We analyzed the interaction of convulxin (Cvx), a 72-kDa protein isolated from the venom of Crotalus durissus terrificus, with human platelets. Cvx is a potent platelet agonist that induces an increase in the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), granule exocytosis and aggregation. \textsuperscript{125}I-Labeled Cvx binds specifically and rapidly to platelets at binding sites of high and moderate affinity. Platelets adhere to immobilized Cvx in a time-dependent but cation-independent manner. Platelet exocytosis and aggregation induced by Cvx were inhibited by an anti-integrin \(\alpha_\text{IIb}\beta_\text{Ia}\) monoclonal antibody (6F1) and by the Fab fragments of a polyclonal anti-glycoprotein VI (GPVI) antibody. Both the adhesion of platelets to Cvx and the Cvx-induced increase in [Ca\textsuperscript{2+}] were inhibited by anti-GPVI Fab fragments but not by 6F1. Ligand blotting assay showed that \textsuperscript{125}I-Cvx binds to a 57-kDa platelet protein with an electrophoretic mobility identical to that of GPVI. In addition, we observed the following: (i) \textsuperscript{125}I-Cvx binds to GPVI immunoprecipitated by the anti-GPVI antibody from a platelet lysate, and (ii) Cvx inhibits the binding of anti-GPVI IgG to GPVI. Taken together, these results demonstrate that GPVI behaves as a Cvx receptor and that the \(\alpha_\text{IIb}\beta_\text{Ia}\) integrin appears to be involved in the later stages of Cvx-induced platelet activation, i.e. exocytosis and aggregation.

Many snake venom proteins are known to interact with platelets. Some behave in vitro as cell agonists, and others are inhibitors of platelet activation induced by physiological agents (1). Several groups can be distinguished according to their mechanism of action and molecular structure: inhibitory and activating proteases, glycoproteins (aggregoserpinins) and lectins (thrombolectins) that stimulate platelets, or peptides and (ii) Cvx inhibits the binding of anti-GPVI IgG to GPVI. Taken together, these results demonstrate that GPVI behaves as a Cvx receptor and that the \(\alpha_\text{IIb}\beta_\text{Ia}\) integrin appears to be involved in the later stages of Cvx-induced platelet activation, i.e. exocytosis and aggregation.

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Snake Venom Platelet Activator

previously (10). Control human IgG and Fab fragments used in these studies were prepared from the plasma of healthy donors according to the procedures described above. Anti-Cvx antibody was kindly provided by Dr. M. Leduc (Unité des Venins Institut Pasteur). Calf skin collagen type I, obtained from Stago (Asnières, France), was used according to the manufacturer’s instructions.

Platelet Preparation—Blood from healthy human volunteers was collected by venipuncture on acid-citrate-dextrose anticoagulant (ACD-A) or on trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 110 × g for 15 min. Platelet secretion and adhesion were determined using platelets labeled in PRP (ACD-A) incubated with 0.6 μM [125I]-hydroxytryptamine (Amersham, Les Ulis, France) and 2 μCi/ml [3]H (CIS International, Gil-sur-Yvette, France), for 30 min at 37 °C respectively. Platelets were sedimented at 1100 × g for 15 min after acidification of the PRP to pH 6.5 with ACD-A and addition of 25 μg/ml apyrase (Sigma) and 100 nM prostaglandin E1 (Sigma) and resuspended in washing buffer (103 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5 mM glucose, 36 mM citric acid) at pH 6.5 containing 3.5 mg/ml BSA (Sigma), 25 μg/ml apyrase, and 100 nM prostaglandin E1 (11). After sedimentation, the platelets were washed twice in this buffer and resuspended at 3 × 109/ml in the reaction buffer composed of 5 mM Hepes, 137 mM NaCl, 2 mM KCl, 1 mM MgCl2, 12 mM NaHCO3, 0.3 mM NaH2PO4, 5.5 mM glucose, pH 7.4, containing 3.5 mg/ml BSA. Formaldehyde-fixed platelets were prepared by incubating 1 volume of citrated PRP or washed platelets for 30 min at 37 °C, pH 7.4, containing 30% (w/v) formaldehyde for 18 h at room temperature and in the dark. Fixed platelets were washed three times in PBS before resuspension in PBS.

Platelet Aggregation and Secretion—PRP or washed platelets were preincubated for 3 min at 37 °C in the presence of buffer or antibodies before aggregation was initiated by Cvx or collagen. Experiments were performed under stirring conditions at 37 °C in a Chrono-Log aggregometer (Chrono-Log Corp, Haverton, PA). Release of [125I]-HT was measured as described previously (11).

Platelet Adhesion—Platelet adhesion was measured on microtitration plates. Typical experiments were performed as follows. Collagen (2 μg) in 100 μl of 20 mM acetic acid, Cvx (1.4 μg) in 100 μl of PBS, or BSA (5 μg) in 100 μl of PBS were immobilized on Immulon II plates (Dynatech, St-CLOUD, France) for 2 h at room temperature. Plates were then saturated with 2 mg/ml BSA in PBS for 1 h, and washed with PBS and with reaction buffer. [35S]Labeled platelets (2 × 106/ml in reaction buffer, 100 μl) were added to the wells in the presence or absence of 300 μM Arg-Gly-Asp-Ser (RGDS) peptide (Bachem Biochimie, Voisins-le-Brévon, France) or 2 mM EDTA. A certain number of experiments were performed in the presence of antibodies, as indicated in the text. Wells were emptied and washed three times with reaction buffer after different incubation times at room temperature. One hundred μl of 2% SDS (w/v) was subsequently added to each well, and the samples were counted for 30Cr.

Intracellular Ca2+ Concentration—Intracellular free calcium ([Ca2+]i) transients were monitored by fura-2 fluorescence. Platelets were suspended in washing buffer after centrifugation of the PRP and loaded with 2 μM fura-2-acetoxyethylmester (fura-2-AM, Sigma) for 60 min at 37 °C, centrifuged again, washed twice, and resuspended in reaction buffer. The platelets (2 × 106/ml) were then preincubated with 2 mM CaCl2 in the absence or presence of different antibodies for 3 min at 37 °C prior to addition of Cvx or collagen. Fluorescence was measured at 37 °C using two excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm on an Hitachi H-2000 spectrophotometer (Scienecetec, Les Ulis, France). Maximal and minimal fluorescence were determined after platelet lysis with 1% (v/v) Triton X-100 and the addition of 2 mM EGTA, respectively. Ca2+ concentrations were calculated using a Kd of 224 nm for the interaction between fura 2 and Ca2+ (12).

Binding of 125I-Labeled Cvx to Platelets—Washed or formaldehyde-fixed platelets (3 × 109/ml) were incubated at 37 °C in 400 μl of various solutions of 125I-labeled Cvx. The platelets were transferred to tubes containing 500 μl of 20% (w/v) sucrose in PBS after different incubation times, and centrifuged for 5 min at 12,000 × g. Supernatants were aspirated, and the tips of the tubes were counted for 125I to determine the fraction of Cvx bound to platelets. Nonspecific binding was determined in the presence of a 100-500-fold excess of unlabeled Cvx.

Ligand Blotting Assay and Immunoblotting—Platelet lysates were prepared by solubilization of washed platelets (107/ml) with 2% (w/v) SDS in 20 mM Tris-HCl, 150 mM NaCl, 3 mM EDTA, 5 mM N-ethylmaleimide (Sigma), for 5 min at 100 °C. Proteins (7 μg) were separated on 10% acrylamide slab gels (13), and transferred to nitrocellulose mem-

branes (Bio-Rad, Ivry-sur-Seine, France). The membranes were then soaked with 5% (w/v) nonfat dry milk in PBS, incubated with 125I-labeled Cvx (50 ng/ml) in PBS, pH 7.4, containing 0.5% (w/v) dry milk and 0.2% (v/v) Tween 20, washed, and exposed to X-AR films (Kodak). Nitrocellulose sheets were incubated alternatively with 9 μg/ml anti-GPVI IgG in PBS, pH 8.6, containing 0.1% dry milk and 0.01% Tween 20, followed by 125I-labeled protein A or peroxidase-coupled protein A revealed by chemiluminescence (Amersham).

Immunoprecipitation—Platelet lysates were obtained by solubilization of washed platelets (5 × 109/ml) with 1% (v/v) Nonidet P-40 in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 15 μg/ml leupeptin (Sigma), 50 kallikrein-inactivating units of aprotinin, 1 mM phenylmethylsulfonyl fluoride (Sigma), 2 mM benzamidine HCl, and 2 mM EDTA (lysis buffer), at 4 °C for 30 min followed by centrifugation at 13,000 × g at 4 °C for 30 min. Lysates were precleared by incubation with protein A-Sepharose for 30 min at 4 °C and centrifugation to avoid nonspecific precipitation. Cleared lysates were incubated with 200 μg/ml anti-GPVI IgG for 30 min at room temperature and then protein A-Sepharose at 4 °C overnight. Samples were centrifuged at 12,000 × g, and the immunoprecipitates were washed three times with the lysis buffer. Immunoprecipitated proteins were eluted by 2% SDS in Laemmli buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes, which were probed using 125I-Cvx or anti-GPVI IgGs, as described above.

RESULTS

Cvx Induces Activation of Human Platelets—Washed human platelets aggregated in response to Cvx (Fig. 1) as reported previously for rabbit platelets (6). Aggregation was also observed in PRP and was preceded by a change in cell shape. The effect of Cvx was tested on formaldehyde-fixed platelets to verify that Cvx induced true platelet aggregation rather than passive agglutination. No modification of light transmittance was observed for suspensions of fixed platelets, indicating that Cvx does not agglutinate platelets (data not shown). The threshold concentration of Cvx that typically induced platelet aggregation was between 15 and 35 pM. Important heterogeneity in platelet sensitivity to Cvx was observed with platelets from normal individuals however. Secretion of [14C]5-Ht from dense granules paralleled aggregation. The dose-response curve was sigmoid and characterized by a very abrupt slope (Fig. 2A). Aggregation was prevented by 2 mM EDTA and by 300 μM RGDS peptide, which block fibrinogen binding to integrin αIIbβ3. In contrast, neither cell shape change nor [14C]5-HT release were inhibited by EDTA or RGDS peptide (Fig. 2B).

Along with granule exocytosis and cell aggregation, Cvx in-

![Fig. 1. Convulxin-induced platelet aggregation. Purified Cvx was added (arrowhead) to washed platelets at 3 × 109/ml (a–d) or to PRP (e). Incubations were performed at 37 °C under stirring in an aggregometer cuvette and light transmittance through the cell suspension was recorded. Cvx concentrations were: a, 100 pm; b, 50 pm; c, 25 pm; d, 12.5 pm; e, 3.5 pm. Aggregation curves presented here are representative of five experiments performed with different platelet preparations.](image-url)
and 8.6

concentration was 200 pM. Results are from one representative exper-

Additional experiments were per-

formaldehyde-fixed platelets were used. Affinities were slightly

platelets indicated two classes of binding sites, one of high

with Cvx (Fig. 4). Platelets also bound to immobilized collagen

produced a dose-dependent increase in [Ca2+]i. Following a lag

Human Platelets Adhere to Immobilized Cvx—51Cr-Labeled

platelets were found to bind to microtitration plates coated

with Cvx (Fig. 4). Platelets also bound to immobilized collagen

under similar static conditions. Platelet adhesion was a func-

tion of the coating concentration in both cases and reached

a maximum for Cvx and collagen concentrations ≥ 10 μg/ml

(data not shown). A total of 43 ± 2% of the platelets were bound

to immobilized Cvx after 1 h of incubation when the proportion

of platelets bound to immobilized collagen was 2-fold lower and

less than 0.5% were bound to immobilized BSA. The number of

platelets bound to Cvx and collagen decreased to 12.7 ± 0.5%

and 8.6 ± 0.8%, respectively, due to inhibition of platelet ag-

gregation when experiments were performed in the presence

of 300 μM RGDS (Fig. 4). These results indicate that platelet

aggregation is more important in Cvx-coated wells than colla-

gen-coated wells and confirms that Cvx has a high potency for

inducing platelet activation. The proportion of platelets bound
to Cvx remained unchanged in the presence of EDTA (2 mM) as

in the presence of RGDS, but platelet adhesion to collagen was

prevented by EDTA (Fig. 4), as reported previously (14).

125I-Cvx Binds to Human Platelets—125I-Cvx bound to

washed platelets in a time- and concentration-dependent man-

ner (Fig. 5). Nonspecific binding measured in the presence of

a 100-fold excess of unlabeled Cvx represented less than 10% of

the total radioactivity added. Association of 125I-Cvx with hu-

man platelets at 37 °C was rapid and reached a steady state at

15 min (Fig. 5A). Addition of a 500-fold excess of unlabeled Cvx

15 min after the addition of 125I-Cvx to platelets showed, in

contrast, that dissociation was very slow, with less than 1% of

the bound 125I-Cvx being displaced per minute in the presence

of a 100-fold excess of unlabeled Cvx (data not shown). Scat-

chard plot analysis of specific binding of 125I-Cvx to human

platelets indicated two classes of binding sites, one of high

affinity and low capacity and one of moderate affinity and
capacity (Table I). Comparable results were obtained when

formaldehyde-fixed platelets were used. Affinities were slightly

lower in this case, but receptor number was increased for both

classes of sites (Table I). Additional experiments were per-

formed in the presence of sugars such as 45 mM galactose or

mannose or in the presence of 10 μg/ml WGA, suspected pre-

viously to interact with the Cvx binding site on platelets (15),
to rule out the possibility that Cvx binding to platelets might be
due to a lectin-like activity. None of the above compounds
modified Cvx binding to platelets (data not shown).

Integrin αβ6 and GPVI Are Involved in Cvx-induced Platelet

Activation—Specific antibodies to known membrane glycopro-
teins were tested for their effect on Cvx-induced platelet activa-
tion to discover whether these glycoproteins were involved in
Cvx interaction with platelets. We tested antibodies to glyco-
proteins already identified as receptors for different cell ago-
nists or ligands, such as the GPIb-IX complex (receptor for
von Willebrand factor), the integrin αβ6, and GPVI (receptors
for collagen), and GPIV (CD 36), which has a less clearly
defined function and may act as a collagen and/or throm-
bospondin receptor (16). Monoclonal antibodies with inhibitory
activity against GPIbα (SZ2), GPIV (FA6-152), and GPV
(SW16) had no effect on Cvx-induced platelet aggregation,
platelet adhesion to Cvx, or Cvx binding to platelets (data not
shown). Cvx-induced platelet aggregation and secretion were
both inhibited, in contrast by 6F1, a monoclonal antibody
against integrin αβ6, and by polyclonal anti-GPVI Fab frag-
ments (Fig. 6, A and B). Inhibition by 6F1 was con-
Another monoclonal anti-3 was total for low concentrations of Cvx (data not shown). The concentrations of 6F1, Gi9, and anti-GPVI Fab fragments that inhibited Cvx-induced platelet aggregation also inhibited collagen-induced platelet aggregation (data not shown and Refs. 9 and 10). The increase in [Ca\textsuperscript{2+}], concentration induced by Cvx was prevented by preincubation of platelets with the anti-GPVI Fab fragments (Fig. 6C). 6F1 (1 \mu g/ml) did not decrease the signal induced by Cvx in contrast but completely inhibited Ca\textsuperscript{2+} mobilization induced by collagen (Fig. 6C).

The effects of 6F1 and anti-GPVI Fab-fragments on platelet adhesion to immobilized Cvx and collagen were also analyzed. All experiments were performed in the presence of 300 \mu M RGDS to avoid platelet aggregation. None of the antibodies modified the number of \textsuperscript{125}I-labeled platelets bound to immobilized Cvx after a 60-min incubation, whereas 6F1 inhibited platelet adhesion to collagen by 90\% (data not shown), as shown by others (9). We used shorter incubation times (5 min for Cvx and 15 min for collagen) to increase the chances of observing an inhibition since binding of Cvx to platelets is rapid, whereas dissociation is very slow. Approximately the same percentage of platelets adhered to the two proteins under these conditions. Platelet adhesion to Cvx at 5 min was not significantly reduced by the presence of 6F1 (even at concentrations up to 10 \mu g/ml), but platelet adhesion to collagen at 15 min was clearly inhibited by 6F1 at a concentration as low as 0.5 \mu g/ml (Fig. 7). Gi9 also failed to inhibit platelet adhesion to Cvx, but it inhibited platelet adhesion to collagen (data not shown). The anti-GPVI Fab fragments (0.2 mg/ml) inhibited platelet adhesion to Cvx significantly in contrast, and also inhibited platelet adhesion to collagen at 15 min (Fig. 7).

**Cvx Binds to GPVI**—The possibility that Cvx recognizes a specific platelet protein was tested using a ligand blotting assay, since Cvx has a very high affinity for platelets. Binding of \textsuperscript{125}I-Cvx was observed to occur on a single band of 57,000 \textsuperscript{a} protein recognized by \textsuperscript{125}I-Cvx was similar to that reported for Cvx-induced platelet aggregation but was less potent than 6F1, inhibition being 30\% and 80\% with 1 \mu g/ml and 5 \mu g/ml of Gi9, respectively. The anti-GPVI Fab fragments, at 0.2 mg/ml, produced a ~50\% inhibition of platelet aggregation and secretion induced by 30–40 \textsuperscript{pM} Cvx. As with 6F1, inhibition of platelet aggregation and secretion by the anti-GPVI Fab fragments was overcome by increasing the concentration of Cvx. Control experiments showed that nonimmune human Fab had no effect on Cvx-induced platelet aggregation and secretion (data not shown). The concentrations of 6F1, Gi9, and anti-GPVI Fab fragments that inhibited Cvx-induced platelet aggregation also inhibited collagen-induced platelet aggregation (data not shown and Refs. 9 and 10). The increase in [Ca\textsuperscript{2+}], concentration induced by Cvx was prevented by preincubation of platelets with the anti-GPVI Fab fragments (Fig. 6C). 6F1 (1 \mu g/ml) did not decrease the signal induced by Cvx in contrast but completely inhibited Ca\textsuperscript{2+} mobilization induced by collagen (Fig. 6C).

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**Cvx Binds to GPVI**—The possibility that Cvx recognizes a specific platelet protein was tested using a ligand blotting assay, since Cvx has a very high affinity for platelets. Binding of \textsuperscript{125}I-Cvx was observed to occur on a single band of 57,000 \textsuperscript{a} protein recognized by \textsuperscript{125}I-Cvx was similar to that reported for Cvx-induced platelet aggregation but was less potent than 6F1, inhibition being 30\% and 80\% with 1 \mu g/ml and 5 \mu g/ml of Gi9, respectively. The anti-GPVI Fab fragments, at 0.2 mg/ml, produced a ~50\% inhibition of platelet aggregation and secretion induced by 30–40 \textsuperscript{pM} Cvx. As with 6F1, inhibition of platelet aggregation and secretion by the anti-GPVI Fab fragments was overcome by increasing the concentration of Cvx. Control experiments showed that nonimmune human Fab had no effect on Cvx-induced platelet aggregation and secretion (data not shown). The concentrations of 6F1, Gi9, and anti-GPVI Fab fragments that inhibited Cvx-induced platelet aggregation also inhibited collagen-induced platelet aggregation (data not shown and Refs. 9 and 10). The increase in [Ca\textsuperscript{2+}], concentration induced by Cvx was prevented by preincubation of platelets with the anti-GPVI Fab fragments (Fig. 6C). 6F1 (1 \mu g/ml) did not decrease the signal induced by Cvx in contrast but completely inhibited Ca\textsuperscript{2+} mobilization induced by collagen (Fig. 6C).

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Cvx Binds to GPVI—The possibility that Cvx recognizes a specific platelet protein was tested using a ligand blotting assay, since Cvx has a very high affinity for platelets. Binding of \textsuperscript{125}I-Cvx was observed to occur on a single band of \textit{M} = 57,000 \pm 1000 (Fig. 8A) after separation of proteins from whole platelet lysates by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes. Labeling of this band was not observed when the incubation was performed in the presence of a 100-fold excess of unlabeled Cvx, or 5 \mu g/ml anti-Cvx antibody, indicating that binding was specific. No binding of labeled Cvx was observed when the platelet proteins were reduced with 5\% \textit{β}-mercaptoethanol before electrophoresis. Binding was not inhibited in the presence of 5 \mu g/ml WGA, and was still observed after pretreatment of platelets with 5 \textit{nM} thrombin, or 3 \mu g/ml protease of \textit{Serratia marcescens} or 1 \mu M \textit{α}-chymotrypsin, respectively (data not shown). The latter findings indicated that this 57-kDa protein was neither a binding site for WGA nor degraded to any great extent by these proteases. We also observed a protein band migrating at the same position in membrane extracts obtained after cellular fractionation (data not shown). It appeared likely that this 57-kDa platelet protein corresponded to GPVI since the size of the protein recognized by \textsuperscript{125}I-Cvx was similar to that reported for GPVI (10, 15, 16) and platelet activation by Cvx was inhibited by the anti-GPVI Fab fragments. Immunoprecipitates of platelet lysates obtained with the anti-GPVI IgG were probed with \textsuperscript{125}I-Cvx to test this possibility. Fig. 8B shows that the immu-
Platelets preincubated with 0.5 mg/ml control platelets without antibodies (closed bars) or 0.5 mg/ml 6F1 (dotted bars). The results are expressed as the percentage of control release measured in the absence of antibodies. The values are from one representative experiment out of five. C, increase in [Ca$^{2+}$] was induced by the addition of 80 pM Closer 2 (upper panel) or 5 mg/ml collagen (lower panel) to control platelets without antibodies (1), platelets preincubated with 0.5 mg/ml 6F1 (2), 1 mg/ml 6F1 (3), 200 mg/ml anti-GPVI Fab fragments (4).

The present study demonstrates that Cvx is a very potent activator of human platelets and that the membrane glycoproteins GPVI and integrin $\alpha_2\beta_1$ are both involved in the activation pathway. Cvx at picomolar concentrations induces extensive activation of stirred platelets, resulting in exocytosis of dense granules and aggregation. Although some authors (6) have reported that Cvx-induced activation of rabbit platelets is calcium-dependent, our results show that Cvx-induced platelet activation of human platelets occurs in the absence of external Ca$^{2+}$ and is in agreement with the findings of Sano-Martins and Daimon (15). However, Cvx induces an increase in [Ca$^{2+}$]. The kinetic of Cvx-induced Ca$^{2+}$ mobilization is slow when compared with the very rapid response induced by other agonists such as thrombin (19), and the lag preceding Ca$^{2+}$ mobilization is more similar to that observed with collagen (see Fig. 6). It has been demonstrated recently that Cvx induces the activation of phospholipase C$\gamma$ by a protein-tyrosine kinase-dependent pathway. Tyrosine phosphorylation and activation of PLC$\gamma$2 have already been reported in collagen-induced platelet activation (20, 21), and a relationship between the mechanisms of collagen- and Cvx-induced platelet activation has been suspected previously since rabbit platelets exposed to convulxin become refractory to subsequent exposure to collagen (6). Our results provide further evidence for similarities between Cvx and collagen interaction with human platelets. Indeed, integrin $\alpha_2\beta_1$ and GPVI are already known to act as co-receptors for collagen-induced platelet activation. Thus, platelet adhesion to immobilized collagen, platelet aggregation induced by collagen fibers, and collagen-induced phosphorylation of the non-receptor tyrosine kinase Syk and of PLC$\gamma$2 are inhibited by anti-$\alpha_2\beta_1$ monoclonal antibodies (9, 22). Human polyclonal anti-GPVI IgG, on the other hand, activates normal platelets but not GPVI-deficient platelets, and the Fab fragments from these IgGs inhibit collagen-induced platelet acti-

**DISCUSSION**

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**FIG. 7. Effect of the anti-$\alpha_2\beta_1$ integrin and anti-GPVI antibodies on platelet adhesion to Cvx and to collagen.** $^{35}$Cr-Labeled platelets in reaction buffer containing 300 mg/ml RGDS were preincubated with reaction buffer (open bars), 1 mg/ml 6F1 (hatched bars), 200 mg/ml anti-GPVI Fab fragments (dotted bars). Adhesion to Cvx-coated wells was measured after 5 min of incubation, whereas adhesion to collagen-coated wells was measured after 15 min of incubation. Results are expressed as the percentage of control platelet adhesion measured in the absence of antibodies. Each value was determined in triplicate, and the results are the mean ± S.E. of at least three independent experiments.
by the F(ab')2 fragments of the anti-GPVI IgG (27). The αβ1 integrin is also important for Cvx-induced platelet activation since the anti-αβ1 antibodies, 6F1 and to a lesser extent Gi9, inhibited Cvx-induced platelet aggregation and exocytosis as well as collagen induced platelet aggregation (9). However, the role played by αβ1 in platelet activation by Cvx appears to differ from that observed in collagen-induced platelet activation. Indeed, while platelet adhesion to collagen is mediated by αβ1 in a Mg²⁺-dependent manner (9, 14), platelet adhesion to Cvx is Mg²⁺-independent and is not affected by the anti-αβ1 antibody 6F1. In addition, 6F1 inhibited collagen-induced increase in [Ca²⁺]i but not Cvx-induced increase in [Ca²⁺]i. The mechanism by which 6F1 prevents Cvx-induced aggregation and secretion is still unclear, and, as for collagen, the mechanism by which αβ1 and GPVI cooperate in producing a total platelet response to Cvx remains to be determined.

Equilibrium binding studies have identified two types of Cvx-binding sites on human platelets. The Kd value measured for the very high affinity binding sites (8 × 10⁻¹¹ M) is consistent with that reported for rabbit platelets (8) and with the concentration of Cvx that induces platelet activation (see Fig. 1). The slight differences in Kd and Bmax values noted between washed and fixed platelets may be due to modifications in the distribution of glycoproteins following Cvx-induced activation of washed platelets (28) or from modifications due to platelet fixation with formaldehyde. Data from the present study did not permit identification of the nature of the high and moderate affinity binding sites. The anti-αβ1 integrin monoclonal antibody 6F1 did not significantly modify ¹²⁵I-Cvx binding to platelets (data not shown) nor did it reduce platelet binding to immobilized Cvx, even under conditions of pre-equilibrium (short incubation) or at low Cvx concentrations (<100 pM). In addition, the scarcity of Fab fragments from the anti-GPVI IgG, which inhibited the binding of platelets to immobilized Cvx, prevented their use in equilibrium binding studies. The estimated number of surface αβ1 sites varies among platelets from different individuals from 1000 to 2000 (29), i.e. within the range for the moderate affinity binding sites for Cvx. The number of surface GPVI sites has not been reported to date.

Platelet activation by Cvx may thus occur via activation of an unidentified signaling pathway coupled to GPVI and/or αβ1, or from the cross-linking of these two membrane receptors by Cvx. Several recent observations support the second hypothesis. Indeed, clustering of platelet surface glycoproteins appears to be a main mechanism by which platelet activation occurs. Thus, homotypic cross-linking of the Fcγ RI IgG receptor by specific antibodies (30), or GPVI by anti-GPVI F(ab')2 fragments (27), as well as heterotypic cross-linking of αβ1 with Fcγ-RII (22) activate protein-tyrosine kinases and PLCγ2. It has been reported previously, using a nondenaturing medium, that Cvx can be present in at least three forms: the 72-kDa (12.5 kDa) Cvx subunits linked by disulfide bonds; and the multimerized 144-kDa and 300-kDa forms of this trimer, organized in a ring-shaped structure (31, 32). All of these molecular forms of Cvx are potentially multivalent and may engage in multiple interactions at the platelet surface, resulting in glycoprotein cross-linking and cell signaling.

Electron microscopy studies have also indicated that Cvx-treated platelets have a decreased capacity to bind WGA. This suggested that Cvx and WGA might share common binding sites(s) on platelets (15). However, no modification in Cvx binding to platelets or GPVI, respectively, was observed in the presence of WGA. It is likely that the decrease in WGA binding, described in earlier studies, might have been due to an activation-
dependent, Cvx-induced redistribution of platelet membrane glycoproteins. Such a mechanism would be comparable to the thrombin-induced internalization of GPIb, which is known to constitute a major binding site for WGA (28).

It is noticeable that the threshold concentration of Cvx required for platelet activation is very low (3 ng/ml), as compared to collagen (10 μg/ml), or the triple helical collagen-like synthetic peptides (>20 ng/ml), which are the simplest structures known to induce platelet activation as collagen (33). Cvx does not appear to be structurally related to collagen. Furthermore, it is not a calcium-dependent lectin, since its interaction with platelets is Ca2+- and sugar-independent. However, Cvx shares structural characteristics with proteins of this family (8). As snake toxins are usually derived from parental physiologically active proteins, it is tempting to hypothesize that Cvx may be analogous to an as yet unidentified molecule in man, associated with a new platelet activation pathway, which might be of physiological importance.

In conclusion, the results of the present work demonstrate that platelet membrane GPVI is a platelet Cvx receptor and that integrin α2β1 is required for Cvx-induced platelet aggregation. Definition of the respective roles played by these glycoproteins in the signaling events evoked by convulxin is therefore of interest.

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Note added in proof—Since the submission of this manuscript, Polgár et al. (34) reported, in agreement with our results, that Cvx binds to GPVT.

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