Platelet Adhesion to Native Type I Collagen Fibrils

ROLE OF GPVI IN DIVALENT CATION-DEPENDENT AND -INDEPENDENT ADHESION AND THROMBOXANE A₂ GENERATION

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Three glycoproteins (GPs), namely GPIa-IIa, GPVI, and GPIV, have been recently implicated in platelet-collagen adhesive interactions. We have employed antibodies to these GPs to investigate further their role in platelet adhesion to immobilized monomeric and polymeric fibrillar collagen under static conditions in the presence and the absence of Mg²⁺. In the presence of Mg²⁺, each antibody inhibited platelet adhesion to fibrillar collagen from 70 to 85%, especially during the early phase (<15 min), but the inhibitory effects diminished dramatically to 25% or less by 60 min. Combination of anti-GPVI with anti-GPIa-IIa antibodies completely inhibited platelet adhesion at 60 min. Anti-GPIV and anti-GPIa-IIa or anti-GPVI antibodies in combinations were more effective in inhibiting adhesion than was anti-GPIa-IIa or anti-GPVI alone. In the absence of Mg²⁺, anti-GPVI completely inhibited adhesion at 60 min, while anti-GPVI antibody inhibited adhesion by about 50% and minimal effects were seen with anti-GPIa-IIa, suggesting that GPIa-IIa does not play a significant role in the divalent cation-independent platelet adhesion to immobilized fibrillar collagen. Under either divalent cation-dependent or -independent conditions, platelets adhered to fibrillar collagen were able to secrete contents of both α-granules and dense granules and generate thromboxane A₂ (TXA₂), but platelets adhering to acid soluble monomeric collagen neither secreted their granular contents nor generated TXA₂. Although anti-GPVI antibodies were not able to inhibit Mg²⁺-dependent adhesion, they completely inhibited TXA₂ generation under both divalent cation-dependent and -independent conditions. With the other antibodies, TXA₂ generation corresponded with the amount of adhesion observed. These results suggest that GPVI is directly associated with the TXA₂ generating system during platelet-collagen interaction.

Collagen has been identified to be the most thrombogenic of all the macromolecular constituents present in the extracellular matrix underlying the subendothelium. Besides supporting adhesion, it is also capable of inducing secretion and the subsequent platelet aggregation that is crucial in the maintenance of hemostatic function (1). Elucidation of the mechanism by which unactivated platelets initially recognize fibrillar collagen and initiate the subsequent platelet activation is essential for the understanding of hemostatic functions.

Both monomeric and fibrillar collagens effectively support platelet adhesion, whereas the polymerization of the monomeric collagen is required to activate platelets and induce secretion of their granule contents (2–6). Over the years, numerous candidates have been proposed for platelet-collagen receptors, probably reflecting the range of techniques that have been used to study this phenomenon (6). Seven criteria have been proposed to establish the role of putative collagen receptors for platelets (6, 7). Three glycoproteins (GPs)¹ have been accepted as major collagen receptors on platelets that meet most if not all of the seven criteria proposed for a collagen receptor; these are α₂β₁ integrin (GPIa-IIa), CD36 (GPIV, also known as GPIIIb), and GPVI. However, the role played by individual GPs in the overall adhesive interactions of platelets with collagen and their possible interdependence are not fully understood. Another collagen receptor has recently been identified in human platelets. A recombinant receptor protein (54 kDa), obtained by using a prokaryotic expression system, reacted specifically with type I collagen but not with type III collagen (8).

Four patients have been described with mild bleeding disorders whose platelets were found to lack GPIa and to be unresponsive to collagen but aggregated normally to other agonists (9–12). Subsequent studies in two patients revealed, however, that the defect in these platelets was not in their ability to adhere to collagen following contact but in their ability to spread on collagen following their initial adherence (10, 13). Further evidence for the participation of GPIa-IIa has been shown by the complete inhibition of Mg²⁺-dependent platelet adhesion to monomeric collagen by prior incubation of platelets with monoclonal antibodies directed against GPIa-IIa (14–16). On the other hand, adhesion to native collagen fibers has a considerable divalent cation-independent component (17) that is not inhibited by anti-GPIa-IIa antibodies (18).

Fab fragments of polyclonal antibodies against GPIV have been shown to partly inhibit platelet adhesion to fibrillar collagen (7). The role of GPIV in adhesive interactions of platelets with collagen was further confirmed (19) by the use of platelets of the Naka-negative phenotype, which constitutively lack GPIV (20). In comparison with control platelets, Naka-negative platelets showed significantly reduced platelet adhesion to collagen (14–16). On the other hand, adhesion to native collagen fibers has a considerable divalent cation-independent component (17) that is not inhibited by anti-GPIa-IIa antibodies (18).

¹The abbreviations used are: GP, glycoprotein; TXA₂ and TXB₂, thromboxane A₂ and B₂, respectively; PF4, platelet factor 4; β-TG, β-thromboglobulin; Fabs, Fab fragments.
showed reduced adhesion to type IV collagen under both flow and static conditions but responded normally with collagens type I and III (22).

Several Japanese patients with mild bleeding disorders have been described whose platelets failed to aggregate in response to collagen. Analysis of the membrane glycoproteins in these patients revealed that their platelets either lacked GPVI or had very little of it (23–25). One patient developed antibodies to GPVI after platelet transfusion. The IgG from the patient serum induced aggregation of normal platelets but not her own platelets, and the Fab fragments obtained from the patient's IgG inhibited collagen-induced platelet aggregation (26).

We have utilized antibodies to these GPs to obtain more precise information about their roles in the adhesive interactions of platelets with acid-insoluble type I fibrillar collagen under static conditions. We have studied the effects of blocking two receptors simultaneously on divalent cation-dependent and -independent platelet adhesion as well as on the secretion of serotonin, platelet factor 4 (PF4), and tryptase (6, TG) and the generation of TXA2. Our results suggest a direct association of GPVI with the TXA2 generation system.

EXPERIMENTAL PROCEDURES

Reagents—Acid-insoluble equine tendon fibrillar collagen (Chrono Par) was obtained from Chrono-Log Corp. (Broomall, PA). Acid-soluble rat tail type I collagen was purchased from Collaborative Biomedical Products Research (Bedford, MA). [3H]Serotonin (5-hydroxytryptamine creatinine sulfate; 58 mCi/mmol) and [51Cr] (Na2CrO4, 250 mCi/mg) were purchased from Amersham Corp. Prostaglandin E1 was from Cayman Chemicals (Ann Arbor, MI). Enzyme immunoassay kits for thromboxane B2 were from PerSeptive Diagnostic, Inc. Kits for PF4 and β-TG were obtained from Diagnostica Stago (France). Bovine serum albumin and other chemicals were purchased from Sigma.

Antibodies—Protein G affinity-purified mouse monoclonal antibody 6F1, directed against human platelet GPIa-IIa and recognizing the GPIa subunit, was generously provided by Dr. Barry S. Coler (Mount Sinai Medical School, New York). An unidentified mouse monoclonal antibody of the IgG subclass (clone MOPC 21) was purchased from Sigma. A monospecific polyclonal antibody to human platelet GPIV (anti-GPIV 916) was raised in New Zealand White rabbits as described previously (19). A monospecific antibody against GPVI was purified from the plasma of a patient with idiopathic thrombocytopenic purpura who had developed an autoantibody against GPVI (26). Fab fragments (Fabs) were prepared from the IgG fraction of the patient's plasma, from normal human plasma, and from the rabbit anti-GPIV serum by digestion with agarose-coupled papain utilizing an ImmunoPure Fab preparation kit (Pierce), principally according to the manufacturer's instructions but with slight modifications in the digestion temperature and the mode of shaking. In preliminary experiments using conditions suggested by the manufacturer, the resulting Fabs from the patient's IgG failed to block the aggregation of washed platelets by the intact IgG. In subsequent digestions, therefore, the IgG samples were digested at 35 °C in an oven with gentle rotation. The final products in each case was dialyzed extensively against HEPES-saline, pH 7.4. Fab fragments thus obtained retained their activity as judged by their ability to block aggregation of washed platelets induced by the corresponding intact IgG.

Platelet Preparation—Human blood was collected from healthy individuals who had not ingested any medication for at least 14 days prior to phlebotomy by the two-syringe method of venipuncture using a 19-gauge butterfly needle. Whole blood was collected directly into a syringe containing 3.8% sodium citrate as anticoagulant (9:1 whole blood/anticoagulant, v/v). Platelet-rich plasma was obtained by centrifugation at 150 × g for 20 min at room temperature. Washed platelets were prepared by the citrate wash method (19) with minor modifications. Briefly, citrate-washed platelets were suspended in Tyrode-HEPES buffer (136.7 mM NaCl, 5.5 mM glucose, 2.6 mM KCl, 13.8 mM, NaHCO3, 1.0 mM MgCl2, 0.36 mM NaH2PO4, 0.25% bovine serum albumin, pH 7.4) at a concentration of 2 × 109 platelets/ml. When required, platelets (1 × 109) were labeled with Na2[31Cr]O4 (50 μCi/ml) for 1 h at room temperature followed by washing twice with citrate wash buffer containing 0.5% bovine serum albumin (19). To avoid platelet activation during washing and aggregation during adhesion assays, prostaglandin E1 (250 nmol) was included in all buffers used to prepare washed platelets and in subsequent operations.

Adhesion Assay—Microtiter wells were coated with type I acid-insoluble equine tendon fibrillar collagen or with acid-soluble rat tail type I collagen maintained under acid conditions to ensure maintenance of monomer structure. Divalent cation-free adhesion buffer was prepared by replacing Mg2+ (1 mM) in the Tyrode-HEPES buffer with 50 μM EDTA (19). [51Cr]-Labeled platelets were suspended in divalent cation-free adhesion buffer or in Tyrode-HEPES at a cell concentration of 3 × 107/ml, and adhesion assays were carried out as described previously (19).

Serotonin Release Reaction—For adhesion-induced secretion studies, washed platelets from a single donor were divided into two equal aliquots: one aliquot was labeled with [51Cr], while the other aliquot was labeled in parallel with [3H]serotonin. Washed platelets (1 × 109 cells/ml) were incubated with [3H]labeled serotonin (0.1 μCi/ml, 1 μM) for 60 min at room temperature. The unincorporated radioactivity was removed by washing the platelets twice with platelet wash buffer (19). Platelets were finally suspended in Tyrode-HEPES buffer containing Mg2+ (1 mM) or EDTA (50 μM) and imipramine (1 μM) to stop the reuptake of released serotonin.

An aliquot (50 μl) of serotonin-loaded platelets was added to individual collagen-coated wells. At the desired times, adhesion was stopped by removing nonadhered platelets by washing each well six times by decantation using 200-μl aliquots of Tyrode-HEPES buffer containing 1 mM EDTA (19). The adhered platelets were solubilized in SDS (2%) for 30 min, and their serotonin content was quantitated by counting the lysates in a β-counter (C). At the end of the incubation, an aliquot (50 μl) of unused platelet suspension was solubilized with an equal volume of SDS (2%) and counted (T). An aliquot of the supernatant obtained from the unused platelet suspension was also counted to determine the background radioactivity (B). The adhesion rate (R) (percentage of adhesion × 0.01) was quantitated in parallel experiments using [3H]-labeled platelets. The adhesion-induced serotonin release was calculated by the following equation.

\[
\text{Secretion (%) } = \frac{(T - B) \times R - C}{(T - B) \times R} \times 100 
\]

PF4 and β-TG Release—Levels of both PF4 and β-TG were measured in the supernatants of the adhered platelets by commercially available kits based on enzyme-linked immunosorbent assay. At the desired times, nonadherent platelets were removed, and the wells were washed twice with wash buffer (200 μl). Suspensions of the nonadhered platelets and the two washes were combined and centrifuged at 6000 × g for 2 min to sediment the platelets, and the supernatants were immediately frozen. At the same time, an aliquot (50 μl) of unused platelet suspension was also frozen. Subsequently, all samples were thawed on ice and made 1% with respect to Triton X-100 by adding an equal volume of chilled Triton X-100 (2%) in Tyrode-HEPES buffer. After 1 h on ice, samples were centrifuged at 15,000 × g for 15 min at 4 °C to sediment Triton X-100-insoluble components. PF4 and β-TG levels were measured in the supernatants according to the manufacturer's instructions.

Thromboxane A2 Generation—TXA2 was measured as TXB2, a stable metabolite of TXA2, by a commercially available kit. At the desired times, unadhered platelets and suspending medium were transferred to ice-cold tubes and centrifuged at 6000 × g for 2 min to sediment platelets. Clear supernatants were kept until used. Eicosanoids were extracted from the supernatants in ethyl acetate (27) prior to their quantitation by the immunosorbent assay kit. Briefly, the supernatants (100 μl) were acidified to a pH of 3–3.5 with formic acid (15 μl, 2.5%) and made 0.5 g/ml with respect to NaCl by the addition of 50 mg of solid NaCl to each tube, and eicosanoids were extracted twice with ethyl acetate (2.2 ml/extraction). Organic phases from two extractions were combined and vacuum-dried. Samples were reconstituted in kit buffer for assay and processed further according to the manufacturer's instructions. In preliminary experiments, extraction efficiency was determined by lacing the supernatants with known amounts of tritium-labeled TXB2 before extraction with ethyl acetate; 90–98% of the radioactivity was found in the organic phase.

RESULTS

Platelet Adhesion to Monomeric and Fibrillar Type I Collagen—Platelets can interact with collagen by both divalent cation-dependent and -independent mechanisms (6, 18, 19). Adhesion assays were carried out by a previously published method (7, 28) except that Tris-buffered solution was previously used to minimize...
platelet aggregation was replaced with Tyrode-HEPES buffer, since Tris slightly affects both the morphology (29) and responses of platelets (30). HEPES-based adhesion buffer has been used successfully to study platelet adhesion to collagen and Matrigel-coated plastic wells (31). Divalent cation-dependent platelet adhesion was measured in the presence of 

\[ \text{Mg}^{2+} \]\n
while the divalent cation-independent adhesion was measured in its absence but in the presence of a small amount of EDTA (50 \( \mu \text{M} \)) to chelate minute amounts of divalent cations present in the water and the chemicals used to prepare buffers (19). Typical patterns of time-dependent platelet adhesion to type I acid-insoluble fibrillar collagen and acid-soluble monomeric collagen both under 

\[ \text{Mg}^{2+} \]-dependent and -independent conditions are shown in Fig. 1, a and b, respectively. Under these conditions, at the 60-min time point, the divalent cation-independent platelet adhesion was about one-fourth of the adhesion observed in the presence of 

\[ \text{Mg}^{2+} \]\n
and was similar to that obtained using Tris-based buffers (19). However, platelet adhesion to monomeric collagen was exclusively divalent cation-dependent, since no adhesion was observed in the absence of 

\[ \text{Mg}^{2+} \]\n
(Fig. 1b).

**Effects of Anti-GPIa-IIa, Anti-GPIV, and Anti-GPVI Antibodies on Platelet Adhesion**—In preliminary studies, we determined the dose-dependent effect of each antibody on platelet adhesion to both the acid-insoluble fibrillar type I collagen from equine tendon and to acid-soluble monomeric type I collagen from rat tail. Adhesion was studied for 30 min both in the presence and absence of 

\[ \text{Mg}^{2+} \]. \] Since IgG fractions of both anti-GPIV and anti-GPVI antibodies induced aggregation of the washed platelets, Fab fragments were used in both cases. Maximum inhibition of divalent cation-independent platelet adhesion to acid-insoluble fibrillar type I collagen was obtained at 300 \( \mu \text{g/ml} \) Fab fragments of anti-GPIV and anti-GPVI. 

\[ \text{Mg}^{2+} \]-dependent adhesion was not affected by higher concentrations of these Fabs. Maximal inhibition of 

\[ \text{Mg}^{2+} \]-dependent adhesion to rat tail monomeric collagen was obtained at a concentration of 20 \( \mu \text{g/ml} \) of anti-GPIa-IIa IgG (6F1). In subsequent experiments, therefore, we used 6F1, anti-GPIV (916 Fab), and anti-GPVI (anti-p62 Fab) at 20, 300, and 300 \( \mu \text{g/ml} \), respectively.

In the presence of 

\[ \text{Mg}^{2+} \], all three antibodies effectively inhibited platelet adhesion to acid-insoluble fibrillar collagen at the 15-min time point (Fig. 2a). Both anti-GPIa-IIa and anti-GPVI inhibited adhesion by about 85%, while anti-GPI inhibited adhesion by 70–75%. However, these inhibitory effects diminished with time. At the 60-min time point, anti-GPIa-IIa showed inhibition of $-25\%$ and anti-GPVI showed $-15\%$, while anti-GPI showed inhibition of only $5\%$. In the absence of 

\[ \text{Mg}^{2+} \]\n
(Fig. 2b), anti-GPIa-IIa had minimal effects, while both anti-GPVI and anti-GPI V Fabs effectively inhibited platelet adhesion to fibrillar collagen at all time points examined. At 300 \( \mu \text{g/ml} \), anti-GPVI Fabs inhibited adhesion completely, while anti-GPI V Fabs showed an inhibition of about 50%. Under each set of conditions, unrelated mouse IgG1 (clone MOPC 21) and control Fabs prepared from rabbit and human normal IgG used as negative controls were without effect (data not shown).

We next examined the combined effects of these antibodies both under 

\[ \text{Mg}^{2+} \]-dependent and -independent conditions. In the presence of 

\[ \text{Mg}^{2+} \], combinations of antibodies were signifi-
cantly more effective in inhibiting platelet adhesion as compared with the individual antibody alone. Notably, the combination of anti-GPla-IIa and anti-GPVI completely inhibited adhesion even at 60 min in the presence of Mg$^{2+}$ (Fig. 2a). Combination of anti-GPIV with anti-GPla-IIa or anti-GPVI also enhanced inhibition, but these effects decreased with time, and only ~50% inhibition was observed at 60 min. In the absence of Mg$^{2+}$, preincubation of platelets with anti-GPVI resulted in a complete inhibition of adhesion at all time points (Fig. 2b). Interestingly, anti-GPIV, which had minimal effect on Mg$^{2+}$-dependent platelet adhesion at the 60-min time point, significantly inhibited platelet adhesion at all time points (35–50%) in the absence of Mg$^{2+}$. Contrary to its inhibitory effect in the presence of Mg$^{2+}$, anti-GPla-IIa had a minimal effect on time-dependent adhesion in the absence of Mg$^{2+}$. To conserve the human anti-GPVI antibody, and since it completely inhibited platelet adhesion to acid-insoluble fibrillar collagen under Mg$^{2+}$-free conditions, we did not study the effects of combinations of anti-GPVI Fabs with either anti-GPla-IIa IgG or anti-GPIV Fabs, but we did examine the combined effects of anti-GPla-IIa IgG and anti-GPIV Fabs. The combination of anti-GPla-IIa with anti-GPIV was slightly more effective in inhibiting adhesion than was anti-GPIV alone, but substantial adhesion was seen at all time points, suggesting that in a Mg$^{2+}$-free system GPVI alone can overcome the loss of two major receptors. Under each set of conditions, unrelated mouse IgG1 and control Fabs prepared from rabbit and human normal IgG used as negative controls were without effect (data not shown).

Effects of Anti-GPla-IIa, Anti-GPIV, and Anti-GPVI Antibodies on Platelet Adhesion to Monomeric Type I Collagen—Rat tail acid-soluble monomeric collagen was coated onto microtiter wells as described for acid-insoluble fibrillar collagen under “Experimental Procedures.” Since no adhesion could be seen in the absence of Mg$^{2+}$ (Fig. 1b), we examined first the effect of the three antibodies individually in the presence of Mg$^{2+}$ (Fig. 3). Preincubation of platelets with anti-GPla-IIa IgG resulted in a complete inhibition of adhesion at all time points. Anti-GPVI Fabs, which significantly inhibited adhesion to acid-insoluble type fibrillar collagen at early time points (Fig. 2a), had little or no effect on platelet adhesion to rat tail monomeric collagen. Anti-GPIV Fabs by themselves inhibited adhesion significantly at early time points. The addition of both anti-GPVI Fabs and anti-GPIV Fabs showed no further inhibition, confirming again that GPVI has no role in platelet adhesion to acid-soluble monomeric collagen under static conditions. These results support earlier observations that GPla-IIa is an important adhesion receptor for monomeric collagen (14–16) and that GPVIV may also participate in the adhesive interactions of platelets with monomeric collagen.

**Table I**

<table>
<thead>
<tr>
<th>Time</th>
<th>Platelet adhesion (mean ± S.D.)</th>
<th>Serotonin secretion from adhered platelets (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Mg$^{2+}$-dependent</td>
<td>30 16.5 ± 3.4</td>
<td>57.4 ± 4.4</td>
</tr>
<tr>
<td>60</td>
<td>34.2 ± 4.4</td>
<td>58.8 ± 14.9</td>
</tr>
<tr>
<td>Mg$^{2+}$-independent</td>
<td>30 4.2 ± 2.4</td>
<td>79.5 ± 5.1</td>
</tr>
<tr>
<td>60</td>
<td>9.1 ± 3.2</td>
<td>77.1 ± 13.9</td>
</tr>
</tbody>
</table>

**Adhesion-induced Platelet Release Reaction**—The extent of the platelet release reaction was determined by measuring the amount of $^{14}$C serotonin secreted into the medium from the dense granules and of PF4 and β-TG from the α-granules following adhesion to fibrillar collagen. In the presence of Mg$^{2+}$, adhered platelets secreted ~60% of their dense granule contents into the medium (Table I). In the absence of Mg$^{2+}$, the amount of adhesion was reduced to about one-fourth, but the adhered platelets secreted ~80% of their $^{14}$C serotonin content. These results suggest that Mg$^{2+}$ is not required for the collagen-induced release reaction. Adhesion to monomeric collagen did not induce a significant release reaction (data not shown). The combination of anti-GPla-IIa and anti-GPVI Fabs that completely inhibited Mg$^{2+}$-dependent adhesion also completely inhibited serotonin secretion (Table II). Similarly, anti-GPVI Fabs which inhibited platelet adhesion in Mg$^{2+}$-free buffer also inhibited secretion of serotonin from platelets. Like serotonin secretion, the secretion of PF4 and β-TG was also inhibited by anti-GPVI Fabs by themselves alone and in combination with anti-GPla-IIa (data not shown).

**Adhesion-induced TXA$_2$ Generation**—TXB$_2$, a stable metabolite of TXA$_2$, was measured by immunosorbent assay in the platelet suspending medium after the adhesion as described under “Experimental Procedures.” Both in the presence and absence of Mg$^{2+}$, adhered platelets generated TXA$_2$ in a time-dependent manner (Table III). The absolute amounts of TXA$_2$...
Platelet-Collagen Interactions under Static Conditions

Table II

Effects of anti-GPIa-IIa, anti-GPIV and anti-GPVI on platelet adhesion to fibrillar type I collagen and [14C]serotonin secretion

Platelets were incubated with anti-GPIa-IIa (6F1; 20 μg of IgG/ml), anti-GPIV (916 Fabs; 300 μg/ml), and anti-GPVI (anti-p62 Fabs; 300 μg/ml) for 30 min prior to their addition to microtiter wells. Adhesion and release reactions were measured as described under "Experimental Procedures." In each case, nonimmune mouse IgG or normal rabbit or human Fabs were run as controls. The data represent means ± S.D. of three experiments using platelets from different individuals, and each samples was run in triplicate. The interassay variability was within a 2–6% range.

| Mg²⁺-dependent | Anti-GPIa-IIa | 72.3 ± 3.4 | 73.7 ± 20.9 |
| Anti-GPIV | 93.1 ± 7.2 | 78.6 ± 18.5 |
| Anti-GPVI | 87.8 ± 6.1 | 62.5 ± 18.2 |
| Anti-GPIa-IIa + anti-GPIV | 50.0 ± 8.6 | 63.2 ± 16.7 |
| Anti-GPVI + anti-GPVI | 58.5 ± 14.9 | 72.0 ± 6.8 |
| Anti-GPIa-IIa + anti-GPVI | 0.8 ± 0.4 | ND² |
| Mg²⁺-independent | Anti-GPIa-IIa | 74.9 ± 10.4 | 77.5 ± 18.2 |
| Anti-GPIV | 41.0 ± 5.1 | 87.7 ± 20.6 |
| Anti-GPIa-IIa + anti-GPIV | 28.9 ± 5.7 | 70.9 ± 15.8 |
| Anti-GPVI | 0.8 ± 0.4 | ND² |

⁰ ND, not detectable.

Table III

Time-dependent TXA₂ generation by platelets adhered to type I fibrillar collagen under static conditions

Adhesion assays were terminated at the desired time, and levels of TXA₂ released from the adhered platelets were measured by enzyme immunoassay as described under "Experimental Procedures." The values given are means ± S.D. from three experiments each run in duplicate. The interassay values varied 2–9%.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Platelet adhesion (mean ± S.D.)</th>
<th>TXA₂ generation (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺-dependent</td>
<td>15</td>
<td>5.6 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>15.8 ± 4.8</td>
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<tr>
<td></td>
<td>60</td>
<td>31.5 ± 3.8</td>
</tr>
<tr>
<td>Mg²⁺-independent</td>
<td>15</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.2 ± 1.2</td>
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<td></td>
<td>60</td>
<td>8.7 ± 2.6</td>
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</table>

DISCUSSION

The purpose of this study was to investigate in detail the roles of GPIa-IIa, GPIV, and GPVI in platelet adhesion to collagen under static conditions. Type I monomeric collagen interacts primarily with platelet membrane GPIa-IIa in a Mg²⁺-dependent fashion without release of dense granule contents (6). On the other hand, interaction with polymeric collagen has a strong divalent cation-independent element that is not inhibitable by anti-GPIa-IIa antibodies (18). We have evaluated, therefore, both acid-insoluble polymeric type I collagen and acid-soluble monomeric type I collagen as substrates to study platelet-collagen adhesive interactions.

In our adhesion assay, 1) platelet adhesion to acid-soluble monomeric collagen was Mg²⁺-dependent, since no adhesion was seen in the absence of Mg²⁺; 2) it was completely inhibited by preincubation of platelets with anti-GPIa-IIa antibody; and 3) an insignificant release reaction from the adhered platelets was seen. These results confirm earlier observations made by Santoro (6). The lack of effect of anti-GPVI Fabs alone on platelet adhesion to monomeric collagen suggests that GPVI does not play a significant role in these interactions. Anti-GPVI Fabs by themselves were able to significantly inhibit adhesion only during early time points.

Platelets adhere to polymeric fibrillar collagen via both Mg²⁺-dependent and -independent mechanisms (18). In fact, our results suggest that one-fourth of the total adhesion is Mg²⁺-independent. Mg²⁺-independent adhesion was not inhibited by anti-GPIa-IIa antibody, but it was completely inhibited by anti-GPVI Fabs and partially (≈50%) inhibited by anti-GPIV Fabs. Moroi et al. (23) described a GPVI-deficient patient whose platelets failed to adhere to collagen fibrils in the presence of EDTA, which is consistent with our results and further confirms that GPVI is a primary receptor in divalent cation-independent platelet adhesion to collagen. In addition, 50% inhibition seen with anti-GPVI Fabs confirms our earlier observation that GPVI-mediated adhesion is Mg²⁺-independent (28). These results suggest that both GPVI and GPIV interact with the immobilized collagen in a Mg²⁺-independent fashion. It is, of course, important to emphasize that these Mg²⁺-independent reactions go forward in the presence of Mg²⁺ but are not dependent on it.

All three antibodies individually inhibited adhesion (70–85%) during the early phases of Mg²⁺-dependent adhesion, but at the 60 min time point only very limited inhibition was observed in each case. This loss of inhibition by the 60-min time...
point suggests that all three receptors may be involved during the early phases, but in the absence of one receptor the other two are ultimately sufficient to overcome the absence of the third. This may explain why patients with deficiency of either GPIa or GPVI do not exhibit a severe bleeding tendency. However, when two receptors were blocked simultaneously, the combination of anti-GPVI Fab and anti-GPIa-IIa antibody completely abolished adhesion, while the other two combinations, namely anti-GPIa-IIa/anti-GPVI and anti-GPV/anti-GPVI, were more effective than each individual antibody alone. These results suggest, that under static conditions and in the presence of extracellular Mg$^{2+}$, all three receptors participate in adhesive interactions of platelets with polymeric type I collagen but that GPIa-IIa and GPVI play the major roles in these interactions.

Upon binding to collagen fibers, platelets spread, secrete their granular contents, and generate TXA$_2$. Some of the secreted products, including TXA$_2$, are themselves proaggregatory in that they bind back to platelets and induce aggregation resulting in the growth of thrombus size. The role of individual collagen receptors in various activation processes has not so far been elucidated. We have attempted to clarify the role of each receptor by measuring the adhesion-induced release reaction from dense granules and $\alpha$-granules and the generation of TXA$_2$. Minimal serotonin, PF4, and $\beta$-TG release (about 15%) and no TXA$_2$ generation was observed when monomeric type I collagen was used as a substrate (data not shown). In contrast, once adhered to fibrillar type I collagen, platelets were able to secrete 60–80% of their dense and $\alpha$-granule contents and generate TXA$_2$ under both divalent cation-dependent and -independent conditions. It is interesting to note that relatively more TXA$_2$ was generated by the adhered platelets in the absence of Mg$^{2+}$. To the best of our knowledge, this is the first report to evaluate Mg$^{2+}$ effects on adhesion-induced TXA$_2$ generation. These results further confirm that only native collagen fibers can induce the release reaction and demonstrate for the first time that polymeric fibrillar collagen is essential for TXA$_2$ generation.

To explore further the role of the various collagen receptors in TXA$_2$ generation, we carried out antibody blocking experiments. TXA$_2$ generation paralleled the amount of adhesion when GPIa-IIa and GPVI were blocked with their respective antibodies alone or in combination (Fig. 4), suggesting that these receptors are not directly involved in TXA$_2$ generation during adhesion to collagen under static conditions. On the other hand, anti-GPVI Fab totally abolished TXA$_2$ generation in the presence of Mg$^{2+}$, but adhesion was inhibited by only $\sim$15%. TXA$_2$ generation was not seen in all cases where GPVI was blocked. These results are in agreement with those of Ryo et al. (25) and Sugiyama et al. (26), who showed that platelets lacking GPVI did not generate TXA$_2$ upon stimulation with collagen under stirring conditions. The failure to detect TXA$_2$ generation in GPIa-deficient platelets (9) probably reflects the absence of collagen-induced aggregation in these patients or a combined deficiency of other receptors such as GPV and GPVI, which could affect platelet responses (11). These results strongly suggest a direct association between GPV and TXA$_2$ generation during platelet-collagen adhesive interactions.

In summary, platelets have both divalent cation-dependent (integrin; GPIa-IIa) and -independent (non-integrin; GPV and GPVI) receptors for fibrillar type I collagen. GPV and GPIa-IIa seem to be the primary receptors for immobilized fibrillar type I collagen under static conditions, and GPV may accelerate the rate of adhesion once platelets establish contact with collagen fibers via GPV and GPIa-IIa. Adhered platelets secrete dense and $\alpha$-granule contents and induce the generation of TXA$_2$ independent of divalent cations. GPV-mediated signaling seems to play an important role in TXA$_2$ generation. The steps involved in the interaction of collagen with GPV and the formation of TXA$_2$ are not known and require further study.

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REFERENCES

FIG. 4. Effects of anti-GPIa-IIa, anti-GPVI, and anti-GPV antibodies on TXA$_2$ generation by the adhered platelets. $^3$Cr-Labelled platelets were suspended either in Mg$^{2+}$-containing buffer (Fig. 4a) or Mg$^{2+}$-free buffer (Fig. 4b) and incubated with the various antibodies for 30 min at room temperature prior to their addition to the microtiter wells as described in the legend of Fig. 2. After a 60-min incubation, TXA$_2$ generation was measured in the supernatant as described under "Experimental Procedures." Solid bars represent percentage of adhesion relative to corresponding control values, and hatched bars show adhesion-induced TXA$_2$ formation. Values given are the means ± S.D. of three experiments each run a duplicate.

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