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Multiphasic generation of diacylglycerol in thrombin-activated human platelets

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The diacylglycerol (DAG)/protein kinase C pathway plays an important role in platelet aggregation and granule secretion. In this study, we examined the detailed kinetics of DAG formation in response to platelet stimulation. Both α- and γ-thrombin caused multiphasic generation of DAG mass, with DAG production reaching peaks at 0.3–0.6 min intervals. A sub-threshold concentration of γ-thrombin (1.5 nM) produced oscillations of DAG, but peak DAG levels rapidly returned to baseline (unstimulated) values. Intermediate concentrations of γ-thrombin (8–30 nM) resulted in prominent phases of DAG production whose troughs became significantly elevated compared with baseline levels. This delayed accumulation of DAG coincided in time with the onset of secretion and irreversible aggregation. In contrast, stimulation of platelets with collagen resulted in delayed single-phase DAG production. The kinetics of DAG production in stimulated platelets may control both the timing and the degree of DAG accumulation. This may ensure that protein kinase C is activated optimally at the onset of secondary aggregation and secretion. This is the first report of oscillating DAG production in a biological system.

INTRODUCTION

The production of *sn*-1,2-diacylglycerol (DAG) is a critical mechanism of signal transduction that leads to the activation of protein kinase C (Kishimoto *et al.*, 1980; Nishizuka, 1986). The DAG pathway of cell regulation is currently thought to be regulated by a rapid on/off mechanism whereby phospholipase C (PLC)-induced hydrolysis of PtdInsP_2 initiates DAG production, whereas the actions of DAG kinase and DAG lipase terminate the signal (Majerus *et al.*, 1986; Bishop & Bell, 1988).

Platelet activation can be conveniently divided into two parts: primary aggregation and secondary aggregation. Primary aggregation includes a shape change and reversible platelet aggregation. In response to stronger activation, primary aggregation is followed by secondary aggregation, which consists of irreversible aggregation and secretion (Huang & Detwiler, 1986). PtdInsP_2 hydrolysis and DAG production are the earliest signalling events in thrombin-stimulated platelets (Billah & Lapetina, 1982; Rittenhouse-Simmons, 1979) and are associated with primary aggregation, which occurs within 0.5 min after thrombin stimulation. However, studies from our laboratory and others have shown that protein kinase C activation is important in secondary aggregation and secretion (Hannun *et al.*, 1987; Kaibuchi *et al.*, 1983), which occur 0.5–2.0 min after primary aggregation. This suggests that protein kinase C may be regulated by complex signalling events that ensure DAG production beyond the primary phase of platelet activation.

Therefore the detailed kinetics of DAG production in agonist-stimulated platelets were examined in this study. DAG production in thrombin-stimulated human platelets occurred in multiple phases. DAG levels oscillated but did not accumulate in response to sub-threshold concentrations of thrombin. Higher concentrations of thrombin resulted in progressive and delayed accumulation of DAG. A similar delay in DAG accumulation was observed in response to collagen stimulation, but this occurred without any discernible oscillation in DAG levels. The complex kinetics of DAG generation in response to agonist may be an important mechanism that allows DAG to accumulate at the onset of secondary aggregation.

EXPERIMENTAL

Materials

Purified human γ-thrombin was a gift from Dr. John W. Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A.). Luciferin–luciferase (Chronolume) and collagen were obtained from Chronolog Corp. Prostaglandin I_2 (PGI_2), human α-thrombin, ceramide and *Bacillus cereus* PLC were obtained from Sigma. β-Octyl glucoside was purchased from Calbiochem. *sn*-1,2-Dioleoylglycerol was prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) by PLC digestion (Mavis *et al.*, 1972), followed by extraction in diethyl ether and quantification by ester analysis (Stern & Shapiro, 1953). Dioleoylphosphatidylglycerol was from Avanti. DAG kinase from *Escherichia coli* was kindly given by Dr. Carson Loomis and Dr. Robert Bell (Department of Biochemistry, Duke University, Durham, NC, U.S.A.). [γ-^{32}P]ATP was from New England Nuclear, and sodium ATP from Pharmacia.

Methods

Preparation of platelets. Platelets were obtained from normal drug-free adults for studies with α-thrombin by a modification of the procedure of Siess *et al.* (1983). Briefly, 90–135 ml of blood was anticoagulated with ACD (acid citrate dextrose buffer: 85 mM-sodium citrate/111 mM-dextrose/71 mM-citric acid) and centrifuged at 200 *g* for 20 min to obtain platelet-rich plasma (PRP). PGI_2 (5 ng/ml) was added, and the PRP was centrifuged at 800 *g* for 15 min to pellet the platelets. The platelet-poor plasma (PPP) was aspirated, the platelet pellet was suspended in 5 ml of Tyrode–Hepes buffer (134 mM-NaCl/12 mM-$NaHCO_3$/2.9 mM-KCl/0.36 mM-NaH_2PO_4/5 mM-Hepes/5 mM-

Abbreviations used: DAG, *sn*-1,2-diacylglycerol; PGI_2, prostaglandin I_2; PLC, phospholipase C; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

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glucose, pH 7.4) with 1 mM-EGTA, 300 ng of PGI_2/ml and 5% PPP, and the suspension was centrifuged at 600 *g* for 10 min. This wash procedure was then repeated, and the platelet pellet was finally resuspended in Tyrode–Hepes buffer with 1 mM-EGTA but without PGI_2. The platelet concentration was adjusted to $(2.5–5) \times 10^8$/ml with the same buffer after determining the platelet concentration with a Coulter Counter. The platelet suspension was then incubated at 25 °C for 90–120 min to allow resolution of the inhibitory effects of the PGI_2.

γ-Thrombin, a proteolysed form of α-thrombin, was also employed to correlate platelet activation with the DAG response. γ-Thrombin does not bind fibrinogen and thus does not initiate coagulation (Berliner, 1984; Bing *et al.*, 1977). It binds to one of the two putative thrombin receptors (McGowan & Detwiler, 1986), but still induces platelets to aggregate, secrete, and undergo many of the biochemical changes associated with α-thrombin stimulation. For these reasons, γ-thrombin has proved to be a useful agonist for platelet studies, especially in the presence of plasma (Hannun *et al.*, 1987). In these experiments, a platelet pellet was obtained from PRP by the above procedure, then suspended in 5 ml of Tyrode–Hepes buffer with 300 ng of PGI_2/ml and without EGTA. The platelets were then diluted 6–8-fold with Tyrode–Hepes buffer without PGI_2 and with 0.7% PPP to 2.5×10^8 platelets/ml, and left at 25 °C for 2 h to allow resolution of the inhibitory effects of PGI_2. Platelets were not washed further. PPP was included in the final suspending medium because platelets prepared by this method had low DAG baseline levels that allowed sensitive measurement of DAG production while still retaining the ability to undergo physiological shape change and aggregation similar to platelets in PRP.

Measurement of DAG production in response to α-thrombin. Washed platelets were warmed to 37 °C in a water bath. At zero time, 0.8 ml of the platelet suspension was placed in 3 ml of chloroform/methanol (1:2, v/v). The washed platelets were then stimulated with α-thrombin, and 0.8 ml samples were placed in 3 ml of chloroform/methanol at the indicated time points.

Simultaneous measurements of aggregation, ATP secretion and DAG mass in response to γ-thrombin. The platelet suspension was warmed to 37 °C in a water bath and stirred. Samples (0.8 ml, equal to 2×10^8 platelets) for zero-time points were placed in 3 ml of chloroform/methanol (1:2, v/v) for lipid extraction. The platelets were then stimulated with γ-thrombin, and a sample was immediately placed in a Chronolog Lumi-aggregometer for measurement of aggregation and ATP secretion with luciferin–luciferase. At the indicated times, samples (0.8 ml) of the platelet suspension were placed in chloroform/methanol.

DAG mass measurements. Lipids were extracted by the method of Bligh & Dyer (1959), and total phospholipid phosphate was determined from 0.2 ml of the final organic phase (Ames & Dubin, 1960; Van Veldhoven & Mannaerts, 1987). A 1 ml portion of the final organic phase was dried under N_2, and the DAG mass in this lipid extract was measured by using *E. coli* DAG kinase and [γ-^{32}P]ATP as described by Preiss *et al.* (1986). Specific radioactivity of the [γ-^{32}P]ATP was measured as described by Walsh & Bell (1986), and ranged from 31200 to 91800 c.p.m./nmol. Conversion of diolein standards was always linear, with a correlation coefficient of 0.999 or greater. In experiments done to examine the variability of the DAG kinase assay, the lipid-containing chloroform phase was divided in half and DAG mass was measured in the lipid extract of each portion. DAG mass was always corrected for total phospholipid phosphate.

Statistical tests. Data analysis and standard deviations were calculated by using Lotus 1-2-3 (Lotus Development Corp.) and Excel (Microsoft Corp.) software. Paired *t* tests were performed as described by Iman & Conover (1983).

RESULTS

Multiphasic DAG production in response to α-thrombin

When washed platelets were stimulated with α-thrombin, at least three phases of DAG production were observed in six out of seven separate experiments (Fig. 1). Although multiple phases of DAG production were consistently observed in platelets stimulated with α-thrombin, a statistical analysis of the data was

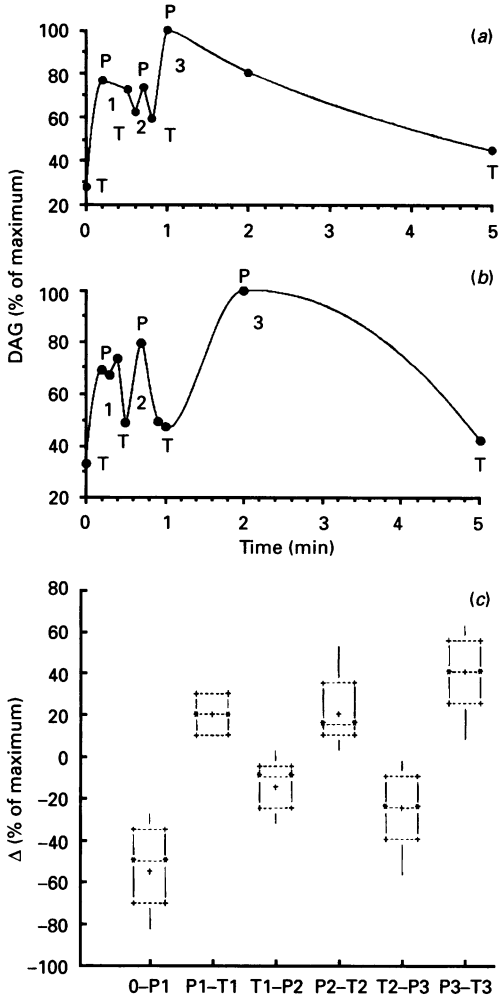

Fig. 1. Kinetics of DAG production in platelets stimulated with α-thrombin

Platelets prepared as described under 'Methods' were stimulated with 1 unit of α-thrombin/ml. DAG mass was quantified at the indicated time points by the DAG kinase assay. DAG was corrected for total phospholipid phosphate (PL) and plotted as percentage of maximum DAG/PL achieved. (*a*) and (*b*) depict two independent experiments which are representative of seven experiments using platelets obtained from four different donors. Phases of DAG production are numbered 1–3. The peaks of each phase are marked by P, and the troughs by T. (*c*) shows the means (+), medians (*---*) and 25th (+---+) to 75th (+---+) percentiles of each group of differences from all seven experiments. The 25th to 75th percentiles represent the interquartile range; single vertical lines represent range of all values. Differences are: 0–P1, 0 to peak 1; P1–T1, peak 1 to trough 1; T1–P2, trough 1 to peak 2; P2–T2, peak 2 to trough 2; T2–P3, trough 2 to peak 3; P3–T3, peak 3 to trough 3. Trough-to-peak differences are negative; peak-to-trough differences are positive. The differences between each peak (P) and its flanking troughs (T) are significant by the paired *t* test ($P < 0.025$; for four of six differences $P < 0.005$). In six experiments the average S.D. of duplicate or triplicate measurements of DAG levels in unstimulated platelets were 5% of maximum.

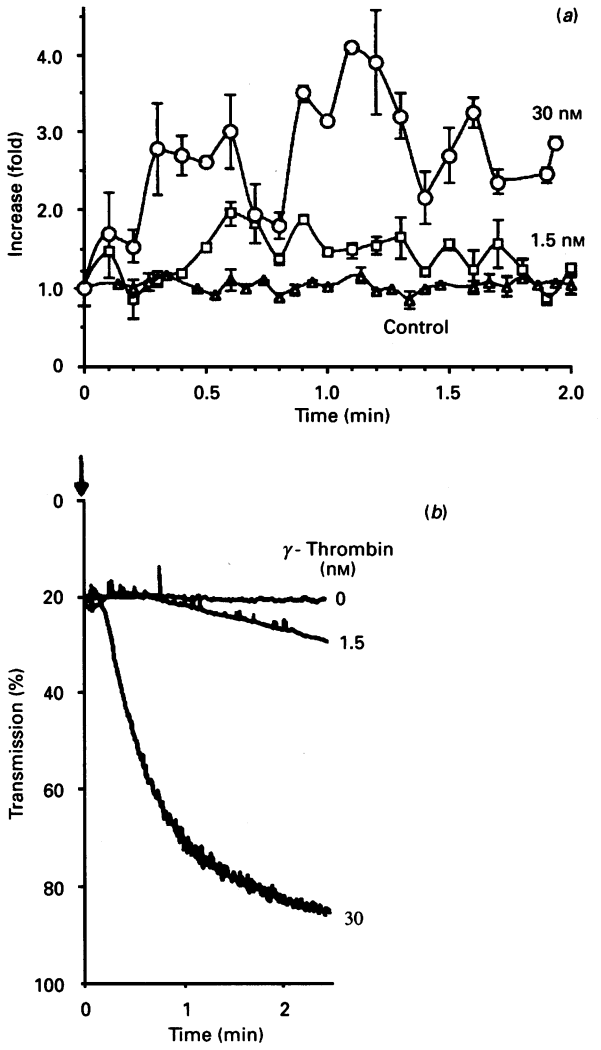

Fig. 2. (*a*) Multiphasic DAG generation in response to dose of γ-thrombin, and (*b*) aggregation responses of platelets whose DAG responses were measured in (*a*)

DAG masses were measured in platelets stirred at 37 °C and stimulated with 0 (control), 1.5 nM- or 30 nM-γ-thrombin. DAG levels were corrected for total PL and plotted as fold increase over baseline. Duplicate DAG mass measurements were performed on the lipid extracts obtained at 0.1 min (6 s) intervals; error bars indicate S.D. (*b*) Platelets were stimulated with 0, 1.5 nM- or 30 nM-γ-thrombin, and aggregation was measured by percentage of light transmission in a lumiaggregometer. Arrow indicates addition of thrombin.

required to determine whether the differences between the peaks and troughs of these phases were significant. This analysis was complicated by the lack of synchrony between the individual experiments. For example, in the experiment depicted in Fig. 1(*a*) a peak in DAG production occurred 1 min after thrombin stimulation, whereas in the experiment shown in Fig. 1(*b*) a trough occurred 1 min after stimulation. Thus averaging experiments from Figs. 1(*a*) and 1(*b*) would result in large S.D. values, which would obscure the underlying multiphasic behaviour. Therefore points were compared on the basis of whether they represented a peak or trough in DAG mass rather than on the basis of the time interval after stimulation. The differences between each consecutive trough and peak were calculated. The differences obtained for all seven experiments are graphically illustrated in Fig. 1(*c*). The differences between troughs and peaks are negative, whereas those between peaks and trough are positive. The interquartile range of each trough-to-peak group of differences is always less than zero, whereas the interquartile range of each peak-to-trough group of differences is always greater than zero. If these differences were insignificant, they would cluster around zero, rather than away from zero as they do in Fig. 1(*c*). Since each experiment represents an independent stimulation, and since all these differences were found to be normally distributed by the Lilliefors test (Iman & Conover, 1983), a paired *t* test was performed to determine whether these differences achieved statistical significance. All differences had *P* values of less than 0.025; four of the six differences were found to have *P* values of less than 0.005. Therefore the differences between the peaks and troughs of these phases of DAG production achieved statistical significance in these seven experiments. Moreover, the differences between troughs and peaks were greater than variations in DAG assay. Duplicate or triplicate measurements of DAG levels in unstimulated platelets were performed in 6 of 7 of these experiments, and the average S.D. was only 5% of maximum. In contrast, the smallest average difference between any peak and trough was 12% of maximum. Since platelet DAG levels reflect the opposing effects of production and metabolism, these studies suggest there are repeated waves of DAG generation accompanied by rapid metabolism.

Multiphasic DAG production in response to γ-thrombin

γ-Thrombin stimulation of platelets also caused multiphasic DAG production (Fig. 2*a*). To determine whether this multiphasic response was specifically induced by thrombin and/or was due to variation in the DAG kinase assay, duplicate DAG mass measurements were performed on each lipid sample obtained at 0.1 min (6 s) intervals, and means and S.D.s of these determinations plotted (Fig. 2*a*). Unstimulated platelets showed little change in DAG (Fig. 2*a*). A sub-threshold concentration of γ-thrombin (1.5 nM) produced oscillating DAG production, with major peaks at 0.1, 0.6, 0.9 and 1.5 min (Fig. 2*a*). Stimulation of platelets with 30 nM-γ-thrombin, which induced a full biological response, also resulted in oscillations in DAG production that showed higher amplitude per phase and a net accumulation of DAG (Fig. 2*a*). There is no overlap of the error bars when peak DAG levels are compared with trough levels in γ-thrombin-stimulated platelets (Fig. 2*a*), indicating that the changes in DAG levels observed are greater than the variability of the DAG kinase assay. These changes in DAG levels were not caused by variations in phospholipid, since total phospholipid remained essentially constant (results not shown).

Multiphasic production of DAG may serve a critical physiological function in controlling platelet responses by allowing delayed net accumulation of DAG above a critical cellular level. Platelets stimulated with 30 nM-γ-thrombin exhibited a progressive increase in trough DAG levels (Fig. 2*a*). The delayed accumulation of DAG above baseline appeared to correlate with the onset of secondary aggregation (Fig. 2*b*). Delayed DAG accumulation did not occur in unstimulated platelets or in those stimulated with a sub-threshold dose (1.5 nM) of γ-thrombin (Fig. 2*a*); these platelets also did not undergo secondary aggregation (Fig. 2*b*).

In order to define better the kinetics of DAG production, measurements of DAG mass were conducted on a time scale that would allow definitive resolution of discrete phases of DAG production. Therefore DAG mass was measured at 2 s intervals after platelet stimulation with γ-thrombin. Multiple discrete phases of DAG production became apparent, with peak increases in DAG reaching 2–3-fold over baseline at 0.2, 0.6 and 1.2 min after stimulation (Fig. 3). Again, these phasic changes in DAG were greater than the variability of the DAG kinase assay (Fig.

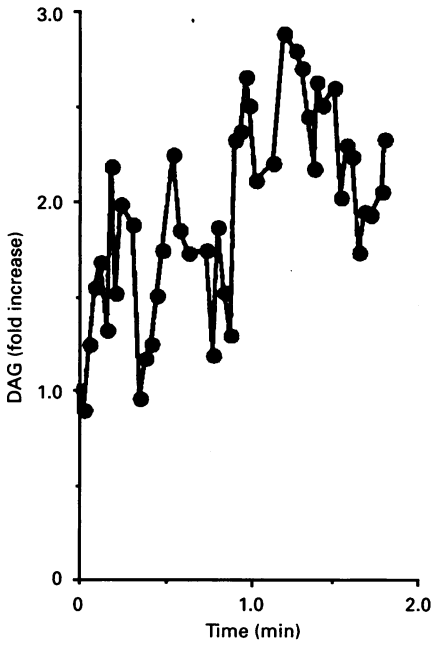

Fig. 3. Rapid kinetics of DAG production in platelets stimulated with γ-thrombin

DAG mass was measured at 2 s intervals. DAG levels were standardized for total phospholipid phosphate and plotted as fold increase over baseline. Baseline at zero time was 0.91 pmol of DAG/nmol of phospholipid. Variation in baseline DAG levels was 1.00±0.11 (S.D.)-fold, based on duplicate to quadruplicate determinations of DAG levels in unstimulated platelets in six experiments.

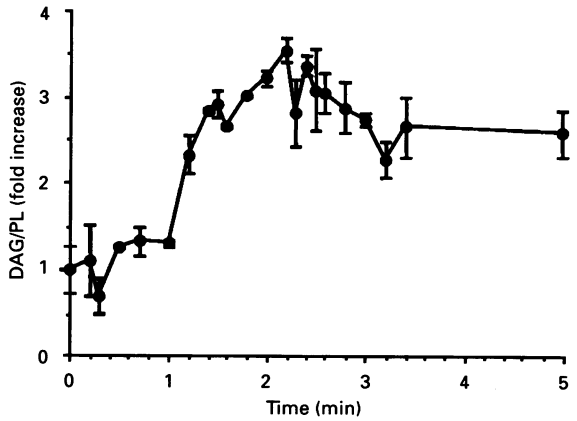

Fig. 4. Uniphasic DAG production in response to collagen

DAG levels were measured in platelets stirred at 37 °C and simulated with 2.5 μg of collagen/ml. DAG levels were corrected for total phospholipid phosphate (PL) and plotted as fold increase over baseline. Duplicate DAG mass measurements were performed on the lipid extracts as described under 'Methods'; error bars indicate S.D. This one experiment is representative of three independent experiments.

3). After 0.8 min, DAG levels remained persistently elevated above baseline (Fig. 3). This sustained DAG production coincided with secondary aggregation and secretion (results not shown). It should be noted that the phasic DAG response was observed in platelets from the majority of donors tested. However, the amplitude, frequency and the exact timing of peaks and troughs varied among different donors.

Collagen causes uniphasic DAG production

Stimulation of platelets with collagen also resulted in delayed accumulation of DAG, but in a single phase (Fig. 4). We were unable to detect multiphasic DAG production in collagen-activated platelets at high or sub-threshold concentrations (results not shown). These results suggest that multiphasic DAG production is dependent on the agonist. They also underscore the different signalling mechanisms employed by thrombin and collagen: DAG generation in response to collagen appears to be almost entirely dependent on arachidonic acid metabolism (Rittenhouse & Allen, 1982).

DISCUSSION

In this study we show that thrombin activation of human platelets results in oscillation of DAG levels. Several lines of evidence document the authenticity of these oscillations: (1) they are consistently observed in multiple experiments; (2) they are statistically significant; (3) the oscillations are greater than the variation observed in the DAG kinase assay; (4) the rapid time-course experiment (Fig. 3) more clearly delineated both peaks and troughs, because these phases were composed of multiple determinations; (5) these phases of DAG production could not be accounted for by variations in platelet number or total lipid extracted, since all DAG measurements were corrected for phospholipid, a measure of total lipid extracted; and (6) the oscillations were observed when platelets were stimulated by thrombin, but not when platelets were stimulated by collagen. If these multiphasic DAG responses represented an artifact of the DAG kinase assay or a peculiarity of the platelet preparation, then they should have also been observed in collagen-stimulated platelets.

This is the first report of multiphasic DAG generation, although biphasic DAG production has been noted in platelets (Werner & Hannun, 1988, 1991; Nakashima *et al.*, 1991) and other cell systems (Griendling *et al.*, 1986; Wright *et al.*, 1988). It is interesting that one study of DAG production in platelets observed an inconsistent 'late secondary rise in DAG mass' after thrombin stimulation (Bishop *et al.*, 1990), which resulted in large S.E.M. values when these experiments were averaged. This may be the result of out-of-phase multiphasic DAG production.

These changes in DAG are reminiscent of oscillations in cyclic AMP observed in synchronized populations of *Dictyostelium* cells (Gerisch & Wick, 1975; Tomchik & Devreotes, 1981) and similar to oscillations of free cytosolic Ca^{2+} that have been observed with single-cell recordings (Berridge *et al.*, 1988; Jacob *et al.*, 1988). The advantages of frequency-modulated systems of signal transduction have been discussed in relation to intracellular Ca^{2+} oscillations (Berridge *et al.*, 1988; Jacob *et al.*, 1988). Similarly, multiphasic frequency- and/or amplitude-modulated DAG levels in platelets may allow tight control of stimulus–response coupling. Such a system has several advantages. First, it allows discrimination between low and high concentrations of agonists (e.g. see Fig. 2). Thus platelets could be exposed to sub-threshold concentrations of agonist without undergoing irreversible aggregation and secretion, since DAG would oscillate, but not accumulate to an extent necessary for secondary aggregation and secretion. At higher concentrations of agonist, the phases of DAG production would become larger and begin to overlap, leading to DAG accumulation, secondary aggregation and secretion. Second, it may explain the synergistic action of multiple platelet activators. For example, if a sub-threshold concentration of thrombin initiates multiphasic DAG production

without accumulation, the subsequent addition of a second agonist, at a concentration which by itself would be sub-threshold, might augment the DAG response, leading to DAG accumulation, aggregation and secretion. Third, such a system would allow accumulation of DAG in response to the continued presence of agonists. In fact, it has been shown that persistent stimulation of the thrombin receptor in platelets is required for continued phosphatidic acid production (Holmsen *et al.*, 1981, 1984) and inositol phosphate formation (Huang & Detwiler, 1987). Finally, a multiphasic mechanism of DAG generation allows delayed DAG accumulation at 0.5–1.0 min after stimulation. This time interval appears to be the optimal time for the action of DAG and for the activation of protein kinase C during secondary rather than primary aggregation (Hannun *et al.*, 1987; Werner & Hannun, 1991).

The mechanisms involved in the multiphasic generation of DAG are unknown, but, given the complexity of signal-transduction mechanisms in platelets and their interrelationships, several potential mechanisms may be involved. These may constitute feedback loops in DAG production and/or DAG metabolism. For example, DAG production may activate protein kinase C-mediated inhibition of PLC (Watson & Lapetina, 1985; Bishop *et al.*, 1990) and inhibition of further DAG formation. Rapid metabolism of DAG (Bishop & Bell, 1988) would then result in relief of the inhibition of PLC. In the continued presence of thrombin (Huang & Detwiler, 1987), this then leads to further DAG formation. Such a mechanism may result in cyclic production of DAG. Positive-feedback loops may also contribute to multiphasic DAG production. For example, agonist activation of receptors may lead to sequential activation of a PtdInsP_2-specific PLC, followed by activation of PtdIns$P$-, PtdIns-(Griendling *et al.*, 1986), and possibly phosphatidylcholine-specific PLCs. There is evidence for an initial PLC-mediated hydrolysis of PtdInsP_2 followed by hydrolysis of PtdIns in platelets (Nakashima *et al.*, 1991), but no evidence for PLC-mediated phosphatidylcholine hydrolysis in platelets (Bishop *et al.*, 1990; Nakashima *et al.*, 1991). This scheme would be consistent with present evidence of an initial Ins(1,4,5)P_3-mediated monophasic rise in cytosolic Ca^{2+} (Tarver *et al.*, 1987; Johnson *et al.*, 1985). Also, different platelet-derived agonists may activate different PLCs with different substrate specificities. For example, arachidonic acid metabolites such as thromboxane A_2 have been shown to activate PLC (Banga *et al.*, 1986). Multiphasic DAG production may depend on the ability of an agonist to cause DAG production by more than one mechanism. For example, thrombin can cause DAG production by both direct activation of PLC through the thrombin receptor (Siess *et al.*, 1984; Huang & Detwiler, 1987) and indirect activation of PLC via arachidonic acid metabolism (Siess *et al.*, 1984). On the other hand, collagen-induced DAG production occurs primarily through arachidonic acid metabolism and subsequent PLC activation (Rittenhouse & Allen, 1982). This may explain why DAG production is multiphasic in response to thrombin and uniphasic in response to collagen. Because multiphasic DAG generation was observed in a washed plasma-free platelet system, it is unlikely that plasma factors play a role in this multiphasic response.

A major limitation of these studies results from our current inability to measure DAG levels in single cells. Therefore, the above results may reflect either the integration of sequential signalling events in single platelets or sequential activation of discrete sub-populations of platelets. Although we cannot distinguish between these two possibilities, several studies of second messengers in platelets can be considered as indirect evidence that addresses this question. Flow-cytometry studies have been done which demonstrate that sub-populations of platelets exist which exhibit different Ca^{2+} responses (Davies *et al.*, 1988, 1990), suggesting that DAG responses might also reflect the responses of different sub-populations of platelets. However, these studies also show that one platelet sub-population exhibits maximal Ca^{2+} responses to sub-saturating doses of thrombin, whereas others do not respond at all (Davies *et al.*, 1988), suggesting that, although sub-populations of platelets exist, they are not activated sequentially. On the other hand, several lines of evidence argue against sequential activation of sub-populations of platelets. First, although collagen resulted in similar activation of platelets and in delayed DAG production in this study, this was not accompanied by discrete phases in DAG levels. Although this demonstrates that oscillation of DAG is not a mechanism universal to all platelet agonists, it argues against sequential activation of sub-populations of platelets. Second, flow-cytometry studies have shown that platelets exhibit uniform pH responses to thrombin (Davies *et al.*, 1990), indicating that some platelet responses occur uniformly rather than in distinct sub-populations. Third, we find that PtdInsP_2 hydrolysis is complete within the first 30 s of thrombin addition (results not shown), again arguing against sequential activation of platelet sub-populations. These last results further suggest that the subsequent phases of DAG production are not derived from PtdInsP_2 hydrolysis, and are consistent with the work of others (Nakashima *et al.*, 1991; Agranoff *et al.*, 1983). In light of these studies, it should not be surprising to find a dissociation between platelet Ca^{2+} and DAG responses, for the lipids implicated as sources for the later phases of DAG production would not release the Ca^{2+}-elevating second messenger InsP_3. Clearly, further investigation is indicated to examine the mechanisms of multiphasic DAG production and to determine if other cell systems employ oscillating DAG production as a signal-transduction mechanism.

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