0026-895X/92/020382-05$03.00/0

MOLECULAR PHARMACOLOGY, 41:382-386

Quantitative Analysis of Diacylglycerol Second Messengers in Human Platelets: Correlation with Aggregation and Secretion

MARK H. WERNER, ALICJA E. BIELAWSKA, and YUSUF A. HANNUN

Division of Hematology and Oncology, Department of Medicine, Duke University School of Medicine, Durham, North Carolina 27710

Received August 27, 1991; Accepted November 19, 1991

SUMMARY

The action of many agonists results in rapid production of *sn*-1,2-diacylglycerol (DAG). Using platelets as a model system, we previously identified a delayed phase of DAG accumulation that is temporally associated with secondary aggregation and secretion. In the present study, we examined the quantitative relationship between this delayed DAG accumulation and platelet aggregation and secretion. To quantitate the low levels of DAG in platelets, we used the sensitive DAG kinase assay and simultaneously compared DAG levels with aggregation and ATP secretion. In platelets stimulated by γ-thrombin or collagen, there was a dose response between concentration of agonist and DAG accumulation. Significantly, a dose response was observed between DAG accumulation and extent of aggregation and secretion in platelets stimulated by either agonist. A concentration of either γ-thrombin or collagen that caused secondary aggregation and secretion was associated with DAG accumulation above 0.2 pmol of DAG/nmol of phospholipid. Subthreshold concentrations of γ-thrombin or collagen resulted in DAG levels less than 0.2 pmol/nmol of phospholipid. Thus, these data suggest that a threshold level of DAG must be achieved for platelet activation to occur. Moreover, secretion was blocked when DAG production was blocked with aspirin or when protein kinase C was inhibited with sphingosine. We conclude that endogenously formed DAG plays a critical role in regulating secondary aggregation and secretion and, therefore, represents an important target for future antiplatelet agents.

DAG has emerged as a critical second messenger in the activation of PKC (1, 2). There is, however, a paucity of studies that attempt to establish quantitative relationships between endogenous DAG levels and cellular responses. Such studies are essential to determine the physiologic roles of DAG.

Because platelet stimulation is associated with DAG production and PKC activation, it follows that the DAG/PKC pathway may play an important role in some of the functional responses of activated platelets. The activation of PKC by exogenous DAG has been shown to act in synergy with calcium ionophores to produce platelet secretion (3, 4). Inhibition of platelet PKC with sphingosine had no effect on the early agonist-stimulated response of platelet shape change but completely inhibited the later responses of secondary aggregation and secretion (5). These experiments with exogenously applied pharmacologic agents suggested that the DAG/PKC pathway may play a necessary role in aggregation and secretion.

The observation that DAG production was rapid and transient (6) raised some question as to whether DAG was the second messenger regulating PKC-mediated aggregation and secretion, especially at lower (and perhaps more physiologic) concentrations of agonist, which result in a longer delay in the development of secondary aggregation and secretion. However, in recent studies in which we measured the kinetics of DAG production in response to γ-thrombin and collagen, we observed delayed accumulation of DAG, which coincided with both PKC activation and the biologic responses of secondary aggregation and secretion (7).

Although these studies suggest a role for DAG in mediating secondary aggregation, it has not yet been determined whether endogenous DAG production can be quantitatively correlated with biologic responses. Moreover, earlier studies on DAG levels in platelets have primarily relied on metabolic labeling of DAG with precursor arachidonic acid, thus possibly limiting these studies to a subgroup of DAG molecular species. Because of these considerations, we used an assay that measures total mass of DAG to determine whether endogenous DAG production in response to thrombin or collagen could be quantitatively correlated with platelet aggregation and secretion.

Experimental Procedures

Materials

Purified human γ-thrombin was a gift of Dr. John W. Fenton II (Division of Laboratories and Research, New York State Department

This work was supported in part by National Institutes of Health Grants HL-43707 and CA-47741. M.H.W. is a fellow of the North Carolina Affiliate of the American Heart Association, and Y.A.H. is an established investigator of the American Heart Association.

ABBREVIATIONS: DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; Me_2SO, dimethylsulfoxide; PGI_2, prostaglandin I_2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of Health, Albany, NY). Collagen and luciferin-luciferase (Chronolume) were obtained from Chrono-log Corporation. PGI_2, aspirin, sphingosine, ceramide, and *Bacillus cereus* phospholipase C were obtained from Sigma. Aspirin, which was freshly prepared for each experiment, was dissolved in Me_2SO (final concentration of Me_2SO was 0.5%). Me_2SO alone at these concentrations had no effect on aggregation. β-Octylglucoside was purchased from Calbiochem and recrystallized. *sn*-1,2-Dioleoylglycerol (diolein) was prepared from 1,2-dioleoyl-*sn*-glycerol-3-phophocholine (Avanti Polar Lipids) by phospholipase C digestion (8) followed by extraction in ether; the final diolein concentration was determined by ester analysis (9). DAG kinase from *Escherichia coli* was a generous gift from Drs. Carson Loomis and Robert Bell (Department of Biochemistry, Duke University, Durham, NC). [γ-^{32}P]ATP was purchased from New England Nuclear, and sodium ATP from Pharmacia.

Methods

Preparation of platelets. Platelets were obtained from healthy drug-free adults as described (10). Briefly, 90–135 ml of blood were added to acid-citrate-dextrose, in a 9:1 ratio (v/v), and centrifuged to obtain platelet-rich plasma. PGI_2 (5 ng/ml) was added to the platelet-rich plasma, which was then centrifuged at 800 × *g* for 15 min. The platelet pellet was then suspended in 5 ml of Tyrode-HEPES buffer with 300 ng/ml PGI_2. The platelet concentration was measured with a Coulter counter and adjusted to 2.5×10^8/ml with Tyrode-HEPES buffer without PGI_2 and with 0.7% platelet-poor plasma. The platelet suspension was then allowed to sit for 120 min, to allow resolution of the inhibitory effects of the PGI_2. In experiments with aspirin, platelets were incubated with aspirin for at least 15 min (unless otherwise indicated) at 25°.

Collagen and γ-thrombin, a proteolyzed form of α-thrombin, were used to correlate aggregation and secretion with the DAG response. γ-Thrombin binds to one of two putative thrombin receptors (11) and induces platelets to aggregate, secrete, and undergo many of the biochemical changes associated with α-thrombin stimulation. However, γ-thrombin does not initiate coagulation because it does not bind fibrinogen (12). For these reasons, γ-thrombin has been the agent of choice to study thrombin activation of platelets in the presence of serum components (5, 13).

Simultaneous measurements of aggregation, ATP secretion, and DAG mass levels. The platelet suspension was placed in a 37° water bath and stirred. Samples (0.8 ml, equal to 2×10^8 platelets) for zero time points were placed in 3 ml of 1:2 (v/v) chloroform/methanol for lipid extraction. The platelets were then stimulated with γ-thrombin or collagen, and an aliquot was immediately placed in a Chrono-log Lumi-Aggregometer. Aggregation was measured as percentage of light transmission; ATP secretion was measured with luciferin-luciferase and expressed as nmol of ATP or as a percentage of full deflection under standardized conditions. ATP secretion was measured at the point at which it attained its maximum. The time at which maximum secretion occurred varied inversely with the dose of agonist (range of 0.5–4.7 min with γ-thrombin and 1.8–3.0 min with collagen). At the indicated time points after stimulation, 0.8-ml aliquots of the platelet suspension were placed in chloroform/methanol. In some experiments, duplicate 0.8-ml platelet aliquots were obtained.

DAG mass measurements. Lipids were extracted by the method of Bligh and Dyer (14), and the final organic phase was divided into aliquots for measurement of total phospholipid phosphate (15, 16) and DAG. The total mass of DAG in the lipid extracts was measured using recombinant *E. coli* DAG kinase and [γ-^{32}P]ATP, as described (7, 17). The specific activity of the ATP, which was always precisely measured (18), ranged from 31,200 to 91,800 cpm/nmol. Conversion of diolein standards was invariably linear, with a correlation coefficient that was typically 0.999. This assay is superior to radiolabeling with arachidonic acid because it is more sensitive and because it quantitates *sn*-1,2-DAGs regardless of their fatty acid composition. The sensitivity of the DAG kinase assay is particularly important when measuring base-line DAG levels in platelets, which are about 5–10-fold less than levels found in other cells (17).

Data analysis. Data were analyzed using Lotus 1-2-3 (Lotus Development Corp.) and Excel (Microsoft Corp.) software.

Measurement of 40-kDa protein phosphorylation. Platelets prepared as described above were labeled with [^{32}P]orthophosphate and stimulated. Phosphorylation of the 40-kDa protein was measured 2 min after stimulation as described (19).

Results

When platelets are stimulated with γ-thrombin at a concentration sufficient to produce aggregation and secretion, delayed DAG accumulation occurs (7). If DAG accumulation is an important mechanism for the regulation of secondary aggregation and secretion, then the magnitude of DAG accumulation should correlate with the magnitude of aggregation and secretion. The extent of DAG accumulation correlated with the concentration of γ-thrombin (Fig. 1). Aggregation, secretion, and DAG accumulation were all of the highest magnitude when platelets were stimulated with a high concentration of γ-thrombin (50 nM) (Fig. 1A). At an intermediate concentration of γ-thrombin (8 nM), less DAG accumulation occurred, and this correlated with a slower rate of aggregation and less secretion (Fig. 1B). When platelets were stimulated with a low concen-

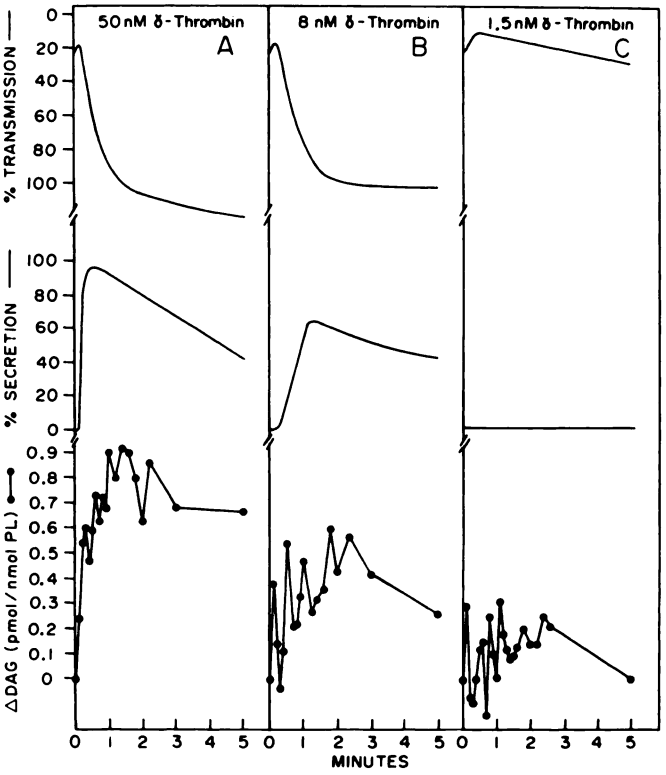

Fig. 1. Correlation of aggregation, secretion, and DAG production with dose of γ-thrombin. Platelets were prepared as described in Experimental Procedures and were warmed to 37° and stirred. They were then stimulated with γ-thrombin. Aggregation and secretion were measured in a lumi-aggregometer by percentage of light transmission and ATP secretion, respectively. Lipids were extracted for measurement of DAG mass as described in Experimental Procedures. *ΔDAG*, change in DAG, in pmol/nmol of total phospholipid (*PL*), from base-line (unstimulated) levels. Base-line DAG/phospholipid levels were 0.54 ± 0.09 (A), 0.36 ± 0.01 (B), and 0.50 ± 0.08 (C) pmol/nmol of phospholipid (mean ± standard deviation of duplicate determinations). These three independent experiments are representative of three or more experiments.

tration of γ-thrombin (1.5 nM), which caused shape change alone, minimal DAG production was observed, and secretion did not occur (Fig. 1C).

The relationship between the concentration of γ-thrombin and DAG mass levels was further examined. Because the delayed phase of DAG accumulation in response to γ-thrombin most often occurred from 0.5 to 2.5 min after stimulation, delayed DAG accumulation was calculated by averaging DAG production over this interval. DAG accumulation correlated with the concentration of γ-thrombin (Fig. 2).

In turn, there was a prominent correlation between DAG levels and both the magnitude of aggregation (Fig. 3A) and the magnitude of secretion (Fig. 3B). Two interesting features emerged from this analysis. First, there appeared to be a threshold of DAG accumulation, above 0.2 pmol/nmol of phospholipid, that was required for secondary aggregation and secretion to occur (Fig. 3). Secondary aggregation and secretion occurred in 12 of 14 experiments in which DAG accumulation was ≥0.2 pmol/nmol of phospholipid, whereas secondary aggregation and secretion did not occur in any of the five experiments in which DAG accumulation was <0.2 pmol/nmol of phospholipid. We also examined DAG accumulation over an earlier time interval, to determine whether there was a similar threshold of DAG accumulation before the development of maximum aggregation. When DAG accumulation was calculated by averaging DAG production over 0.2 to 1 min, secondary aggregation and secretion was associated with DAG accumulation of >0.2 pmol/nmol of phospholipid in eight of 12 experiments, whereas, in four of five experiments in which DAG accumulation was <0.2 pmol/nmol of phospholipid, secretion did not occur (data not shown). Therefore, subthreshold levels of agonist led to subthreshold accumulation of DAG, no irreversible aggregation, and no secretory response. Second, above the 0.2 pmol/nmol of phospholipid threshold of DAG, a quantitative correlation between aggregation, secretion, and DAG accumulation was observed, which appeared to plateau at high DAG levels (Fig. 3).

To determine whether a similar quantitative relationship existed in response to other platelet agonists, we also studied the relationship between DAG accumulation, aggregation, and secretion in platelets stimulated with collagen. A correlation

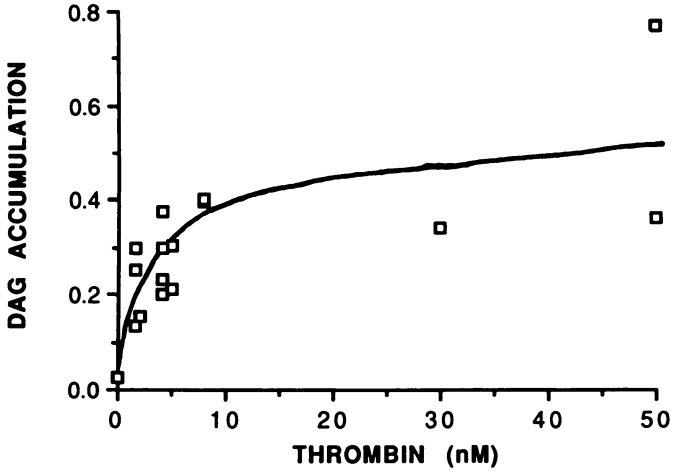

Fig. 2. Relationship of γ-thrombin dose to DAG accumulation. Platelets were stimulated with the indicated dose of γ-thrombin, and DAG production above base-line, in pmol/nmol of phospholipid, was averaged from 0.5 to 2.5 min after stimulation. Each *symbol* represents an independent experiment.

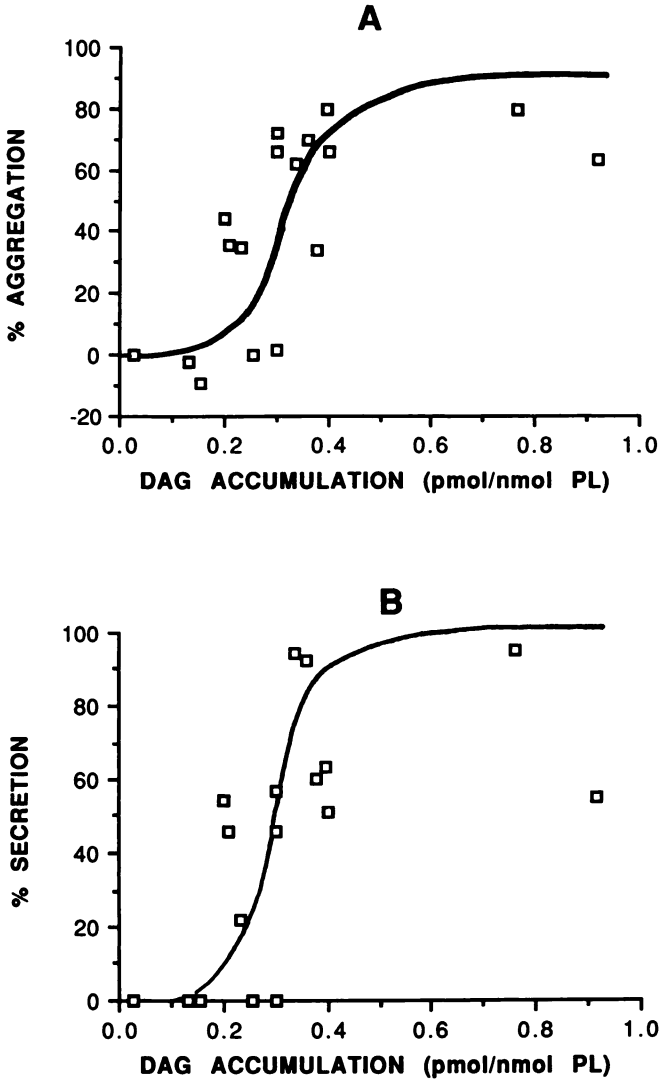

Fig. 3. A, Correlation of aggregation and DAG accumulation in γ-thrombin-stimulated platelets. Platelets were stimulated with γ-thrombin, and aggregation was measured by percentage of light transmission at 2 min after stimulation. B, Correlation of ATP secretion and DAG accumulation in γ-thrombin-stimulated platelets. Platelets were stimulated with γ-thrombin, and the maximum ATP secretion achieved was quantitated as described in Experimental Procedures. DAG accumulation was measured by averaging DAG production above base line from 0.5 to 2.5 min after stimulation. Each *symbol* represents an independent experiment.

was observed between collagen dose and DAG accumulation (Fig. 4). A sharp increase in DAG accumulation occurred when collagen concentrations exceeded 0.2 μg/ml, such that collagen concentrations below 0.2 μg/ml did not result in any significant DAG accumulation (data not shown). Because DAG accumulation in response to collagen most often occurred from 0.8 to 3 min after stimulation (7), DAG accumulation was measured by averaging DAG production over base line from 0.8 to 3 min. A steep dose-response curve was also observed when either aggregation or secretion was compared with DAG accumulation (Fig. 5). Again, irreversible aggregation and secretion occurred when DAG accumulation exceeded 0.2 pmol/nmol of phospholipid (Fig. 5). Thus, in either γ-thrombin- or collagen-stimulated platelets, secondary aggregation and secretion occurred when DAG accumulation exceeded the same threshold of 0.2

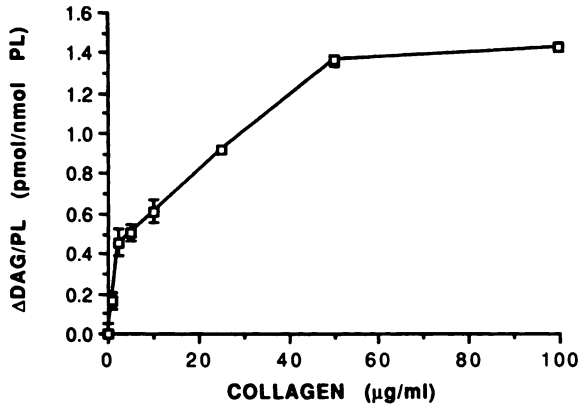

Fig. 4. Relationship between collagen dose and DAG production. Platelets were stimulated with the indicated dose of collagen, and DAG mass was measured in duplicate 2 min after stimulation. *ΔDAG*, change in DAG from base-line levels. *Error bars*, standard deviations; *symbols without error bars* had standard deviations smaller than the size of the symbols.

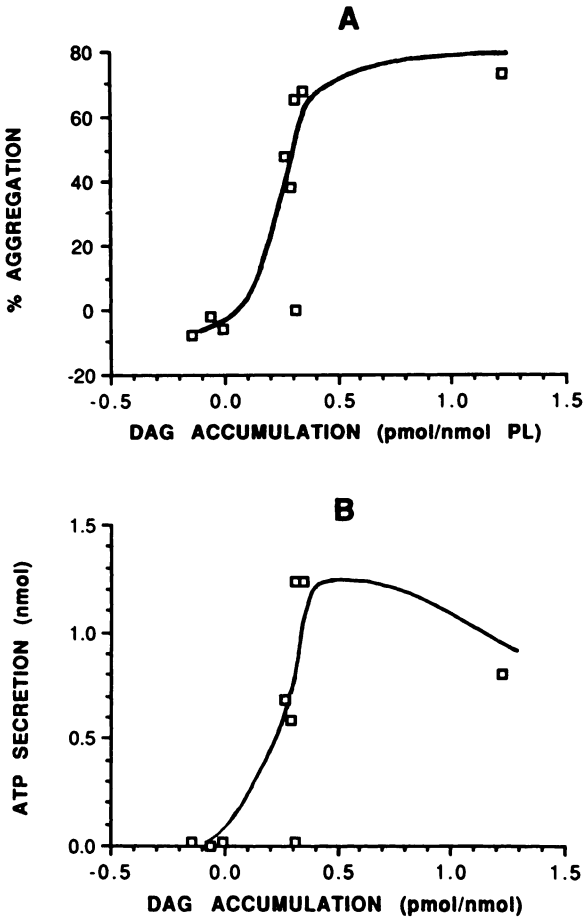

Fig. 5. A, Correlation between DAG accumulation and platelet aggregation in collagen-stimulated platelets. B, Correlation between DAG accumulation and platelet secretion in collagen-stimulated platelets. Platelets were stimulated with collagen, and then aggregation at 3 min, ATP secretion, and DAG mass were measured. DAG accumulation was measured by averaging DAG production above base line from 0.8 to 3 min after stimulation. Each *symbol* represents an independent experiment.

pmol/nmol of phospholipid, despite the fact that these different agonists are thought to use different molecular mechanisms of action (20, 21).

The crucial role of DAG production and PKC activation in the induction of secondary aggregation and secretion is also supported by studies using inhibitors. Aspirin, a cyclooxygenase inhibitor, causes inhibition of DAG accumulation (10, 22), probably by interrupting feedback effects of thromboxane A_2 on phospholipase C (22). Aspirin inhibition of DAG production in response to γ-thrombin was associated with inhibition of 40-kDa protein phosphorylation and secretion (Table 1). On the other hand, sphingosine, which inhibits platelet PKC as measured by 40-kDa protein phosphorylation (5), also caused inhibition of secretion, even though DAG production was not inhibited (Table 1). Thus, secretion can be prevented by direct inhibition of PKC activation (sphingosine) or by inhibition of DAG accumulation above a threshold level (aspirin).

Discussion

The role of second messengers in mediating specific biologic responses is difficult to establish. The biologic effects of exogenously applied second messengers or their inhibitors provide important clues as to second messenger function. In addition, it is important to compare quantitatively the mass of a second messenger formed under physiologic conditions with biologic responses. For example, cAMP production is associated with inhibition of platelet aggregation and secretion (23, 24), but the degree of platelet inhibition is not proportional to the level of cAMP (25). Increases in free intracellular calcium have been shown to be related to platelet aggregation and secretion (26, 27). However, these studies did not quantitatively relate the magnitude of calcium increase to the magnitude of secretion. Also, these studies showed that different agonists could produce different biologic responses even though they increased the intracellular calcium concentration to a similar extent (26, 27). Therefore, these studies suggested that other second messenger(s) may play an important role in mediating aggregation and secretion.

Our studies provide evidence that DAG is this other second messenger. We observed a quantitative relationship between DAG accumulation, aggregation, and secretion. DAG accumulation above a threshold (0.2 pmol/nmol of phospholipid) was quantitatively associated with secondary aggregation and secretion, regardless of whether the agonist was γ-thrombin or collagen (Figs. 3 and 5). In contrast, secondary aggregation in response to collagen was found to occur at a concentration of

TABLE 1

Effects of the platelet inhibitors aspirin (ASA) and sphingosine on DAG accumulation, 40-kDa protein phosphorylation, and secretion in platelets stimulated by γ-thrombin

Platelets were stimulated with γ-thrombin in the absence or presence of the indicated inhibitors, and average DAG mass in the accumulative phase, 40-kDa protein phosphorylation, and secretion were measured as described in Experimental Procedures.

Agent	DAG	40-kDa protein phosphorylation	Secretion
	pmol/nmol of phospholipid	*cpm*	
4 nM γ-Thrombin	0.301	1349	+
8 nM γ-Thrombin	0.398	1360	+
8 nM γ-Thrombin + 5 mM ASA	0.382	1170	+
4 nM γ-Thrombin + 5 mM ASA	0.080	493	−
8 nM γ-Thrombin + 10 μM sphingosine	0.431	270	−

intracellular calcium that was not associated with secondary aggregation in thrombin-stimulated platelets (27). The direct correlation between DAG accumulation and platelet biologic responses provides additional evidence of the critical importance of the DAG/PKC pathway to platelet aggregation and secretion. The major role of the DAG/PKC pathway is also supported by experiments that demonstrated that aggregation and secretion could be blocked by inhibition of either DAG production or PKC activation (Table 1). This is consistent with previous studies that demonstrated that the PKC inhibitor sphingosine blocked secondary aggregation in response to a variety of agonists (5) and that low doses of exogenous DAG overcame aspirin inhibition of secondary aggregation (10).

There are several lines of evidence suggesting that activation of the DAG/PKC pathway is specifically related to secondary aggregation and secretion, rather than to other platelet responses. First, when DAG and phorbol esters are exogenously added to platelets, they do not cause platelet shape change (4, 7, 10). Second, the greatest amount of DAG accumulation occurs during secondary aggregation, rather than during shape change (7). Third, despite the fact that sphingosine inhibits secondary aggregation and secretion, it does not affect agonist-induced shape change (5). Lastly, the ability of exogenous DAG and phorbol ester to activate platelets is greatly potentiated by low doses of calcium ionophores (3, 4), which induce both shape change and an increase in free cytosolic calcium. This implies that DAG/PKC mediation of secondary aggregation and secretion requires some antecedent "priming" events that occur independently of the DAG/PKC pathway. The lack of correlation of DAG production and PKC activation with shape change underscores the specificity of this pathway for secondary aggregation and secretion.

The central role of DAG in platelet biology has important mechanistic and pharmacologic implications. First, the requirement for a threshold level of DAG accumulation for secondary aggregation and secretion may allow platelets to discriminate between subthreshold and suprathreshold concentrations of agonist. Thus, a subthreshold concentration of agonist produces only a subthreshold accumulation of DAG, which is unable to mediate secondary aggregation, whereas a suprathreshold concentration of agonist induces DAG accumulation sufficient to mediate secondary aggregation and secretion. Second, the existence of a final common pathway of platelet activation (DAG accumulation and PKC activation) could facilitate synergy of different platelet agonists. Thus, the combination of two different agonists that individually produce only subthreshold amounts of DAG accumulation could produce an additive or synergistic accumulation of DAG; this would then cause secondary aggregation and secretion. Third, the development of inhibitors that affect the DAG/PKC pathway may lead to the development of more potent platelet inhibitors. Because inappropriate platelet activation has been implicated in the etiology of myocardial infarction (28) and stroke (29), the development of drugs that modulate the DAG/PKC pathway may lead to important advances in the treatment of these diseases.

Acknowledgments

We would like to thank Dr. Wasiuddin Khan for careful review of the manuscript and Marsha Haigood for expert secretarial assistance.

References

1. Bishop, W. R., and R. M. Bell. Functions of diacylglycerol in glycerolipid metabolism, signal transduction and cellular transformation. *Oncogene Res.* **2**:205–218 (1988).
2. Nishizuka, Y. Studies and perspectives of protein kinase C. *Science (Washington D. C.)* **233**:305–312 (1986).
3. Kaibuchi, K., Y. Takai, M. Sawamura, M. Hoshijima, T. Fujikura, and Y. Nishizuka. Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. Biol. Chem.* **258**:6701–6704 (1983).
4. Rink, T. J., A. Sanchez, and T. J. Hallam. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature (Lond.)* **305**:317–319 (1983).
5. Hannun, Y. A., C. S. Greenberg, and R. M. Bell. Sphingosine inhibition of agonist-dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event of the signal transduction pathway. *J. Biol. Chem.* **262**:13620–13626 (1987).
6. Rittenhouse-Simmons, S. Production of diglyceride from phosphatidylinositol in activated human platelets. *J. Clin. Invest.* **63**:580–587 (1979).
7. Werner, M. H., and Y. A. Hannun. Delayed accumulation of diacylglycerol in platelets as a mechanism for regulation of onset of aggregation and secretion. *Blood* **78**:435–444 (1991).
8. Mavis, R. D., R. M. Bell, and P. R. Vagelos. Effect of phospholipase C hydrolysis of membrane phospholipids on membranous enzymes. *J. Biol. Chem.* **247**:2835–2847 (1972).
9. Stern, I., and B. Shapiro. A rapid and simple method for the determination of esterified fatty acids and for total fatty acids in blood. *J. Clin. Pathol.* **6**:158–160 (1953).
10. Werner, M. H., L. Senzel, A. Bielawska, W. Khan, and Y. A. Hannun. Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Mol. Pharmacol.* **39**:547–556 (1991).
11. McGowan, E. B., and T. C. Detwiler. Modified platelet responses to thrombin. *J. Biol. Chem.* **261**:739–746 (1986).
12. Berliner, L. J. Structure-function relationships in human α- and γ-thrombins. *Mol. Cell. Biochem.* **61**:159–172 (1984).
13. Charo, I. F., R. D. Feinman, and T. C. Detwiler. Interrelations of platelet aggregation and secretion. *J. Clin. Invest.* **60**:866–873 (1977).
14. Bligh, E. G., and W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **37**:911–917 (1959).
15. Ames, B. N., and D. T. Dubin. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235**:769–775 (1960).
16. Van Veldhoven, P. P., and G. P. Mannaerts. Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161**:45–48 (1987).
17. Preiss, J., C. R. Loomis, W. R. Bishop, R. Stein, J. E. Niedel, and R. M. Bell. Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. *J. Biol. Chem.* **261**:8597–8600 (1986).
18. Walsh, J. P., and R. M. Bell. *sn*-1,2-Diacylglycerol kinase of *Escherichia coli*. *J. Biol. Chem.* **261**:6239–6247 (1986).
19. Hannun, Y. A., C. R. Loomis, A. H. Merrill, Jr., and R. M. Bell. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *J. Biol. Chem.* **261**:12604–12609 (1986).
20. Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka. A role of calcium-activatied phospholipid-dependent protein kinase in human platelet activation. *J. Biol. Chem.* **258**:2010–2013 (1983).
21. Siess, W., P. Cuatrecasas, and E. G. Lapetina. A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated human platelets. *J. Biol. Chem.* **258**:4683–4686 (1983).
22. Rittenhouse, S. E., and C. L. Allen. Synergistic activation by collagen and 15-hydroxy-9a,11a-peroxidoprosta-5,13-dienoic acid (PGH_2) of phosphatidylinositol metabolism and arachidonic acid release in human platelets. *J. Clin. Invest.* **70**:1216–1224 (1982).
23. Friedman, F., and T. C. Detwiler. Stimulus-secretion coupling in platelets: effects of drugs on secretion of adenosine 5′-triphosphate. *Biochemistry* **14**:1315–1320 (1975).
24. Huang, E. M., and T. C. Detwiler. Stimulus-response coupling mechanisms, in *Biochemistry of Platelets* (D. R. Phillips and M. A. Shuman, eds.). Academic Press, Inc., Orlando, 1–68 (1986).
25. Tsien, W.-H., S. P. Sass, and H. Sheppard. The lack of correlation between inhibition of aggregation and cAMP levels with canine platelets. *Thromb. Res.* **28**:509–519 (1982).
26. Rink, T. J., S. W. Smith, and R. Y. Tsien. Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. *FEBS Lett.* **148**:21–26 (1982).
27. Ware, J. A., P. C. Johnson, M. Smith, and E. W. Salzman. Effect of common agonists on cytoplasmic ionized calcium concentration in platelets. *J. Clin. Invest.* **77**:878–886 (1986).
28. Trip, M. D., V. M. Cats, F. J. L. van Capelle, and J. Vreeken. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *N. Engl. J. Med.* **322**:1549–1554 (1990).
29. Joseph, R., K. M. A. Welch, S. Grunfeld, S. B. Oster, and G. D'Andrea. Baseline and activated platelet cytoplasmic ionized calcium in acute ischemic stroke: effect of aspirin. *Stroke* **19**:1234–1238 (1988).

Send reprint requests to: Yusuf A. Hannun, Division of Hematology/Oncology, Box 3355, Duke University Medical Center, Durham, NC 27710.