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Diacylglycerol Overcomes Aspirin Inhibition of Platelets: Evidence for a Necessary Role for Diacylglycerol Accumulation in Platelet Activation

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SUMMARY

Aspirin, an inhibitor of cyclooxygenase, inhibits platelet aggregation in response to many stimuli. Previous studies suggested an important and necessary role for protein kinase C (PKC) in platelet aggregation and secretion. Therefore, the effects of aspirin on *sn*-1,2-diacylglycerol (DAG), the endogenous activator of PKC, were investigated. Specifically, we sought to determine whether inhibition of DAG production is critical for aspirin action on platelets. Total DAG mass was measured using the DAG kinase assay. At low doses of γ-thrombin (4 nM), aspirin (5 mM) completely inhibited secondary aggregation; this inhibition was associated with near-complete inhibition of DAG production. Inhibition of collagen-induced aggregation by aspirin (50 μM) was also associated with complete inhibition of collagen-stimulated DAG production and secondary aggregation. Concomitantly, aspirin reduced phosphorylation of the 40-kDa protein, a specific PKC substrate strongly suggesting inhibition of PKC in response to aspirin. To determine the physiologic significance of the inhibition of DAG production by aspirin, reconstitution studies were conducted with dioctanoylglycerol (diC_8), a cell-permeable DAG. Under conditions in which aspirin completely inhibited secondary aggregation induced by γ-thrombin, collagen, or arachidonic acid, diC_8 overcame aspirin inhibition of agonist action and reconstituted secondary aggregation. DiC_8 exerted these effects at low concentrations (2–3 μM), which caused minimal aggregation of control platelets. Phorbol 12,13-dibutyrate, a phorbol ester that directly activates PKC, mimicked the effects of diC_8 in overcoming aspirin inhibition of collagen-induced platelet activation. However, subthreshold concentrations of the calcium ionophore ionomycin, arachidonic acid, or γ-thrombin were unable to overcome aspirin inhibition of collagen-induced platelet aggregation, suggesting that the ability to overcome aspirin inhibition is not shared by other second messengers and is not due to nonspecific synergy. These studies constitute evidence that inhibition of DAG production and subsequent PKC activation are crucial to the antiaggregatory effects of aspirin. They also support the hypothesis that DAG production and PKC activation may be the final common pathway for induction of secondary aggregation.

Platelet aggregation and secretion[1] are important events in physiologic and pathologic hemostasis. These biologic events are thought to depend upon agonist-mediated activation of two key enzymes, PLC and PLA_2. PLC cleaves PIP_2 into DAG (1), which activates PKC (2), and IP_3, which increases free cytosolic calcium (3, 4), resulting in activation of calcium- and calmodulin-dependent kinases. PLA_2 releases arachidonic acid, which is then metabolized to potent aggregating agents such as TxA_2 (5).

The PLC and PLA_2 pathways are closely interrelated. DAG can be metabolized via diacylglycerol lipase to arachidonic acid (6). Arachidonic acid can indirectly activate PLC, regardless of whether the arachidonic acid is endogenously released by agonist or exogenously added (7–9). This activation depends on active cyclooxygenase and the generation of thromboxane, because it is inhibited by cyclooxygenase inhibitors (7–9). Also, the stable prostaglandin H_2 analog and thromboxane agonist U-46619 (10) is able to activate PLC in aspirin-treated platelets (7, 11), strongly implicating thromboxanes as mediators of the effects of arachidonic acid. In addition, both calcium and DAG may activate PLA_2 (7, 8, 12). This complex interplay of several

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[1] Platelet activation can be 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 (44).

ABBREVIATIONS: PLC, phospholipase C; DAG, *sn*-1,2-diacylglycerol; G protein, GTP-binding protein; diC_8, dioctanoylglycerol; PLA_2, phospholipase A_2; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate; PGI_2, prostaglandin I_2; TxA_2, thromboxane A_2; PRP, platelet-rich plasma; PPP, platelet-poor plasma; Me_2SO, dimethyl sulfoxide; PDBu, phorbol 12,13-dibutyrate; ACD, acid citrate dextrose buffer (85 mM sodium citrate, 111 mM dextrose, 71 mM citric acid); Tyrode-HEPES buffer, 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mM glucose, pH 7.4.

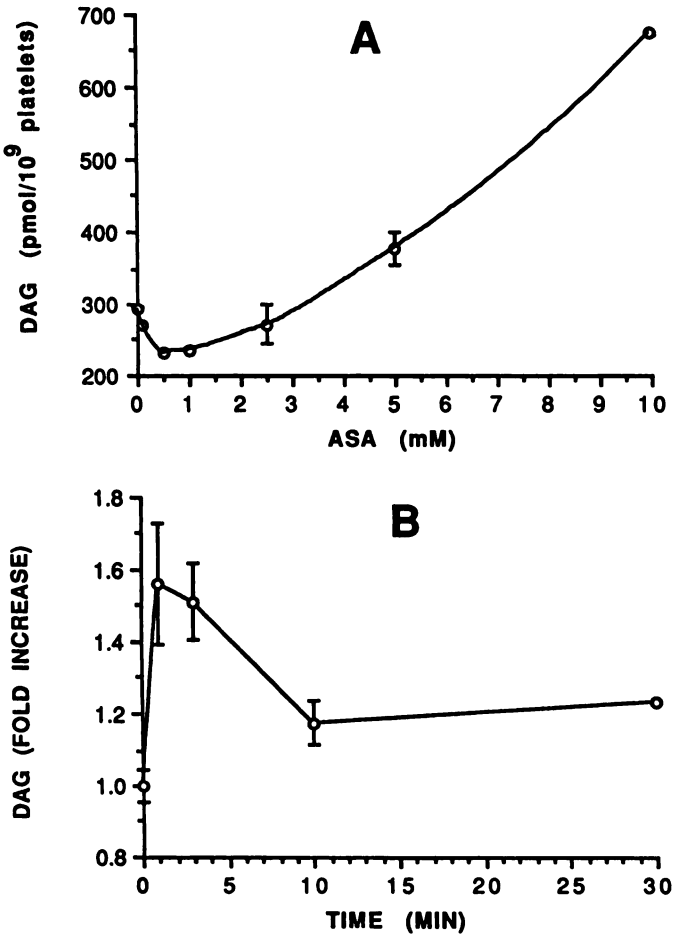

Fig. 1. A, Dose response of aspirin-induced changes in DAG levels. Platelets were prepared as described in Experimental Procedures and were incubated with the indicated concentrations of aspirin (*ASA*) for 5 min. Duplicate samples (except for 0.1 mM aspirin, which represents a single determination) were then obtained for measurement of DAG mass using the DAG kinase assay. Means and standard deviations were calculated for each duplicate sample and are represented by *error bars*; means without error bars had standard deviations smaller than the size of the *symbols*. B, Time course of aspirin-associated changes in DAG. Platelets were incubated with 5 mM aspirin, and duplicate samples were obtained at the indicated time points for measurement of DAG mass. *Error bars*, standard deviations.

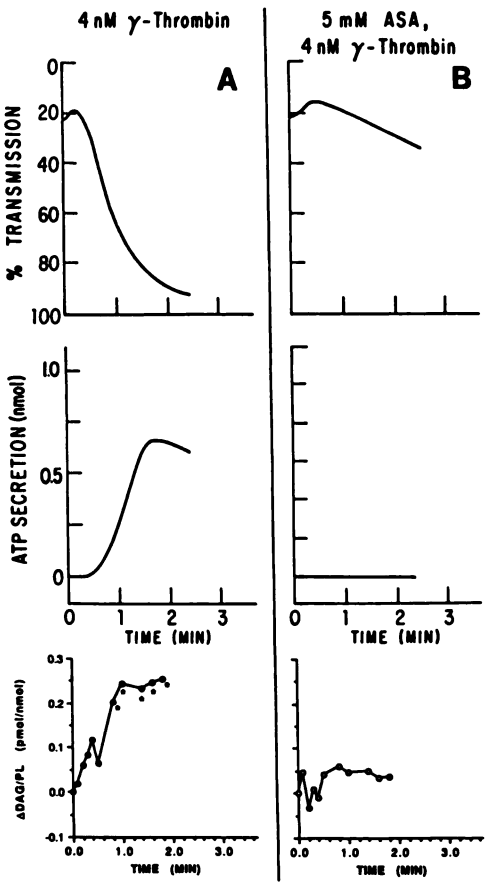

Fig. 2. Effect of aspirin on γ-thrombin-mediated aggregation, secretion, and DAG production. Platelets incubated without (A) or with (B) 5 mM aspirin (*ASA*) were stimulated with 4 nM γ-thrombin. Aggregation, ATP secretion, and DAG mass were measured as described in Experimental Procedures. Δ*DAG*, change in DAG, in pmol/nmol of phospholipid phosphate (*PL*), from unstimulated platelets at time 0. The *points* represent the average of three independent experiments. *, Significant elevations in DAG above levels generated in platelets stimulated in the presence of aspirin ($p < 0.05$ by two-sample *t* test in all cases). The baseline DAG level in unstimulated platelets was 0.72 ± 0.23 pmol/nmol of phospholipid phosphate (mean ± standard deviation), whereas the baseline DAG level in platelets incubated with 5 mM aspirin before stimulation was 1.10 ± 0.28 pmol/nmol of phospholipid phosphate (mean ± standard deviation).

second messengers has prevented the determination of the role of individual second messenger(s) in platelet responses.

Recent studies have examined the role of the DAG/PKC pathway in platelet activation. Exogenous cell-permeable DAGs have been shown to induce minimal secretion from dense granules but require increased intracellular calcium for a full secretory response (13). DAG and phorbol esters have also been shown to stimulate fibrinogen binding (14), which plays an important role in the formation of platelet aggregates (15). At concentrations that preferentially inhibited PKC, sphingosine was shown to completely inhibit secondary aggregation and secretion in response to a variety of agonists, without affecting platelet shape change. DAG and phorbol esters overcame this inhibition by sphingosine (16). These studies suggested a necessary, but insufficient, role for PKC in secondary aggregation. Although these studies suggested a necessary role for DAG in secondary aggregation, they did not establish such a role. This is because PKC activity may be modulated by agents other than DAG [such as arachidonic acid (17) and PIP_2 (18)] and because DAG may have actions other than activation of PKC (12, 19).

If DAG is indeed functioning as a common and necessary second messenger for platelet aggregation and secretion, then the modulation of platelet DAG levels would be an important target for the action of inhibitors of aggregation and secretion. According to this hypothesis, platelet inhibitors could prevent aggregation by either directly or indirectly inhibiting agonist-stimulated DAG production, because this would prevent the PKC activation necessary for secondary aggregation.

We investigated this hypothesis by studying the effects of the cyclooxygenase inhibitor aspirin (20) on agonist-stimulated DAG levels and by studying the ability of DAG to specifically overcome aspirin inhibition of secondary aggregation. It is well established that aspirin treatment inhibits platelet aggregation in response to many stimuli (21), but previous studies have noted variable inhibition by aspirin of thrombin-stimulated DAG production (1, 7, 22). Aspirin, at a concentration about 10 times higher than that achieveable *in vivo* with conventional

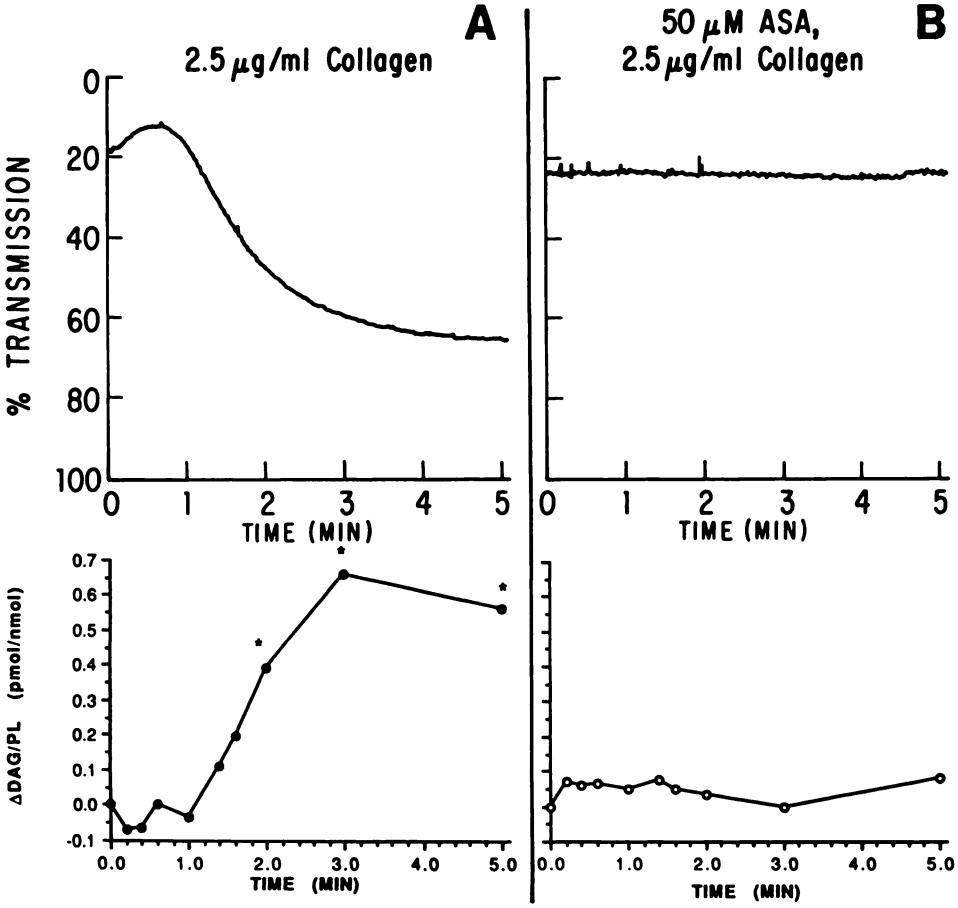

Fig. 3. Effect of aspirin (*ASA*) on collagen-mediated aggregation and DAG production. Platelets incubated without (A) or with (B) 50 μM aspirin were stimulated with 2.5 μg/ml collagen, and aggregation and DAG mass were measured as described in Experimental Procedures. *ΔDAG*, change in DAG from unstimulated platelets at time 0. The baseline DAG level in unstimulated platelets was 0.55 ± 0.04 pmol/nmol of phospholipid phosphate (*PL*) (mean ± standard deviation); the baseline DAG level in aspirin-treated platelets before stimulation was 0.51 ± 0.02 pmol/nmol of phospholipid phosphate (mean ± standard deviation). The *points* represent the mean of duplicate determinations for one experiment, which is representative of two independent experiments. *, Significant elevations in DAG above levels generated in platelets stimulated in the presence of aspirin ($p < 0.025$ by two-sample *t* test in all cases).

doses of aspirin (23), was found to inhibit collagen-induced DAG production in another study (11). These studies did not specifically investigate whether aspirin inhibition of DAG production was related to aspirin inhibition of secondary aggregation. In addition, these studies did not determine whether concentrations of aspirin achieveable *in vivo* could affect agonist-stimulated DAG production. Although aspirin inhibition of DAG production might be a crucial molecular mechanism mediating aspirin inhibition of aggregation, it is also quite possible that 1) aspirin inhibition of DAG production is an epiphenomenon unrelated to aspirin inhibition of aggregation or 2) DAG requires another second messenger (such as Ca^{2+}) to overcome aspirin inhibition of aggregation. Therefore, we undertook a study to examine the role of suppression of DAG levels in mediating aspirin inhibition of platelet aggregation. We first measured the effects of aspirin on both DAG production and aggregation in response to γ-thrombin and collagen and then determined whether exogenous DAG could overcome the inhibitory effects of aspirin. These studies support a role for DAG as a common and necessary second messenger in platelet aggregation. They also suggest that modulation of DAG responses may be an important target for antiplatelet agents.

Experimental Procedures

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). Luciferin-luciferase (Chronolume), arachidonic acid, and collagen were obtained from Chronolog Corporation. Arachidonic acid was also obtained from Biomol. PGI_2, aspirin, indomethacin, ceramide, and *Bacillus cereus* PLC 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%) or in Tyrode-HEPES buffer, whose pH was then adjusted to 7.4. Me_2SO alone at these concentrations did not affect aggregation. β-Octylglucoside and ionomycin were purchased from Calbiochem. *sn*-1,2-Dioleoylglycerol was prepared from 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids) by PLC digestion (24), followed by extraction in ether and quantitation by ester analysis (25). DAG kinase from *Escherichia coli* was a kind gift from Drs. Carson Loomis and Robert Bell (Department of Biochemistry, Duke University, Durham, NC). [γ-^{32}P]ATP was from New England Nuclear, and sodium ATP was from Pharmacia. diC_8 was from Avanti. This was dissolved in Me_2SO; the final concentration of Me_2SO never exceeded 0.75%. Me_2SO alone at these concentrations did not affect platelet aggregation in response to agonists (data not shown).

Methods

Preparation of platelets. Platelets were obtained from healthy drug-free adults using a modification of the procedure of Siess *et al.* (9). Briefly, 90–135 ml of blood were added to ACD in a 9:1 ratio (v/v) and centrifuged at 200 × *g* for 20 min, to obtain PRP. PGI_2 (5 ng/ml) was added and the PRP was centrifuged at 800 × *g* for 15 min. The PRP-derived platelet pellet was suspended in 5 ml of Tyrode-HEPES buffer with 300 ng/ml PGI_2. The platelet concentration was determined with a Coulter counter and was adjusted to 2.5×10^8 cells/ml with Tyrode-HEPES buffer without PGI_2 and with 0.7% PPP. The platelets were then allowed to sit for 120 min to allow resolution of the inhibitory effects of the PGI_2. In aggregation experiments employing collagen, arachidonic acid, or ionomycin, 1 mM $MgCl_2$ was added to the suspend-

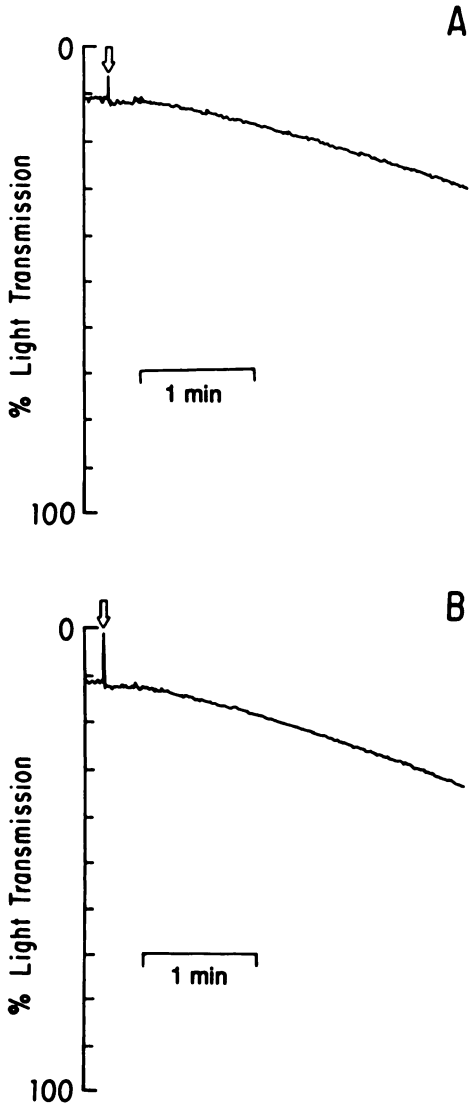

Fig. 4. Aspirin effects on diC_8-mediated aggregation. Platelets incubated without (A) or with (B) 50 μM aspirin were stimulated with 4 μM diC_8 ⇓, and aggregation was measured.

ing medium. Additionally, 1 mM $CaCl_2$ was added to the medium in experiments that examined the action of ionomycin. In experiments examining the effects of aspirin, platelets were incubated with aspirin for at least 15 min (unless otherwise indicated) at 25°. Platelets were not further washed and PPP was included in the final suspending medium because platelets prepared by this method had low DAG baseline levels, which allowed sensitive measurement of DAG mass levels, but still retained the ability to undergo physiologic shape change and aggregation similar to platelets in PRP.

Collagen and γ-thrombin, a proteolyzed form of α-thrombin, were used to correlate platelet activation with the DAG response. γ-Thrombin does not initiate coagulation because it does not bind fibrinogen (26). γ-Thrombin binds to one of two putative thrombin receptors (27) and induces platelets to aggregate, secrete, and undergo many of the biochemical changes associated with α-thrombin stimulation, without the induction of coagulation. For these reasons, γ-thrombin has been the agent of choice to study thrombin effects on platelets in the presence of serum components (16, 28).

Simultaneous measurements of aggregation, ATP secretion, and DAG mass levels. The platelet suspension was stirred in a 37° water bath. Samples (0.8 ml, equal to 2×10^8 platelets) for zero time points were placed in 3 ml of chloroform/methanol (1:2) for lipid extraction. The platelets were then stimulated with γ-thrombin or collagen, and an aliquot was immediately placed in a Chronolog Lumi-Aggregometer for measurement of aggregation and ATP secretion with luciferin-luciferase. At the indicated times, 0.8-ml aliquots of the platelet suspension were placed in chloroform/methanol. In some experiments, duplicate 0.8-ml platelet aliquots were placed in chloroform/methanol at the indicated time points. Similar studies were performed with platelets that had been incubated with aspirin.

DAG mass measurements. Lipids were extracted by the method of Bligh and Dyer (29), and total phospholipid phosphate was determined as described (30, 31). The total mass of DAG in the lipid extracts was measured using *E. coli* DAG kinase and [γ-^{32}P]ATP, as described (32). 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 specific activity of the ATP ranged from 31,200 to 91,800 cpm/nmol. Conversion of diolein standards was invariably linear, with a correlation coefficient of 0.999 or greater. The sensitivity of the DAG kinase assay is particularly important when measuring baseline DAG levels in platelets, which are about 10-fold less than those found in other cells (32).

Statistical tests. Standard deviations were calculated using Lotus 1-2-3 (Lotus Development Corp.) and Excel (Microsoft Corp.) software. Data analysis was performed using two-sample *t* tests, as described (33).

Measurement of 40-kDa protein phosphorylation. Platelets prepared as described above were labeled with [^{32}P]orthophosphate and stimulated, and measurement of 40-kDa protein phosphorylation was performed as described (34).

Results

Aspirin has effects on DAG levels in resting platelets. We found that low millimolar concentrations of aspirin were required to inhibit activation of platelets by γ-thrombin. Therefore, the effects of aspirin on baseline DAG levels were investigated. When platelets were incubated with aspirin for 5 min, a biphasic dose response was observed between aspirin and resting levels of DAG mass (Fig. 1A). DAG levels declined from 295 pmol/10^9 platelets to 233 pmol/10^9 platelets in response to 0–0.5 mM aspirin. At aspirin concentrations of 0.5–10 mM, DAG levels proportionately increased, reaching a level of 633 pmol/10^9 platelets at 10 mM aspirin. The increases in DAG levels also were time dependent (Fig. 1B). After the addition of 5 mM aspirin, DAG levels rapidly increased 1.6-fold by 1 min, declined after 3 min, but still remained 1.2-fold elevated for at least 60 min after addition of aspirin. Thus, aspirin has effects on DAG homeostasis in unstimulated platelets. The biologic significance of these changes was not determined, but they emphasize the necessity of measuring total mass of DAG, rather than pools specifically labeled with arachidonic acid.

Aspirin inhibits DAG production in activated platelets in parallel with its inhibition of secondary aggregation. Because aspirin inhibits the formation of cyclooxygenase metabolites of arachidonic acid, it could reduce the possible positive feedback of thromboxanes on PLC, thus reducing PLC-mediated DAG formation in stimulated platelets (7, 8). This positive feedback may be important for platelet activation. Earlier studies showed no or moderate effects of aspirin on DAG levels in α-thrombin-stimulated platelets (1, 22). We, therefore, sought to determine whether aspirin inhibited DAG accumulation in thrombin-activated platelets and whether these effects occurred in parallel with the ability of aspirin to inhibit secondary aggregation and secretion. At high doses (5 mM), aspirin was able to completely inhibit aggregation and

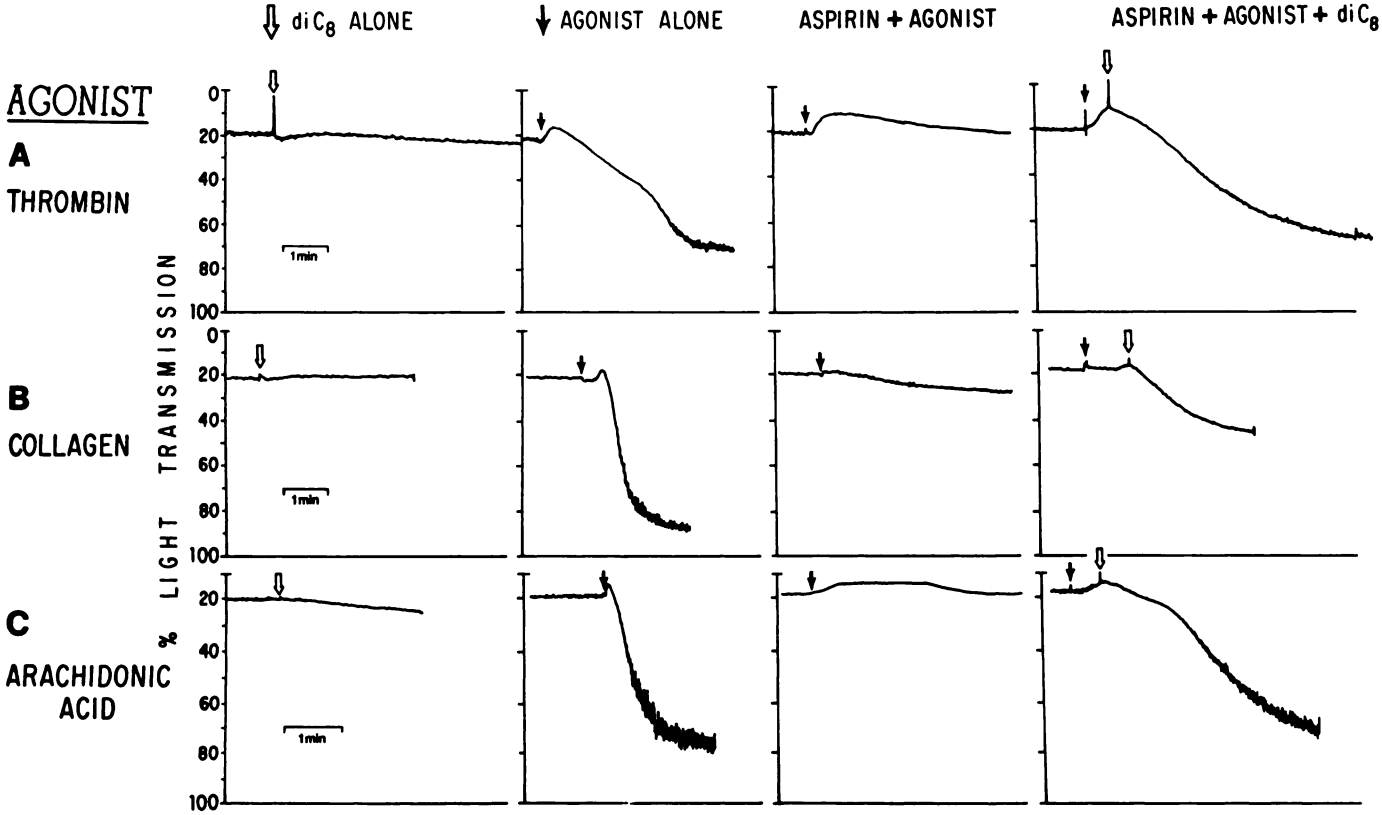

Fig. 5. Overcoming of aspirin inhibition of γ-thrombin-, collagen-, and arachidonic acid-stimulated aggregation by diC_8. A, A subthreshold concentration of diC_8 (3 μM) (⇓) was added, and then aggregation in response to 4 nM γ-thrombin (↓), in the absence and presence of 5 mM aspirin, was determined. Platelets were then incubated with 5 mM aspirin and stimulated by 4 nM γ-thrombin (↓). DiC_8 (3 μM) (⇓) was added 0.5 min after γ-thrombin. B, Platelets were stimulated with a subthreshold concentration of diC_8 (3 μM, ⇓), and then aggregation in response to 2.5 μg/ml collagen (↓), without and with 50 μM aspirin, was measured. Platelets were incubated with 50 μM aspirin and were then stimulated with 2.5 μg/ml collagen (↓); diC_8 was added (⇓) 1 min after collagen. C, A subthreshold dose (2 μM) (⇓) of diC_8 was added, and then aggregation in response to 100 μM arachidonic acid (↓), in the absence and presence of 50 μM aspirin, was measured. Then, platelets incubated with 50 μM aspirin were stimulated with 100 μM arachidonic acid (↓), followed 0.5 min later by 2 μM diC_8 (⇓).

secretion in response to 4 nM γ-thrombin (Fig. 2A). Other authors have also found that high doses of aspirin are required to inhibit thrombin-induced aggregation (21). Whereas 4 nM γ-thrombin stimulated the accumulation of DAG, aspirin inhibition of aggregation was associated with near-complete inhibition of DAG accumulation (Fig. 2B). At 8 nM γ-thrombin, 5 mM aspirin was unable to prevent secondary aggregation; DAG accumulation also was not inhibited (data not shown).

The effects of aspirin on collagen-stimulated platelets were determined next. Along with thrombin, collagen appears to be a major physiologic platelet agonist, but its predominant mechanism of action appears to differ from that of thrombin. Collagen-stimulated formation of inositol phosphates (35) and DAG (11, 36) is significantly inhibited by aspirin or indomethacin, whereas several investigators have found that thrombin-stimulated production of these two second messengers is not inhibited by aspirin or indomethacin (7, 36, 37). Whereas these studies suggest that collagen activation of PLC is primarily indirect, other studies suggest that collagen can activate PLC directly via a receptor-linked G protein (38). For these reasons, it became important to determine whether aspirin inhibition of collagen-induced platelet aggregation was also closely associated with inhibition of delayed DAG accumulation. Moreover, studies with collagen allowed us to use much lower concentrations of aspirin that do not alter baseline DAG levels. Addition of collagen (2.5 μg/ml) to platelets caused a time-dependent increase in DAG mass of 0.66 pmol/nmol phospholipid above baseline (Fig. 3A), representing a 2.2-fold increase in DAG. Aspirin, at a low concentration (50 μM), completely inhibited both aggregation and DAG production in response to collagen (Fig. 3B).

Therefore, in platelets stimulated with either γ-thrombin or collagen, inhibition of DAG accumulation correlated with inhibition of secondary aggregation and secretion. These studies suggest that the positive feedback of thromboxanes on PLC accounts for most of the DAG generated in response to collagen or low doses of γ-thrombin.

Aspirin does not inhibit DAG-mediated aggregation. If inhibition of DAG production is a final common critical event that occurs distal to the site of action of aspirin, then aspirin should not affect DAG-mediated aggregatory responses. To test this hypothesis, we stimulated platelets with a concentration of the cell-permeable DAG diC_8 which induced an aggregatory response. After incubation of platelets with a concentration of aspirin that completely inhibited collagen-induced aggregation (50 μM), platelets were stimulated with the same concentration of diC_8. Fig. 4 demonstrates that diC_8-mediated aggregation is not affected by aspirin. This is consistent with DAG action occurring distal to that of aspirin.

Exogenous DAG reconstitutes secondary aggregation

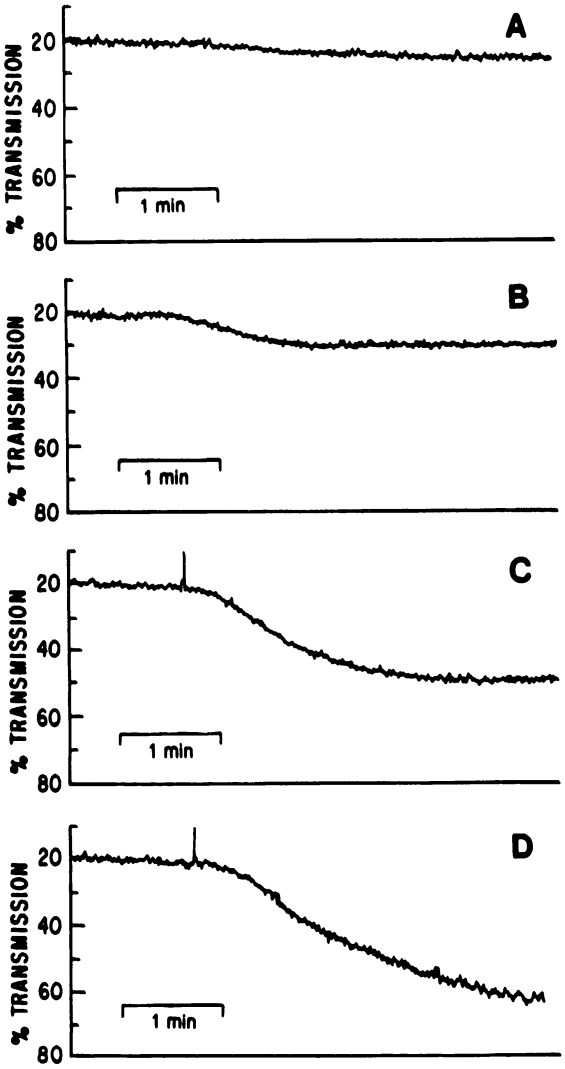

Fig. 6. Dose dependence of ability of diC_8 to reconstitute secondary aggregation. Platelets incubated with 50 μM aspirin were stimulated by 2.5 μg/ml collagen, followed 1 min later by diC_8. A, 0.5 μM diC_8; B, 1 μM diC_8; C, 2 μM diC_8; D, 4 μM diC_8.

in aspirin-inhibited platelets. If aspirin inhibition of secondary aggregation is independent of its ability to inhibit DAG production, then exogenous DAG should not overcome inhibition by aspirin. We, therefore, investigated whether exogenously added DAG could reconstitute secondary aggregation in platelets whose aggregatory responses had been inhibited by aspirin. In order to ensure that the observed DAG effect could only be a synergistic effect and not one related to secondary aggregation, which can be caused by high concentrations of DAG alone, we used low concentrations of diC_8, which in the absence of aspirin caused minimal or no aggregatory responses. This subthreshold concentration of diC_8 was then tested for its ability to reconstitute secondary aggregation in platelets whose ability to undergo secondary aggregation in response to γ-thrombin, collagen, or arachidonic acid had been completely blocked by aspirin. These experiments are illustrated in Fig. 5. Even though diC_8 was used in low subthreshold concentrations (2–3 μM), it was still able to overcome aspirin inhibition of γ-thrombin, collagen, and arachidonic acid and reconstitute secondary aggregation (Fig. 5). Additional experiments demonstrated that the ability of diC_8 to overcome aspirin inhibition

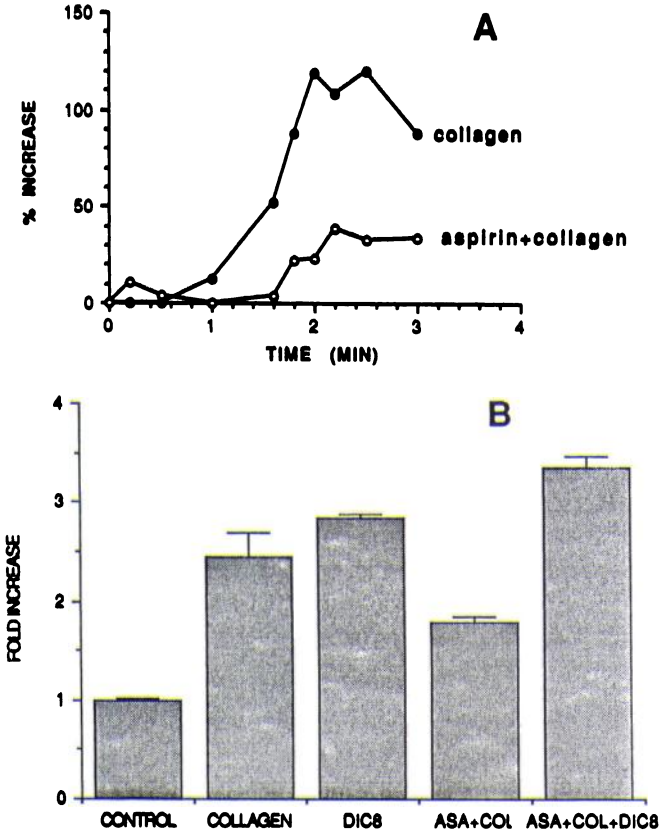

Fig. 7. A, Effects of aspirin on 40-kDa protein phosphorylation in collagen-stimulated platelets. Platelets were prepared and labeled with ^{32}P as described in Experimental Procedures, incubated without or with 50 μM aspirin, and then stimulated with 2.5 μg/ml collagen. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, stained, dried, and autoradiographed. The ^{32}P-labeled 40-kDa phosphoprotein was then excised and counted. Data are expressed as percentage of increase over control (unstimulated) 40-kDa phosphoprotein cpm. Before stimulation, 40-kDa protein phosphorylation was 602 cpm in untreated platelets and 353 cpm in aspirin-treated platelets. B, Ability of diC_8 to reconstitute 40-kDa protein phosphorylation in aspirin-inhibited platelets. Platelets were prepared and labeled with ^{32}P as described in Experimental Procedures, incubated with or without 50 μM aspirin (*ASA*), and then stimulated with collagen (2.5 μg/ml), diC_8 (3 μM), or collagen followed 45 sec later by diC_8. Platelets were placed in sodium dodecyl sulfate sample buffer 3 min after stimulation. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, stained, dried, and autoradiographed. The 40-kDa phosphoprotein was then excised and counted. Data are expressed as fold increase over control (unstimulated) 40-kDa phosphoprotein cpm. *Bars*, mean ± standard deviation of three independent stimulations. *COL*, collagen. Samples with collagen were greater than control ($p < 0.001$) and aspirin plus collagen ($p < 0.01$), whereas samples with aspirin plus collagen were less than aspirin plus collagen plus diC_8 ($p < 0.001$) by two-sample *t* tests. Forty-kilodalton protein phosphorylation in control (unstimulated) platelets was 102 ± 1 cpm (mean ± standard deviation); 40-kDa protein phosphorylation in aspirin-treated unstimulated platelets was 94 ± 8 cpm (mean ± standard deviation).

of collagen-induced aggregation was dose dependent (Fig. 6), providing further evidence that this reconstitution of secondary aggregation was a direct action of diC_8.

In other studies, we have observed the importance of delayed DAG accumulation at the time of secondary aggregation. These experiments utilized subthreshold doses of diC_8 or γ-thrombin, which alone were unable to cause secondary aggregation. Addition of diC_8 after γ-thrombin resulted in secondary aggregation and secretion. DiC_8 was most potent at producing second-

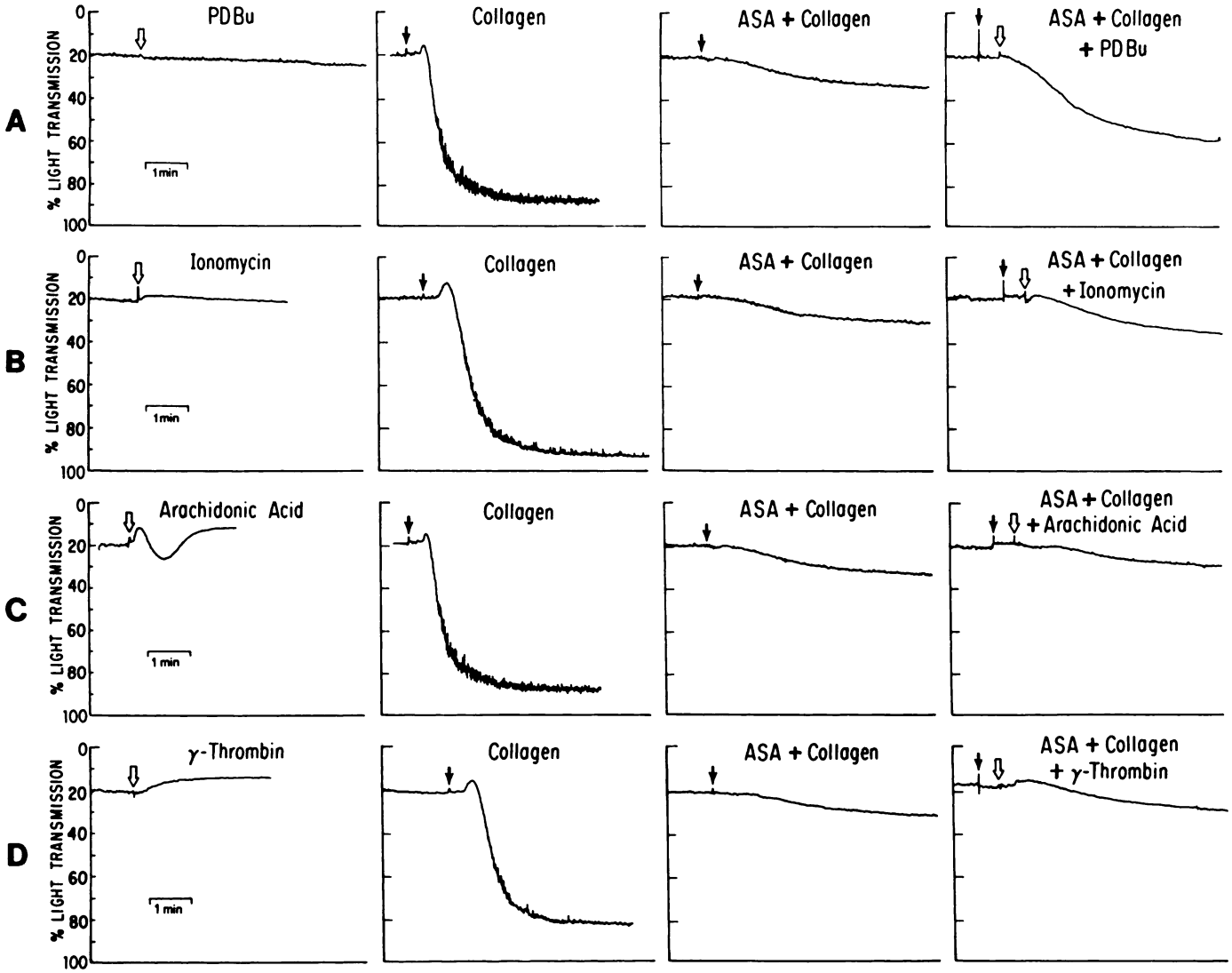

Fig. 8. Ability of other platelet activators to reconstitute secondary aggregation in aspirin-inhibited platelets. A, A subthreshold concentration of PDBu (5 nM) (⇓) was added, and then aggregation in response to 1.25 μg/ml collagen (↓), without and with prior incubation with 50 μM aspirin (*ASA*), was measured. Then, platelets were incubated with 50 μM aspirin and stimulated with 1.25 μg/ml collagen (↓); 5 nM PDBu (⇓) was added 0.5 min after collagen. B, Aggregation in response to a subthreshold concentration (50 nM) (⇓) of ionomycin was measured, and then platelets were incubated without or with 50 μM aspirin and stimulated with 1.5 μg/ml collagen (↓). Platelets were incubated with 50 μM aspirin and stimulated with 1.5 μg/ml collagen (↓); 50 nM ionomycin (⇓) was added 0.5 min after collagen. C, Aggregation in response to a subthreshold concentration (2.5 μM) (⇓) of arachidonic acid was measured; this response was then compared with the aggregatory responses of platelets stimulated by 1.25 μg/ml collagen (↓) after prior incubation without or with 50 μM aspirin. Platelets were then incubated with 50 μM aspirin and stimulated by 1.25 μg/ml collagen (↓), followed by the addition of 2.5 μM arachidonic acid (⇓). D, Aggregation of platelets in response to a subthreshold concentration (1.5 nM) (⇓) of γ-thrombin was measured. Platelets were then incubated without or with 50 μM aspirin and stimulated with 2.5 μg/ml collagen (↓). After incubation with 50 μM aspirin, platelets were stimulated with 2.5 μg/ml collagen (↓) followed by 1.5 nM γ-thrombin (⇓).

ary aggregation when it was added 0.5 min after rather than simultaneously with γ-thrombin.[2] Therefore, experiments were performed to determine whether the ability of diC_8 to overcome aspirin inhibition of aggregation exhibited a time dependence. Platelets were incubated with a concentration of aspirin (50 μM) that completely inhibited collagen-induced aggregation. Platelets were then stimulated with collagen. A subthreshold concentration of diC_8 (2 μM) was added 0, 0.5, 1, or 2 min after collagen stimulation. DiC_8 was most potent at reconstituting secondary aggregation when added at a delayed interval after rather than simultaneously with collagen (data not shown). This provides further evidence that DAG accumulation at a delayed interval after agonist stimulation is critical for secondary aggregation to occur.

In order to determine whether this action of aspirin occurred primarily through cyclooxygenase inhibition, we also tested the ability of diC_8 to reconstitute secondary aggregation in platelets whose aggregatory response had been blocked by indomethacin, another cyclooxygenase inhibitor. At low concentrations (4 μM), which are achieveable *in vivo*, indomethacin completely blocked aggregation in response to 2 μg/ml collagen. Addition of a low concentration of diC_8 (3 μM) after collagen overcame the indomethacin inhibition and reconstituted secondary aggregation (data not shown). Thus, not only was aspirin inhibition of platelet aggregation strongly correlated with inhibition of DAG accumulation, but also adding back DAG reconstituted

[2] M. Werner and Y. Hannun, manuscript in preparation.

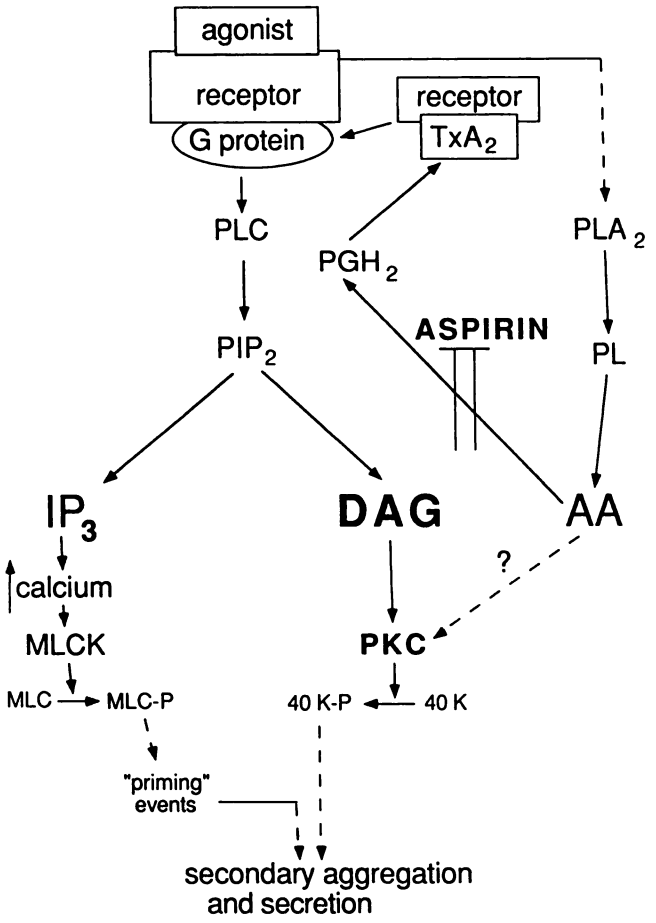

Fig. 9. Model of biochemical regulation of platelet aggregation. *AA*, arachidonic acid; *40 K*, 40-kDa substrate of PKC; *40 K-P*, phosphorylated 40-kDa substrate of PKC; *MLCK*, myosin light chain kinase; *MLC*, myosin light chain; *MLC-P*, phosphorylated myosin light chain; *PL*, phospholipids. Aspirin blocks conversion of arachidonic acid to prostaglandin H_2 (*PGH_2*). *Solid arrows*, well established pathways. *Broken arrows*, possible pathways suggested by this and other studies. *?*, other possible functions. See text for details.

the biologic response of secondary aggregation when aggregation had been blocked by either aspirin or indomethacin.

Aspirin-mediated inhibition of DAG production is associated with decreased PKC activity. DAG activation of PKC is well described (2, 39), but it has also been reported that DAG facilitates membrane fusion (19) and activates PLA_2 (12) and PLC (40). Therefore, we sought to determine whether the inhibitory effects of aspirin on DAG production were associated with decreased PKC activation. Measurements of phosphorylation of the 40-kDa protein, a PKC substrate, were performed in platelets stimulated with collagen in the absence or presence of aspirin. Similar to DAG production, 40-kDa protein phosphorylation was inhibited by aspirin (Fig. 7A). Consistent with these results, it has been shown that indomethacin inhibits 40-kDa protein phosphorylation in response to collagen (35).

In order to determine whether the ability of diC_8 to overcome aspirin inhibition of platelet aggregation correlated with reconstitution of PKC activity, we evaluated the phosphorylation of the 40-kDa protein as an indicator of PKC activity. The addition of diC_8 (3 μM) reconstituted 40-kDa protein phosphorylation in aspirin-treated platelets that had been stimulated with 2.5 μg/ml collagen (Fig. 7B). These results strongly suggest that the inhibitory effects of aspirin are mediated through reduction of DAG production and subsequent reduced activation of PKC.

It should be noted that diC_8 alone does not cause significant aggregation, even though it causes 40-kDa protein phosphorylation comparable to that produced by a dose of collagen that does cause aggregation (Fig. 7B). In addition, previous studies done in the absence of aspirin have shown that addition of subthreshold diC_8 after a subthreshold dose of agonist results in secondary aggregation and secretion.[2] Taken together, these experiments strongly suggest that subthreshold agonist is able to "prime" platelets to the action of diC_8. These results are consistent with the hypothesis that the DAG/PKC pathway is necessary but not sufficient for platelet aggregation. The nature of the priming event is poorly defined but may be determined by the intracellular calcium signal, because calcium ionophores potentiate aggregation and secretion in response to cell-permeable DAG (13, 41).

The ability of the DAG/PKC pathway to overcome aspirin inhibition of aggregation is not shared by other platelet activators. It is possible that the ability of DAG to overcome aspirin inhibition of platelet aggregation is a nonspecific effect shared by any second messenger or any agonist that can activate platelets. Therefore, we investigated the abilities of subthreshold concentrations of other second messengers and agonists to reconstitute secondary aggregation in collagen-stimulated platelets in which secondary aggregation had been completely inhibited by aspirin.

PDBu, a phorbol ester that activates PKC, was used to determine whether direct PKC activation could overcome aspirin inhibition. PDBu was used at a concentration (5 nM) that has been shown to activate PKC. This concentration of PDBu caused no platelet aggregation (Fig. 8A). This concentration of PDBu was able to overcome aspirin inhibition and reconstitute secondary aggregation (Fig. 8A). This supports the hypothesis that exogenous DAG is able to overcome aspirin inhibition of platelet aggregation by activating PKC and that the DAG effect is not due to other possible targets.

The calcium ionophore ionomycin was used to determine whether aspirin inhibition could be overcome by a subthreshold increase in another second messenger, i.e., free cytosolic calcium. In these experiments, 1 mM calcium was added to the medium to maximize calcium influx in response to ionomycin. The addition of calcium to the medium did not affect collagen-induced aggregation or aspirin inhibition of collagen-induced aggregation (Fig. 8B). At a concentration of 50 nM, which has been shown to cause significant calcium mobilization (42, 43), ionomycin initiated a mild shape change (Fig. 8B). At this concentration, ionomycin did not reconstitute secondary aggregation in collagen-stimulated platelets whose aggregatory responses had been blocked by aspirin. This implies that free cytosolic calcium cannot reconstitute secondary aggregation in aspirin-inhibited platelets.

Arachidonic acid was evaluated to determine whether it could overcome aspirin inhibition directly or possibly through its lipoxygenase metabolites. Arachidonic acid was also used at a subthreshold concentration (2.5 μM), which caused only a reversible shape change (Fig. 8C). Like ionomycin, arachidonic acid was unable to reconstitute secondary aggregation in aspirin-inhibited platelets.

Finally, we investigated whether the effects of diC_8 were a result of synergy between platelet agonists (44). γ-Thrombin

was used to determine whether another platelet agonist could synergize with collagen to overcome aspirin inhibition. Again, γ-thrombin was used at a subthreshold concentration (1.5 nM), which caused only a reversible shape change (Fig. 8D). This subthreshold concentration of γ-thrombin did not reconstitute secondary aggregation in aspirin-inhibited platelets (Fig. 8D).

All these compounds were used at low concentrations, which produced minimal aggregatory responses in non-aspirin-treated platelets, similar to the aggregatory responses observed at the doses of diC_8 used in the previous experiments (Figs. 5 and 8). With the exception of PDBu, none of these compounds, including the calcium ionophore ionomycin, were able to reconstitute secondary aggregation in collagen-stimulated platelets that had been preincubated with aspirin (Fig. 8). These data support the hypothesis that the DAG/PKC pathway is the final common pathway through which aspirin inhibits secondary aggregation.

Discussion

In this study, we demonstrate that aspirin inhibits γ-thrombin- and collagen-mediated accumulation of DAG mass at the time during which secondary aggregation would normally occur. Aspirin inhibited platelet DAG production in response to collagen at a concentration (50 μM) 10-fold lower than that previously reported (11). This aspirin concentration is attainable *in vivo* with conventional doses of aspirin (23). We found aspirin to be much less potent in inhibiting γ-thrombin-induced DAG production; the dose of aspirin required to inhibit a relatively low dose of γ-thrombin was 100 times greater than that required to inhibit collagen-mediated DAG production. A slightly higher concentration of γ-thrombin overcame aspirin inhibition of both DAG accumulation and secondary aggregation. This is consistent with another report in which thrombin-stimulated DAG production was found to be partially inhibited by aspirin when low concentrations of thrombin were used (22). This probably explains why some authors (1, 7, 36) who used high concentrations of thrombin observed that aspirin or indomethacin did not inhibit thrombin-induced DAG production, and this is consistent with reports that thrombin and collagen activate platelets through different molecular mechanisms (36, 45). The high concentrations of aspirin required to inhibit γ-thrombin-induced aggregation and DAG production may indicate that other inhibitory effects besides cyclooxygenase inhibition are necessary for aspirin to inhibit γ-thrombin-induced aggregation and DAG production. However, even though γ-thrombin and collagen activate platelets by different molecular mechanisms, aspirin inhibition of DAG accumulation is a common signal transduction event that closely parallels its inhibition of secondary aggregation and secretion in response to both these agonists.

We also demonstrate that aspirin inhibition of DAG production has major biologic significance. This is based on the following evidence. First, a subthreshold concentration of diC_8 could restore secondary aggregation in aspirin-inhibited platelets. The observation that aspirin did not inhibit the aggregatory effects of diC_8 indicates that aspirin exerts its effects by altering DAG production but not DAG action. Second, aspirin inhibition of DAG production was associated with inhibition of 40-kDa protein phosphorylation. Thus, aspirin inhibition of DAG production is associated with inhibition of PKC activity and platelet aggregation, and this aggregation defect can be overcome by increases in DAG. This indicates that aspirin inhibition of DAG production is not a minor parallel event of aspirin action but has crucial biochemical and biologic consequences.

Multiple observations strongly suggest that the ability of diC_8 to overcome aspirin inhibition is specific to the DAG/PKC pathway. First, the PKC activator PDBu was able to overcome aspirin inhibition in a manner similar to that of diC_8. Second, under the same conditions in which diC_8 reconstituted secondary aggregation in aspirin-treated platelets, it also reconstituted phosphorylation of the 40-kDa protein, a PKC substrate. Third, ionomycin and arachidonic acid did not overcome aspirin inhibition of platelet aggregation. Fourth, a subthreshold dose of γ-thrombin was unable to overcome aspirin inhibition of collagen-stimulated aggregation, indicating that the ability of diC_8 to overcome aspirin inhibition is not due to a nonspecific synergistic effect.

A model (Fig. 9) of the biochemical regulation of platelet aggregation can be hypothesized based on these studies and those discussed above. A platelet agonist binds to its receptor and directly activates PLC, probably through a G protein. PLC hydrolyzes PIP_2 into DAG and IP_3; the latter causes a rapid release of calcium from intracellular stores. This increase in free cytosolic calcium primes the platelet (46), perhaps by activating calcium/calmodulin-dependent kinases such as myosin light chain kinase (47), which may play a role in the development of shape change (48). If shape change is the functional correlate of priming, it is unlikely that the DAG/PKC pathway plays a role in priming, because neither exogenous cell-permeable DAG nor phorbol ester cause shape change (41). After priming, the platelet is ready to respond to DAG accumulation and PKC activation. The DAG generated from initial PLC activation may be insufficient to cause aggregation and secretion. Activation of the PLA_2/arachidonic acid pathway (via G proteins or by elevated calcium) leads to the generation of thromboxanes. TxA_2 activates PLC via its own receptor, resulting in further DAG production. This delayed production and accumulation of DAG further activates PKC, which phosphorylates protein substrates and thus completes the process of platelet activation, with the induction of secondary aggregation and secretion.

Thus, both the PLC and PLA_2 pathways contribute to DAG production and platelet activation; the contribution of each pathway varies according to the platelet agonist and its concentration. High concentrations of thrombin can potently activate PLC directly, without requiring the release of arachidonic acid, as indicated by its relative insensitivity to aspirin. Collagen, on the other hand, is much more dependent on arachidonic acid release and metabolism for DAG production. Some of these mediators, such as arachidonic acid and the TxA_2 receptor, may have other functions besides those depicted in the figure. For example, arachidonic acid activates PKC *in vitro* (17). However, the present studies indicate that the release of arachidonic acid by collagen cannot sufficiently activate PKC to produce secondary aggregation and secretion when arachidonic acid metabolism is blocked by aspirin.

The role of DAG as a mediator of secondary aggregation has important therapeutic implications. Although aspirin has been shown to reduce the risk of stroke (49) and myocardial infarction (50), these salutary effects are relatively small. This may be related to its low potency as an inhibitor of thrombin-induced DAG production; we observed that 100 times more

aspirin was required to inhibit thrombin-induced DAG production and platelet aggregation than was required to inhibit similar responses induced by collagen. Effective inhibition of DAG production in platelets stimulated by any agonist may, therefore, represent a promising strategy upon which to develop future, more effective, antithrombotic agents.

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