Hauser & Eichberg, 1975), (*b*) chloroform/methanol/4M-NH_3 (9:7:2, by vol.) for the resolution of PtdIns4*P* and PtdIns(4,5)P_2 (Lloyd *et al.*, 1972, 1973), (*c*) upper layer of ethyl acetate/iso-octane/acetic acid/water (9:5:2:10, by vol.) (Hong & Levine, 1976) for PtdA, and (*d*) chloroform/methanol/conc. HCl (87:13:0.5, by vol.) for diacylglycerol. The double development proposed by Skipski *et al.* (1968) also was used in order to check on the purity of the diacylglycerols obtained using the previous system. The acids contained in the solvents were neutralized by exposure to NH_3 fumes. Resolved compounds were detected using a primulin spray (Sigma) and/or by autoradiography.

G.l.c. of the fatty acid methyl esters

The silica scrapings were treated for 10min with 2M-sodium methoxide at 50°C in the presence of 5μg of methyl heptadecanoate as an internal standard. After neutralization of the mixture the fatty methyl esters were extracted into hexane. The dried esters were then dissolved in 50μl of CS_2. G.l.c. was performed in a Hewlett-Packard (Avondale, PA, U.S.A.) chromatograph model 5730A fitted with two 6ft×$\frac{1}{8}$in (1.83m×3mm) glass columns containing 10% SP-2330 on 100/200 Chromosorb W AW (Supelco, Bellefonte, PA, U.S.A.). The chromatograph was operated in a differential mode with a temperature gradient as follows: 2min at 170°C, increasing at 4°C/min to 220°C and holding for 8min. Injection and flame ionization ports were at 250°C. The carrier gas was N_2 (20ml/min). Quantification of the different fatty acid methyl esters was performed with an automatic integrator (Hewlett-Packard model

3390A) using methyl heptadecanoate as the internal standard.

Results

Fatty acid composition of PtdIns(4,5)P_2, PtdIns4P, PtdIns, PtdA and diacylglycerols in gel-filtered platelets

The masses obtained by fatty acid analysis of each individual platelet lipid are in close agreement with those obtained by others (Cohen & Derksen, 1969; Broekman *et al.*, 1980; Perret *et al.*, 1983; Mahadaveppa & Holub, 1983) (Table 1). All the inositol-containing phospholipids displayed an almost identical fatty acid composition; stearic and arachidonic acids were present in equimolar quantities and accounted for 86.6% of the fatty acids in PtdIns, 76.9% in PtdIns4*P* and 85.4% in PtdIns(4,5)P_2. The remaining fatty acids were mainly oleic and palmitic acid, with small amounts of linoleate in PtdIns4*P*. In non-stimulated platelets small amounts of PtdA were found. Similar quantities already have been reported by Vickers *et al.* (1982) in rabbit platelets. However this PtdA contained very low amounts of ^{32}P compared with the specific radioactivity of PtdIns and of that of PtdIns4*P* and PtdIns(4,5)P_2 (results not shown). Furthermore the fatty acid content was different from that of the inositol phospholipids. Taken together this might indicate that we detected some of the PtdA involved in synthesis *de novo* of neutral phospholipids. Finally, we were able to detect minute amounts of diacylglycerols in resting platelets. The main fatty acid was oleate (34%) with palmitate, stearate and linoleate being present in equivalent amounts. This fatty acid distribution is close to that observed by Marcus *et al.* (1969) in triacylglycerols, suggesting that gel-filtered platelets contain very small amounts of diacylglycerols involved in neutral lipid metabolism.

Effect of thrombin on the fatty acid content of inositol-containing phospholipids, PtdA and diacylglycerols

G.l.c. was used to follow both the mass and fatty acid composition of each individual lipid. The most striking observation was that in all the lipids involved in the PtdIns response stearic and arachidonic acids behaved in a totally parallel manner (Figs. 1, 2, 3 and 4). PtdIns(4,5)P_2 mass decreased 5–10s after the addition of thrombin, due to a simultaneous disappearance of stearate and arachidonate (Fig. 1*a*). Likewise, an increase of these two fatty acids was responsible for the subsequent increase in mass. These variations paralleled those observed by ^{32}P and [^{3}H]-arachidonate measurements (results not shown). The same parallelism between the hydrolysis of stearic and arachidonic acid accounted for the reduction of the mass of PtdIns4*P*, which was observed as soon as 10s after addition of thrombin but subsequently reached a plateau (Fig. 1*b*). Interestingly, the other fatty acids present in PtdIns4*P* were not affected. A decrease in the mass of PtdIns was detected at 5s, accelerated between 10 and 30s and continued throughout the experiment (Fig. 2). This hydrolysis affected mainly stearic and arachidonic acids with a slight participation of oleic acid. On the other hand, a sharp and rapid increase of stearoylarachidonoylglycerol was observed (Fig. 3*a*). After reaching a maximum at 5s, the level of diacylglycerol fell rapidly but was still elevated at the end of the experiment. Very little arachidonate was detected in diacylglycerol in resting cells but it appeared upon thrombin stimulation and its increase was closely paralleled by stearic acid. The other fatty acids remained at a constant level (Fig. 3*b*). Thus, we observed

Table 1. *Amounts and fatty acid composition of inositol-containing phospholipids, PtdA and diacylglycerol, in non-stimulated platelets*

The lipids from gel-filtered platelets were extracted, resolved by t.l.c. and trans-esterified with sodium methoxide. The mass of each individual lipid was obtained as the sum of all the fatty acid amounts determined by g.l.c. Fatty acid data are percentages of the total fatty acids. Results are means ± S.E.M. of four to eight independent experiments.

	Mass (nmol/10^9 cells) of:				
	PtdIns	PtdIns4*P*	PtdIns(4,5)P_2	PtdA	Diacylglycerol
	18.8 ± 0.3	3.1 ± 0.5	1.1 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
	Fatty acid composition (%)				
Fatty acid					
Palmitate	2.1 ± 0.7	7.3 ± 1.6	6.0 ± 1.4	14.0 ± 4.0	18.8 ± 3.7
Stearate	41.7 ± 1.1	39.7 ± 1.5	45.4 ± 1.9	33.2 ± 4.5	18.0 ± 6.0
Oleate	9.2 ± 2.7	13.5 ± 1.3	9.0 ± 1.2	27.0 ± 3.8	34.1 ± 9.8
Linoleate	Traces	1.8 ± 1.0	Traces	Traces	16.6 ± 5.7
Arachidonate	46.9 ± 2.3	37.2 ± 3.5	40.0 ± 2.0	24.0 ± 1.0	6.9 ± 6.5

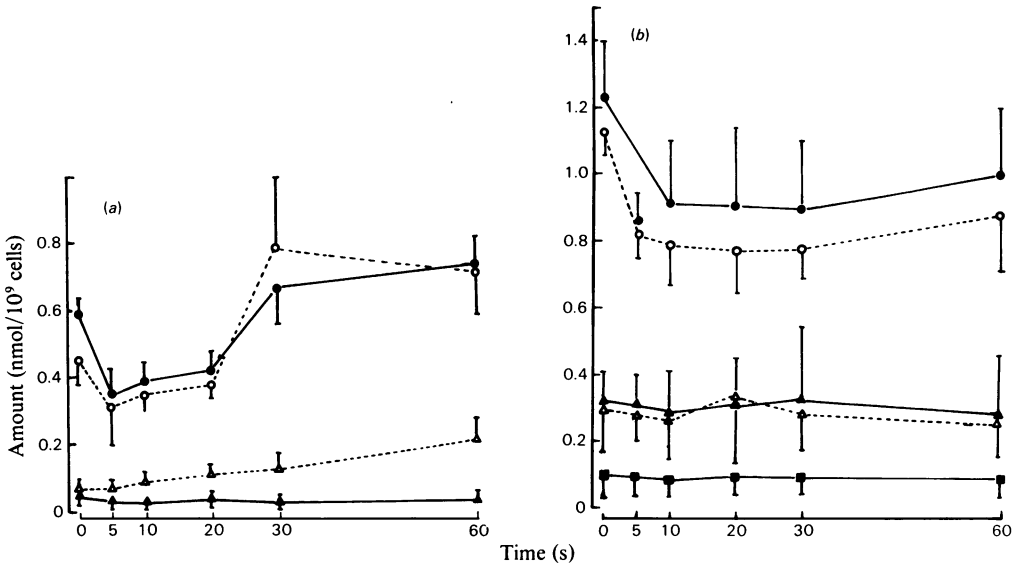

Fig. 1. *Changes in the amounts of fatty acids (a) in PtdIns(4,5)P_2 and (b) in PtdIns4P after stimulation by thrombin*
Human gel-filtered platelets were stimulated by 5 i.u. of thrombin/ml and lipids were extracted and trans-esterified as described under 'Experimental'. Fatty acid amounts were estimated by g.l.c. Results are mean ± S.E.M. for four independent experiments. ○, Arachidonic acid; ●, stearic acid; △, oleic acid; ■, linoleic acid; ▲, palmitic acid.

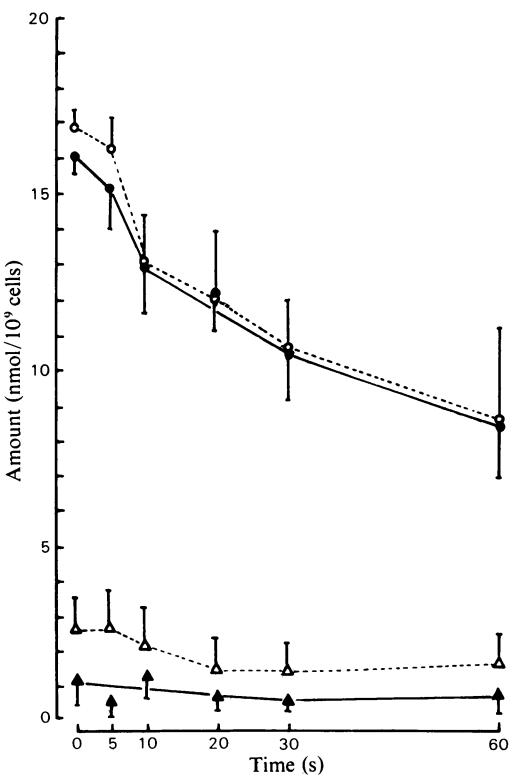

Fig. 2. *Changes in the amounts of fatty acids in PtdIns after addition of thrombin*
See legend to Fig. 1 for details.

a specific increase of stearoylarachidonoylglycerol concomitant with the initial hydrolysis of stearoylarachidonoyl-PtdIns, -PtdIns4*P* and -PtdIns(4,5)P_2. The increase of phosphatidic acid was slower than that of diacylglycerols and reached a plateau at 30s (Fig. 4). Here also, the major change was an increase in stearic and arachidonic acid in almost equimolar quantities.

In all the lipids studied oleic acid followed the variations of arachidonic and stearic acids but its relative contribution was minor. Linoleic and palmitic acids were not appreciably modified. Since arachidonate and stearate vary in a parallel manner and in equimolar amounts throughout the whole cycle we can assume that they are utilized as a common backbone, stearoylarachidonoylglycerol, during the PtdIns cycle.

Mass recovery in inositol phospholipids and related compounds during thrombin stimulation

Table 2 reports the actual changes in mass of all the lipids involved in the PtdIns response at different times after addition of thrombin. Interestingly, the hydrolysis of PtdIns and PtdIns4*P* was not compensated for by an increase of PtdA and diacylglycerol, or later on by that of PtdIns(4,5)P_2. At 60s after addition of thrombin, only 38% of the hydrolysed PtdIns was accounted for by increases in diacylglycerol, PtdA and PtdIns(4,5)P_2. Using autoradiography of ^{32}P-

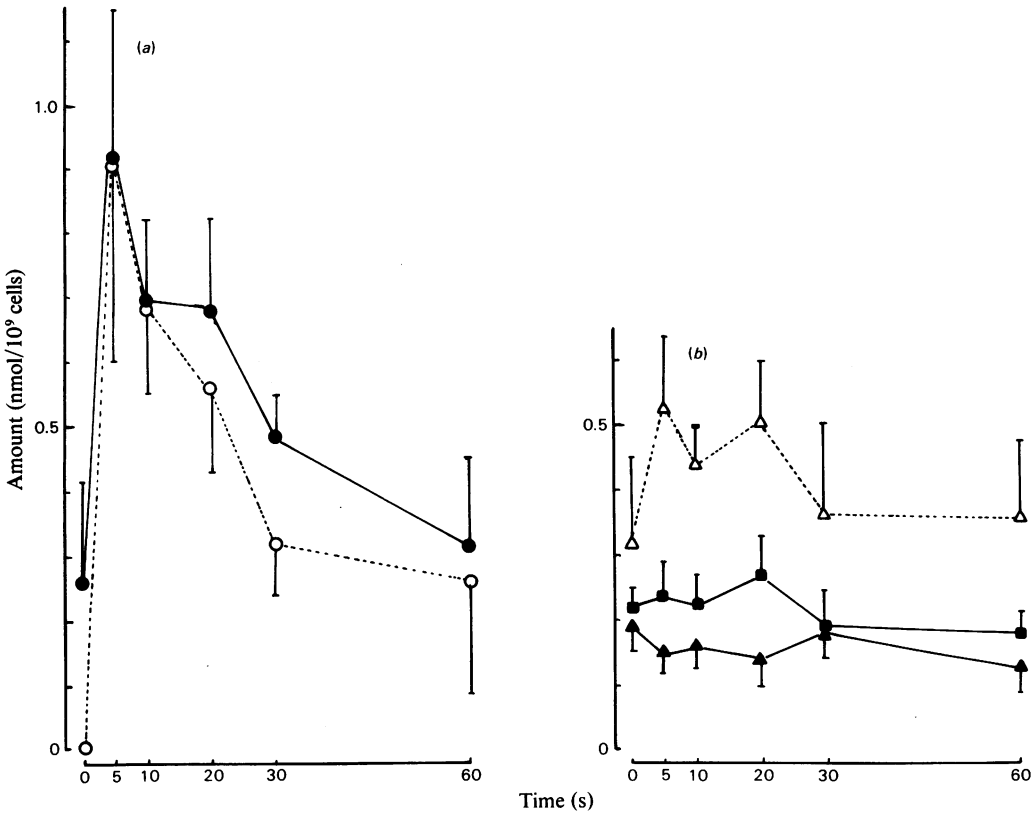

Fig. 3. *Changes in the amounts of fatty acids in diacylglycerols after addition of thrombin*
(*a*) Stearic and arachidonic acids; (*b*) other fatty acids. See the legend to Fig. 1 for details.

Table 2. *Variations of the amounts of inositol-containing phospholipids, PtdA and diacylglycerols during thrombin stimulation*
Results are calculated from Figs. 1–4 as the difference in amounts of a given lipid before and after stimulation by thrombin (5i.u./ml) and are expressed as nmol/10^9 platelets.

	Mass (nmol/10^9 platelets)				
Time (s) ...	5	10	20	30	60
PtdIns	−0.9	−3.5	−4.6	−5.7	−6.6
PtdIns4*P*	−0.7	−0.8	−0.8	−0.8	−0.6
PtdIns(4,5)P_2	−0.3	−0.3	−0.1	+0.6	+0.5
PtdA	+0.7	+1.0	+2.1	+2.5	+2.2
Diacylglycerol	+0.7	+0.5	+0.5	+0.3	+0.2
Total of losses	1.9	4.6	5.5	6.5	7.2
Total of gains	1.4	1.5	2.6	3.2	2.9
Lost inositol lipid not recovered as either diacylglycerol or PtdA (%)	26	67	53	51	60

labelled platelet extracts we could not detect appreciable amounts of lysoPtdIns. The only lyso derivative appearing upon thrombin stimulation was lysoPtdA. However, as we previously reported, its amount was less than 10% of the total PtdA obtained under these conditions (Mauco *et al.*, 1978) and it was below the sensitivity (0.1 nmol/10^9 cells) of the g.l.c. procedure used in the present studies. No monoacylglycerol production was detected.

Discussion

We report here on the fatty acid composition of all the components of the phosphatidylinositol cycle that were present in measurable amounts in

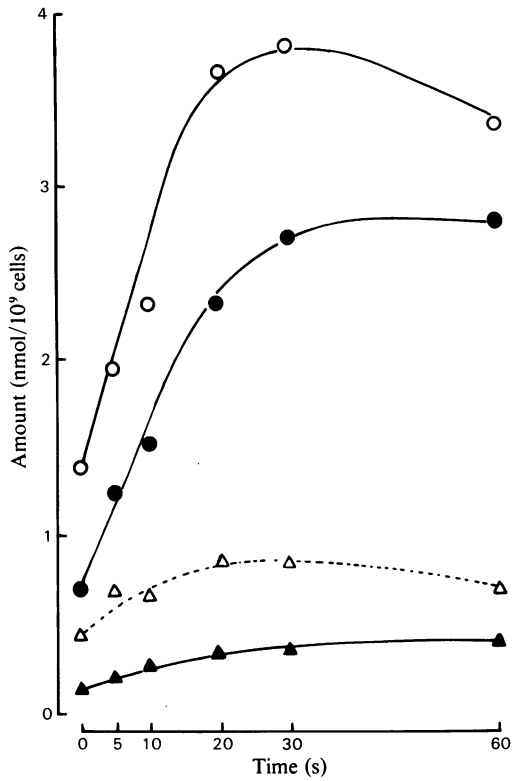

Fig. 4. *Changes in the amounts of fatty acids in PtdA after addition of thrombin*
See the legend to Fig. 1 for details. Results are the mean of two independent determinations.

human platelets. Our approach relied on g.l.c. This allowed us to follow not only the fatty acid content of each of these lipids but also their mass. Our results confirm and extend some of previously published studies on the amounts of lipids present in resting as well as thrombin-stimulated human platelets (Cohen & Derksen, 1969; Marcus *et al.*, 1969; Broekman *et al.*, 1980, 1981; Vickers *et al.*, 1982; Perret *et al.*, 1983). However, for the first time, all the elements of the cycle have been measured in the same platelet preparation. Furthermore, the characterization of the fatty acid content of PtdIns4*P* and PtdIns(4,5)P_2 is reported for the first time. Table 1 shows that these lipids comprise 18% of the inositol lipids of platelets.

The most interesting observation was that an identical response is observed in all the elements of the phosphatidylinositol cycle. Arachidonate and stearate are the main contributors of the changes observed upon thrombin stimulation; since their stoichiometry is constantly equal to 1 throughout the whole cycle it seems likely that stearic and arachidonic acids are part of an entity, stearoyl-arachidonoylglycerol. A corollary of this is that the key enzyme(s) for the phosphatidylinositol response appears to be the phospholipase(s) C suggested by Michell (1975) for PtdIns breakdown. In thrombin-stimulated platelets the phospholipase C activity seems to act not only on PtdIns (Mauco *et al.*, 1979*a*; Rittenhouse-Simmons, 1979; Siess & Lapetina, 1983; Lenstra *et al.*, 1984) but also on PtdIns4*P* (Rittenhouse, 1983; Graff *et al.*, 1984) and PtdIns(4,5)P_2 (Agranoff *et al.*, 1983; Rittenhouse, 1983). Our data are consistent with a possible hydrolysis of all the inositol phospholipids by such phosphodiesterases, since all are decreased after thrombin stimulation and this decrease can be attributed to a simultaneous reduction of stearate and arachidonate as one would expect after phospholipase C action. Low & Weglicki (1983) found that myocardial phospholipase C hydrolysed polyphosphoinositides as well as PtdIns. However, it must be noted that our data do not provide definitive evidence for such activities, since we did not measure the simultaneous production of inositol 1-phosphate, 1,4-bisphosphate and 1,4,5-trisphosphate, which can be expected after phospholipase C cleavage of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, respectively. Also we cannot discount totally the simultaneous deacylation of the phospholipids by a phospholipase A and a lysophospholipase. Phospholipase A_2 activities have been reported to act on PtdIns (Billah *et al.*, 1981) or PtdA (Billah & Lapetina, 1982). The appearance of the small amounts of lysophosphatidic acid also could be attributed to phosphorylation of monoacylglycerols (Call & Rubert, 1973; Mauco *et al.*, 1978). We did not detect any formation of lysoPtdIns.

Our data do provide evidence for the metabolic link between the three inositol-containing phospholipids; the persistence of the stearoyl-arachidonoylglycerol structure in PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 is in keeping with an interconversion of each one into the other. Such phosphorylations and dephosphorylations have been documented in brain (Kai *et al.*, 1966; Irvine *et al.*, 1984) and erythrocytes (Koutouzow & Marche 1982; Quist, 1982; Quist & Barker, 1983). Furthermore, Berridge (1984) has proposed that there is no direct hydrolysis of PtdIns but rather hydrolysis of PtdIns(4,5)P_2, which is rapidly resynthesized from PtdIns and PtdIns4*P*.

Our studies also allowed us to estimate the extent of recycling of the common diacylglycerol backbone. Indeed, the rate of hydroysis of the diacylglycerols formed during thrombin stimulation has never before been measured at the same time as the reincorporation of the diacylglycerol into PtdIns and its phosphorylated derivatives. We observed that with 5i.u. of thrombin/ml only about 40% of

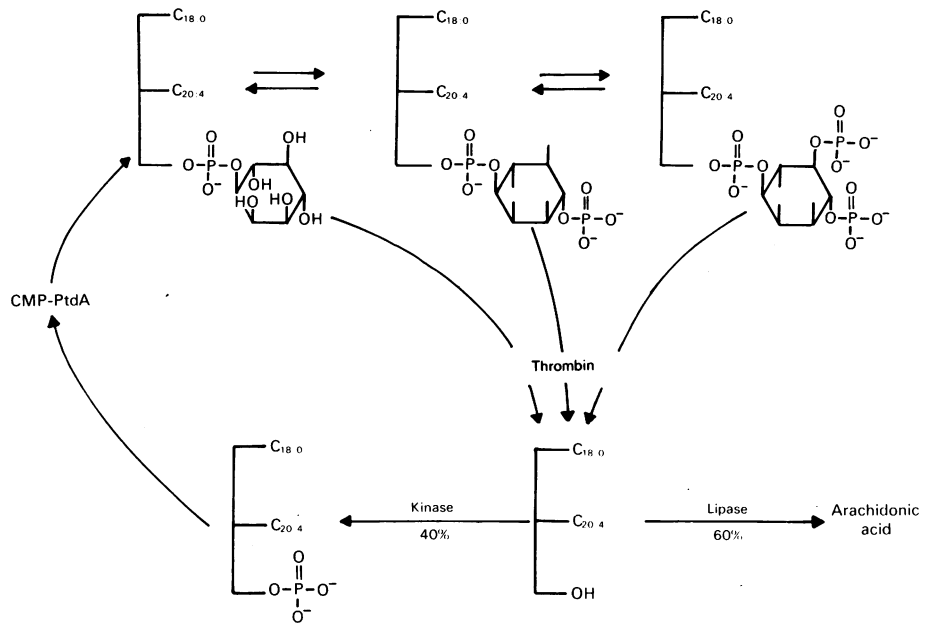

Fig. 5. *A pivotal role for diacylglycerol in human platelet response to thrombin*
Thrombin stimulation of the phosphodiesteratic cleavage [phospholipase(s) C] of inositol-containing phospholipids induces a transient accumulation of stearoylarachidonoylglycerol at 5s. This can be metabolized within the next 1min by two pathways: (*a*) phosphorylation into PtdA and subsequent activation as CMP-PtdA leading to the resynthesis of PtdIns and presumably of its phosphorylated derivatives, PtdIns4*P* and PtdIns(4,5)P_2, or (*b*) deacylation by the diacylglycerol lipase (and monoacylglycerol lipase) producing free arachidonate (and the same amount of stearate).

the hydrolysed phospholipids were conserved as diacylglycerol, PtdA or PtdIns(4,5)P_2 after 60s of stimulation. The remainder of the intermediate diacylglycerol would have released its fatty acids into the medium, presumably by the action of a lipase (or lipases). Previously, we reported that lysed platelets hydrolyse monoacylglycerols 20 times faster than they do diacylglycerols (Mauco *et al.*, 1978), explaining why few, if any, monoacylglycerols accumulate during thrombin stimulation (Prescott & Majerus, 1981). Thus, the sequential action of phospholipase C and lipase may produce arachidonic acid as previously suggested (Mauco *et al.*, 1978, 1979*b*; Bell *et al.*, 1979). The participation of this pathway could be as much as 4–5nmol of arachidonate/10^9 cells in 60s.

As shown in Fig. 5, thrombin-stimulated hydrolysis of phospholipids by phospholipase C activities can be proposed for all three inositol-containing lipids. Then the produced diacylglycerols can undergo at least two different transformations: either deacylation, producing arachidonic acid, or phosphorylation into PtdA. PtdA is presumably recycled into PtdIns according to the classical PtdIns cycle (Michell, 1975). This scheme shows also the utilization of the stearoylarachidonoylglycerol structure throughout the cycle as discussed above. Taken together, these data support the hypothesis of a multifunctional role for inositol phospholipid hydrolysis to diacylglycerols. We propose here that diacylglycerols play a pivotal role in the response of platelets to physiological agonists and are conserved by the stearoylarachidonoylglycerol backbone. Moreover, diacylglycerols can supply 4–5nmol of the approx. 20nmol/10^9 platelets of arachidonate that becomes available when human platelets are stimulated with 5i.u. of thrombin/ml (Bills *et al.*, 1977).

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