THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 22, Issue of August 5, pp. 15568-15577, 1992
Printed in U.S.A.

Fibrinogen Binding to Purified Platelet Glycoprotein IIb-IIIa (Integrin $\alpha_{IIb}\beta_3$) Is Modulated by Lipids*

(Received for publication, December 23, 1991)

Susan S. Smyth‡, Cheryl A. Hillery‡§¶, and Leslie V. Parise‡‖

From the ‡Department of Pharmacology, Center for Thrombosis and Hemostasis and the §Departments of Medicine and Pediatrics, University of North Carolina, Chapel Hill, North Carolina 27599

Soluble fibrinogen binding to the glycoprotein IIb-IIIa complex (integrin $\alpha_{IIb}\beta_3$) requires platelet activation. The intracellular mediator(s) that convert glycoprotein IIb-IIIa into an active fibrinogen receptor have not been identified. Because the lipid composition of the platelet plasma membrane undergoes changes during activation, we investigated the effects of lipids on the fibrinogen binding properties of purified glycoprotein IIb-IIIa. Anion exchange chromatography of lipids extracted from platelets exposed to thrombin or other platelet agonists resolved an activity that increased fibrinogen binding to glycoprotein IIb-IIIa. A monoester phosphate was important for activity, and phosphatidic acid coeluted with the peak of activity. Purified phosphatidic acid dose-dependently promoted a specific interaction between glycoprotein IIb-IIIa and fibrinogen which possessed many but not all of the properties of fibrinogen binding to activated platelets. Phosphatidic acid appeared to increase the proportion of fibrinogen binding-competent glycoprotein IIb-IIIa complexes without altering their affinity for fibrinogen. The effects of phosphatidic acid were a result of specific structural properties of the lipid and were not mimicked by other phospholipids. Lysophosphatidic acid, however, was a potent inducer of fibrinogen binding to glycoprotein IIb-IIIa. These results demonstrate that specific lipids can affect fibrinogen binding to purified glycoprotein IIb-IIIa and suggest that the lipid environment has the potential to influence fibrinogen binding to its receptor.

The binding of the plasma protein fibrinogen to the glycoprotein IIb-IIIa (GP[1] IIb-IIIa or $\alpha_{IIb}\beta_3$) complex mediates platelet aggregation (Phillips *et al.*, 1988; Plow and Ginsberg, 1989). GP IIb-IIIa exists as a Ca^{2+}-dependent heterodimer (Brass *et al.*, 1985; Fitzgerald and Phillips, 1985); each subunit is predicted to have a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic tail (Poncz *et al.*, 1987; Fitzgerald *et al.*, 1987). Although the GP IIb-IIIa complex is present on the surface of resting platelets, it serves as a fibrinogen receptor only after platelet activation (Bennett and Vilaire, 1979).

GP IIb-IIIa belongs to a family of cell surface receptors, the integrins, that are composed of noncovalently associated α- and β-subunits (Hynes, 1987). In addition to their structural similarities, integrins share several functional properties, including the ability to mediate cell to cell and cell to extracellular matrix interactions. A common feature of many members of the integrin family is their ability to bind proteins containing the tripeptide sequence Arg-Gly-Asp (RGD) (Ruoslahti and Pierschbacher, 1987). Fibrinogen binding to GP IIb-IIIa can be inhibited by both RGD-containing peptides (Plow *et al.*, 1985) and the synthetic peptide His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (H12), corresponding to the carboxyl-terminal segment of the γ-chain of fibrinogen (Plow *et al.*, 1984). Many integrins constitutively bind ligands; however, accumulating evidence indicates that the binding activity of members of both the β_1 (Shimizu, 1990) and β_2 (Wright and Meyer, 1986; Dustin and Springer, 1989) class of integrins may be modulated by cellular activation, in a manner similiar to that of GP IIb-IIIa.

Despite advances in delineating the structure and function of the GP IIb-IIIa complex, little is known about the mechanisms by which GP IIb-IIIa acquires fibrinogen binding activity on the surface of activated platelets. Investigations of the mechanism of activation of GP IIb-IIIa have been hampered by the observation that, when removed from intact platelets, GP IIb-IIIa does not possess the levels of fibrinogen binding observed in activated platelets. GP IIb-IIIa in isolated platelet membranes (Phillips and Baughan, 1983), reconstituted in phospholipid vesicles (Parise and Phillips, 1985b), or expressed recombinantly in a variety of cell types (Bodary *et al.*, 1989; O'Toole *et al.*, 1991) cannot bind stoichiometric quantities of soluble fibrinogen. However, the capacity to bind fibrinogen is an intrinsic property of the receptor because certain GP IIIa-specific monoclonal antibodies (mAb)

* This work was supported by National Institutes of Health Grant R29HL38405. Additional support was provided by a grant-in-aid from the American Heart Association (to L. V. P.), a fellowship from the Pharmaceutical Manufacture's Association (to S. S. S.), Predoctoral Training Grant GM07040 (to S. S. S.), and Research Fellowship in Hematology/Oncology HL07149-15 (to C. A. H.). An abstract of this work was presented at the 1991 meeting of the American Society of Hematology, Denver, Colorado. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: The Blood Center of Southeastern Wisconsin, 1701 W. Wisconsin Ave., Milwaukee, WI 53233.

‖ This work was done during the tenure of an Established Investigatorship from the American Heart Association. To whom correspondence and reprint requests should be sent. Dept. of Pharmacology, CB 7365, University of North Carolina, Chapel Hill, NC 27599.

[1] The abbreviations used are: GP, glycoprotein; BSA, bovine serum albumin; DAG, diacylglycerol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; lyso-PA, lysophosphatidic acid; mAb, monoclonal antibody(ies); PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PGI_2, prostacyclin; PMA, phorbol 12-myristate 13-acetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(O'Toole *et al.*, 1991) and RGD-containing peptides (Du *et al.*, 1991) can induce high levels of fibrinogen binding to recombinant and purified GP IIb-IIIa. To date, receptor-mediated activation of the fibrinogen binding properties of GP IIb-IIIa has been observed only in intact and saponin-permeabilized platelets (Shattil and Brass, 1987).

Platelet agonists, including thrombin, ADP, and thromboxane A_2, which increase the fibrinogen binding properties of platelets, elicit polyphosphoinositide hydrolysis and/or Ca^{2+} mobilization (Rittenhouse-Simmons, 1979; Leung *et al.*, 1983; Seiss *et al.*, 1985) and therefore are thought to release the two second messengers diacyglycerol (DAG) and inositol trisphosphate. Although treatment of permeabilized platelets with either phorbol esters or Ca^{2+} ionophores converts GP IIb-IIIa to a functional fibrinogen receptor (Shattil and Brass, 1987), neither direct phosphorylation of GP IIb-IIIa (Hillery *et al.*, 1991) nor changes in intracellular Ca^{2+} levels (Shattil and Brass, 1987) appear to be the final mediator of GP IIb-IIIa activation. Platelet activation is accompanied by stimulation of phosphatidylinositol (PI)-specific phospholipase C, phosphatidylcholine (PC)-specific phospholipases C and D, and arachidonate-releasing phospholipase A_2. In addition to their water-soluble products, the action of these enzymes results in generation of DAG, phosphatidic acid (PA), and lysophospholipids. Additionally, production of D-3 phosphate-containing inositol lipids is stimulated in agonist-treated platelets (Nolan and Lapetina, 1990; Kucera and Rittenhouse, 1990).

Three lines of evidence suggest that lipids may be important in modulating the ligand binding function of integrins. The avidity of members of the β_2 family of integrins, including $\alpha_M\beta_2$ and $\alpha_L\beta_2$, is enhanced by a lipid factor (termed *integrin-modulating factor-1*) extracted from activated polymorphonuclear leukocytes (Hermanowski-Vosatka *et al.*, 1992). Integrin-modulating factor-1 enhances the ligand binding properties of $\alpha_M\beta_2$ and $\alpha_L\beta_2$ in intact cells and purified $\alpha_M\beta_2$. In addition, the binding specificity and affinity of isolated vitronectin receptor ($\alpha_v\beta_3$), which shares the same β-chain as GP IIb-IIIa but has a different α-chain, is altered by insertion into liposomes of different compositions (Conforti *et al.*, 1990). Finally, in human melanoma cells $\alpha_v\beta_3$ exists in a calcium-dependent complex with the disialoganglioside GD2, and the addition of GD2 to purified $\alpha_v\beta_3$ enhances its binding to RGD-containing peptides (Cheresh *et al.*, 1987). In this study, we examined the ability of lipids to enhance fibrinogen binding to purified GP IIb-IIIa. We have found that fractionated lipids from agonist-treated platelets as well as purified PA and lyso-PA can increase fibrinogen binding to mAb-immobilized GP IIb-IIIa.

EXPERIMENTAL PROCEDURES

Materials—Glycerophosphates and monoacylglycerol were purchased from Sigma. Phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) were purified from rat brain Folch fraction I and supplied by Dr. Andrew Morris, University of North Carolina. Bovine brain phosphatidylserine (PS), egg PC, egg PA, 1-oleoyl,2-hydroxyglycero-3-phosphate and 1,2-dicaproyl-*sn*-glycero-3-phosphate were purchased from Avanti Polar Lipids, Alabaster, AL.

Antibodies—The GP IIIa-specific mAb AP3 (Newman *et al.*, 1985) was kindly supplied by Dr. Peter Newman, the Blood Center of Southeastern Wisconsin; the GP IIb-specific mAb Tab (McEver *et al.*, 1980) was the generous gift of Dr. Rodger McEver, Oklahoma Medical Research Foundation. Although their determinants are extracellular, neither AP3 nor Tab interferes with fibrinogen binding to GP IIb-IIIa on activated platelets. The GP IIb-IIIa complex-specific mAb 10E5, which inhibits platelet aggregation and fibrinogen binding (Coller *et al.*, 1983), was generously supplied by Dr. Barry Coller, SUNY-Stony Brook. The anti-fibrinogen mAb 1D4, which cross-reacts with amino acids 241–476 on the Aα chain, and mAb Shainoff, which cross-reacts with amino acids 15–21 on the Bβ chain, were obtained from Dr. B. Kudryk, New York Blood Center.

Platelet Isolation and Lipid Extraction—Fresh human platelets were obtained from acid citrate dextrose-anticoagulated venous blood of adult donors by the procedure of Mustard *et al.* (1989) and washed twice with Ca^{2+}- and Mg^{2+}-free Tyrode's buffer (138 mM NaCl, 12 mM $NaHCO_3$, 10 mM KCl, 5.5 mM glucose, 0.36 mM Na_2HPO_4, 10 mM Hepes, pH 7.4) containing 150 nM prostacylin (PGI_2) and 0.35% bovine serum albumin (BSA). The platelets were resuspended in Ca^{2+}- and Mg^{2+}-free Tyrode's buffer ($0.5–1 \times 10^9$ platelets/ml) at 37 °C and treated with 0.2–0.5 unit/ml thrombin for 30 s, 0.68 μM U46619 for 1 min, 5 μM phorbol 12-myristate 13-acetate (PMA) for 5 min, or 150 nM PGI_2 for 1 min. For steady-state ^{32}P labeling of phospholipids, the platelets were incubated with $^{32}P_i$ for 4 h, as described previously (Hillery *et al.*, 1991), before exposure to agonists. Lipids were extracted as described by Allan and Michell (1978), dried under N_2, and resuspended in 1 ml of $CHCl_3$.

For separation by anion exchange chromatography, platelet lipid extracts were applied to a 1-ml DEAE-cellulose column (Whatman) that was prepared by the procedure of Low (1990) and equilibrated in $CHCl_3$. The column was washed sequentially with 10 column volumes of $CHCl_3$, 9 volumes of 9:1 $CHCl_3$:MeOH (v/v), 9 volumes of 7:3 $CHCl_3$:MeOH (v/v), 9 volumes of 1:1 $CHCl_3$:MeOH (v/v), 10 volumes of 20:9:1 $CHCl_3$:MeOH:H_2O (v/v), and 10 volumes each of 20:9:1 $CHCl_3$:MeOH:H_2O (v/v/v) with 100, 200, and 500 mM ammonium acetate. Each fraction was reextracted as described above and the $CHCl_3$ phase stored at −20 °C until further use. To separate polyphosphoinositides, the whole lipid extract was applied to neomycin-linked glass beads (0.5-ml bed volume) and eluted as described (Morris *et al.*, 1990). Thin layer chromatography (TLC) was performed as described by Ferrell and Huestis (1984) with the first dimension in 60:30:6 $CHCl_3$:MeOH:NH_4OH (v/v/v) followed by 60:10:6 $CHCl_3$:MeOH:CH_3COOH (v/v/v). Phospholipids were stained with I_2; radiolabeled phospholipids were detected by autoradiography. Phospholipid phosphorus was quantitated with Malachite Green by the method of Itaya and Ui (1966).

Protein Purification and Iodination—Fibrinogen was purified from fresh-frozen plasma by the glycine precipitation method of Kazal *et al.* (1963). Contaminating fibronectin was removed by gelatin-Sepharose chromatography. The concentration of fibrinogen was determined spectrophotometrically at 280 nm, assuming an extinction coefficient (0.1%) of 1.51 and a molecular mass of 340,000 daltons. Fibrinogen was centrifuged ($11,600 \times g$) for 5 min before use. Fibrinogen (300 μg) or antibody (100 μg) was radioiodinated by incubation with 1 mCi of $Na^{125}I$ (Amersham Corp.) and 0.05 mg/ml chloramine T for 5 min at room temperature. The reaction was stopped with 0.05 mg/ml sodium metabisulfite, and unincorporated $Na^{125}I$ was removed by gel filtration on a Pharmacia PD-10 (G-25 M) column (Pharmacia LKB Biotechnology Inc.). The specific activity of ^{125}I-labeled protein was $> 1 \times 10^{18}$ cpm/mol. Chloramine T-catalyzed iodination does not alter the ability of fibrinogen to bind to activated platelets (Marguerie *et al.*, 1979).

Glycoprotein IIb-IIIa was purified from washed, outdated platelets lysed in 1% Triton X-100, 0.15 M NaCl, 1 mM $CaCl_2$, 20 mM Tris, pH 7.4, 50 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotonin by modification of the procedure of Fitzgerald *et al.* (1985). Glycoproteins were isolated at room temperature by concanavalin A affinity chromatography in 0.1% Triton X-100, 0.15 M NaCl, 1 mM $CaCl_2$, 20 mM Tris, pH 7.4. The concanavalin A-retained glycoproteins were eluted with the same buffer containing 300 mM α-methyl D-mannose and applied to sequential heparin-Sepharose, gelatin-Sepharose, and Sephacryl S-300 columns. The concentration of GP IIb-IIIa was determined by the Pierce BCA protein assay (Pierce Chemical Co.) with BSA as the standard. A molecular mass of 265,000 daltons for the GP IIb-IIIa complex was assumed.

Fibrinogen Binding to Purified, mAb-immobilized GP IIb-IIIa—Microtiter wells (Immulon 2 Removawell strips, Dynatech Labs, Chantilly, VA) were incubated with 50 μl of the GP IIIa-specific mAb AP3 (15 μg/ml) in buffer A (100 mM NaCl, 0.05% NaN_3, 50 mM Tris, pH 7.4) overnight at 4 °C. In some experiments, the GP IIb-specific mAb Tab served as the capturing antibody. To lower nonspecific binding, the wells were blocked in buffer A containing 0.5% BSA for 1 h at 4 °C. After washing, the wells were incubated for 6–18 h at 4 °C with 50 μl of purified GP IIb-IIIa (20 μg/ml) in buffer A containing 0.035% Triton X-100 and 1 mM $CaCl_2$. Uncaptured GP IIb-IIIa was removed by washing the wells with buffer A containing 1 mM $CaCl_2$. Binding reactions were conducted in a total volume of 100 μl containing ^{125}I-labeled ligand, 100 mM NaCl, 1 mM $CaCl_2$, 50

mM Tris, pH 7.4, and 0.5% BSA. To test the effects of lipids on the fibrinogen binding activity of GP IIb-IIIa, lipids were dried, resuspended in buffer A, sonicated for 5 min in a water bath sonicator (Laboratory Supplies Inc., Piscataway, NJ), and added to the binding reaction. Unless otherwise indicated, the binding reactions proceeded for 1 h at room temperature, at which time the wells were washed three times with 300 μl of buffer A containing 1 mM $CaCl_2$, and the amount of radioactivity associated with the well was quantitated. Nonspecific binding was defined by performing the assay either in microtiter wells that were coated with BSA instead of AP3 or in AP3-coated wells that had been incubated with buffer A containing Triton X-100 but lacking GP IIb-IIIa.

Enzymatic Degradation of PA—PA was dried, resuspended in Tris/NaCl buffer (100 mM NaCl, 50 mM Tris, pH 7.4) to 400 μM or 4 mM, and incubated with 0.01–10 units/ml of alkaline phosphatase (Boehringer Mannheim), snake venom phospholipase A_2, or *Vibrio* phospholipase B (Sigma) for 1 h at 37 °C. For alkaline phosphatase reactions, PA was resuspended in buffer at pH 8.0, and the buffer was readjusted to pH 7.4 after the incubation. In some instances, the reactions were reextracted, separated by two-dimensional TLC, and visualized by staining with I_2 or by detecting phospholipid phosphorus with the reagent of Dittmer and Lester (1964).

Production of [^{32}P]Lyso-PA and Binding of [^{32}P]Lyso-PA to mAb-immobilized GP IIb-IIIa—[^{32}P]Lyso-PA was generated by DAG kinase-catalyzed phosphorylation of monoacylglycerol in the presence of [^{32}P-γ]ATP (Du Pont-New England Nuclear; specific activity 2.2 × 10^{16} cpm/mol) essentially as described (Preiss *et al.*, 1986), except that membranes were replaced with monoacylglycerol. After a 3-h incubation at 37 °C, lipids were extracted as described above. [^{32}P] Lyso-PA binding to mAb-immobilized GP IIb-IIIa was performed under the same conditions as fibrinogen binding. Nonspecific binding was determined by measuring the binding of [^{32}P]lyso-PA to wells lacking GP IIb-IIIa.

Fibrinogen Binding to GP IIb-IIIa Incorporated into Phospholipid Vesicles—GP IIb-IIIa was exchanged into the dialyzable detergent octyl glucoside and incorporated into phospholipid vesicles containing PS:PC (2:1 molar ratio) or PS:PC:PA (2:1:0.4 molar ratio) as described by Parise and Phillips (1985b). Fibrinogen binding to GP IIb-IIIa incorporated in phospholipid vesicles was determined by incubating the vesicles in 0.1 M NaCl, 1 mM $CaCl_2$, 0.5% BSA, 0.02% NaN_3, 50 mM Tris, pH 7.4, in the presence of ^{125}I-fibrinogen for 1 h at room temperature. Saturability and specificity of the reactions were demonstrated by including 1.5 μM nonradioactive fibrinogen in the reaction. Nonspecific binding was determined by incubating phospholipid vesicles lacking GP IIb-IIIa with ^{125}I-fibrinogen. The reactions were terminated by filtration, the filters were washed three times with 1 ml of ice-cold buffer (0.1 M NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, 50 mM Tris, pH 7.4), and the radioactivity associated with the filters was quantitated. Parallel reactions, in which ^{125}I-mAb 10E5 replaced ^{125}I-fibrinogen, were conducted to quantitate the amount of GP IIb-IIIa available for binding.

Fibrinogen Binding to Purified Platelet Membranes—Washed platelets were resuspended in 150 mM NaCl, 10 mM Tris, pH 7.4, placed in the pressure chamber of a Parr cell disruption bomb (Parr Instrument Co., Moline, IL), equilibrated with N_2 (1200 p.s.i.) for 30 min, and discharged into the same buffer with a final concentration of 0.1 mM phenylmethylsulfonyl fluroide, 10 μg/ml aprotinin, and 0.1 mg/ml leupeptin. The suspension was centrifuged (100,000 × *g*) for 30 min at 4 °C, and platelet membranes were isolated by sucrose density sedimentation as described (Phillips and Baughan, 1983). The membranes were treated with various concentrations of phospholipase D (Sigma) for 30 min at 37 °C. The ability of phospholipase D to transfer the phosphatidyl moiety of PC to certain nucleophiles such as ethanol was used to quantitate the hydrolysis of PC (Dowson, 1967; Meulen and Haslam, 1990). Phospholipase D-catalyzed formation of phosphatidylethanol was measured by including 300 mM [^{14}C] ethanol (ICN Biomedicals, Costa Mesa, CA) in some reactions, extracting lipids, and quantitating incorporation of the ^{14}C label into the lipid fraction. Total phospholipid phosphorus in the membranes was quantitated with Malachite Green as described above and averaged 1,350 nmol of phosphate/mg of protein. PC was assumed to represent 40% of total lipid phosphorus (Holmsen, 1990). The production of PA by phospholipase D was verified by extracting lipids from platelet membranes that had been treated with buffer or 1 unit/ml of phospholipase D, separating the lipids by two-dimensional TLC, and visualizing phospholipids with the reagent of Dittmer and Lester (1964). Fibrinogen binding to the membranes was performed as described for fibrinogen binding to phospholipid vesicles. The reactions were terminated by filtration. Nonspecific binding was determined by including 1.5 μM nonradiolabeled fibrinogen in the reactions.

Calculations—In each experiment, all assays were performed in triplicate. Data are plotted as mean ± S.E. Unless otherwise indicated, graphs represent the results from three experiments.

RESULTS

Fibrinogen Binding Properties of mAb-immobilized GP IIb-IIIa—The goal of these studies was to determine whether lipids can modulate the fibrinogen binding activity of purified GP IIb-IIIa. To address this question, we used a microtiter plate assay in which GP IIb-IIIa was immobilized by the GP IIIa-specific mAb AP3. The amount of GP IIb-IIIa captured by the mAb AP3 was between 0.32 and 0.45 pmol/well, as quantitated by measuring the binding of the GP IIb-IIIa complex-specific mAb 10E5. These values correlated well with the values obtained for ^{125}I-GP IIb-IIIa binding to the mAb AP3-coated wells (0.33 pmol/well). When incubated with ^{125}I-fibrinogen in three experiments, only 15.3 ± 3.6% of the GP IIb-IIIa was capable of binding fibrinogen. Du and co-workers (1991) obtained similar results with GP IIb-IIIa from detergent lysates of fresh platelets. Thus, as observed by others using a variety of systems (Phillips and Baughan, 1983; Parise and Phillips, 1985b), the majority of purified GP IIb-IIIa does not retain the fibrinogen binding properties of activated platelets.

Effects of Lipid Extracts from Resting and Activated Platelets on Fibrinogen Binding to Purified GP IIb-IIIa—Lipids were extracted from washed platelets treated with platelet activators (thrombin, the thromboxane A_2-mimetic U46619, or PMA) or a platelet activation-inhibitor (PGI_2) and tested for their effects on fibrinogen binding to GP IIb-IIIa either before or after fractionation of the extracts by anion exchange chromatography. Although ^{125}I-fibrinogen binding was not enhanced by the addition of unfractionated lipid extracts to the assay (25 nmol of phospholipid phosphorus/well), the binding was increased 3-fold by a fraction obtained following DEAE-cellulose chromatography of lipid extracts from thrombin- (Fig. 1*A*) but not PGI_2-treated platelets (Fig. 1*B*). The peak of this activity eluted from the column in solvent containing 100 mM ammonium acetate (fraction 7). A factor with a similiar anion exchange chromatography profile but less activity was produced within 10 s of platelet exposure to thrombin (data not shown). DEAE-chromatography also resolved an activity from lipid extracts of U46619- and PMA-treated platelets, which increased the ^{125}I-fibrinogen binding by 3- and 3.5-fold, respectively (data not shown). Several fractions (*e.g.* fractions 1 and 2, Fig. 1) appeared to lower fibrinogen binding. This effect was probably nonspecific since these fractions lowered fibrinogen binding to wells lacking GP IIb-IIIa as well as to wells containing GP IIb-IIIa.

The activity in the peak fraction from thrombin-stimulated platelets contained a critical monoester phosphate, as evidenced by the ability of alkaline phosphatase treatment (1 unit/ml) to reduce the fibrinogen binding activity by 58%. The inability of alkaline phosphatase to abolish the activity may be caused by substrate inaccessibility and/or the presence of additional active factors without free phosphate groups. Phosphate-containing lipids in the active DEAE column fractions were identified by prelabeling platelets to steady-state with $^{32}P_i$. Thin layer chromatography of fractions 6–9 revealed that a ^{32}P-labeled lipid comigrating with authentic PA was substantially increased in fraction 7 from thrombin-stimulated platelets, whereas ^{32}P-labeled lipids comigrating with PS and PI were present in equal amounts in fractions from either thrombin- or PGI_2-treated platelets (Fig. 2). Alkaline

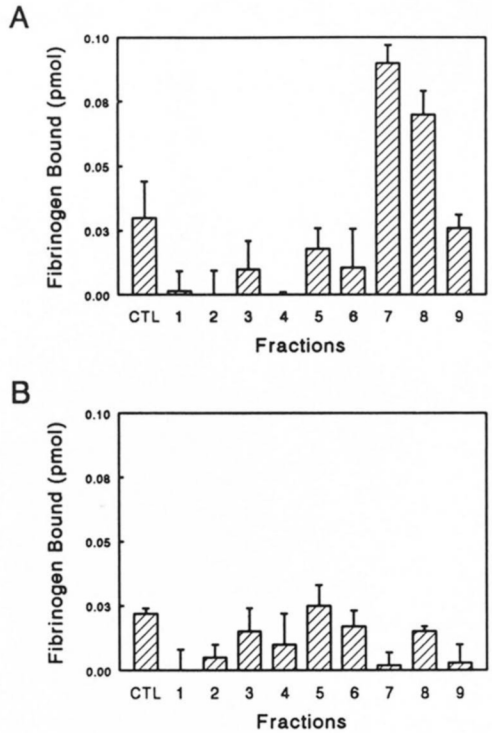

FIG. 1. **Effect of DEAE column fractions on ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa.** Lipids were extracted from platelets, which had been treated with either *A*, 0.5 unit/ml thrombin for 30 s or *B*, 150 nM PGI_2 for 1 min at 37 °C, and passed over DEAE-cellulose columns as described under "Experimental Procedures." The columns were washed with *fraction 1*, $CHCl_3$; *fraction 2*, $CHCl_3$:MeOH 9:1; *fraction 3*, $CHCl_3$:MeOH 7:3; *fraction 4*, $CHCl_3$:MeOH 1:1; *fractions 5* and *6*, $CHCl_3$:MeOH:H_2O 20:9:1; *fraction 7*, 100 mM ammonium acetate in $CHCl_3$:MeOH:H_2O; *fraction 8*, 200 mM ammonium acetate in $CHCl_3$:MeOH:H_2O; and *fraction 9*, 500 mM ammonium acetate in $CHCl_3$:MeOH:H_2O. Each fraction was dried, resuspended in 0.5 ml of buffer A, sonicated, and assayed for effects on the binding of 25 nM ^{125}I-fibrinogen to immobilized GP IIb-IIIa. Control (*CTL*) is the amount of ^{125}I-fibrinogen bound in the absence of added lipids. The data represent specific binding and are combined results from two experiments. Nonspecific binding was defined as the amount of ^{125}I-fibrinogen bound to wells lacking GP IIb-IIIa in the presence of the fractions. Similar results were obtained with lipids extracted from platelets treated with the thromboxane A_2-mimetic U46619 or PMA.

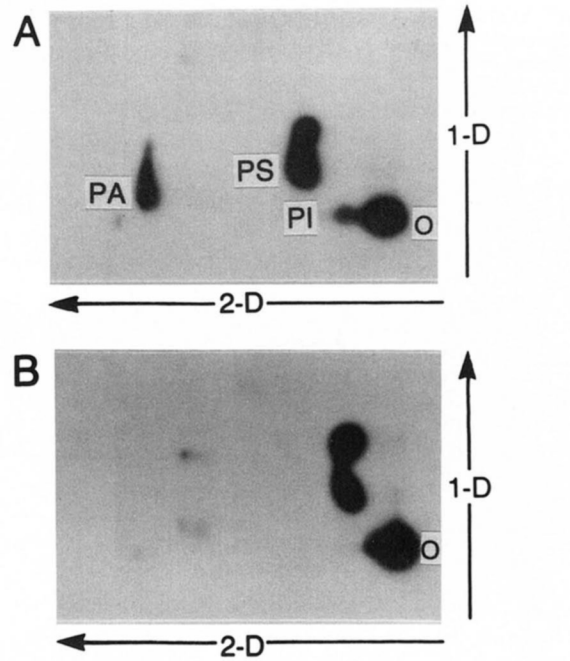

FIG. 2. **TLC analysis of ^{32}P-labeled lipids in the active DEAE column fraction (fraction 7).** To identify phosphate-containing components in fraction 7 from the DEAE column, platelets were incubated with $^{32}P_i$ before exposure to *A*, thrombin; or *B*, PGI_2. Lipids were extracted and fractionated by DEAE chromatography as described in the legend to Fig. 1. The lipids in fraction 7 were further resolved by TLC and visualized by autoradiography. The positions of lipid standards (PS, PI, PA) detected by I_2 are indicated. *1-D* and *2-D*, first- and second-dimension, respectively.

TABLE I

Effects of phospholipids, diacylglycerol, and glycerophosphates on the fibrinogen binding activity of GP IIb-IIIa

All compounds were prepared in 400 μM stock solutions, pH 7.4. Final concentrations in the binding assay were 100 μM. Binding reactions were conducted for 1 h at room temperature in the presence of 50 nM ^{125}I-fibrinogen. The results are expressed as the percent of specific fibrinogen binding induced by 100 μM PA.

Treatment	^{125}I-Fibrinogen bound
	% maximal
PA	100 ± 5.4
PS	23.1 ± 7.7
PC	21.1 ± 5.5
PIP	28.5 ± 15.4
PIP_2	23.1 ± 3.8
DAG	16.2 ± 7.3
α-Glycerophosphate	31.1 ± 3.0
β-Glycerophosphate	15.2 ± 4.6
No lipid	36.9 ± 5.3

phosphatase treatment (1 unit/ml) of fraction 7 from thrombin-stimulated platelets decreased the radioactivity associated with the TLC spot corresponding to PA by 38% (110,800 dpm without enzyme treatment *versus* 67,500 dpm with treatment). When a standard of authenthic PA was separated on the DEAE column, 65% of the lipid was recovered in fraction 7 and 35% in fraction 8. Thus, PA is one anionic lipid with a monoester phosphate that increases with platelet activation and is found in the active DEAE column fraction.

A second class of anionic lipids with monoester phosphates that undergo substantial changes with platelet activation is polyphosphoinositides. Although ^{32}P-labeled lipids comigrating with authentic PIP and PIP_2 were present in DEAE column fraction 8, the activity was not retained by neomycin-linked glass beads under appropriate conditions for polyphosphoinositide adsorption to this matrix (data not shown). Moreover, neither purified PIP nor PIP_2 in concentrations from 1 μM to 1 mM increased fibrinogen binding to purified GP IIb-IIIa (Table I).

Characterization of the Effects of Purified PA on Fibrinogen Binding to Purified GP IIb-IIIa—Since the evidence presented above suggests that PA is one component in the active DEAE column fraction, we examined the effects of purified PA on fibrinogen binding to GP IIb-IIIa. PA increased the binding of ^{125}I-fibrinogen to mAb-immobilized GP IIb-IIIa in a concentration-dependent manner, with a maximal effect at 1 mM (Fig. 3). The results were not a nonspecific effect of lipids in general as other lipids, including PS and PC, did not possess similar activity (Table I). Examination of the radioactivity bound to GP IIb-IIIa in the presence and absence of PA by polyacrylamide gel electrophoresis and autoradiography demonstrated that the ^{125}I label was associated with the Aα, Bβ, and γ chains of fibrinogen. Moreover, the lipids did not alter the mobility of GP IIb or GP IIIa separated by polyacrylamide gel electrophoresis, suggesting that PA does not alter the covalent structure of the proteins (data not shown).

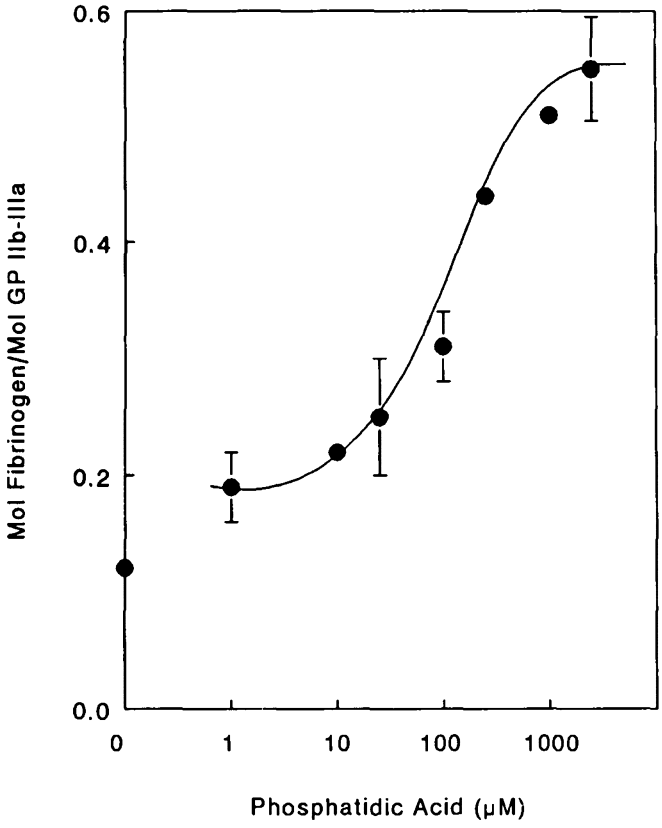

FIG. 3. **Concentration dependence of PA-induced ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa.** Binding of 50 nM ^{125}I-fibrinogen to wells containing GP IIb-IIIa captured by mAb AP3 was determined in the presence of various concentrations of PA as described under "Experimental Procedures." The amount of GP IIb-IIIa present was quantitated by measuring the binding of 200 nM ^{125}I-mAb 10E5. ^{125}I-mAb 10E5 binding was observed only when GP IIb-IIIa was added to mAb AP3-coated plates, was inhibited by Ca^{2+} chelators, and was saturable. Varying the concentrations of PA did not alter the levels of ^{125}I-mAb 10E5 bound. The data are expressed as mol of fibrinogen bound/mol of GP IIb-IIIa, and specific binding is shown. When GP IIb-IIIa was captured with the GP IIb-specific mAb Tab similar results were obtained.

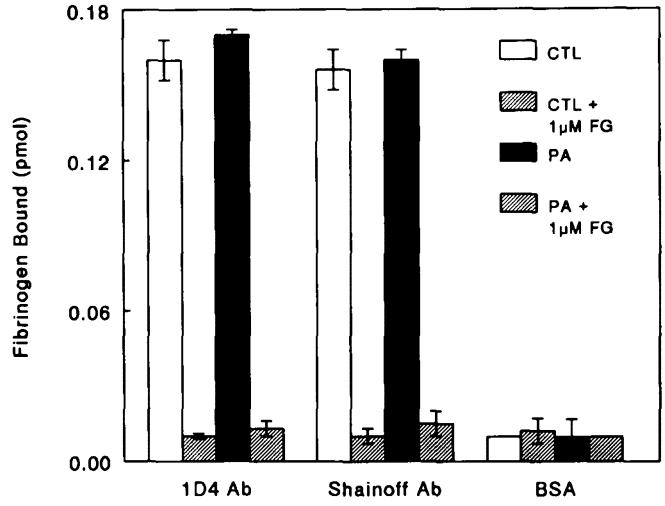

FIG. 4. **PA does not alter ^{125}I-fibrinogen binding to anti-fibrinogen mAb.** Microtiter wells were coated with 50 μl of the anti-fibrinogen mAb 1D4 or Shainoff (0.015 mg/ml) or BSA (0.015 mg/ml). ^{125}I-Fibrinogen (15 nM) was incubated with the wells for 1 h at room temperature without (*open bars*) or with (*solid bars*) the addition of 1 mM PA. The wells were washed three times, and the radioactivity associated with the well was quantitated. ^{125}I-Fibrinogen binding in the presence or absence of PA was inhibited by the inclusion of 1.5 μM fibrinogen (*FG*) in the assay (*hatched bars*). Data are graphed as pmol of total ^{125}I-fibrinogen bound. Results from one experiment performed in triplicate are presented.

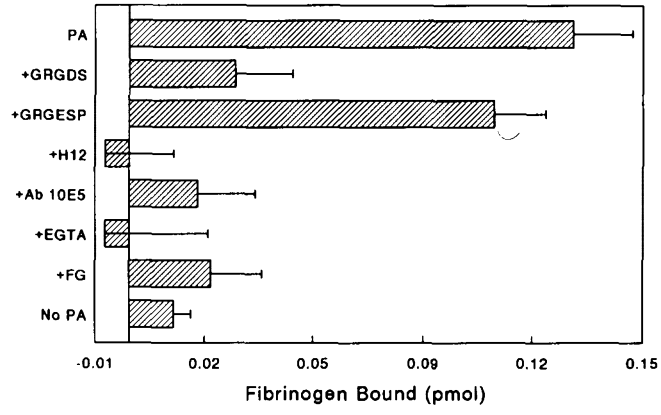

FIG. 5. **Inhibition of PA-induced ^{125}I-fibrinogen binding by agents that inhibit platelet aggregation.** Immobilized GP IIb-IIIa was incubated with 50 nM ^{125}I-fibrinogen and 1 mM PA. The specificity of PA-induced binding was verified by including 0.5 mM GRGDS peptide, 0.5 mM GRGESP peptide, 0.5 mM H12 peptide, 1.5 μM mAb 10E5, 5 mM EGTA, or 1.5 μM fibrinogen (*FG*) in the assay as indicated. Basal levels of binding of ^{125}I-fibrinogen to immobilized GP IIb-IIIa, measured in the absence of PA and inhibitors, is indicated (*No PA*). Data are graphed as pmol of ^{125}I-fibrinogen specifically bound; nonspecific binding was defined by incubating wells lacking GP IIb-IIIa with ^{125}I-fibrinogen and the indicated agents.

Three lines of evidence indicate that PA did not increase fibrinogen binding nonselectively, but promoted a specific interaction between GP IIb-IIIa and fibrinogen. First, the effects of PA depended on the presence of GP IIb-IIIa, and PA did not alter the amount of GP IIb-IIIa captured by mAb AP3 (data not shown). Second, when wells were coated with the anti-fibrinogen mAb Shainoff or 1D4, PA did not alter the levels of fibrinogen bound to these mAb (Fig. 4), suggesting that PA was not increasing the amount of fibrinogen bound to GP IIb-IIIa by inducing molecular interactions between fibrinogen molecules; if PA were nonspecifically promoting fibrinogen binding, its effect should be independent of the protein to which fibrinogen is binding. Third, the PA-induced fibrinogen binding was inhibited by agents known to inhibit fibrinogen binding to GP IIb-IIIa, including nonradioactive fibrinogen, divalent cation chelation, mAb 10E5, the peptides GRGDS and H12, but not the control peptide GRGESP (Fig. 5). Thus, PA-stimulated fibrinogen binding to mAb-immobilized GP IIb-IIIa possesses many of the properties of fibrinogen binding to the GP IIb-IIIa complex on activated platelets. However, PA increased fibrinogen binding to Ab-immobilized GP IIb-IIIa containing the β_{3Cam} subunit with properties similar to those described above (data not shown). The Cam variant of GP IIb-IIIa, which contains a substitution of Asp^{119} by Tyr in GP IIIa, does not bind RGD peptides and cannot be activated to bind fibrinogen when expressed on intact cells (Loftus *et al.*, 1990).

Determination of Kinetic and Equilibrium Binding Constants—Binding of ^{125}I-fibrinogen (30 nM) in the presence of 25 μM, 100 μM, or 1 mM PA reached steady state within approximately 60 min at room temperature (Fig. 6). The association rate constant (k_1) for fibrinogen binding mediated by PA was 4.5×10^5 M^{-1} min^{-1}. Altering the concentration of PA in the assay did not affect the k_1. Nonradioactive fibrinogen (1.5 μM) added at 60 min dissociated 77% of the radiolabeled fibrinogen within 30 min. Dissociation over the first

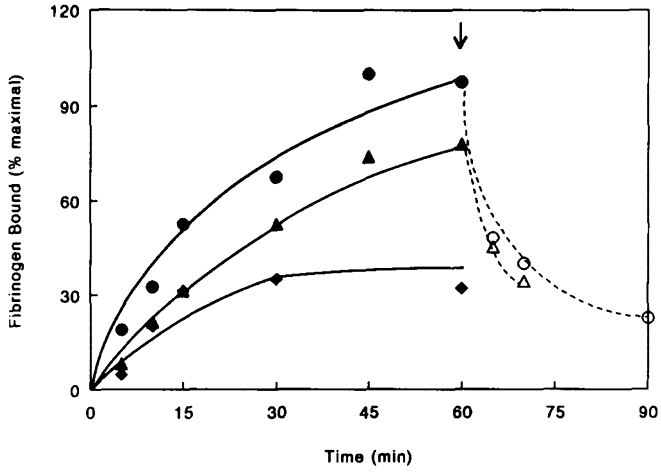

FIG. 6. **Time course and reversal of ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa in the presence of PA.** Microtiter wells containing GP IIb-IIIa were incubated with 30 nM ^{125}I-fibrinogen for the indicated times (*solid symbols*) in the presence of 25 μM (*diamonds*), 100 μM (*triangles*), or 1 mM PA (*circles*). Nonradioactive fibrinogen was added after 60 min (*arrow*) to displace bound ^{125}I-fibrinogen (*open symbols*). Data are plotted as percent maximal binding, defined as the amount of ^{125}I-fibrinogen specifically bound in the presence of 1 mM PA after 1 h at room temperature.

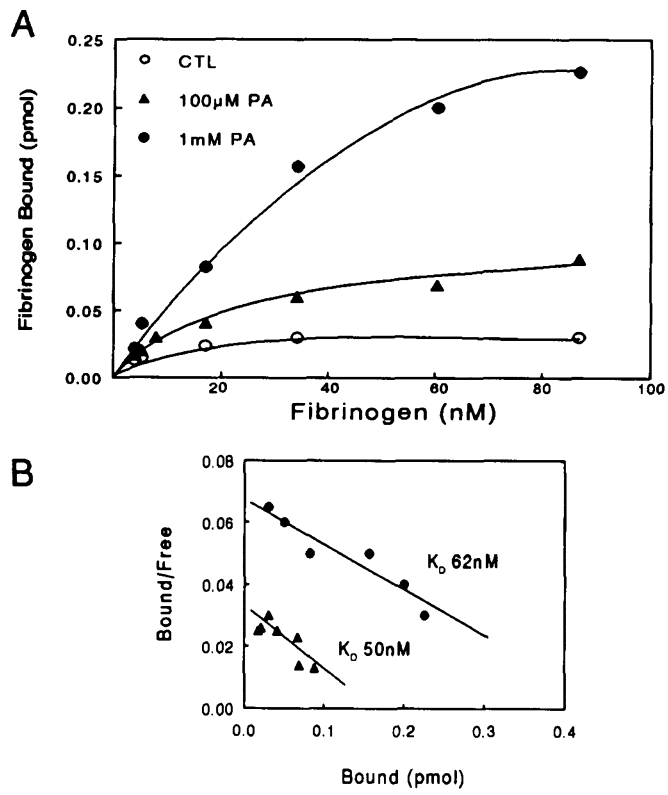

FIG. 7. **Saturation binding isotherm and Scatchard analysis of ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa in the presence of PA.** *A*, microtiter wells containing GP IIb-IIIa were incubated with no added (*open circles*), 100 μM (*closed triangles*), or 1 mM (*closed circles*) PA and concentrations of ^{125}I-fibrinogen as indicated. Data are plotted as pmol of ^{125}I-fibrinogen specifically bound and *B*, replotted as pmol specifically bound *versus* bound ligand divided by free ligand according to the method of Scatchard (1949). The dissociation constants (K_D) were 50 and 62 nM in the presence of 100 μM and 1 mM PA, respectively. The amount of GP IIb-IIIa captured by mAb AP3 was quantitated by measuring the binding of ^{125}I-mAb 10E5. Maximal binding capacities of 0.42 and 1.1 mol of fibrinogen bound/mol of GP IIb-IIIa were obtained, respectively.

10 min in the presence of 100 μM and 1 mM PA occurred with an average rate constant (k_2) of 4.42×10^{-2} min^{-1}. A kinetically derived dissociation constant ($K_D = k_2/k_1$) of 100 nM was obtained.

Equilibrium binding studies revealed that fibrinogen binding in the presence of 100 μM or 1 mM PA was saturable (Fig. 7). A Scatchard transformation of the saturation binding isotherms (Fig. 7*B*) indicated that the ^{125}I-fibrinogen binding promoted by PA occurs apparently to a single class of sites, presumably GP IIb-IIIa. Dissociation constants for fibrinogen binding in the presence of 100 μM and 1 mM PA were 50 and 62 nM, respectively, in agreement with the kinetically derived K_D. The apparent maximal binding capacity of GP IIb-IIIa was 0.42 mol of fibrinogen/mol of GP IIb-IIIa in the presence of 100 μM PA and 1.1 mol of fibrinogen/mol of GP IIb-IIIa in the presence of 1 mM PA. The binding isotherm obtained in the absence of PA was not analyzed by the method of Scatchard since the amount of ^{125}I-fibrinogen bound did not increase appreciably with increasing concentrations of ^{125}I-fibrinogen. Our results suggest that PA increases the proportion of fibrinogen-binding competent GP IIb-IIIa complexes and has little or no effect on the affinity of GP IIb-IIIa for fibrinogen.

Structural Requirements of PA Activity—The structural requirements for PA activity were evaluated by testing the effects of enzymatically treated PA and derivatives of PA on fibrinogen binding to GP IIb-IIIa. Preincubation of PA with alkaline phosphatase or phospholipase B for 1 h at 37 °C dose-dependently decreased the ability of PA to induce fibrinogen binding to GP IIb-IIIa (Fig. 8, *A* and *B*). These observations were supported by the finding that neither purified DAG, a product of alkaline phosphatase, nor α-glycerophosphate, a product of phospholipase B, was capable of inducing fibrinogen binding to GP IIb-IIIa (Table I). β-Glycerophosphate was also without effect (Table I). Preincubation of a maximal concentration of PA (1 mM) with up to 1 unit/ml phospholipase A_2 had no effect on the ability of PA to induce fibrinogen binding (Fig. 8*C*, *solid line*). Under these conditions, resolution of the products by TLC revealed that phospholipase A_2 was active in converting PA to lyso-PA. Higher doses of phospholipase A_2 (10–100 units/ml) lowered the activity of 1 mM PA and reduced the total amount of PA and lyso-PA in the reaction. One interpretation of these results is that at high concentrations, phospholipase A_2 generates glycerophosphates which, in turn, inhibit PA-induced fibrinogen binding (see below). However, preincubation of a submaximal concentration of PA (100 μM) with phospholipase A_2 increased both the levels of lyso-PA in the reaction and the ability to promote fibrinogen binding (Fig. 8*C*, *dashed line*).

These results suggested that lyso-PA is a potent activator of fibrinogen binding to GP IIb-IIIa. Indeed, we found that a synthetic lyso-PA (1-oleoyl,2-hydroxyglycero-3-phosphate) induced fibrinogen binding to GP IIb-IIIa in a dose-dependent manner, with an EC_{50} of 50 μM (Fig. 9, *solid line*), which was slightly more potent than commercially obtained PA, which had an EC_{50} of 100 μM. It should be noted that at concentrations > 1 mM, the synthetic lyso-PA increased fibrinogen binding to the microtiter wells in the absence of GP IIb-IIIa. The critical micellar concentration of lyso-PA is reported to be 1.3 mM at pH 7.4 (Jalink *et al.*, 1990), and this increase in nonspecific binding may represent interactions of fibrinogen with lyso-PA micelles.

Examination of the purified PA by TLC revealed that no detectable lipid chromatographed with lyso-PA, and approximately 10% or less of the lipid chromatographed with PC.

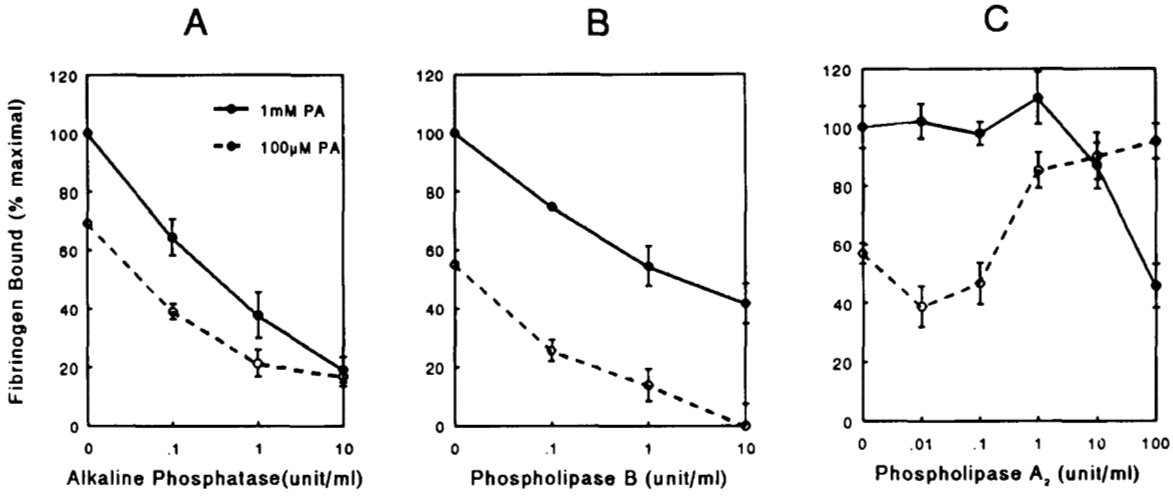

FIG. 8. **Enzymatic alterations in the ability of PA to induce ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa.** PA, resuspended and sonicated in Tris/NaCl buffer, was preincubated with various concentrations of *A*, alkaline phosphatase; *B*, phospholipase B; or *C*, phospholipase A_2 as described under "Experimental Procedures" before addition to binding reactions containing 50 nM ^{125}I-fibrinogen. Final concentrations of PA in the binding assays were 100 μM (*dashed lines*) or 1 mM (*solid lines*). Data are graphed as a percent of specific fibrinogen binding induced by 1 mM PA.

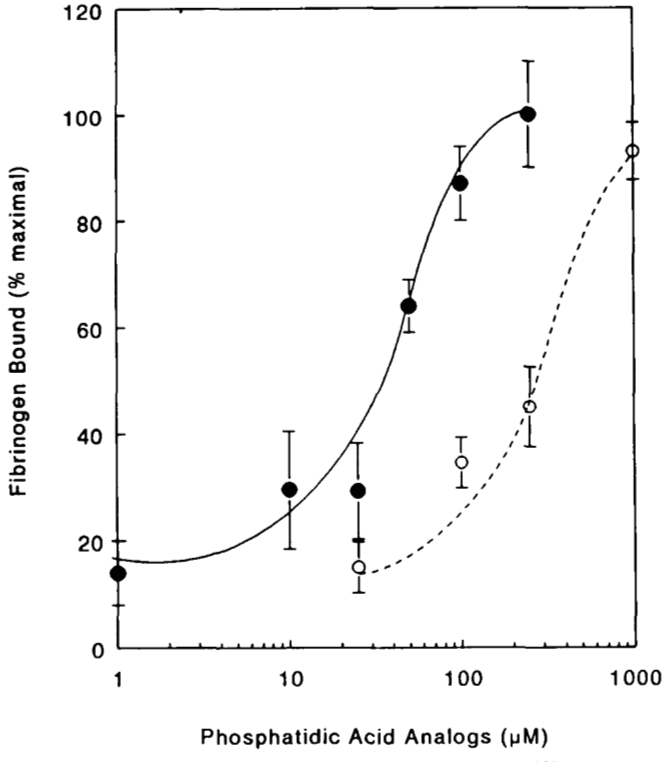

FIG. 9. **Concentration-dependent induction of ^{125}I-fibrinogen binding to mAb-immobilized GP IIb-IIIa by PA analogs.** Microtiter wells containing immobilized GP IIb-IIIa were incubated with various concentrations of a synthetic lyso-PA (1-oleoyl,2-hydroxyglycero-3-phosphate; *solid line*) or a synthetic PA (1,2-dicaproyl-*sn*-glycero-3-phosphate; *dashed line*) and 50 nM ^{125}I-fibrinogen for 1 h as described under "Experimental Procedures." The data are graphed as percent maximal binding induced by 1 mM PA.

Although we cannot rule out the possibility that the effects ascribed to PA are caused by low levels of contaminating lyso-PA, this is unlikely since the dose-response curves suggest that at least 10% of the lipid in the PA would have to be lyso-PA to produce the observed effects.

Although these results may provide insight into the structural requirements for PA activity and potential mechanisms of PA interaction with GP IIb-IIIa, it is possible that the effects of PA are caused by the macromolecular form assumed by the lipid in aqueous solution. Therefore, several chemical forms of PA that assume different macromolecular structures were compared. Synthetic 1,2-dicaproyl-*sn*-glycerol-3-phosphate, which forms micelles when sonicated in aqueous solution, induced fibrinogen binding with an EC_{50} of 250 μM (Fig. 9, *dashed line*) a slightly higher EC_{50} than that of PA, which forms bilayers when sonicated in aqueous solution (Cullis *et al.* 1983). Moreover, lyso-PA was active at concentrations below its critical micellar concentration, conditions in which lipid monomers are expected to predominate (Fig. 9). Based on these results, we believe that it is the chemical structure and not the macromolecular structure assumed by PA in solution that is more important in determing its ability to promote fibrinogen binding.

Our studies imply that there is an interaction between PA or lyso-PA and GP IIb-IIIa. We therefore examined the ability of [^{32}P]lyso-PA to bind directly to GP IIb-IIIa. [^{32}P]Lyso-PA bound specifically and dose-dependently to GP IIb-IIIa immobilized by mAb AP3 (Fig. 10*A*). Moreover, [^{32}P]lyso-PA binding to GP IIb-IIIa was inhibited by nonradiolabeled lyso-PA, PA, and α-glycerophosphate, but not DAG or β-glycerophosphate (Fig. 10*B*). The inhibition of binding by α-glycerophosphate suggested that this compound might alter PA-induced fibrinogen binding. When tested, α-glycerophosphate did inhibit PA-induced fibrinogen binding to mAb-immobilized GP IIb-IIIa, whereas DAG or monoacylglycerol failed to inhibit binding (data not shown).

Effect of PA on the Fibrinogen Binding Activity of GP IIb-IIIa Reconstituted in Phospholipid Vesicles and in Purified Platelet Membranes—Having established an effect of PA on the fibrinogen binding properties of mAb-immobilized GP IIb-IIIa, we examined the effect of PA on fibrinogen binding to GP IIb-IIIa reconstituted into phospholipid vesicles. The glycoprotein was incorporated by detergent dialysis into phospholipid vesicles composed of PS:PC or PS:PC:PA. As expected based on previous studies (Parise and Phillips, 1985a), different amounts of GP IIb-IIIa were either incorporated into and/or recovered with vesicles of different lipid compositions (see Fig. 11, legend). Therefore, the fibrinogen binding results are expressed per mol of accessible GP IIb-IIIa as

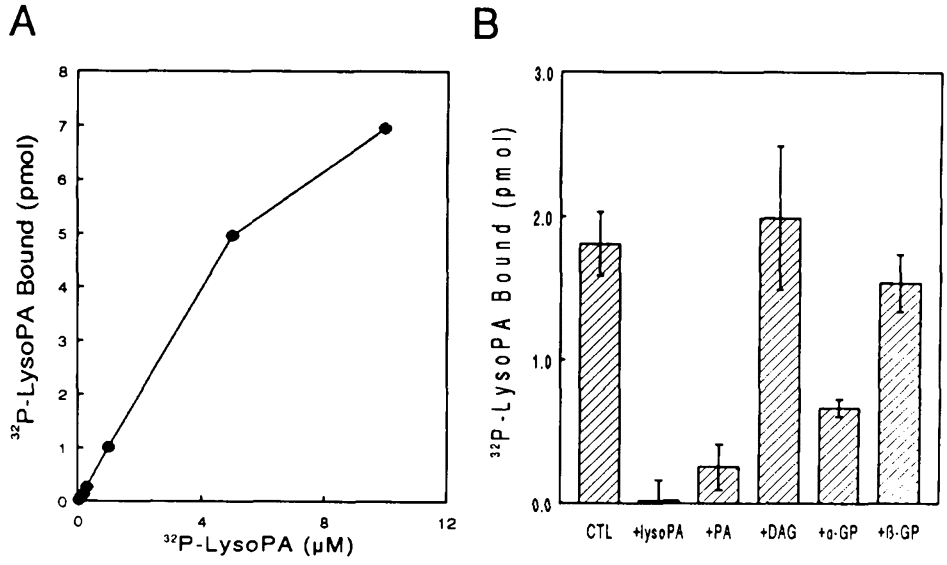

FIG. 10. **[^{32}P]Lyso-PA binding to mAb-immobilized GP IIb-IIIa.** *A*, microtiter wells containing GP IIb-IIIa were incubated with various concentrations of [^{32}P]lyso-PA for 1 h at room temperature as described under "Experimental Procedures." *B*, [^{32}P]lyso-PA (1 μM) binding was determined in the absence (indicated as *CTL*) or the presence of nonradiolabeled lyso-PA (100 μM), PA (100 μM), diacylglycerol (100 μM), or glycerophosphates (100 μM) as indicated. The data are expressed as pmol of [^{32}P] lyso-PA specifically bound. Nonspecific binding was determined by quantitating the amount of [^{32}P]lyso-PA binding to wells lacking GP IIb-IIIa.

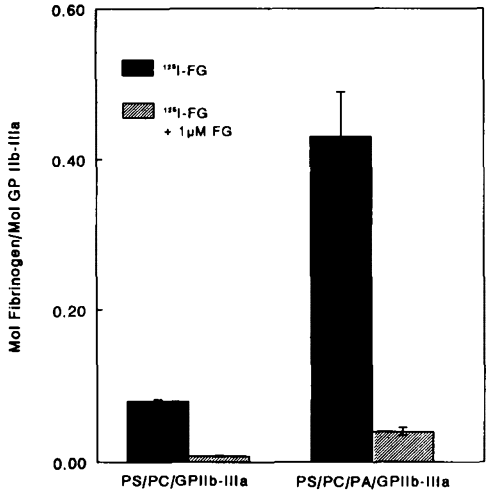

FIG. 11. **Specific ^{125}I-fibrinogen binding to GP IIb-IIIa incorporated into phospholipid vesicles.** Phospholipid vesicles composed of PS:PC or PS:PC:PA were formed with GP IIb-IIIa as described under "Experimental Procedures." The phospholipid vesicles were incubated with 50 nM ^{125}I-fibrinogen or 200 nM ^{125}I-mAb 10E5 for 1 h at room temperature. Where indicated, 1.5 μM fibrinogen (*FG*) was included in the reactions with ^{125}I-fibrinogen (*hatched bars*). Data are graphed as mol of ^{125}I-fibrinogen bound/mol of ^{125}I-mAb 10E5 bound. The amount of GP IIb-IIIa acessible for ligand binding was 2.5-fold lower in the PS:PC:PA vesicles as determined by measuring the binding of ^{125}I-mAb 10E5. Nonspecific binding was determined by measuring the binding of ^{125}I-ligand to phospholipid vesicles formed without GP IIb-IIIa.

determined by measuring the binding of ^{125}I-10E5. Vesicles composed of PS:PC:PA bound 4.2-fold more fibrinogen/mol of GP IIb-IIIa than did vesicles formed with PS:PC only (Fig. 11). Furthermore, ^{125}I-fibrinogen binding to the vesicles was specific and saturable, as evidenced by the ability of nonradioactive fibrinogen to inhibit ^{125}I-fibrinogen binding. In contrast, the addition of exogenous PA to GP IIb-IIIa incorporated in PS:PC vesicles did not alter fibrinogen binding. These results suggest that PA in a membrane environment, either by directly interacting with GP IIb-IIIa or by altering the vesicle structure, enhances fibrinogen binding to GP IIb-IIIa.

Finally, PA was generated in purified platelet membranes to determine the effects on fibrinogen binding to the membranes. Less than 10% of the GP IIb-IIIa in the membranes retains the ability to bind fibrinogen; these low levels of fibrinogen binding can be increased by certain manipulations of the membranes (Smyth and Parise, 1992). Incubation of platelet membranes with 1–10 units/ml of phospholipase D resulted in the hydrolysis of 10–30% of the PC (control levels, ~550 nmol of PC/mg of protein). When incubated with 30 nM ^{125}I-fibrinogen, control membranes bound 5.2 pmol of fibrinogen/mg of protein, whereas membranes treated with 1 and 10 units/ml phospholipase D bound 4.4 and 3.2 pmol of fibrinogen/mg of protein, respectively. No change in the amount of GP IIb-IIIa, as judged by ^{125}I-mAb 10E5 binding (300 pmol/mg of protein), was observed. Thus, we have not been able to demonstrate that elevations of PA can induce fibrinogen binding to GP IIb-IIIa in isolated platelet membranes. Although the reasons for a lack of effect in this more complicated environment are unknown, the results may have interesting implications with regard to the physiologic mechanism of GP IIb-IIIa activation (see "Discussion") or may indicate that the PA produced in this manner may not have the proper accessibility to GP IIb-IIIa or may disrupt the normal membrane structure.

DISCUSSION

The preeminence of integrins in mediating diverse cellular adhesive processes, such as platelet aggregation and wound healing, is well established. In contrast, relatively little is known about the mechanisms by which the ligand binding properties of certain integrins are modulated during cellular activation. In an attempt to identify factors that may regulate the binding function of the major platelet integrin, GP IIb-IIIa, we investigated the effects of lipids on the fibrinogen binding properties of purified GP IIb-IIIa. We report that fractionated lipids from thrombin-activated platelets containing PA, as well as purified PA, and lyso-PA can increase fibrinogen binding to purified, mAb-immobilized GP IIb-IIIa.

DEAE chromatograpy of lipid extracts from thrombin-, U46619-, or PMA-activated, but not PGI_2-inhibited platelets, resolves a lipid fraction that increases fibrinogen binding to mAb-immobilized GP IIb-IIIa. The factor from thrombin-stimulated platelets appears to have a monoester phosphate group that is important for at least part of its activity (~60%). A likely candidate for this activity is PA, since it is the major ^{32}P-labeled lipid that increases in the active fractions. The possibility that active factors without alkaline phosphatase-

sensitive groups might also contribute to this activity requires further investigation. The sensitivity of our activity to alkaline phosphatase distinguishes it from integrin-modulating factor-1 isolated by Hermanowski-Vostaka *et al.* (1992) from lipid extracts of activated polymorphonuclear leukocytes.

Purified PA appears to promote a specific and saturable interaction between mAb-immobilized GP IIb-IIIa and soluble fibrinogen that has many properties similar to those of fibrinogen binding to GP IIb-IIIa on the surface of activated platelets. Specifically, PA-mediated fibrinogen binding to purified GP IIb-IIIa is inhibited by calcium chelation, an RGD-containing peptide, a peptide corresponding to the γ-chain of fibrinogen, and a GP IIb-IIIa complex-specific mAb. Maximally effective concentrations of PA induce a 1:1 molar ratio of fibrinogen binding to GP IIb-IIIa. The affinity constant for fibrinogen binding to GP IIb-IIIa in the presence of PA (~55 nM) is in the range of reported values for the affinity constant for fibrinogen binding to activated platelets (50–500 nM; for review see Plow and Ginsberg, 1989). Analysis of fibrinogen binding in the presence of PA indicates that the lipid increases the proportion of fibrinogen-binding competent GP IIb-IIIa complexes with little or no effect on the affinity of GP IIb-IIIa for fibrinogen. In one regard, the PA-induced fibrinogen binding differed from fibrinogen binding to GP IIb-IIIa on intact platelets: namely, PA increased fibrinogen binding to the Cam variant of GP IIb-IIIa, which is unable to recognize ligands on platelets. The ability of PA to increase fibrinogen binding to the Cam variant of GP IIb-IIIa suggested that PA may enhance binding by interacting with fibrinogen. Although we cannot unequivocably eliminate this possibility, we were unable to detect an effect of PA on fibrinogen binding to anti-fibrinogen antibodies (Fig. 4), suggesting that if PA is acting on fibrinogen the effect is specific for the fibrinogen-GP IIb-IIIa interaction.

The ability of PA to promote fibrinogen binding to GP IIb-IIIa is not a general property of negatively charged lipids. Although the loss of activity of the platelet lipid extract and purified PA with alkaline phosphatase treatment suggests that the monoester phosphate is important, the negative charge provided by the phosphate or the phosphate *per se* is not sufficient. Other negatively charged lipids (PS, PI, the monoester phosphate-containing PIP and PIP_2) or the water-soluble analogs of PA (α- and β-glycerophosphate) were without effect. Neutral lipids (DAG, PC) were also inactive. At least one fatty acid chain is also required for the activity, since lyso-PA, but not α-glycerophosphate, is a potent inducer of fibrinogen binding to GP IIb-IIIa. However, fatty acids of different chain lengths in PA or active analogs are effective (Fig. 9).

The specific chemical structure of the lipid appears to be more important than the macromolecular forms assumed by the active lipids in solution for this activity since lipids expected to form monomers, bilayers, or micelles in solution were active. Lyso-PA monomers may account for the apparent increase in potency of lyso-PA relative to PA. This relationship might not necessarily hold with GP IIb-IIIa incorporated into membranes, in which both lyso-PA and PA would be soluble. The monomeric nature of lyso-PA also suggested that this lipid would be applicable in direct binding studies, and we found that [^{32}P]lyso-PA bound directly and specifically to GP IIb-IIIa.

PA and lyso-PA are generated rapidly and in significant quantities upon cellular activation (Exton, 1990). PA levels rise within seconds of exposure of platelets to agonists and peak between 60 and 120 s. In thrombin-stimulated platelets, approximately 90% of the newly synthesized PA (2–4 nmol/1 $\times 10^9$ platelets) arises from the sequential generation of DAG by PI-specific phospholipase C and phosphorylation of the DAG by DAG kinase, whereas 10% is generated directly by phospholipase D-mediated hydrolysis of PC (Huang *et al.*, 1991). The rate of *de novo* synthesis does not change in response to thrombin (Neufield and Majerus, 1983). Lyso-PA represents approximately 10% of accumulated PA and is produced largely by the action of phospholipase A_2 on PA (Billah *et al.*, 1981). In at least two situations, platelet aggregation can be dissociated from the production of PA via PIP_2 hydrolysis. First, PA formation from DAG is partially inhibited by DAG kinase inhibitors under conditions in which platelet aggregation is enhanced (Chaffoy de Courcelles *et al.*, 1989). Second, platelet aggregation can be inhibited by protein kinase C inhibitors, such as staurosporin, which have no effect on PA production (Watson *et al.*, 1988). Furthermore, when added to platelets, both PA (Kroll *et al.*, 1989) and lyso-PA (Benton *et al.*, 1982) induce platelet aggregation, apparently by receptor-mediated activation of PI-specific phospholipase C. This involvement in stimulus activation coupling complicates efforts to measure a direct effect of exogenous PA on GP IIb-IIIa function. Thus, at this time any effects that PA may have on the fibrinogen binding activity of GP IIb-IIIa in platelets are unknown.

Our studies indicate that fibrinogen binding to GP IIb-IIIa incorporated into PA-containing lipid vesicles is enhanced, suggesting that PA can affect GP IIb-IIIa within a membrane environment. However, we have been unable to enhance fibrinogen binding to GP IIb-IIIa by generating PA in isolated platelet membranes. These results may suggest that activation of GP IIb-IIIa requires both the release of a negative regulator (O'Toole *et al.*, 1991; Steiner *et al.*, 1991) as well as the action of a positive element. The future combination of approaches involving purified GP IIb-IIIa, isolated platelet membranes, and whole platelets should potentially allow the elucidation of mechanisms of GP IIb-IIIa activation.

In summary, our results demonstrate that PA, lyso-PA, and lipid(s) from activated platelets can affect the fibrinogen binding properties of purified GP IIb-IIIa. These lipids may directly regulate the binding activity of the glycoprotein or the lipids may provide an environment which restores fibrinogen binding activity to GP IIb-IIIa. Further studies are needed to determine whether the specific lipids identified here actually affect the fibrinogen binding capacity or any other function of GP IIb-IIIa in whole platelets. However, the identification of these lipids, as well as the specific peptides (Du *et al.*, 1991) and antibodies (O'Toole *et al.*, 1990) previously described, provide a number of distinct tools to aid in understanding mechanisms and alterations in GP IIb-IIIa structure that allow fibrinogen binding to occur.

Acknowledgments—We are indebted to Andrew Morris for his advice and expertise, to Anne Criss for many helpful discussions, to Dr. Mark Werner for advice on DAG kinase, to Dr. Barry Coller for his gift of mAb 10E5, to Dr. Peter Newman for mAb AP3, to Dr. Rodger McEver for mAb Tab, to Dr. Bohdan Kudryk for anti-fibrinogen mAb, to Dr. John Fenton for thrombin, and to Dr. Joseph Loftus and Dr. Mark Ginsberg for Chinese hamster ovary cells expressing recombinant GP IIb-IIIa and GP IIb-IIIa containing the β_{3Cam} subunit.

REFERENCES

Allan, D., and Michell, R. H. (1978) *Biochim. Biophys. Acta* **508,** 277–286
Bennett, J. S., and Vilaire, G. (1979) *J. Clin. Invest.* **64,** 1393–1401
Benton, A. M., Gerrard, J. M., Michiel, T., and Kindom, S. E. (1982) *Blood* **60,** 642–649
Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1981) *J. Biol. Chem.* **256,** 5399–5404
Bodary, S. C., Napier, M. A., and McLean, J. W. (1989) *J. Biol. Chem.* **264,** 18859–18862

Brass, L. F., Shattil, S. J., Kunicki, T. J., and Bennett, J. S. (1985) *J. Biol. Chem.* **260,** 7875–7881
Cheresh, D. A., Pytela, R., Pierschbacher, M. D., Klier, F. G., Ruoslahti, E., and Reisfeld, R. A. (1987) *J. Cell Biol.* **105,** 1163–1173
Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) *J. Clin. Invest.* **72,** 325–338
Conforti, G., Zanetti, A., Pasquali-Ronchetti, I., Quaglino, D., Jr., Neyroz, P., and Dejana, E. (1990) *J. Biol. Chem.* **265,** 4011–4019
Cullis, J., Kruijff, B., Hope, M. J., Nayar, R., Farren, S., Tilcock, C., Madden, T. D., and Bally, M. B. (1983) in *Membrane Fluidity in Biology* (Aloia, R. C., ed) pp. 39–81, Academic Press, New York
de Chaffoy de Courcelles, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y., and De Clerck, F. (1989) *J. Biol. Chem.* **264,** 3274–3285
Dittmer, J. D., and Lester, R. L. (1964) *J. Lipid Res.* **5,** 126–127
Dowson, R. M. C. (1967) *Biochem. J.* **265,** 205–210
Du, X., Plow, E. F., Frelinger, A. L., O'Toole, T., Loftus, J. C., and Ginsberg, M. H. (1991) *Cell* **65,** 409–416
Dustin, M. L., and Springer, T. A. (1989) *Nature* **341,** 619–624
Exton, J. H. (1990) *J. Biol. Chem.* **265,** 1–4
Ferrell, J. E., and Huestis, W. H. (1984) *J. Cell Biol.* **98,** 1992–1998
Fitzgerald, L. A., and Phillips, D. R. (1985) *J. Biol. Chem.* **260,** 11366–11374
Fitzgerald, L. A., Leung, B., and Phillips, D. R. (1985) *Anal. Biochem.* **151,** 169–177
Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Lo, S., and Phillips, D. R. (1987) *J. Biol. Chem.* **262,** 3936–3939
Hermanowski-Vosatka, A., Van Strijp, J. A. G., Swiggard, W., and Wright, S. (1992) *Cell* **68,** 341–352
Hillery, C. A., Smyth, S. S., and Parise, L. V. (1991) *J. Biol. Chem.* **266,** 14663–14669
Holmsen, H. (1990) in *Hematology* (Williams, W., Beutler, E., Erslev, A., and Lichtman, M., eds) pp. 1182–1199, McGraw-Hill, New York
Huang, R., Kucera, G. L., and Rittenhouse, S. E. (1991) *J. Biol. Chem.* **266,** 1652–1655
Hynes, R. O. (1987) *Cell* **48,** 549–554
Itayai, K., and Ui, M. (1966) *Clin. Chem. Acta* **14,** 361–366
Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) *J. Biol. Chem.* **265,** 12232–12239
Kazal, L. A., Amsel, S., Miller, O. P., and Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med.* **113,** 989–994
Kroll, M. H., Zavoico, G. B., and Schafer, A. I. (1989) *J. Cell. Physiol.* **139,** 558–564
Kucera, G. L., and Rittenhouse, S. E. (1990) *J. Biol. Chem.* **265,** 5345–5348
Leung, N. L., Vickers, J. D., Kinlough-Rathbone, R. L., Relmers, H., and Mustard, J. F. (1983) *Biochem. Biophys. Res. Commun.* **113,** 483–490
Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L., Ginsberg, M. H. (1990) *Science* **249,** 915–918
Low, M. (1990) in *Methods in Inositide Research* (Irvine, R., ed) pp. 145–151, Raven Press, New York
Marguerie, G. A., Plow, E. F., and Edgington, T. S. (1979) *J. Biol. Chem.* **254,** 5357–5363
McEver, R. P., Baenziger, N. L., and Majerus, P. W. (1980) *J. Clin. Invest.* **66,** 1311–1381
Meulen, J. V. D., and Haslam, R. J. (1990) *Biochem. J.* **271,** 693–700
Morris, A. J., Waldo, G. L., Downes, C. P., and Harden, T. K. (1990) *J. Biol. Chem.* **265,** 13501–13507
Mustard, J. F., Kinlough-Rathbone, R. L., and Packham, M. A. (1989) *Methods Enzymol.* **169,** 3–21
Neufield, E. J., and Majerus, P. W. (1983) *J. Biol. Chem.* **258,** 2461–2467
Newman, P. J., Allen, R., Kahn, R. A., and Kunicki, T. J. (1985) *Blood* **65,** 227–232
Nolan, R. D., and Lapetina, E. G. (1990) *J. Biol. Chem.* **265,** 2441–2445
O'Toole, T. E., Loftus, J. C., Du, X., Glass, A. A., Ruggeri, Z. M., Shattil, S. J., Plow, E. F., and Ginsberg, M. H. (1990) *Cell Regul.* **1,** 883–893
O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S., Plow, E. F., and Ginsberg, M. H. (1991) *Science* **254,** 845–847
Parise, L. V., and Phillips, D. R. (1985a) *J. Biol. Chem.* **260,** 1750–1756
Parise, L. V., and Phillips, D. R. (1985b) *J. Biol. Chem.* **260,** 10698–10707
Phillips, D. R., and Baughan, A. K. (1983) *J. Biol. Chem.* **258,** 10240–10246
Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) *Blood* **71,** 831–843
Plow, E. F., and Ginsberg, M. H. (1989) *Prog. Hemostasis Thromb.* **9,** 117–156
Plow, E. F., Srouji, A. H., Meyer, D., Marguerie, G., and Ginsberg, M. H. (1984) *J. Biol. Chem.* **259,** 5388–5391
Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., and Ginsberg, M. H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8057–8061
Poncz, M., Eisman, R., Heidenreich, R., Silver, S., Vilaire, G., Surrey, S., Schwartz, E., and Bennett, J. (1987) *J. Biol. Chem.* **262,** 8476–8482
Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Neidel, J. E., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 8597–8600
Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63,** 580–587
Ruoslahti, E., and Pierschbacher, M. D. (1987) *Science* **238,** 491–497
Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
Seiss, W., Boehlig, B., Weber, P. C., and Lapetina, E. G. (1985) *Blood* **65,** 1141–1148
Shattil, S. J., and Brass, L. F. (1987) *J. Biol. Chem.* **262,** 992–1000
Shimizu, Y., VanSeventer, G. A., Horgan, K. J., and Shaw, S. (1990) *Nature* **345,** 250–253
Smyth, S. S., and Parise, L. V. (1992) *J. Cell. Biochem.* **16F,** 162 (abstr.)
Steiner, B., Kouns, W. C., Jennings, L. K., and Hadvary, P. (1991) *Blood* **78,** 363 (abstr.)
Watson, S. P., McNally, J., Shipman, J. L., and Godfrey, P. P. (1988) *Biochem J.* **249,** 345–350
Wright, S. D., and Meyer, B. C. (1986) *J. Immunol.* **136,** 1759–1764