Signal-transducing Mechanisms Involved in Activation of the Platelet Collagen Receptor Integrin $\alpha_2\beta_1$*

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Evidence was obtained about the mechanism responsible for platelet integrin $\alpha_\beta$ activation by determining effects of various inhibitors on soluble collagen binding, a parameter to assess integrin $\alpha_2\beta_1$ activation, in stimulated platelets. Agonists that can also activate platelet glycoprotein Ib/IIIa are able to activate integrin $\alpha_2\beta_1$, but those operating via glycoprotein Ib cannot. Activation of $\alpha_2\beta_1$ induced by low thrombin or collagen-related peptide concentrations was almost completely inhibited by apyrase, and the inhibitors wortmannin, 4-amino-5-(chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, bisindolylmaleimide I, and SQ29548 significantly inhibited it. Activation induced by high thrombin or collagen-related peptide concentrations was far less sensitive to these inhibitors. However, only wortmannin markedly inhibited ADP-induced integrin $\alpha_2\beta_1$ activation, and this was not ADP concentration-dependent. These results suggest that at the low agonist concentrations, the released ADP would be a primary inducer of integrin $\alpha_2\beta_1$ activation, while at the high agonist concentrations, there would be several pathways through which integrin $\alpha_2\beta_1$ activation can be induced. Kinetic analyses revealed that ADP-induced platelets had about the same number of binding sites ($B_{max}$) as thrombin-induced platelets, but their affinity ($K_d$) for soluble collagen was 3.7-12.7-fold lower, suggesting that activated integrin $\alpha_2\beta_1$ induced by ADP is different from that induced by thrombin. The data are consistent with an activation mechanism involving released ADP and in which there exists two different states of activated integrin $\alpha_2\beta_1$; these activated forms of integrin $\alpha_2\beta_1$ would have different conformations that determine their ligand affinity.

Integrins comprise a family of heterodimeric cell surface proteins that mediate intracellular and cell-to-extracellular interactions. In humans, at least 15 different $\alpha$-subunits and eight different $\beta$-subunits have been identified to date. The various permutations of the $\alpha$- and $\beta$-subunit complexes yield integrin dimers with diverse ligand specificities and biological activities. There is tissue-specific expression of each type of integrin; some integrins are only expressed in a certain tissue, while others are more universal.

Integrin $\alpha_{\text{Ib}}\beta_3$ (platelet glycoprotein (GP)$^1$ Ib/IIIa) is only expressed in platelets and megakaryocytes, but integrin $\alpha_2\beta_1$ (platelet GP Ia/IIa) is known to be present in many cell types (1). The GP IIb/IIIa complex is present as a nonactive heterodimer in resting platelets and becomes activated when platelets are induced by agonists (2–4); activated GP IIb/IIIa possesses high affinity for its ligand, fibronogen. GP IIb/IIIa is one of the most abundant proteins in the platelet membrane, and its binding reaction with fibronogen was shown to be one of the most important reactions in platelet aggregation. On the other hand, although integrin $\alpha_2\beta_1$ was indicated to be a receptor for collagen from studies on a patient’s platelets lacking this protein (5), neither soluble ligand binding to integrin $\alpha_2\beta_1$ nor the activation of the integrin had not been clearly demonstrated until recently. In our previous paper, we showed that upon agonist stimulation of platelets, integrin $\alpha_2\beta_1$ is activated to a form with high affinity for soluble collagen (6). These results suggested that integrin $\alpha_2\beta_1$ might be converted to its activated form through a mechanism similar to that responsible for the activation of GP IIb/IIIa.

The activation mechanism of GP IIb/IIIa has been examined by many investigators, but is yet not fully explained. Recombinant proteins having various mutational changes in the cytoplasmic domains of GP IIb, GP IIIa, or both, with different conformational states of the extracellular portion of the integrin (7–9), showed different abilities to bind fibronogen in response to activation. These results suggested that transformation of the extracellular domain to a conformation with high affinity for fibronogen would be regulated by interactions involving the cytoplasmic domain(s) of GP IIb/IIIa (10, 11). Several proteins were indicated to interact with the cytoplasmic tails of GP IIb/IIIa, including integrin-associated protein (12), $\beta_3$-endonexin (13), CD98 (14), and calcium- and integrin-binding protein (15). However, none of these proteins was indicated to function as a regulator of GP IIb/IIIa activity in platelets. As to the $\beta_1$-integrins, the cytoplasmic domain of the $\alpha_\beta$-chain has been indicated to act as a negative regulator (16), and the NPXY motif of the $\beta$-cytoplasmic domain was indicated to be critical for inside-out signaling (8). Furthermore, several proteins were reported to interact with the cytoplasmic domains of integrin $\alpha_2\beta_1$ and suggested to regulate its function; these are calreticulin (17), integrin-linked kinase 1 (18), and ICAP-1 (19) in addition to cytoskeletal proteins. The contributions of these factors to platelet function remain to be analyzed.

Phosphorylation of the cytoplasmic domain of GP IIb/IIIa was also suggested to control the affinity of the integrin (20), but other studies suggested that the phosphorylation of GP IIb/IIIa is related to the interaction with the cytoskeleton; i.e. outside-in signaling (21, 22). The activated GP IIb/IIIa binds

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1 The abbreviations used are: GP, glycoprotein; BIMI, bisindolylmaleimide; CRP, collagen-related peptide; CP/CPK, creatine phosphate/creatine phosphokinase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PP2, 4-amino-5-chlorophenyl-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; vWF, von Willebrand factor.
with fibrinogen, and this interaction also stimulates platelets (outside-in signaling), which severely complicates the analyses of the activation mechanism of GP IIb/IIIa. However, this is not the case for integrin αβ₁, where collagen is not secreted from platelets after they are activated; thus, this allows us to neglect the effect of outside-in signaling, making it particularly amenable to the analysis of the integrin activation mechanism.

Our previous study demonstrated that platelet integrin αβ₁ is activated to a form with high affinity for soluble collagen after platelets are stimulated by various agonists (6). Although many cells were observed to increase their adhesive activity to the integrin ligands after cell activation with stimuli (23, 24), the activation of integrins, especially of β₁-integrins, was ascribed to avidity changes, since there had not been any clear evidence for the affinity change of β₁-integrins using soluble ligand binding (25). Our demonstration of the activation of integrin αβ₁ associated with avidity change suggested the existence of an activation mechanism that would induce a conformational change in the integrin. A similar activation mechanism was indicated for integrin GP IIb/IIIa (integrin αIIbβ₃) of platelets, and many investigations have been performed to describe this activation mechanism, designated as inside-out signaling (4, 26).

In this paper, we analyzed the effects of various inhibitors and agonists on the activation of integrin αβ₁. The results indicated the following. 1) All of the agonists that induce GP IIb/IIIa-dependent platelet aggregation induced integrin αβ₁ activation. 2) An ADP scavenger, apyrase, almost completely inhibited integrin αβ₁ when platelets were stimulated with a low concentration of an agonist (thrombin or collagen-related peptide (CRP)); and other inhibitors, wortmannin, PP2, bisindolylmaleimide I (BIMI), and SQ29548, inhibited the activation significantly under this condition. 3) When platelets were stimulated with higher concentrations of agonists, these inhibitors had no significant effect, except for the case of wortmannin, which had an inhibitory effect on ADP-induced activation. 4) Integrin αβ₁ activated with ADP and integrin αβ₁ activated by a high concentration of thrombin showed different Kᵣ values but had the same number of binding sites per platelet. These results suggest that released ADP participates in the activation of integrin αβ₁ and suggest the presence of two different states of activated integrin αβ₁ that have different conformations.

**EXPERIMENTAL PROCEDURES**

**Preparation of Soluble Collagen**—Bovine type III collagen (Koken Co., Ltd., Tokyo, Japan) was labeled with Na¹²⁵I by using IODO-BEADS (Pierce) as described in our previous report (6) and stored at 4 °C until use. To ensure that the preparation used for the binding study was composed of only soluble collagen, prior to each study, the sample-containing tubes were frozen at −80 °C, and then the tips of the tubes, containing the pellet of platelets/platelet-bound¹²⁵I-labeled soluble collagen, were cut off with scissors. The radioactivities of the pellets were determined in a γ-counter.

**Assessment of ³²P Incorporation into Platelet Proteins**—Platelet-rich plasma was incubated with 10 μM indomethacin for 20 min and then added with prostaglandin I₂ (0.1 μM) buffer (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH₂PO₄, 12 mM NaHCO₃, 5.5 mM glucose, pH 6.5). The washed platelets were finally suspended in buffer A (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH₂PO₄, 12 mM NaHCO₃, 5.5 mM glucose, and 5 mM HEPES, pH 7.4) containing 2% bovine serum albumin (Sigma) to a concentration appropriate for each study.

**Determination of Soluble Collagen Concentration**—The concentration of the¹²⁵I-labeled collagen was determined by the Non-Interfering Protein Assay™ (Geno Technology, Inc., St. Louis, MO), with unlabeled collagen as the standard protein. The molar concentration of soluble collagen was calculated with 3 × 10⁵ as the molecular weight of collagen.

**Binding of Soluble Collagen**—Detailed descriptions of the binding procedures and establishment of conditions have been given in the previous report (6); the following is a brief description of the binding method used in the present studies, with more detailed descriptions of the conditions provided in the figure legends. Each total binding mixture (final volume of 50–80 μl, pH 7.4) contained activating agent or no activating agent (control, “resting” platelets), 2 mM MgCl₂,¹²⁵I-labeled soluble collagen (prepared as described above), agent to be tested (if used), bovine serum albumin, and sufficient buffer A to adjust the mixture to the final volume. Nonspecific binding was determined in identical mixtures that contained, in addition, 5 mM EDTA. Binding was initiated by adding the washed platelets (final concentration of 4 × 10⁶ cells/ml), either untreated or preincubated with an agent for 10 min, and very briefly mixed. The binding reaction was then allowed to proceed for 80 min at room temperature, without any further agitation. At the end of the binding time, each mixture was layered over a 250-μl pad of 20% sucrose, 0.2% bovine serum albumin, buffer A in a narrow tipped, 0.4-ml microcentrifuge tube (Assist, Tokyo, Japan) and centrifuged at 10,000 × g in a microcentrifuge (model MX-150, TIMH-2 rotor; Tomy, Tokyo, Japan) for 5 min at 10 °C. The sample-containing tubes were frozen at −80 °C, and then the tips of the tubes, containing the pellet of platelet-bound¹²⁵I-labeled soluble collagen, were cut off with scissors. The radioactivities of the pellets were determined in a γ-counter.

**Assessment of ³²P Incorporation into Platelet Proteins**—Platelet-rich plasma was incubated with 10 μM indomethacin for 30 min and then added with prostaglandin I₂ (0.1 μM) followed by centrifugation at 1000 × g for 10 min. The sedimented platelets were suspended with 1–2 ml of 6.85 mM citrate, 130 mM NaCl, 4 mM KCl, 5.5 mM glucose buffer, pH 6.5; and then the suspension was applied to a column of Sepharose 4B. The column was preequilibrated with buffer P (145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM glucose, 25 mM HEPES, and 0.5 mM EGTA, pH 7.4), and the gel-filtered platelets were obtained in the pass-through fraction. The platelet suspension was incubated with about 25 MBq of ³²P-phosphate (Amersham Pharmacia Biotech)/ml for 1 h at room temperature. ³²P-labeled platelets were washed twice with buffer P, and the platelet count was adjusted to 5 × 10⁶/ml with buffer P. To this platelet suspension, MgCl₂ and CaCl₂ were each added to a final concentration of 1 mM. ³²P-Platelets were preincubated for 2–3 min in the absence (control “resting” platelets) or presence of the following inhibitors: piceatannol (final concentration of 0.1 mM), staurosporine (1 μM), wortmannin (0.1 μM), cytochalasin D (0.1 μM), BIMI (10 μM). Then the preincubated platelets were incubated with one of the following agonists: thrombin (final concentration, 0.1 units/ml), SR142333 (0.1 μM), U73122 (5 μM), U46619 (1 μM), NCI 866 (0.05 μM), cytochalasin D (0.1 μM), and BIMI (10 μM). After 2 min, the reaction was stopped by the addition of an equal volume of 2× Laemmli buffer (0.125% Trit, 4% SDS, 20% glycerol, 0.6 μg/ml bromphenol blue solution, pH 6.8); the mixture was heated for 2–3 min at 100 °C. A 20-μl aliquot of each sample was analyzed by SDS-gel electrophoresis after the reduction with 5% mercaptoethanol, and the bands of phosphorylated proteins were visualized with a BAS 2000 Bio-Imaging Analyzer (Fuji Film Co., Tokyo, Japan).

**Assessment of Tyrosine Phosphorylation**—Gel-filtered platelets were prepared as described above, except buffer A was used instead of buffer P. The final platelet counts were adjusted to 5 × 10⁶/ml, and MgCl₂ and CaCl₂ were each added to the final concentration of 1 mM. After platelets were incubated with various concentrations of PP2 or Me₂SO (as a control) for 2–5 min, thrombin (final concentrations, 0.05 and 0.2 units/ml) or CRP (0.05 and 0.4 μg/ml) was added, and incubation was carried out for 2 min at room temperature. At the end of the incubation, each sample was added with an equal volume of 2× Laemmli buffer containing 5% mercaptoethanol and 2 mM Na₂VO₃, and then heated for 2–3 min at 100 °C. Samples were subjected to SDS-gel electrophoresis and electroblotted to a nitrocellulose membrane, and the tyrosine-phosphorylated bands were visualized by using anti-phosphotyrosine antibody, RC20:HRPO, and ECL Western blotting detection reagent (Amersham Pharmacia Biotech).

**Data Analyses**—In the soluble binding experiments, specific binding was determined by subtracting the nonspecific binding determined in the presence of 5 mM EDTA from the total binding. In the kinetics studies, nonlinear regression analyses by the software Prism (Version 3.0; GraphPad Software, Inc., La Jolla, CA) was used to determine the binding parameters; other statistical analyses were also performed with the same program.
Signal Transduction Mechanisms in Integrin \( \alpha_2\beta_1 \) Activation

Fig. 1. Comparison of soluble collagen binding in platelets activated by various agonists. Platelets were induced by various agonists, and the activation of integrin \( \alpha_2\beta_1 \) was monitored by soluble collagen binding, as described under “Experimental Procedures.” Specific binding (mean ± S.E., six replicate binding mixtures) is reported as the percentage relative to the specific binding induced in thrombin (0.1 units/ml)-activated platelets. Each set of bars shows the specific binding induced by one or more concentrations of a particular agonist (in which case concentrations are given in parentheses from left to right); n refers to the number of different platelet samples for which the binding was determined; none, no added agonist (n = 4); thrombin (Throm) (0.1 unit/ml; determined for each platelet sample); ADP (2 \( \mu \)M, n = 1; 5 \( \mu \)M, n = 3; 10 \( \mu \)M, n = 5; 20 \( \mu \)M, n = 1); CRP (0.2 \( \mu \)g/ml, n = 5; 0.5 \( \mu \)g/ml, n = 4; 3.5 \( \mu \)g/ml, n = 1); TS2/16 (5 \( \mu \)g/ml, n = 4); PMA (0.2 \( \mu \)M, n = 1; 2 \( \mu \)M, n = 4; 10 \( \mu \)M, n = 1); U46619 (1 \( \mu \)M, n = 4; 5 \( \mu \)M, n = 1); A23187 (1 \( \mu \)M, n = 1; 5 \( \mu \)M, n = 1; 10 \( \mu \)M, n = 1, 25 \( \mu \)M, n = 2); vWf/bot (1 mg/ml ristocetin plus 0.5 \( \mu \)g/ml vWf, n = 2; 1 mg/ml ristocetin plus 2.5 \( \mu \)g/ml vWf, n = 2; vWf/bot (2.0 \( \mu \)g/ml botrocetin plus 10 \( \mu \)g/ml vWf, n = 2); Albo (1.35 \( \mu \)g/ml alboagregin, n = 2).

RESULTS

Induction of Soluble Collagen Binding by Various Activators—Fig. 1 shows that integrin \( \alpha_2\beta_1 \) activation, as monitored by soluble collagen binding, is induced by most agonists that do not exert their action through interaction with GP Ib: thrombin, ADP, CRP, TS2/16 (integrin \( \alpha_2\beta_1 \)-activating antibody), U46619 (thromboxane \( \Delta_2 \) mimetic), and A23187 (Ca²⁺ ionophore). In contrast, vWf in the presence of ristocetin or botrocetin or the agonist alboagregin B (Fig. 1), which activate platelets through interaction with GP Ib, did not induce any binding. In this figure, it is notable that the binding induced by ADP is invariably only 50–60% that induced by thrombin at a given concentration of soluble collagen. The bindings induced by CRP, PMA, U46619, and A23187 show some variability with each platelet preparation; and in many preparations, the bindings approached the level induced by 0.1 units/ml thrombin at the optimal agonist concentrations. There is decreased binding at the highest concentration of CRP, since this peptide can compete with soluble collagen for the binding to activated integrin \( \alpha_2\beta_1 \), as shown previously (6). The binding induced by TS2/16 was equal to or greater than that induced by 0.1 unit/ml thrombin.

Concentration Dependence of the Effects of Inhibitors on Integrin \( \alpha_2\beta_1 \) Activation—To establish the concentration range at which three types of inhibitors might exert their effects on integrin \( \alpha_2\beta_1 \) activation, the concentration dependence curves were first determined under our standard agonist conditions: 0.1 unit/ml thrombin, 0.2 \( \mu \)g/ml CRP, or 10 \( \mu \)M ADP, concentrations routinely used by other investigators. As shown in Fig. 2A, the phosphatidylinositol 3-kinase inhibitor wortmannin inhibited both thrombin- and CRP-induced binding to approximately 50% and was much more inhibitory toward the ADP-induced binding, which was diminished to about 10%. Neither the PKC inhibitor BIM (Fig. 2B) nor the protein-tyrosine kinase inhibitor PP2 (Fig. 2C) had significant effects under these conditions. Whether this pattern of inhibition was similar at all agonist concentrations was determined in detailed experiments that will be described below.

Effect of ADP-Trapping Agents on Integrin \( \alpha_2\beta_1 \) Activation—Conditions were established to trap essentially all of the ADP that may be secreted by agonist-induced platelets, because activation of integrin \( \alpha_2\beta_1 \) may occur indirectly through stimulation of pathways, leading to secretion of ADP or thromboxane \( \Delta_2 \). Two trapping systems were tested: apyrase and the creatine phosphate/creatine phosphokinase system (CP/CPK). The maximum inhibition of binding was obtained with 3–5 units/ml apyrase, which decreased the binding to about 30–40% relative to the control without apyrase (Fig. 3A). Although the CP/CPK system gives similar results for the CRP-induced platelets, there is a precipitous drop in the thrombin-induced binding at CPK concentrations higher than 25 units/ml (Fig. 3B); this may be due to some nonspecific effects on the platelets and/or the binding system, indicating that CP/CPK is unsuitable as an ADP trap for the present experiments. Thus, 5 units/ml apyrase was used as the ADP-trapping system in the following experiments.
Inhibitor Effects on Integrin α₂β₁ Activation Induced by Various Thrombin Concentrations—Four types of inhibitors (wortmannin (phosphatidylinositol 3-kinase inhibitor), BIMI (PKC inhibitor), PP2 (protein-tyrosine kinase inhibitor), and SQ29548 (thromboxane A₂ antagonist)) were tested for their ability to inhibit integrin α₂β₁ activation induced by various concentrations of thrombin in the presence of ADP. Wortmannin and PP2 strongly inhibited soluble collagen binding induced by low thrombin concentrations (<0.05 unit/ml), whereas they only decreased binding about 20–30% at the higher thrombin concentrations (>0.1 unit/ml). SQ29548 produced only mild inhibition at both low and high thrombin concentrations, but it was still more inhibitory at low thrombin, like the other inhibitors.

Apyrase almost completely inhibited the activation at low thrombin and inhibited it about 50–60% at high thrombin; thus, released ADP is a primary contributor to the activation of integrin α₂β₁. The simultaneous presence of apyrase and another inhibitor (wortmannin, BIMI, or PP2) resulted in more inhibition than that with apyrase alone; sometimes this inhibition approached 100%.

These results indicate that integrin α₂β₁ is activated differently by low and high concentrations of thrombin. Integrin α₂β₁ is activated mainly by secreted ADP when platelets are activated with a low concentration of thrombin. On the other hand, integrin α₂β₁ would be activated through several pathways by high concentrations of thrombin.

Inhibitor Effects on Integrin α₂β₁ Activation Induced by Various CRP Concentrations—As with the thrombin-induced integrin α₂β₁ activation described above, the inhibition patterns were dependent on the CRP concentration used to activate the platelets. BIMI was highly inhibitory at low CRP (<110 ng/ml) and produced 0–20% inhibition at higher CRP (Fig. 5, upper graphs). Wortmannin was quite inhibitory at all CRP concentrations, although there was less inhibition as the CRP concentrations increased.
**FIG. 4.** Inhibitor effects on soluble collagen binding of platelets induced by various concentrations of thrombin. The effects of four types of inhibitors, wortmannin, BIMI, PP2, and SQ29548, in the absence or presence of apyrase, on the soluble collagen binding to platelets induced by various thrombin concentrations was determined. A, wortmannin and BIMI; B, PP2 and SQ29548. Platelets were preincubated for 15 min with an inhibitor or buffer (no inhibitor) before being added to the binding mixtures containing various thrombin concentrations, which contained no apyrase or 5 units/ml apyrase. A1 and A2, no inhibitor (■—■), 100 nm wortmannin (•—•), 100 nm wortmannin plus 5 units/ml apyrase (○—○), 10 μM BIMI (□—□), 10 μM BIMI plus 5 units/ml apyrase (●—●), B1 and B2, no inhibitor (■—■), no inhibitor plus 5 units/ml apyrase (□—□), 10 μM SQ29548 (▲—▲), 10 μM SQ29548 plus 5 units/ml apyrase (●—●), 25 μM PP2 (◆—◆), 25 μM PP2 plus 5 units/ml apyrase (◊—◊). A1 and B1 show the specific binding in terms of the actual radioactivity (cpm) of 125I-labeled soluble collagen, and graphs A2 and B2 show the specific binding as a percentage of the binding in the absence of any added inhibitor and apyrase.

As with the thrombin-induced platelets, apyrase severely reduced the soluble collagen binding, even at the high concentrations of CRP. The combination of apyrase and wortmannin, BIMI, or PP2 resulted in nearly total inhibition of binding. If SQ29548 is combined with apyrase, the inhibition is similar to that by apyrase alone, further indicating the low effect of this compound.

These results also suggested that activation induced by low CRP concentrations is mediated mainly by secreted ADP and that induced by a high CRP concentrations involve different activation mechanisms.

**Inhibitor Effects on Integrin αβ̂ Activation Induced by Various ADP Concentrations**—In contrast to the inhibition patterns of thrombin- and CRP-induced platelets, the ADP-activated platelets showed characteristically different patterns in which the inhibition by wortmannin, BIMI, and PP2 were not ADP concentration-dependent (Fig. 6). Only wortmannin strongly inhibits ADP-induced activation, decreasing binding to 20–25% throughout the entire range of ADP concentrations (Fig. 6, upper graphs). PP2 produced moderate inhibition of about 30% (Fig. 6, lower graphs). BIMI and SQ29548 were not inhibitory at any ADP concentration (Fig. 6, upper and lower graphs, respectively). As would be expected, apyrase completely inhibited the activation at all tested ADP concentrations; these results indicate that the 5 units/ml of apyrase employed as the ADP trap was sufficient to completely inhibit the effect induced by as much as 50 μM ADP.

**Effects of Combinations of Inhibitors**—We determined the effects of various combinations of the inhibitors wortmannin, BIMI, and PP2 on the soluble collagen binding induced by high (by our definition) concentrations of thrombin (0.1 units/ml), CRP (0.2 μg/ml), and ADP (10 μM) (Fig. 7). For thrombin-induced binding, the combination of BIMI and wortmannin produced inhibition greater than additive inhibition (apparently synergistic effect), whereas combinations including PP2 with BIMI or wortmannin produced no further inhibition. For the CRP-induced binding, the BIMI-wortmannin combination also produced greater than additive inhibition, which was much greater than that observed in thrombin-induced platelets. Combinations of BIMI or wortmannin with PP2 caused marked, synergistic inhibition, in contrast to the thrombin-activated platelets; and the combination of all three inhibitors produced complete inhibition of the CRP-induced binding. For ADP (10 μM)-induced platelets, only wortmannin was inhibitory, and its combination with either or both of the other two inhibitors produced no further inhibition; this is consistent with the pattern observed in Fig. 6.

These results indicate that there is more than one activation pathway involved when platelets are activated by a high concentration of thrombin or CRP.

**Effects of Inhibitors on 32P Incorporation into Platelet Proteins**—The results on 32P incorporation into agonist-activated platelets and the effects of inhibitors on this incorporation are shown in Fig. 8. As seen by the autoradiographic patterns, both thrombin and PMA induced strong phosphorylation of P47 pleckstrin and myosin light chain, indicating the activation of PKC. Staurosporine and BIMI almost completely inhibited pleckstrin phosphorylation in platelets activated by these ago-
FIG. 5. Inhibitor effects on soluble collagen binding of platelets induced by various concentrations of CRP. The effects of wortmannin, BIMI, PP2, and SQ29548, in the absence or presence of apyrase, on the soluble collagen binding to platelets induced by various CRP concentrations was determined. A, wortmannin and BIMI; B, PP2 and SQ29548. Platelets were preincubated for 15 min with an inhibitor or buffer (no inhibitor) before being added to the binding mixtures containing various concentrations of CRP, in the absence or presence of apyrase. A1 and A5, no inhibitor (—), no inhibitor plus 5 units/µl apyrase (——); 100 nm wortmannin (●—●), 100 nm wortmannin plus 5 units/ml apyrase (❖—❖), 10 µM BIMI (○—○), 10 µM BIMI plus 5 units/ml apyrase (□—□), B1 and B2, no inhibitor (—), no inhibitor plus 5 units/µl apyrase (——), 10 µM SQ29548 (▲—▲), 10 µM SQ29548 plus 5 units/ml apyrase (△—△), 25 µM PP2 (▼—▼), 25 µM PP2 plus 5 units/ml apyrase (▼—▼). A1 and B1 show the specific binding in terms of the actual radioactivity (cpm) of 125I-labeled soluble collagen, and A2 and B2 show the specific binding as a percentage of the binding in the absence of any added inhibitor and apyrase.

Effects of Other Inhibitors on Thrombin-, CRP-, and ADP-induced Integrin α5β1 Activation—Table I summarizes the effects of other inhibitors that we tested on platelets induced by low and high concentrations of thrombin and CRP and 10 µM ADP, which was examined at one concentration because the effects of the inhibitors did not depend on the ADP concentration (Fig. 6). Stauroporine (general inhibitor of protein kinases) inhibited soluble collagen binding induced by both low and high concentrations of thrombin or CRP; the inhibition was greater against the low agonist-induced binding; it was also inhibited against ADP-induced binding. Cherlorythrine chloride (protein kinase C inhibitor) had no effect on the soluble collagen binding induced by any of the agonists. Cytochalasin D inhibited the bindings induced by both low and high concentrations of thrombin or CRP, with greater effect on the high agonist-induced binding; it inhibited the ADP (10 µM) binding by almost 60%. U73122 (phospholipase C inhibitor) inhibited the low CRP-induced binding by about 40% but had little effect on the bindings induced by high CRP, thrombin at either concentration, and 10 µM ADP. Calyculin almost completely inhibited the soluble collagen binding induced by any of the agonist at any concentration; this suggested the strong involvement of dephosphorylation in regulating the integrin activation that occurs through many platelet pathways.

Concentration-dependent Inhibition of Protein Tyrosine Phosphorylation by PP2—Platelets were first preincubated with different concentrations of PP2 and then induced by thrombin or CRP; the tyrosine phosphorylation was analyzed by immunoblotting after SDS-polyacrylamide gel electrophoresis (Fig. 9). Under both high and low concentrations of thrombin and CRP (low concentrations shown in gels on the left; high concentrations in gels on the right), PP2 at concentrations of 10 µM and higher strongly inhibited the tyrosine phosphorylation of proteins that migrated at the same positions as Syk and c-Src, as indicated in the figure. These data demonstrate that PP2 actually inhibits tyrosine phosphorylation, even in the presence of high agonist concentrations, although it had little effect on integrin α5β1 activation induced by the same concentrations.

Comparison of Thrombin- and ADP-induced Activation of Integrin α5β1 through Kinetic Analyses—Because of the interesting phenomenon that ADP-induced platelets always showed about half the amount of soluble collagen binding as thrombin-induced ones, kinetic analyses were performed to quantitatively define the binding characteristics of each type of activated binding. Representative graphs of the data obtained for thrombin (0.1 units/ml) and ADP (10 µM)-induced soluble collagen binding (data of experiment 7 in Table II), obtained with the same platelets on the same day, are shown in Fig. 10; these graphs exhibit a very obvious difference in slope. Table II...
Fig. 6. Inhibitor effects on soluble collagen binding of platelets induced by various concentrations of ADP. The effects of wortmannin, BIMI, PP2, and SQ29548, in the absence of presence of apyrase, on the soluble collagen binding to platelets induced by various ADP concentrations was determined. A, wortmannin and BIMI; B, PP2 and SQ29548. Platelets were preincubated for 15 min with an inhibitor or buffer (no inhibitor) before being added to the binding mixtures containing various concentrations of ADP in the absence or presence of apyrase. A1 and A2, no inhibitor (■— ■), 100 nM wortmannin (● — ●), 100 nM wortmannin plus 5 units/ml apyrase (○ — ○), 10 μM BIMI (□ — □), 10 μM BIMI plus 5 units/ml apyrase (△ — △), B1 and B2, no inhibitor (■— ■), no inhibitor plus 5 units/ml apyrase (——), 10 μM SQ29548 (▲ — ▲), 10 μM SQ29548 plus 5 units/ml apyrase (○ — ○). B1 and B2 show the specific binding in terms of the actual radioactivity (cpm) of 125I-labeled soluble collagen, and A2 and B2 show the specific binding as a percentage of the binding in the absence of any added inhibitor and apyrase.

shows the binding parameters calculated from nonlinear regression analyses of thrombin- and ADP-induced binding obtained in seven different experiments. In all cases, both thrombin-induced and ADP-induced platelets show a very similar number of binding sites, Bmax, which ranged from about 600 to 1600 sites/platelet, depending on the platelet donors. In marked contrast, the dissociation constant, Ka, for ADP-induced binding is always larger than that for thrombin-induced platelets in all of the experiments, although the ratio Ka ADP/Ka (thrombin) may range in value from 3.66 to 12.74. These data indicate that the activated integrin αIIbβ3 induced by thrombin is a state with higher binding affinity than that induced by ADP.

**DISCUSSION**

*Induction of Soluble Collagen Binding by Various Activators*—In this study, we measured soluble collagen binding to platelets as an indicator of the activation of integrin αIIbβ3, since resting platelets show very little if any soluble collagen binding (6). As indicated in Fig. 1, many platelet agonists, ADP, thrombin, CRP, FMA, U46619, and A23187, induced integrin αIIbβ3 activation, although the agonists for GP Ib-dependent aggregation, ristocetin, botrocetin, and alboaggregin B, could not. These results suggest that the activation of integrin αIIbβ3 is induced during the process of platelet activation. The inhibition of the activation by prostaglandin I2 also supports this hypothesis (6). These properties of the activation of integrin αIIbβ3 are similar to those of GP IIb/IIIa activation (4, 28). The extents of collagen binding induced by other stimulators were similar to the one induced by thrombin, with the notable exception of the activation induced by ADP. The collagen binding to ADP-activated platelets was about half that induced by thrombin in most platelet preparations and invariably less.

**Effects of ADP Scavengers on Integrin αIIbβ3 Activation**—Because integrin αIIbβ3 activation may involve or be a consequence of ADP released from activated platelets, we analyzed the effects ADP scavengers on the induction of soluble collagen binding. Two scavenging systems were evaluated: apyrase and the CP/CPK system, both reagents commonly used for inhibiting the effect of ADP. Both apyrase and CP/CPK inhibited the soluble collagen binding induced by thrombin or CRP to 30–40% of the control (no scavenger) level. The residual binding would be due to activation induced by one or more pathways not related to ADP; these other pathways would be operative at the high agonist concentrations, since the collagen binding was almost completely inhibited by apyrase when platelets were activated with low concentrations of agonists, and the addition of other inhibitors to an apyrase-containing mixture produced almost complete inhibition of collagen binding even at high agonist concentrations (Figs. 4 and 5). However, there still exists the possibility that the added apyrase or CP/CPK could not completely inhibit the effect of released ADP near the cell surface (29). These results indicate that the secreted ADP is a major participant in the induction of integrin αIIbβ3 activation, especially at the low concentrations of agonists.

Similar effects of apyrase or CP/CPK on fibrinogen binding to platelets activated by a variety of stimuli were also reported (30, 31), and the contribution of secreted ADP in GPIIb/IIIa activation was also suggested (4, 32). These previous reports also suggested to us that there might be a common activation mechanism for GP IIb/IIIa and integrin αIIbβ3.

**Effects of Wortmannin and SQ29548 on Integrin αIIbβ3 Activation**—While performing preliminary experiments to check the effects of different concentrations of inhibitors on the induction of collagen binding by thrombin, CRP, and ADP, we
noticed that the effects of the inhibitors were variable with the concentrations of agonists. The results of Figs. 4–6 clearly show that effects of inhibitors are agonist concentration-dependent except for the case of ADP. Toward the binding induced by low concentrations of thrombin or CRP, wortmannin, BIMI, and PP2 were strongly inhibitory, sometimes almost completely abrogating the soluble collagen binding. On the other hand, the inhibition by these three agonists showed no agonist concentration dependence when platelets were activated with ADP (Fig. 6).

The thromboxane \( \alpha_2 \) antagonist SQ29548 only weakly affected the integrin \( \alpha_2 \beta_1 \) activation induced by any of the agonists. It only inhibited about 20% of the soluble collagen binding induced by the low concentrations of thrombin or CRP. Previous reports have indicated that the inhibitors of thromboxane synthesis, indomethacin and aspirin, partially inhibited fibrinogen binding to platelets activated with a low concentration of ADP or epinephrine (33). These results suggest that the contribution of released thromboxane \( \alpha_2 \), which is synthesized in the process of platelet activation, would be small.

Wortmannin is an inhibitor of PI 3-kinase. Gao and Shattil reported that wortmannin inhibited PAC-1 binding to thrombin-activated platelets. Their data also showed that wortmannin had no significant inhibitory effects on PAC-1 binding to platelets activated by a high concentration of thrombin (34). Our data on soluble collagen binding are similar to those of their experiments on PAC-1 binding. Wortmannin strongly inhibited the collagen binding induced by a low concentration of thrombin or CRP, but its effects were weaker against platelets activated by high concentrations of the agonists (Figs. 4 and 5). Wortmannin markedly inhibited the soluble collagen binding to ADP-activated platelets (Figs. 2 and 6). This would explain the rather strong inhibitory effect of wortmannin even at the high concentrations of agonists. These results suggested that PI 3-kinase is involved in the activation of integrin \( \alpha_2 \beta_1 \) induced by ADP. From experiments using wortmannin, Zhang et al. (35) concluded that PI 3-kinase contributes to the activation of GP IIb/IIIa. It is interesting that PI 3-kinase was suggested to activate the inside-out signaling of \( \beta_3 \) integrins, at least partially, through cytohesin-1 in Jurkat cells (36).

Effect of Protein Kinase Inhibitors on Integrin \( \alpha_2 \beta_1 \) Activation and Phosphorylation—BIMI is a protein kinase C inhibitor (37). The effect of BIMI on the integrin activation showed an agonist concentration dependence similar to that of wortmannin plus ADP. However, it did not affect the integrin \( \alpha_2 \beta_1 \) activation induced by ADP (Fig. 6). Hers et al. reported that BIMI inhibited fibrinogen binding (GP IIb/IIIa activation) to thrombin-activated platelets but did not inhibit the binding to ADP-activated platelets (38). Their results and ours showed...
In addition to inhibitors on which detailed analyses were performed (Figs. 2–7), the effects of other inhibitors: staurosporine (1 μM), chelerythrine chloride (10 μM), cytochalasin D (10 μM), U73122 (5 μM), and calyculin (0.1 μM) on soluble collagen binding to platelets induced by low and high concentrations of thrombin and CRP and 10 μM ADP were determined. The experiments were performed with the same platelets on the same day. Washed platelets were preincubated with an inhibitor for 10 min before they were used in the binding assay, where thrombin, CRP, or ADP was used as the activator. The specific binding is reported as the percentage relative to the amount of binding induced by a given concentration of agonist in platelets not pretreated with any inhibitor.

| Inhibitor          | Type of inhibitor          | Specific soluble collagen binding |
|--------------------|---------------------------|----------------------------------|
| No inhibitor       |                           | %                               |
| Staurosporine      | Protein kinase (general)   | 100.0%                          |
| Chelerythrine chloride | Protein kinase C<sub>5</sub> | 98.9 ± 2.9                      |
| Cytochalasin D     | Cytoskeleton              | 126.2 ± 4.9                     |
| U73122             | Phospholipase C           | 89.4 ± 7.7                      |
| Calyculin          | Phosphatasease            | 109.1 ± 3.6                     |

A 46619 induced the phosphorylation of pleckstrin and myosin light chain. Other agonists, TS2/16 (10 μg/ml), ADP (10 μM), and collagen (5 μg/ml), did not induce 32P incorporation into pleckstrin (data not shown). The high level of 32P incorporation into pleckstrin by a high concentration of thrombin was completely inhibited by the specific protein kinase inhibitor BIMI and staurosporine, a protein kinase inhibitor with broad specificity. Both inhibitors abolished the phosphorylation induced by the other agonists. These results indicate that BIMI actually completely inhibited the activity of protein kinase C although the integrin activation was only partially inhibited under the same conditions; this suggests the existence of other pathway(s) for integrin αβ<sub>1</sub> activation.

**Effects of Combinations of Inhibitors on Integrin αβ<sub>1</sub> Activation**—To confirm the presence of multiple activation pathways, we analyzed the effects of the combination of inhibitors on the integrin αβ<sub>1</sub> activation (Fig. 7). For platelets activated by a high concentration of thrombin, only wortmannin showed a significant inhibitory effect. The addition of BIMI, which did not have a significant inhibitory effect by itself, together with wortmannin produced greater inhibition than the same concentration of wortmannin alone. Similarly, for platelets activated with a high concentration of CRP, the addition of BIMI or PP2 in combination with wortmannin produced greater inhibition than that observed with wortmannin alone. These synergistic effects of the combination of inhibitors suggested the presence of several independent pathways for the activation of integrin αβ<sub>1</sub>. Furthermore, these results suggested the greater involvement of tyrosine phosphorylation in the activation induced by CRP than that induced by thrombin. In contrast, the integrin activation induced by ADP did not show any synergistic effects by any combination of inhibitors.

**The Effect of Tyrosine Kinase Inhibitors on Integrin αβ<sub>1</sub> Activation and Tyrosine Phosphorylation**—PP2 is an inhibitor specific for tyrosine kinases of the Src family (40). It inhibited the activation of integrin αβ<sub>1</sub>, induced by thrombin and CRP in an agonist concentration-dependent manner but did not inhibit integrin αβ<sub>1</sub> activation induced by ADP. Tyrosine phosphorylation induced by low and high concentrations of agonists was inhibited with similar concentration dependences (Fig. 9). PP2 completely prevented tyrosine phosphorylation when platelets were activated with thrombin or CRP, but at the same concentration it only partially inhibits integrin αβ<sub>1</sub> activation; the nonconsmisurate effect on integrin αβ<sub>1</sub> activation was especially noticeable in platelets activated with a high concentration of agonists, where the integrin activation was only very weakly inhibited. Ezumi et al. (41) mentioned that PP1, a tyrosine kinase inhibitor with a specificity similar to that of PP2, inhibited tyrosine phosphorylation, platelet aggregation,
Soluble collagen binding in platelets induced by thrombin (0.1 unit/ml) or ADP (10 μM) was determined in seven different experiments, each performed with different platelets on a different day. The thrombin and ADP binding assays for each experiment were performed at the same time, with six replicate assays performed at each soluble collagen concentration (12–14 in total) used to determine the binding curve. Kinetic parameters were determined by nonlinear regression analysis of the specific binding data; in all the experiments, both the thrombin and ADP specific binding data showed best fit to a single binding site model.

| Experiment | \( B_{\text{max(ADP)}} \) | \( K_{d(ADP)} \) | \( B_{\text{max(ADP)}}/B_{\text{max(thrombin)}} \) | \( K_{d(ADP)}/K_{d(thrombin)} \) |
|------------|-----------------|------------------|-----------------|------------------|
| 1          | 1193 ± 39       | 12.25 ± 1.14     | 1185 ± 54       | 52.35 ± 4.14     | 0.99             | 4.27             |
| 2          | 594 ± 17        | 5.57 ± 0.77      | 601 ± 52        | 71.01 ± 12.51    | 1.01             | 12.74            |
| 3          | 1255 ± 77       | 9.18 ± 2.51      | 1024 ± 99       | 49.11 ± 12.05    | 0.81             | 7.01             |
| 4          | 1462 ± 43       | 13.32 ± 1.30     | 1620 ± 87       | 48.74 ± 5.76     | 1.11             | 3.66             |
| 5          | 1204 ± 49       | 10.85 ± 1.37     | 1594 ± 109      | 86.19 ± 11.20    | 1.32             | 7.94             |
| 6          | 1393 ± 36       | 10.99 ± 1.05     | 1453 ± 106      | 57.40 ± 9.16     | 1.04             | 5.22             |
| 7          | 813 ± 24        | 7.43 ± 0.82      | 865 ± 51        | 53.24 ± 2.68     | 1.06             | 7.16             |

Fig. 10. Analysis of the kinetics of soluble collagen binding in platelets induced by thrombin and ADP. These agonist-induced bindings show characteristically different affinities. The soluble collagen concentration-dependent binding of soluble collagen to thrombin (0.1 unit/ml)-induced platelets was compared with that of ADP (10 μM)-induced platelets in the same platelet sample on the same day. Specific binding data obtained at various soluble collagen concentrations were analyzed by nonlinear regression analysis to determine the best fit curves and binding parameters. Shown are the binding curves for experiment 7 in Table II. The number of binding sites (\( B_{\text{max}} \)) for thrombin-induced platelets is similar to that of ADP-induced platelets, but the binding affinity (\( K_d \)) of thrombin-activated platelets is over 7-fold higher than that of ADP-activated platelets.

The presence of two different conformational states of activated integrin \( \alpha_{\beta_1} \).—In most of the binding experiments, the extent of soluble collagen binding to ADP-activated platelets was approximately half of that to thrombin-activated platelets. The kinetic analysis showed that ADP-activated platelets have the same number of binding sites per platelet as thrombin-activated platelets, but their affinity for soluble collagen is 3.66–12.74-fold less compared with that of platelets activated by thrombin. Although the difference of \( K_d \) was somewhat variable, the \( K_d \) of ADP-activated platelets was always larger than that of thrombin-activated platelets, and the number of the binding sites was almost the same in both types of activated platelets. Consistent with the recently described polymorphism in the level of integrin \( \alpha_{\beta_1} \) expression in different individuals, the number of binding sites shows a range of values (42, 43).

Each experiment shown in Table II corresponds to one set of assays performed with platelets obtained from the same source, with both the thrombin- and ADP-induced soluble collagen binding assays being performed on the same day. Thus, these results strongly indicate the existence of two different states of activated integrin \( \alpha_{\beta_1} \). In these experiments, we used 0.1 units/ml of thrombin to activate the platelets, a concentration falling in the high range as defined in the present study. Therefore, this would indicate that the integrin \( \alpha_{\beta_1} \) in platelets activated with a high concentration of thrombin has a higher affinity for soluble collagen than the one in ADP-activated platelets. We do not know what the affinity of platelets activated with a low concentration of thrombin would be. However, it would be experimentally difficult to answer this question, since the low thrombin concentration range is a narrow one, and this is further complicated by the fact that the sensitivity for thrombin is different among different individuals; therefore, one might expect that the binding induced by low thrombin would be a mixture of lower and higher affinity binding.

These results enable us to propose a mechanism for the activation of integrin \( \alpha_{\beta_1} \) (Fig. 11). In this model, ADP, released or externally added, is a primary stimulant for inducing the activation of resting integrin \( \alpha_{\beta_1} \), \( R_0 \), to its activated form, \( R_1 \). When platelets are activated with low concentrations of agonists, the released ADP would be a major factor in the induction of this activation. The release reaction would be induced with a minimum level of stimulation, so the release reaction and the following integrin activation would be inhibited effectively by various types of inhibitors such as wortmannin, BIMI, PP2, and SQ29548. The agonists would induce the production of various signals, and in turn these signals would induce conversion of the activated integrin form \( R_1 \) to another activated form, \( R_2 \), which has a higher affinity for soluble collagen than \( R_1 \). This transition would easily occur when platelets are stimulated with high concentrations of agonists other than ADP. This activation would be mediated through several pathways; thus, the inhibition of one of the pathways would not greatly affect the final activation. Protein kinase C, PI 3-kinase, and tyrosine phosphorylation were suggested to be involved in this activation from analyzing the effects of the inhibitors of these reactions.
Signal Transduction Mechanisms in Integrin αβ₁ Activation

This is the first report indicating the presence of two different forms of activated integrin that each have different affinities. Even among the numerous studies on GP IIb/IIIa, there has not been a precise analysis of the characteristics of the activated forms of this integrin, although there is some data showing that ADP-induced platelets bind less PAC-1 than thrombin-activated platelets (34, 35). Our present studies also indicate that ADP, released or added in the extracellular medium, is a primary inducer of integrin activation. This conclusion was reached only after our careful analysis of the effects of various inhibitors with different concentrations of the agonists. Although there have been no such studies on the activation of GPIIb/IIIa, as far as we know, a similar activation mechanism may likely be applicable for the activation of GP IIb/IIIa.

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Fig. 11. Multistate model for the activation of integrin αβ₁. A multistate model for the activation of integrin αβ₁ in platelets induced by various agonist is suggested from the inhibition pattern data presented in the present study. Integrin αβ₁ can exist in three different conformations: a resting, nonactive conformation, R₀, that has no affinity for soluble collagen and activated states R₁ and R₂, where R₁ has higher affinity for soluble collagen than R₀. ADP, either externally added or secreted upon platelet activation, converts R₀ to R₁, through its binding to ADP receptors. When platelets are activated by a low concentration of agonist, the stimulation system of platelets induces the release reaction, and the secreted ADP converts integrin αβ₁, R₀ to R₁. If platelets are activated by high concentrations of agonists, other systems are also strongly activated, including those involving PKC, phosphoinositid-3-kinase (PI-3 Kinase), and protein-tyrosine kinase; these signals induce the conversion of the R₁ state activated integrin αβ₁ to the further activated form R₂.
Signal-transducing Mechanisms Involved in Activation of the Platelet Collagen Receptor Integrin $\alpha_2\beta_1$

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