A Role for Prostaglandins and Thromboxanes in the Exposure of Platelet Fibrinogen Receptors

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ABSTRACT Exposure of fibrinogen receptors by a variety of agonists is a prerequisite for platelet aggregation. Because the synthesis of prostaglandins and thromboxane A_2 also occurs during platelet aggregation we wondered whether these agents participate in the exposure of platelet fibrinogen receptors. Therefore, we measured the binding of human ^{125}I-fibrinogen to gel-filtered normal human platelets after prostaglandin and thromboxane synthesis had been inhibited by aspirin or indomethacin. The fibrinogen binding assay was performed at 37°C but without stirring to prevent the formation of platelet aggregates. Platelet secretion, measured with [^{14}C]serotonin, did not occur during the procedure. Aspirin or indomethacin inhibited fibrinogen binding stimulated by 10 μM epinephrine by 53%, and inhibited fibrinogen binding stimulated by 1–2 μM ADP by 37.1%. However, ADP at concentrations >2 μM returned fibrinogen binding toward control values. Scatchard analysis demonstrated that aspirin decreased the number but not the affinity of the exposed fibrinogen receptors. To determine whether prostaglandins are capable of directly exposing fibrinogen receptors, prostaglandin H_2 was used to stimulate platelets in the fibrinogen binding assay. Prostaglandin H_2 exposed ~54,000 fibrinogen receptors/platelet and corrected the deficit in receptor exposure induced by aspirin. These studies demonstrate that platelet prostaglandins or thromboxane A_2 can play a direct role in the exposure of platelet fibrinogen receptors. In addition, they suggest that the synthesis of prostaglandins and thromboxane A_2 by stimulated platelets may be all that is required for optimal secondary platelet aggregation.

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INTRODUCTION

Platelet aggregation, in vitro and in vivo, occurs in two phases: a primary phase due to direct stimulation by platelet agonists and a secondary phase due to substances produced or released by stimulated platelets (1). The importance of each of these phases for platelet aggregation depends upon the nature of the platelet agonist. For example, the aggregation response to collagen is entirely dependent upon substances produced or released by the platelet, whereas the aggregation response to thrombin is not (2). The platelet products that may be involved in secondary platelet aggregation include ADP released from platelet-dense granules (1), the products of platelet arachidonic acid metabolism (prostaglandin endoperoxides and thromboxane A_2) (3), and the recently described platelet activating factor (4). The relative contribution of these products to secondary platelet aggregation remains an area of controversy. For example, Charo et al. (2) suggested that secondary aggregation may be a direct response to platelet prostaglandins rather than to secreted platelet ADP because they could demonstrate that the onset of secondary platelet aggregation was simultaneous with, and often preceded, the onset of platelet secretion and both could be prevented by inhibition of prostaglandin synthesis. On the other hand, Malmsten et al. (5) have suggested that the aggregation of platelets produced by prostaglandins is mediated by the prostaglandin-stimulated secretion of platelet ADP.

Stimulation of platelets by a variety of agonists exposes a limited number of fibrinogen receptors on the platelet surface (6–8). Exposure of these receptors is a direct consequence of platelet stimulation and appears to be required for platelet aggregation (6, 9). Moreover, it is unrelated to platelet shape change (10) and is independent of platelet secretion (6, 7). Therefore, investigation of the factors involved in fibrinogen receptor exposure permits an examination of the events

leading to platelet aggregation, isolated from other aspects of platelet function. In this study we have investigated the contribution of prostaglandins to the exposure of fibrinogen receptors on normal human platelets. We have demonstrated that the prostaglandins or thromboxane A_2 synthesized by ADP- or epinephrine-stimulated platelets participate directly in the exposure of fibrinogen receptors. Thus, these studies provide further evidence for a direct role of platelet prostaglandins in normal platelet aggregation.

METHODS

Materials. Lyophilized human fibrinogen was purchased from Kabi (AB), Kabi Blood Products Div., Stockholm, Sweden. Sepharose 2B and 4B were obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. Sigma Chemical Co., St. Louis, Mo., supplied the ADP, ATP, epinephrine bitartrate, human albumin-fraction V, indomethacin, imipramine, and *p*-hydroxymercuribenzoate. Acetylsalicylic acid was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis. William F. Nye, Inc., Fairhaven, Mass., supplied methyl silicone oil (DC 200) and hi-phenyl silicone oil (DC550). Carrier-free ^{125}I-sodium iodine, [^{14}C]5-hydroxytryptamine binoxalate, [^{14}C]arachidonic acid, Econofluor, and Protosol were obtained from New England Nuclear, Boston, Mass. Arachidonic acid (>99% pure) was purchased from Nu Chek Prep, Inc., Elysian, Minn. Prostaglandin B_1 was a gift of Dr. John E. Pike, UpJohn Co., Kalamazoo, Mich. All other chemicals were of reagent grade.

Fibrinogen binding to gel-filtered human platelets. The assay used to measure fibrinogen binding by stimulated platelets has been previously described in detail (6). Briefly, Kabi fibrinogen was further purified by 16% ammonium sulfate fractionation (11) and Sepharose 4B gel filtration chromatography and was radiolabeled with ^{125}I by the iodine monochloride technique (12). The specific activity of the radiolabeled fibrinogen was ~2 Ci/mmol. Platelet-rich plasma, obtained by differential centrifugation of whole blood anticoagulated with 0.1 vol of 0.13 M sodium citrate, was gel-filtered on Sepharose 2B with Tyrode's buffer (0.14 M NaCl, 0.0027 M KCl, 0.012 M Na HCO_3, 0.004 M $NaHPO_4$), pH 7.4, containing 10 mg/ml human albumin and 5.5 mM glucose as the elution buffer (13). The fibrinogen binding assay was performed by mixing gel-filtered platelets (10^8/ml) with radiolabeled fibrinogen and 0.5 mM $CaCl_2$. After the platelet suspensions were equilibrated at 37°C, a platelet agonist was added and the incubations were continued at 37°C without stirring. To terminate the incubations, the platelet suspensions were layered on 0.5 ml of a mixture of hi-phenyl silicone and methyl silicone oil (4:1) and the platelets were sedimented through the oil mixture in an Eppendorf centrifuge (model 5412, Brinkman Instruments, Inc., Westbury, N. Y.). After sedimentation, the supernatant buffer and oil were aspirated from the pelleted platelets and the tips of the centrifuge tubes containing the pellets were sliced from the tubes. The tips were then counted for ^{125}I in a Beckman Gamma 8000 counter (Beckman Instruments, Inc., Fullerton, Calif.). Nonspecific fibrinogen binding was assessed by performing the binding assays in the presence of a 10-fold excess of unlabeled fibrinogen. Nonspecific binding accounted for 10–20% of the labeled fibrinogen associated with the stimulated platelets and was unchanged by prior treatment of the platelets with aspirin or indomethacin.

Preparation and isolation of prostaglandin H_2. Prostaglandin H_2 (PGH_2)[1] was prepared by modification of the methods of Nugteren and Hazelhof (14) and Hamberg et al. (15). A microsomal pellet prepared from homogenized sheep seminal vesicles was resuspended in 15 ml of Tris buffer containing 1 mM phenol and 3 mM *p*-hydroxymercuribenzoate. Arachidonic acid (2.5 μmol) containing a tracer quantity of [^{14}C]arachidonic acid was added and the mixture was shaken vigorously for 2 min. The suspension was then acidified with 0.2 M citric acid and extracted twice with diethyl ether. The ether layers were combined, washed with water, dried with anhydrous $MgSO_4$, and evaporated on ice under nitrogen. The residue was redissolved in diethyl ether, and the prostaglandins were separated by thin-layer chromatography (Silica Gel 60 F-254 precoated thin-layer chromatography plates, Merck AG, Darmstadt, West Germany) at −20°C with a solvent system containing ethyl acetate:2,2,4-trimethylpentane:acetic acid (50:50:0.5, vol/vol/vol). Prostaglandin B_1 (R_f = 0.18) was used as a reference compound and was located with 254 nm light. The R_f of PGH_2 was 0.24. The area of the developed chromatogram corresponding to PGH_2 was scraped and the removed silica gel was extracted with acetone.

When the ability of PGH_2 to stimulate fibrinogen binding was measured, an aliquot of the acetone extract was added to a polypropylene tube (Falcon Labware, Div. of Becton-Dickinson & Co., Oxnard, Calif.), the acetone was evaporated under nitrogen, and a platelet suspension was immediately added. The remainder of the fibrinogen binding assay was performed as described in the previous section.

Studies of platelet aggregation and secretion. Platelet aggregation was studied by the method of Born (16) with a Chrono-Log single-channel aggregometer and recorder (Chrono-Log Corp., Havertown, Pa.). Platelet secretion was measured as [^{14}C]serotonin secretion by the method of Jerushalmy and Zucker (17) in the presence of 1 μM imipramine (18).

Statistical analyses for this study were performed with the aid of a Texas Instruments TI58 programmable calculator (Texas Instruments, Inc., Digital Systems Div., Dallas, Tex.).

RESULTS

Inhibition of fibrinogen binding by aspirin and indomethacin. The synthesis of prostaglandins and thromboxane A_2 is required for the complete aggregation of platelets by epinephrine and ADP (2). Because the exposure of fibrinogen receptors is a prerequisite for platelet aggregation (6–10), we postulated that prostaglandins or thromboxane A_2 should also be responsible for a portion of the fibrinogen binding sites exposed by these agents. To test this hypothesis we prevented platelet prostaglandin synthesis with the fatty acid cyclooxygenase inhibitors aspirin or indomethacin before fibrinogen binding was measured. Platelet-rich plasma was incubated for 20 min at 37°C in the presence and in the absence of 1 mM aspirin. Essentially complete inactivation of platelet fatty acid cyclooxygenase by aspirin was confirmed by failure of the platelet enzyme to incorporate [^{3}H-acetyl]-

[1] *Abbreviation used in this paper*: PGH_2, prostaglandin H_2.

aspirin (19). The aspirin-treated and control platelets were gel filtered and used immediately for studies of epinephrine- and ADP-stimulated fibrinogen binding. The platelet suspensions were not stirred to prevent the formation of platelet aggregates and the possible induction of platelet secretion (20). The results of these experiments are listed in Table I. Aspirin treatment decreased fibrinogen binding to platelets stimulated by 10 μM epinephrine by 53% and also decreased fibrinogen binding to platelets stimulated by 1–2 μM ADP by 37.1%. Moreover, addition of 50 μM indomethacin to gel-filtered platelets that had not been treated with aspirin decreased the fibrinogen binding stimulated by 10 μM epinephrine by 53.8%. Platelet secretion, measured as [^{14}C]serotonin secretion, did not occur during these incubations. Because of concern that the incubation of platelets at 37°C with 1 mM aspirin could have effects on platelet function in addition to inhibition of fatty acid cyclooxygenase, the experiments were repeated with platelets exposed to lower concentrations of aspirin in vivo (21). Identical results were seen when platelets were obtained from donors who had ingested 600 mg of aspirin on the day before blood donation. Thus, these data are consistent with a direct role for platelet synthesized prostaglandins and/or thromboxane A_2 in both epinephrine- and ADP-stimulated fibrinogen receptor exposure.

Effect of varying ADP and epinephrine concentrations on fibrinogen binding to aspirin-treated platelets. The extent of aggregation of aspirin-treated platelets stimulated by 10 μM ADP approximated the extent of aggregation of untreated platelets (Fig. 1). The aspirin-treated platelets did not, however, undergo secretion and the aggregates eventually disaggregated. In contrast, the inhibitory effect of aspirin on the extent of aggregation of epinephrine-stimulated platelets could not be overcome with higher concentrations of epinephrine. In view of these data, we examined the ability of increasing concentrations of epinephrine or ADP to expose fibrinogen receptors on aspirin-treated and control platelets. As illustrated in Fig. 2, aspirin inhibited epinephrine-stimulated fibrinogen binding at each concentration of epinephrine tested. In contrast, the effect of aspirin on ADP-stimulated fibrinogen binding was apparent only at ADP concentrations of ≤2 μM and disappeared as the ADP concentration was increased (Fig. 3).

To insure that the effect of aspirin was not due to its interaction with the fibrinogen binding sites, we measured the affinity of the fibrinogen receptors of aspirin-treated and control platelets by Scatchard analysis (22). As illustrated in Fig. 4, ADP exposed 47,000 receptors on control platelets but only 29,500 sites on aspirin-treated platelets. The Scatchard analysis also demonstrated that ADP exposed a single class of fibrinogen receptors on both aspirin-treated and control platelets with dissociation constants of 0.105 and 0.1 μM, respectively. Moreover, when a concentration of ADP

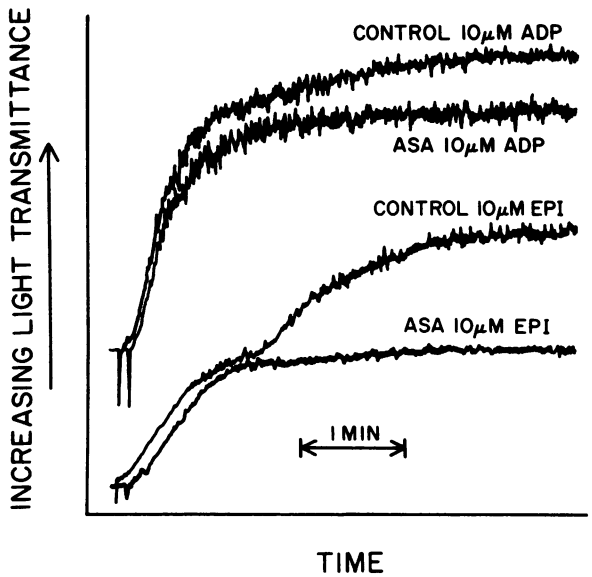

FIGURE 1 Aggregation of aspirin-treated and control platelets by ADP and epinephrine. Platelet-rich plasma was incubated at 37°C for 20 min in the presence or absence of 1 mM aspirin. The incubated platelets were then gel filtered on Sepharose 2B with Tyrode's buffer, pH 7.4, containing 10 mg/ml human albumin and 5.5 mM glucose as the elution buffer. For these aggregation studies, unlabeled fibrinogen (200 μg/ml) and 0.5 mM $CaCl_2$ were added to the gel-filtered platelet suspensions. The control platelets and the aspirin-treated platelets (ASA) were stimulated by 10 μM ADP or 10 μM epinephrine (EPI) at 37°C in a conventional aggregometer.

TABLE I
Effect of Platelet Cyclooxygenase Inhibition on the Exposure of Platelet Fibrinogen Receptors

Platelet agonist	Cyclooxygenase inhibitor	Control specific binding
		%
ADP (1–2 μM)	Aspirin (1 mM)	62.9±3.1
Epinephrine (10 μM)	Aspirin (1 mM)	47.0±3.9
Epinephrine (10 μM)	Indomethacin (50 μM)	46.2±3.9

Platelet-rich plasma was incubated at 37°C for 20 min in the presence or absence of 1 mM aspirin. Platelets were then separated from plasma protein by gel filtration on Sepharose 2B, with Tyrode's buffer, pH 7.4, containing 10 mg/ml human albumin and 5.5 mM glucose as the elution buffer. The ability of these platelets to bind labeled fibrinogen in response to ADP or epinephrine was tested as described under Methods. The effect of indomethacin was tested by adding it to control platelet suspensions immediately before platelet stimulation. Results are expressed as the mean±SEM.

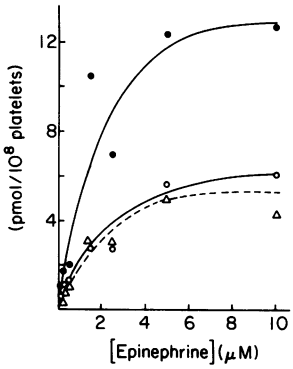

FIGURE 2 Exposure of fibrinogen receptors on control and aspirin-treated platelets by epinephrine. Aspirin-treated and control platelet suspensions were prepared as described in Fig. 1. To measure fibrinogen binding, suspensions of 5×10^7 gel-filtered platelets were incubated with ^{125}I-fibrinogen (200 μg/ml), 0.5 mM $CaCl_2$, and increasing concentrations of epinephrine for 3 min at 37°C. Specific fibrinogen binding was measured as described in Methods. Each point is the mean of triplicate determinations. ●, control platelets; ○, platelets treated with aspirin in vitro; △, dashed line, platelets from the same donor obtained 20 h after the donor ingested aspirin.

was used to expose equal numbers of fibrinogen receptors on control and aspirin-treated platelets, the Scatchard plots could be superimposed. Thus, these data are consistent with an aspirin effect on fibrinogen

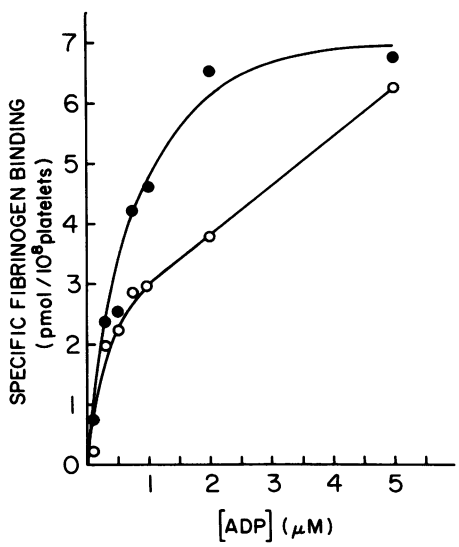

FIGURE 3 Exposure of fibrinogen receptors on control and aspirin-treated platelets by ADP. Aspirin-treated and control platelet suspensions were prepared as described in Fig. 1. The fibrinogen binding assay was performed as described in Fig. 2 except that increasing concentrations of ADP were used as the platelet stimulus. Each point is the mean of triplicate determination. ●, control platelets; ○, aspirin-treated platelets.

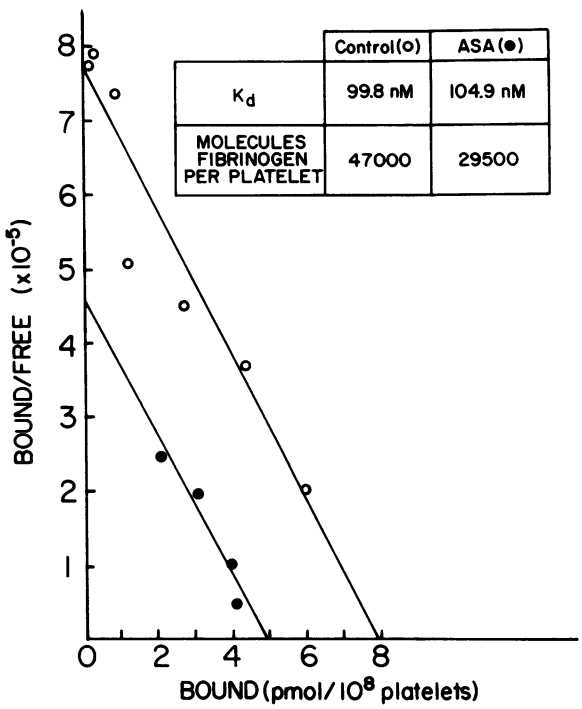

FIGURE 4 Scatchard analysis of ADP-stimulated fibrinogen binding to control and aspirin-treated platelets. Aspirin-treated and control platelet suspensions were prepared as described in Fig. 1. Suspensions of 5×10^7 gel-filtered platelets were incubated with increasing concentrations of ^{125}I-fibrinogen, 0.5 mM $CaCl_2$ and 2 μM ADP for 3 min at 37°C. Specific fibrinogen binding was measured as described in Methods. The data were then analyzed by the method of Scatchard (21) and plotted with the aid of a linear regression analysis. ○, control platelets; ●, aspirin-treated platelets.

receptor exposure rather than on the ability of the exposed receptors to bind fibrinogen.

Exposure of platelet fibrinogen receptors by PGH_2. The inhibition of fibrinogen receptor exposure by aspirin and indomethacin is indirect evidence that prostaglandins or thromboxanes participate in this process. To demonstrate directly that prostaglandins are capable of stimulating fibrinogen binding to platelets, the prostaglandin endoperoxide PGH_2 was prepared biosynthetically and was used to stimulate unstirred gel-filtered platelets in the presence of radiolabeled fibrinogen and $CaCl_2$. Stimulation of platelets with increasing concentrations of PGH_2 resulted in the exposure of a limited number of fibrinogen binding sites (Fig. 5). Although the sensitivity of platelets from different donors to PGH_2 varied, the maximum number of fibrinogen receptors exposed by PGH_2 was 54,650 ±2,245 (SEM, $n = 6$), a value not significantly different from the maximum number of fibrinogen receptors exposed by ADP or epinephrine (6).

Prostaglandin endoperoxides have been shown to initiate platelet secretion (5, 23). Accordingly, we found

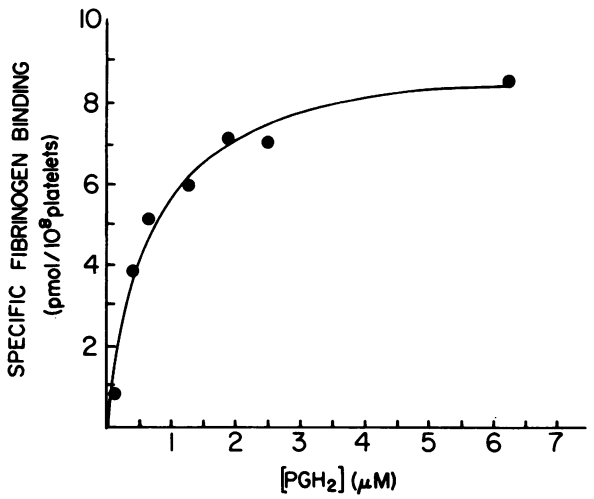

FIGURE 5 Exposure of fibrinogen receptors by PGH_2. Increasing volumes of PGH_2 dissolved in acetone were added to acetone-resistant polypropylene tubes. The acetone was then evaporated under nitrogen. Suspensions of 5×10^7 gel-filtered platelets containing ^{125}I-fibrinogen (200 μg/ml) and 0.5 mM $CaCl_2$ were immediately added to the tubes and specific fibrinogen binding was measured as described in Methods. Each point is the mean of triplicate determinations.

that maximal platelet stimulation by PGH_2 resulted in the secretion of ~40% of the [^{14}C]serotonin from prelabeled platelets. Therefore, the fibrinogen binding produced by PGH_2 could have resulted from direct platelet stimulation by PGH_2 or from PGH_2-stimulated secretion of dense granule ADP. To differentiate between these possibilities, PGH_2-stimulated fibrinogen binding was measured in the presence of ATP, a specific antagonist of ADP-stimulated platelet function (24, 25). In our assay system, ATP inhibited ADP-stimulated fibrinogen binding with a K_i of 3.8±1.1 μM. Moreover, in five experiments, 20 μM ATP inhibited by 90% the fibrinogen binding stimulated by 2 μM ADP, an ADP concentration that could reasonably result from PGH_2-stimulated platelet secretion (26). In contrast, the fibrinogen binding stimulated by 2–4 μM PGH_2 was only inhibited by 6% by the same concentration of ATP. Therefore, it is likely that the majority of the fibrinogen binding stimulated by PGH_2 is a direct consequence of the interaction of the endoperoxide with the platelet.

If aspirin and indomethacin partially inhibit the response of platelets to ADP and epinephrine by preventing prostaglandin synthesis, then exogenous PGH_2 should be able to correct the deficit in fibrinogen receptor exposure produced by these agents. Control and aspirin-treated platelets were stimulated by 10 μM epinephrine both in the presence and in the absence of exogenous PGH_2. The concentrations of PGH_2 used varied from 0.6 to 4.3 μM. Aspirin treatment significantly decreased maximal fibrinogen binding from 7.49 ±1.40 to 3.48±0.64 pmol/10^8 platelets ($P < 0.005$, t test for paired samples, $n = 5$). However, the combination of PGH_2 and epinephrine restored fibrinogen binding to 6.88±0.93 pmol/10^8 platelets, a value not significantly different from the control values. Moreover, the number of fibrinogen receptors exposed by the combination of epinephrine and PGH_2 did not exceed the number of receptors exposed by epinephrine alone.

DISCUSSION

It has been suggested that secondary platelet responses are mediated by the secretion of ADP stored in the platelet-dense granules. This conclusion is based on the failure of collagen, epinephrine, and ADP to elicit secondary aggregation in platelets with an abnormal storage pool of ADP (27) and on the inhibition of secondary aggregation by enzymes that metabolize ADP (28). Recent data, however, have challenged this conclusion. Storage-pool-deficient platelets may have other abnormalities such as defects in the prostaglandin and thromboxane A_2 synthetic pathway (27). Moreover, the mechanism by which ADP scavengers inhibit platelet aggregation has been questioned. Huang and Detwiler (29) demonstrated that inhibition of thrombin- or epinephrine-stimulated platelet aggregation by creatine phosphate/creatine phosphokinase could be overcome by increased concentrations of thrombin or epinephrine. This suggests that creatine phosphate/creatine phosphokinase inhibits platelet function by decreasing the sensitivity of platelets to agonist stimulation rather than by catabolizing secreted ADP. In addition, Mustard et al. (30) and Macfarlane and coworkers (31) have suggested that ADP- and epinephrine-stimulated platelet secretion is an artifact of the low calcium concentration that results when citrate is used as an anticoagulant. Thus, despite the effort that has been expended to understand secondary platelet aggregation, a completely satisfactory explanation is not available. In part, this has resulted from difficulties in finding a simple, unambiguous experimental system.

Our fibrinogen binding assay fulfills the requirements for such a system. Fully reactive platelets can be suspended in a defined medium in the absence of anticoagulants, and the events leading to platelet aggregation can be studied in the absence of platelet secretion. Using this assay, we have demonstrated that the treatment of platelets with the cyclooxygenase inhibitors aspirin and indomethacin decreases the number of fibrinogen receptors exposed by all concentrations of epinephrine and by concentrations of ADP of ≤2 μM. Thus, our data suggest that the prostaglandins and thromboxanes synthesized by stimulated platelets can act in concert with primary agonists to expose fibrinogen receptors. Higher concentrations of

ADP can overcome this effect of cyclooxygenase inhibition, which reinforces the concept that several pathways are available to amplify platelet responses.

Other interpretations of our data are possible, however. It could be argued that although our platelet suspensions were not stirred, small quantities of ADP were still secreted and were not detected by the [^{14}C]-serotonin release assay. Aspirin and indomethacin would simply have inhibited the secretion of this small (but perhaps significant) quantity of ADP. In support of this argument, Plow and Marguerie (32, 33) recently demonstrated that the incubation of unstirred platelets with apyrase or creatine phosphate/creatine phosphokinase for 30 min at 22°C inhibited fibrinogen binding stimulated by ADP, epinephrine, or thrombin. These investigators did not detect [^{14}C]serotonin secretion or cell lysis during their incubations and were unable to identify the source of the hypothesized ADP upon which the enzymes acted. Like Plow and Marguerie, we could not detect [^{14}C]serotonin secretion during our unstirred incubations, even in the presence of stimulating ADP concentrations as high as 10 μM. Assuming that secretion occurred to the extent of the ^{14}C counting error (2%), the potential concentration of secreted ADP would approximate 50 nM (26), an ADP concentration insufficient to produce measurable fibrinogen binding (Fig. 3). Thus, we do not believe that small undetected quantities of secreted ADP could account for our data. Another possible explanation for our results is a direct effect of aspirin or indomethacin on the fibrinogen receptor that would decrease its affinity for fibrinogen. But this possibility is not supported by our Scatchard analyses, which demonstrate nearly identical dissociation constants for the fibrinogen receptors on aspirin-treated and control platelets. In addition, the ability of higher ADP concentrations to overcome the aspirin effect demonstrates that a full complement of fibrinogen receptors of normal affinity can be exposed on aspirin-treated platelets. Finally, aspirin has been shown to modify only a single membrane-associated protein in human platelets (34).

Our demonstration that exogenous PGH_2 stimulates fibrinogen receptor exposure, independent of secreted platelet ADP, is consistent with the ability of the endoperoxide to stimulate platelet aggregation (5, 22). Furthermore, the ability of PGH_2 to restore the response of aspirin-treated platelets to epinephrine supports the suggestion that aspirin affects fibrinogen receptor exposure by inhibiting fatty acid cyclooxygenase. Because the combination of epinephrine and PGH_2 (or ADP and PGH_2, data not shown) could not expose more fibrinogen binding sites than epinephrine (or ADP) alone, it is likely that the range of 44,000–54,000 fibrinogen binding sites per platelet represents the actual number of fibrinogen receptors possessed by human platelets.

In conclusion, we have demonstrated that the products of platelet arachidonic acid metabolism can potentiate the exposure of fibrinogen binding sites by other agonists. Thus, prostaglandin endoperoxides and thromboxane A_2, along with secreted ADP, may play a direct role in normal platelet aggregation. Our studies were not intended to address the role of secreted ADP in secondary aggregation. They do, however, suggest that in the presence of a functional arachidonic acid metabolic pathway, the synthesis of prostaglandin endoperoxides and thromboxanes may be all that is required for optimal secondary platelet aggregation.

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