Rhodocytin (Aggretin) Activates Platelets Lacking $\alpha_2\beta_1$ Integrin, Glycoprotein VI, and the Ligand-binding Domain of Glycoprotein Ib-$\alpha$*

Received for publication, May 1, 2001
Published, JBC Papers in Press, May 14, 2001, DOI 10.1074/jbc.M103892200

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Although $\alpha_2\beta_1$ integrin (glycoprotein Ia/IIa) has been established as a platelet collagen receptor, its role in collagen-induced platelet activation has been controversial. Recently, it has been demonstrated that rhodocytin (also termed aggretin), a snake venom toxin purified from the venom of Calloselasma rhodostoma, induces platelet activation that can be blocked by monoclonal antibodies against $\alpha_2\beta_1$ integrin. This finding suggested that clustering of $\alpha_2\beta_1$ integrin by rhodocytin is sufficient to induce platelet activation and led to the hypothesis that collagen may activate platelets by a similar mechanism. In contrast to these findings, we provided evidence that rhodocytin does not bind to $\alpha_2\beta_1$ integrin. Here we show that the Cre/loxP-mediated loss of $\beta_1$ integrin on mouse platelets has no effect on rhodocytin-induced platelet activation, excluding an essential role of $\alpha_2\beta_1$ integrin in this process. Furthermore, proteolytic cleavage of the 45-kDa N-terminal domain of glycoprotein (GP) Ib-$\alpha$ either on normal or on $\beta_1$-null platelets had no significant effect on rhodocytin-induced platelet activation. Moreover, mouse platelets lacking both $\alpha_2\beta_1$ integrin and the activating collagen receptor GPVI responded normally to rhodocytin. Finally, even after additional proteolytic removal of the 45-kDa N-terminal domain of GPIb-$\alpha$ rhodocytin induced aggregation of these platelets. These results demonstrate that rhodocytin induces platelet activation by mechanisms that are fundamentally different from those induced by collagen.

Collagen is one of the major components of the vessel wall and contributes to platelet activation and adhesion at sites of vascular injury. The interaction between platelets and collagen can either occur indirectly via immobilized von Willebrand factor (vWF)1 binding to platelet receptors glycoprotein (GP) Ib-V-IX and/or activated $\alpha_{IIb}\beta_3$ integrin (1) or by direct recognition of collagen by specific receptors expressed on the platelet surface. Several receptors for collagen have been identified on platelets, most importantly the Ig-like receptor GPVI (2) and $\alpha_2\beta_1$ integrin (3). In contrast to earlier reports, we have recently shown with $\beta_1$ integrin-null platelets that $\alpha_2\beta_1$ integrin is not essential for platelet adhesion to fibrillar collagen. GPVI, however, was found to be indispensable for this process (4).

Although GPVI has been established as the major activating platelet collagen receptor, the way $\alpha_2\beta_1$ integrin modulates the activation process is still unclear. Experimental evidence suggests that collagen contains two distinct epitopes contributing to activation of (murine) platelets. One of these epitopes specifically binds to GPVI, and this interaction is blocked by the anti-GPVI mAb JAQ1 (5). In contrast, activation through the second epitope is not blocked by JAQ1 and involves GPVI, $\alpha_2\beta_1$ integrin, and high concentrations of fibrillar collagen (4). The mechanisms underlying this activation pathway and the role of $\alpha_2\beta_1$ integrin are unclear. In addition to $\alpha_2\beta_1$ and GPVI, other receptors may be involved in this activation pathway. One candidate is GPIb-$\alpha$, because this receptor indirectly interacts with collagen via vWF (1).

Snake venom-derived proteins are frequently used to study mechanisms of platelet activation and aggregation because many of them specifically bind to platelet surface glycoprotein receptors and interfere with their function. Rhodocytin (also termed aggretin (6)), purified from the venom of Calloselasma rhodostoma belongs to the family of C-type lectins and induces aggregation of human as well as mouse platelets (7). Recent studies gave rise to conflicting results on the mechanisms underlying this activation process. Several experiments suggested that rhodocytin activates platelets in a collagen-like manner. First, both processes are sensitive to inhibition of thromboxane A2 formation by treatment with acetylsalicylic acid, and, second, both can be inhibited by mAbs against $\alpha_2\beta_1$ integrin (7, 8). Based on these results, the authors concluded that rhodocytin activates platelets by interacting with $\alpha_2\beta_1$ integrin (8). Others reported that rhodocytin activates platelets through $\alpha_2\beta_1$ integrin and GPIb-$\alpha$ (9), results that were also based on experiments with inhibitory antibodies against both receptors.

Both hypotheses, however, were challenged by our finding that rhodocytin does not bind recombinant, soluble $\alpha_2\beta_1$ (10), and the same result has meanwhile been obtained with wild-body; mAb, monoclonal antibody; PE, R-phycoerythrin; MES, 4-morpholineethanesulfonic acid.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (to B. N.) and the Swedish Research Foundation (to C. B. and R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: vWF, von Willebrand factor; FcR, Fc receptor; FITC, fluoresceine isothiocyanate; GP, glycoprotein; Ab, antibody; mAb, monoclonal antibody; PE, R-phycoerythrin; MES, 4-morpholineethanesulfonic acid.
type αβ₁ integrin isolated from human platelets. Additionally, rhodocytin activates platelets from FcγR chain-deficient mice (7, 9), which lack GPVI (11) and do not respond to collagen (4, 12), suggesting that rhodocytin uses other mechanisms than collagen to induce aggregation. Moreover, we have recently shown that β₁-null platelets fail to express αβ₁ integrin but display no reduced response to fibrillar collagen, indicating that αβ₁ integrin is not a major signaling collagen receptor on platelets (4).

To directly test whether rhodocytin induces platelet activation by mechanisms similar to those induced by collagen, we now examined the effects of this agonist on platelets lacking αβ₁ integrin, GPVI, the ligand-binding domain on GPIbα, or all three of them. The results of these studies demonstrate that none of these receptors is required for platelet activation by rhodocytin.

**EXPERIMENTAL PROCEDURES**

**Animals: Generation of Mice with β₁-null Platelets**—Mice carrying the β₁-null allele in megakaryocytes were generated as described previously (4). Briefly, β₁(+/fl) mice (13) were crossed with transgenic mice carrying the Mx-cre transgene (mx-cre+). Deletion of the β₁ gene was induced in 4-5-week-old (β₁(+/fl)/Mx-cre+) mice by three intraperitoneal injections of 250 μg of polyinosinic-polycytidylic acid at 2-day intervals. Control mice (β₁(+/fl)) received the same treatment and were derived from same litters. For experiments, mice were used at least 2 weeks after polyinosinic-polycytidylic acid injection. The absence of the α₂ and β₁ integrin subunits on the platelets from these mice was always confirmed by flow cytometry and Western blotting as described (4). C57BL/6 mice deficient in the FcγR chain (15) were obtained from Taconics (Germantown, NY). C57BL/6 × SV129 mice deficient in GPV chain-deficiency were kindly provided by F. Lanza (Strasbourg, France) and G. Dickneite (Marburg, Germany), respectively.

**Depletion of Platelet GPVI**—Mice were injected with 100 μg of JAQ1 intraperitoneally, and platelets were isolated on day 7. As reported previously, GPVI was not detectable on those platelets by flow cytometry and Western blotting (16).

**Purification of Rhodocytin**—C. rhodostoma (Malayan pit viper) venom was purchased from Sigma. Dissolved in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, the venom was separated by gel filtration chromatography on a Superose 6 column. The eluate fractions of respective size were pooled. After dilution with Mono S-buffer A (20 mM MES/NaOH, pH 6.5), the pooled fractions were applied to a Mono S HR5/5 column (Amersham Pharmacia Biotech). Not binding to Mono S resin under these conditions, the rhodocytin in the flow-through was dialyzed against Mono S-buffer A (20 mM Tris/HCl, pH 8.0, 0.02% sodium azide) and applied to a Mono Q HR5/5 column (Amersham Pharmacia Biotech). Rhodocytin was eluted from the Mono Q column in a linear NaCl gradient at sodium chloride concentrations above 300 mM. The rhodocytin-containing eluate fraction was concentrated by centrifugal ultrafiltration in a Centricon 10 tube. Finally, the concentrated solution of rhodocytin was purified by gel filtration on a tandem array of TSK G3000 SWXL and TSK G2000 SWXL (TosoHass, Stuttgart, Germany). Purity was determined by SDS-polyacrylamide gel electrophoresis in a 12-18% acrylamide separating gel. Protein concentration was assayed by the BCA method according to the manufacturer’s protocol (Pierce).

**Antibodies**—The rat anti-mouse P-selectin mAb RB40.34 was kindly provided by D. Veetweber (Münster, Germany) and modified in our laboratories. FITC hamster anti-β₁ integrin (Ha31/8), FITC hamster anti-α₂ integrin, and rat anti-β₁ integrin (9EG7) were from BD Pharmingen. Horseradish peroxidase-labeled rabbit anti-FITC, polyclonal rabbit anti-fibrinogen, and polyclonal rabbit anti-vWF were purchased from DAKO. All other antibodies were generated, produced, and modified in our laboratories: JAQ1 (anti-GPVI) (11), JON1 (anti-GPIIb/IIIa) (18), p0p4 (anti-GPIIb) (18), DOM1 (anti-GPV) (19), and ULP1 (anti-CD9) (19).

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*J. A. Eble, unpublished results.*
Platelet Preparation—Mice were bled under ether anesthesia from the retroorbital plexus. The blood was collected in a tube containing 10% (v/v) 7.5 units/ml heparin, and platelet-rich plasma was obtained by centrifugation at 3000 g for 10 min at room temperature. The platelets were washed twice in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO3, 0.3 mM NaH2PO4, 2 mM CaCl2, 5.5 mM glucose, 5 mM Hepes, pH 7.3) containing 0.35% bovine serum albumin and finally resuspended at a density of $2 \times 10^5$ platelets/ml in the same buffer in the presence of 0.02 unit/ml of the ADP scavenger apyrase, a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. Platelets were kept at 37 °C throughout all experiments.

Immunoblotting—Platelets (10^8) were solubilized in 1 ml of lysis buffer (Tris-buffered saline containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotonin, 0.5 μg/ml leupeptin, and 0.5% Nonidet P-40 all from Roche Molecular Biochemicals). After lysis, whole cell extract was run on a 9% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was first incubated with 5 μg/ml FITC-labeled mAb followed by rabbit anti-FITC-horseradish peroxidase (1 μg/ml). The proteins were visualized by ECL.

Flow Cytometry—Washed platelets (2 × 10^6) were incubated with the indicated amounts of agonists for 5 min followed by staining with fluorophore-conjugated Abs (5 μg/ml) for 10 min at 37 °C and immediately analyzed on a FACScalibur (Becton Dickinson). Platelets were gated by forward scatter/side scatter characteristics.

Treatment of Platelets with O-Sialoglycoprotein Endopeptidase—The washed platelets (2 × 10^6/ml) were resuspended in Tyrode’s buffer (1 mM MgCl2, 1 mM CaCl2) and incubated at 37 °C for 30 min with 100 μg/ml O-sialoglycoprotein endopeptidase. Aliquots of the platelet suspensions were analyzed in flow cytometry and Western blotting to estimate markers of platelet activation and alterations in platelet glycoproteins.

Aggregometry—To determine platelet aggregation, light transmission was measured using washed platelets (200 μl with 0.5 × 10^6 platelets/μl). Transmission was recorded on a Fibrin-4 channel aggregometer (LAbor, Hamburg, Germany) over 10 min and was expressed as arbitrary units with 100% transmission adjusted with Tyrode’s buffer.

RESULTS

It has been reported that rhodocytin induces platelet aggregation by interacting with the collagen receptor $\alpha_2\beta_1$ integrin. Based on these results, it was suggested that collagen might also activate platelets via $\alpha_2\beta_1$ integrin (7). These findings were challenged by our result showing that rhodocytin does not bind to a recombinant soluble form of $\alpha_2\beta_1$ integrin (10).

To directly investigate whether rhodocytin activates platelets through mechanisms similar to those induced by collagen, we compared the effects of rhodocytin and fibrillar collagen on mouse platelets lacking the collagen receptors $\alpha_2\beta_1$ integrin and GPVI. As reported previously, FcRγ chain-deficient platelets lack GPVI but express normal amounts of $\alpha_2\beta_1$ integrin (Fig. 1a) and do not respond to collagen (11, 12) (Fig. 1b). In contrast, platelets from mice with a Cre/loxP-mediated deletion of the $\beta_1$ gene in megakaryocytes lack all $\beta_1$ integrins but express normal amounts of GPVI (Fig. 1a). $\beta_1$-null platelets

![Fig. 2](image-url) Rhodocytin activates platelets independently of $\alpha_2\beta_1$ integrin. a, washed platelets from the indicated mice were stimulated with rhodocytin (5 or 50 nM), and light transmission was recorded on a standard aggregometer. b, washed platelets from the indicated mice were left untreated (lower panel) or stimulated with rhodocytin (10 nM, upper panel) and subsequently incubated with anti-fibrinogen-FITC and anti-P-selectin-PE Abs for 10 min at 37 °C and analyzed directly. The platelets were gated by forward scatter/side scatter characteristics and Fl3 positivity (anti-mouse GPIbα-PE/Cy5). The results shown are representative of six individual experiments.
was indistinguishable between platelets. The extent of degranulation and fibrinogen binding completed other receptors, including degradation of GPVI in circulating platelets but does not affect induces a virtually complete internalization and proteolytic (Fig. 2 b).

Mice with GPVI-depleted platelets do not respond to collagen, whereas differently of GPVI/FcR

activation/aggregation. Therefore, we examined the effects of Thus, blocking only one of them would not inhibit platelet platelet activation by interacting with multiple receptors (20). It has been shown that snake venom toxins may induce aggregation of human platelets (7–9). Aggregation occurred in rhodocytin-induced platelet activation. To directly test this hypothesis, we treated platelets with botrocetin (100 μg/mouse). This treatment resulted in complete proteolytic removal of the 45-kDa N-terminal domain of GPIb as demonstrated by flow cytometric analysis (Fig. 4 c). This finding excludes an essential role of α2β1 and GPVI in rhodocytin-induced platelet activation.

A very recent report showed that rhodocytin (aggretin)-induced platelet activation was inhibited by a mAb against the 45-kDa N-terminal domain on GPIbα (9). This domain contains the binding sites for all known ligands, including vWF, thrombin (21), P-selectin (22), and MAC-1 (23), as well as snake venom-derived C-type lectins like jararaca GPIb-BP (24), alboagreggin A (20), and echecitin (25). Based on their results, Navdaev et al. (9) concluded that GPIbα plays an essential role in rhodocytin-induced platelet activation. To directly test this hypothesis, we treated platelets with O-sialoglycoprotein endopeptidase (26). This treatment resulted in complete proteolytic removal of the 45-kDa N-terminal domain of GPIbα as demonstrated by flow cytometric analysis (Fig. 4 a). Interestingly, Western blot analysis revealed that, in addition to cleavage of the 45-kDa N-terminal domain, the truncated remainder of GPIbα (105 kDa) was further cleaved in close vicinity to the transmembrane region of GPIbα, resulting in the release of glycopcalicin lacking the 45-kDa N-terminal region (~85 kDa) (Fig. 4 e). Because of the complete lack of the 45-kDa N-terminal domain of GPIbα, botrocetin-induced vWF binding was abolished in O-sialoglycoprotein endopeptidase-treated platelets (Fig. 4 c). However, these platelets responded normally to
rhodocytin (Fig. 4, d and e), demonstrating that the ligand-binding domain on GPIbα is not essential for this activation process.

Although our results demonstrated that neither the collagen receptors α2β1 integrin and GPVI nor GPIbα are essential for rhodocytin-induced platelet activation, it could not be excluded that rhodocytin binds to all three receptors that independently elicit aggregation. Therefore, we removed the 45-kDa N-terminal region of GPIbα from β1- and β1/GPVI-deficient platelets and examined their response to rhodocytin. As shown in Fig. 5, even the absences of α2β1, GPVI, and the 45-kDa N-terminal domain of GPIbα had no significant effect on rhodocytin-induced platelet activation and aggregation.

**DISCUSSION**

The mechanism underlying platelet activation by rhodocytin (aggretin) has been debated. Several investigators suggested that rhodocytin activates platelets in a collagen-like manner by interacting with α2β1 integrin or α2β1 integrin and GPIbα (7, 20). In contrast to these hypotheses, we reported that rhodocytin does not bind to α2β1 integrin (10).

In the present study we demonstrate that rhodocytin induces activation of murine platelets in the absence of α2β1 integrin, GPVI, and the ligand-binding domain of GPIbα. These are the three major receptors that directly or indirectly interact with collagen. Our findings exclude an essential role for these receptors in rhodocytin-induced activation and demonstrate that the activation process follows mechanisms that are fundamentally different from those induced by collagen. Our present findings are in contrast to those reported by others (7, 9), who showed that mAbs against α2β1 blocked rhodocytin-induced platelet aggregation and concluded that the integrin plays an essential role in the activation process. An explanation for this discrepancy could be that treatment of platelets with mAbs against α2β1 may have different effects than the absence of the receptor. Antibodies may exert steric effects on other cell surface proteins or may elicit inhibitory signals. Previously, results from inhibition studies with some antibodies against α2β1 could not be confirmed in a genetic model in which the β1 gene is deleted in all hematopoietic cells, including megakaryocytes. It was shown that antibodies against α2β1 integrin markedly reduced or abolished platelet adhesion and aggregate formation on collagen in stasis and flow (27) as well as collagen-induced platelet aggregation (7). Using the Cre/loxP technology, we ablated the β1 gene in megakaryocytes and showed that α2β1 integrin is not required for platelet adhesion and thrombus formation on fibrillar collagen under static as well as low and high shear flow conditions. β1-null platelets display a delayed but not reduced aggregation in response to collagen, demonstrating that α2β1 integrin is not a major signaling collagen receptor on platelets (4). The investigations with convulxin provide another example in which antibody inhibition and gene deletion studies showed conflicting results. Whereas the action of convulxin can be inhibited with some antibodies against α2β1 (28), Cre/loxP-mediated ablation of β1 integrin on platelets revealed no detectable role of α2β1 for platelet activation by this agonist (4). Together, these findings strongly suggest that certain antibodies against α2β1 integrin induce inhibitory effects that are not based on blockage of the integrin. Therefore, treatment of platelets with anti-α2β1 antibodies may not be suitable for determining dependence on α2β1 integrin.

Another striking difference between rhodocytin- and collagen-mediated platelet aggregation is the central role of GPVI for collagen but not for rhodocytin. We showed recently that GPVI is the major collagen receptor for platelet activation and that GPVI is essential for collagen-induced platelet aggregation (16). Therefore, GPVI-independent aggregation processes are different from collagen-induced aggregation. Both FeRγ-null platelets, which lack GPVI (11), and GPVI-depleted platelets (16) fail to activate β1 and β3 integrins in response to collagen. Consequently, these platelets neither adhere to collagen nor do they bind adhesive ligands or aggregate in response to this agonist (4, 12, 16). From these data, we conclude that rhodocytin activates platelets by mechanisms that are different from those induced by collagen.

Navdaev and co-workers (9) proposed a mechanism for rhodocytin (aggretin)-induced platelet activation that involves two platelet receptors, α2β1 integrin and GPIbα. The importance of GPIbα for rhodocytin-induced aggregation was concluded from a dose-dependent inhibitory effect of a mAb directed against the thrombin-binding site on GPIbα, which is located in the 45-kDa N-terminal region of the receptor (29). This finding was in contrast to observations made by other investigators who found no role of GPIb in rhodocytin-induced activation (30, 31) or no binding of rhodocytin to GPIb (7). The discrepancies in the binding studies are difficult to explain. They may be related to different experimental conditions used in these studies. In our present study we show that platelets lacking the 45-kDa N-terminal domain on GPIbα respond normally to rhodocytin (Fig. 5). This finding excludes an essential role for the ligand-binding region of GPIbα in the rhodocytin-induced activation process and is in clear contrast to the finding by Navdaev and co-workers (9). Steric hindrance or elicitation of inhibitory signals by the GPIbα-specific antibody could be an explanation for the conflicting results. It is known that occupancy of GPIbα induces tyrosine phosphorylation of different signaling molecules in vitro (32–34) and that dimerization of GPIbα by certain mAbs affects platelet function by yet un-
defined mechanisms in vitro and in vivo (18, 35, 36).

Our results do not exclude the possibility that rhodocytin interacts with an epitope on the GPIb-V-IX complex that is distinct from the 45-kDa N-terminal region of GPIbα. However, in studies using mAbs against different epitopes on either GPIX, GPV, or GPIbα, we were unable to alter rhodocytin-induced activation/aggregation. In addition, GPV-deficient mouse platelets respond normally to rhodocytin (not shown). These results suggest that the GPIb-V-IX complex has no essential role in rhodocytin-induced platelet activation.

Using genetic ablation of αIIbβ3, antibody-mediated depletion of GPV, and proteolytic digestion of GPIb, we show that platelets lacking all three major receptors that directly or indirectly interact with collagen (αIIbβ3, GPV, and GPIb) respond normally to rhodocytin. Because rhodocytin-induced aggregation of human and mouse platelets occurs with a dose-dependent lag time and independently of the FcRγ chain and both pro-

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