Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation

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Collagen-induced platelet aggregation is a complex process and involves synergistic action of integrins, immunoglobulin (Ig)-like receptors, G-protein–coupled receptors and their ligands, most importantly collagen itself, thromboxane A_2 (TXA_2), and adenosine diphosphate (ADP). The precise role of each of these receptor systems in the overall processes of activation and aggregation, however, is still poorly defined. Among the collagen receptors expressed on platelets, glycoprotein (GP) VI has been identified to play a crucial role in collagen-induced activation. GPVI is associated with the FcRγ chain, which serves as the signal transducing unit of the receptor complex. It is well known that clustering of GPVI by highly specific agonists results in platelet activation and irreversible aggregation, but it is unclear whether collagen has the same effect on the receptor. This study shows that platelets from Gαq-deficient mice, despite their severely impaired response to collagen, normally aggregate on clustering of GPVI, suggesting this not to be the principal mechanism by which collagen activates platelets. On the other hand, dimerization of GPVI by a monoclonal antibody (JAQ1), which by itself did not induce aggregation, provided a sufficient stimulus to potentiate platelet responses to Gi-coupled, but not Gq-coupled, agonists. The combination of JAQ1 and adrenaline or ADP, but not serotonin, resulted in $\alpha_{IIb}\beta_3$-dependent aggregation that occurred without intracellular calcium mobilization and shape change in the absence of Gαq or the $P2Y_1$ receptor. Together, these results provide evidence for a cross-talk between (dimerized) GPVI and Gi-coupled receptors during collagen-induced platelet aggregation. (Blood. 2001;97:3829-3835)

Introduction

When the integrity of the vascular endothelium is disrupted, a variety of macromolecular constituents of the subendothelial layer become exposed and accessible to platelets. It is well known that collagen is the most thrombogenic component of the subendothelial layer because it supports not only platelet adhesion but is also a strong platelet activator. Collagen-induced platelet activation is a multistep process and requires a series of extracellular and intracellular events and synergistic action of different receptor systems.[1-3] It is widely accepted that the interaction between platelets and collagen involves firstly adhesion and subsequently, activation, leading to second phase adhesion, secretion, and finally aggregation.[3,4] Platelets express a variety of collagen receptors, including integrin $\alpha_2\beta_1$, glycoprotein (GP) IV, GPVI, and 65- and 85-kd proteins.[2] Although the exact role of each of these receptors in collagen-induced platelet activation is difficult to define, it is commonly accepted that GPVI is critically involved in this process. This is based on the observation that the GPVI-specific agonists, collagen-related peptide (CRP)[5] and convulxin,[6] a snake venom–derived lectin, are powerful platelet activators and that antibodies against GPVI inhibit collagen-induced platelet aggregation.[7,8] Furthermore, platelets from GPVI-deficient patients display severely impaired responses to collagen.[8,9] GPVI is a member of the immunoglobulin superfamily and is associated with the FcRγ chain, which serves as the signal-transducing subunit of the receptor.[10,11] Activation of platelets through GPVI by CRP or by convulxin occurs on clustering of the receptor and subsequent tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) by a src-like kinase.[12,13] Such an activation is independent of classical pathways because it is insensitive to aspirin or to adenosine disphosphate (ADP) scavengers (present study), which are, however, known to influence platelet aggregation induced by collagen. Indeed, it has long been recognized that other agonists than collagen itself are involved in the process of collagen-induced platelet aggregation, particularly ADP and thromboxane A_2 (TXA_2), which are released from activated platelets and amplify the aggregation process. These agonists are absolutely required[14] as evidenced by the effect of aspirin[15] or ADP-selective antiplatelet drugs like clopidogrel[16] or the AR-C compounds[17] on platelet aggregation induced by collagen. Moreover, the main characteristic of patients with storage pool disease, where ADP is lacking in the dense granules, is an impaired response to collagen.[18] Thus, in contrast to the effects of GPVI activators like CRP or convulxin, collagen-induced aggregation appears to critically involve ADP and TXA_2. The receptors for these platelet activators

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couple to several heterotrimeric G proteins, such as Gi, Gq, G_{12}, and G_{13}.[19] The importance of Gq-coupled receptors for collagen-induced platelet aggregation is highlighted by the recent finding that platelets from Gαq-deficient mice are unable to fully aggregate when stimulated with collagen.[20] This effect could be related to a defect in the secretion mechanisms because full aggregation to collagen could be restored by addition of ADP.[21] It has been found that this property of ADP was due to the recently cloned $P2Y_{12}$ receptor[22] and could be mimicked by adrenaline, underlining the role of Gi-coupled receptors in the process of collagen-induced aggregation.[21] On the other hand, the $P2Y_1$ receptor has also been found to play a role in the initial steps of platelet activation by collagen because $P2Y_1$-deficient mouse platelets display inhibition of aggregation to collagen.[23,24] Finally, platelets from Gαq knockout mice still undergo shape change in response to collagen or TXA_2, which is based on $G_{12/13}$ activation and subsequent involvement of the Rho/Rho-kinase pathway resulting in phosphorylation of the myosin light chain.[25,26]

Therefore, GPVI and G-protein–coupled receptors act in concert to exert full platelet aggregation in response to collagen, but the underlying mechanisms have not been completely elucidated. Particularly, the precise role of GPVI is only partly understood. So far, it seems clear that the functional GPVI/FcRγ chain complex is required for collagen-induced aggregation in humans and mice[7,27] and that direct activation of the receptor by cross-linking agents like CRP or convulxin is sufficient to induce full platelet aggregation, suggesting that the latter might not exactly reflect collagen-induced aggregation where G-protein–coupled receptors are involved. Recently, we reported the generation of the first monoclonal antibody (mAb) against mouse GPVI (JAQ1) and demonstrated that the (divalent) mAb, despite its inhibitory effect on collagen-induced platelet aggregation, induced subliminal signaling via the FcRγ chain, which is insufficient to fully activate the cells.[7,28] In the present study, we show that this JAQ1-induced GPVI/FcRγ signaling acts synergistically with Gi-coupled, but not Gq-coupled, receptors to induce activation of the $\alpha_{IIb}\beta_3$-integrin and platelet aggregation that can occur without calcium mobilization and shape change in the absence of Gαq or the $P2Y_1$ receptor. These results provide strong evidence for a cross-talk between GPVI and Gi-coupled receptors during the process of collagen-induced platelet activation. We also show that platelets from Gαq knockout mice, despite their severely impaired response to collagen, fully aggregate on GPVI clustering by JAQ1 cross-linking. Together, these data suggest that collagen might induce dimerization rather than multivalent clustering of GPVI, which then requires cosignals from other, presumably Gi-coupled, receptors to induce platelet aggregation.

Materials and methods

Animals

Mutant mice deficient in Gαq and in the $P2Y_1$ receptor were produced as described.[20,24] Both mutant and wild-type mice were of 129/Sv‖×‖C57BL/6 genetic background.

Chemicals

The ATP analog AR-C69931MX was a generous gift from ASTRA Charnwood (Loughborough, England). The $P2Y_1$ antagonist N6-methyl 2′-deoxyadenosine 3′,5′-bisphophate or MRS2179[29] was synthesized by P. Raboisson (Faculté de Pharmacie, Illkirch, France). ADP, adrenaline, essentially fatty acid free human serum albumin, creatine phosphate (CP), and creatine phosphokinase (CPK) were from Sigma (Saint Quentin-Fallavier, France). Human fibrinogen was from Kabi (Stockholm, Sweden) and fura-2/acetoxymethyl ester (fura-2/AM) was from Calbiochem (Meudon, France). Apyrase was purified from potatoes as previously described.[30] Aspirin was from Synthelabo (Paris, France).

Antibodies

Polyclonal rabbit antibodies to human fibrinogen were purchased from Dako (Glostrup, Denmark) and modified in our laboratories. Rabbit antirat IgG antibodies were also from Dako. The mAbs against mouse GPVI (JAQ1) and mouse $\alpha_{IIb}\beta_3$ have been described previously.[7,31] The anti-mouse $\alpha_{IIb}\beta_3$ antibody, RAM.2,[21] was kindly provided by F. Lanza. Fab fragments from JAQ1 were generated by 12-hour incubation of 10 mg/mL mAb with immobilized papain (Pierce, Bonn, Germany), and the preparations were then applied to an immobilized protein A column followed by an immobilized protein G column (Pharmacia, Uppsala, Sweden) to remove Fc fragments and any undigested IgG. The purity of the Fab fragments was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of the gel.

Platelet aggregation and secretion

Washed mouse platelets were prepared from blood (9 vol) drawn from the abdominal aorta of anesthetized mice into a plastic syringe containing ACD (1 vol). Pooled blood (8 mL) was centrifuged at 1570*g* for 80 seconds at 37°C. Platelet-rich plasma (PRP) was removed and centrifuged at 1570*g* for 15 minutes at 37°C. The platelet pellet was washed twice in Tyrode buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM Hepes, pH 7.3) containing 0.35% human serum albumin and finally resuspended at a density of 2×10^5 platelets/μL in the same buffer in the presence of 0.02 U/mL of the ADP scavenger apyrase (adenosine 5′-triphosphate diphosphohydrolase, EC 3.6.1.5), a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. Platelets were kept at 37°C throughout all experiments.

Aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, ON, Canada). A 450-μL aliquot of platelet suspension was stirred at 1100 rpm and activated by addition of different agonists, with or without antagonists, in the presence of human fibrinogen (0.07 mg/mL), in a final volume of 500 μL. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline level. Secretion was determined as previously described[32] after loading the platelets with [^{3}H]5-HT.

$[Ca^{++}]_i$ measurements

After centrifugation of PRP at 1570*g* for 15 minutes at 37°C, the platelet pellet was resuspended in Tyrode buffer containing no calcium, at a density of 7.5×10^5 platelets/μL, in the presence of 0.02 U/mL apyrase. Platelets were loaded with 15 μM fura-2/AM for 45 minutes at 37°C in the dark, washed in Tyrode buffer containing 0.35% human serum albumin and finally resuspended at 20°C, at a density of 10^5 platelets/μL, in Tyrode buffer containing apyrase and 0.1% essentially fatty acid free human serum albumin. Aliquots of fura-2/AM loaded platelets were transferred to a 10 × 10 mm quartz cuvette and prewarmed to 37°C for 2 minutes and fluorescence measurements were performed under continuous stirring, using a PTI Deltascan spectrofluorimeter (Photon Technology International, Princeton, NJ). The excitation wavelength was alternately fixed at 340 or 380 nm and fluorescence emission was determined at 510 nm.[21]

Flow cytometry

Heparinized PRP was diluted 1:30 with Tyrode buffer. Samples were stimulated with the indicated agonists in the presence of 10 μg/mL JAQ1 or irrelevant rat IgG2a, stained with fluorophore-labeled mAbs for 10 minutes at room temperature, and directly analyzed on a FACScan (Becton Dickinson, Heidelberg, Germany). Platelets were gated by forward/side scatter characteristics and Fl2-positivity (anti-GPIbα^{PE}).[33]

Electron microscopy

A 450-μL aliquot of platelet suspension was fixed in the aggregometer cuvette by addition of an equal volume of fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose, 305 mOsm/L, pH 7.3) previously warmed to 37°C. After 5 minutes at 37°C, the platelets were centrifuged at 700*g* for 20 seconds and the pellet was resuspended in 0.1 M sodium cacodylate buffer. Samples were prepared for scanning electron microscopy by allowing the fixed platelets to adhere for 45 minutes to cover slips preincubated with 10 μg/mL poly-L-lysine. The cover slips were then washed 3 times with 0.9% NaCl and the platelets dehydrated in graded ethanol solutions. After replacement of ethanol by hexadimethyldisilazane, the samples were air-dried, sputtered with gold, and examined under a Hitachi (Tokyo, Japan) scanning electron microscope (5 kV) as described.[21]

Results

JAQ1 potentiates the $P2Y_{12}$ component of platelet aggregation induced by ADP

We recently reported the generation of the first mAb against mouse GPVI (JAQ1) and demonstrated that JAQ1 specifically inhibited collagen-induced platelet aggregation, whereas it had no effect on aggregation induced by thrombin or phorbol myristate acetate (PMA).[7] Further studies showed that JAQ1, despite its inhibitory effect, induces subliminal signaling via the FcRγ chain, which seems to be based on dimerization of GPVI by the mAb.[28] In the present study, we examined the effects of JAQ1 on platelet aggregation induced by other agonists, such as ADP, adrenaline, and serotonin.

As reported previously, JAQ1 (10-180 μg/mL) by itself did not produce any platelet aggregation, whereas on cross-linking with polyclonal antirat IgG antibodies strong and irreversible aggregation occurred (Figure 1A). This aggregation was completely insensitive to aspirin and to ADP scavengers or ADP receptor antagonists, alone or in combination, suggesting it to occur independently of TXA_2 generation and ADP secretion (Figure 1A). As shown in Figure 1B, JAQ1 alone did not cause any change in the intracellular calcium concentration for up to 30 minutes, whereas on cross-linking by antirat IgG antibodies there was a dramatic increase in intracellular calcium concentration detectable.

The reversible aggregation of washed mouse platelets on stimulation with ADP (5 μM) was potentiated and became irreversible in the presence of 10 μg/mL JAQ1 (Figure 2A). This was accompanied by a slight increase in secretion of tritiated serotonin from 0% to 8% (insert). The selective $P2Y_{12}$ receptor antagonist AR-C69931MX (10 μM) completely inhibited platelet aggregation and secretion induced by ADP in the presence of JAQ1 (Figure 2B, lower tracing). However, the shape change response was preserved. It is well known that this concentration of AR-C69931MX blocks platelet aggregation to 5 μM ADP without affecting shape change (not shown). In contrast, the selective $P2Y_1$ receptor antagonist MRS2179 (100 μM), which is known to completely inhibit platelet shape change and aggregation in response to ADP without affecting the Gi-dependent pathways stimulated through the $P2Y_{12}$ receptor, only reduced the velocity of the aggregation induced by ADP in the presence of JAQ1, as can be observed by the prolongation of the lag phase before the rise in light transmission (Figure 2B, upper tracing). In addition, aggregation occurred in the absence of detectable shape change, suggesting this event to be entirely due to the $P2Y_{12}$ receptor when platelets are activated by ADP even in the presence of JAQ1. These results suggested that JAQ1 required activation of a Gi-coupled pathway to exert its potentiating effect.

JAQ1 potentiates Gi-coupled pathways, not Gq-dependent pathways

To address the question of the selectivity of the JAQ1 effect, we compared aggregation in the presence of the Gq-coupled agonist serotonin to aggregation in the presence of the Gi-coupled agonist adrenaline. As shown in Figure 3, JAQ1 only potentiated the response to adrenaline. Again a slight increase of platelet secretion could be measured (5%). Thus, JAQ1 not only potentiated ADP-induced platelet activation but also adrenaline-induced activation. It is noteworthy that, similar to JAQ1, adrenaline by itself is unable to promote platelet aggregation. Intracellular calcium measurement did not detect any calcium rise during platelet aggregation induced by the combination of JAQ1 and adrenaline (not shown) suggesting that there is no participation of phospholipase C in these processes.

JAQ1 potentiates platelet aggregation of Gαq as well as of $P2Y_1$ knockout mice

To confirm that the potentiating effect of JAQ1 does not require calcium signaling, we used platelets from mice lacking the Gαq subunit of G proteins. These platelets display markedly impaired aggregation and secretion responses to almost all agonists.[20] However, exogenous ADP has been found to restore aggregation to collagen through activation of the Gi-coupled $P2Y_{12}$ receptor.[21] We also used platelets from $P2Y_1$ receptor-deficient mice that have been shown not to aggregate in response to 5 μM ADP due to the

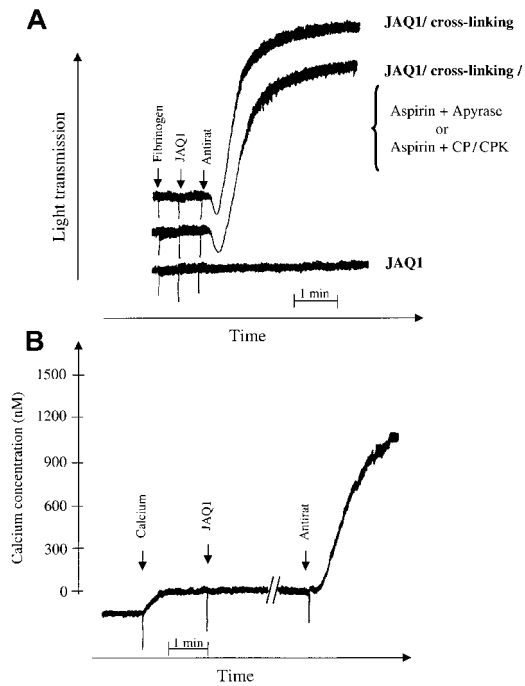

Figure 1. Cross-linking of JAQ1 induces full platelet aggregation and intracellular calcium mobilization. Washed platelets were incubated with JAQ1 (10 μg/mL) alone or JAQ1 followed by polyclonal antirat IgG antibodies (10 μg/mL). (A) JAQ1 alone does not induce aggregation, whereas cross-linking of surface-bound JAQ1 induces strong aggregation that is insensitive to aspirin treatment (1 mM, 15 minutes, 37°C), apyrase (2 U/mL), or CP/CPK (10 mM/20 U/mL). The trace shown was obtained in the presence of aspirin and apyrase. Traces obtained in the presence of aspirin and CP/CPK were similar. (B) JAQ1 only induces intracellular calcium mobilization on cross-linking.

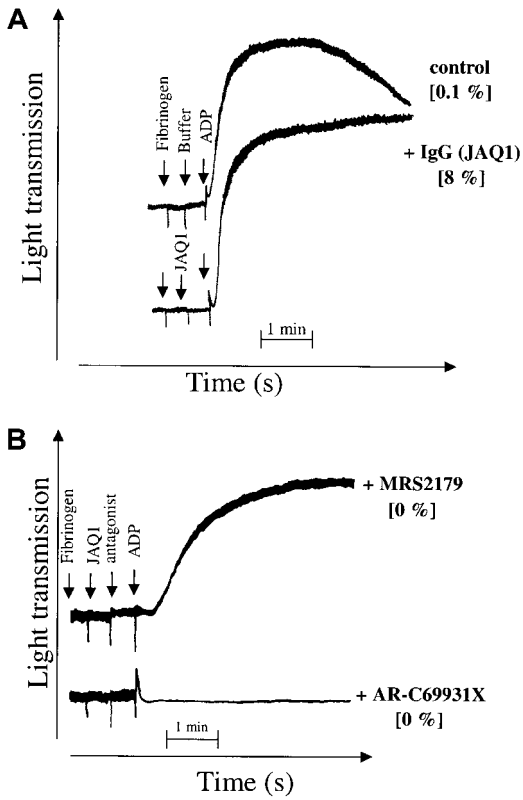

Figure 2. JAQ1 potentiates the $P2Y_{12}$ component of platelet aggregation induced by ADP. (A) Washed platelets were activated with 5 μM ADP in the presence or absence of JAQ1 (10 μg/mL). Tritiated serotonin secretion is indicated in brackets. (B) Washed platelets were incubated with JAQ1 (10 μg/mL), followed by the selective $P2Y_1$ receptor antagonist MRS2179 (100 μM) or the selective $P2Y_{12}$ receptor antagonist AR-C69931X (10 μM), and then activated with ADP (5 μM). Tritiated serotonin secretion is indicated in brackets.

lack of calcium mobilization triggered by $P2Y_1$.[24] In both transgenic mouse platelets, ADP, at the concentration of 100 μM, has been shown to be able to promote partial aggregation without shape change and without calcium signaling.[21,24] Figure 4 shows that in both strains the combination of JAQ1 (10 μg/mL) and ADP (5 or 100 μM) resulted in platelet aggregation without shape change as evaluated by light transmission. This effect was more pronounced at the highest concentration of ADP. Similar results were obtained using 10 μM adrenaline (not shown).

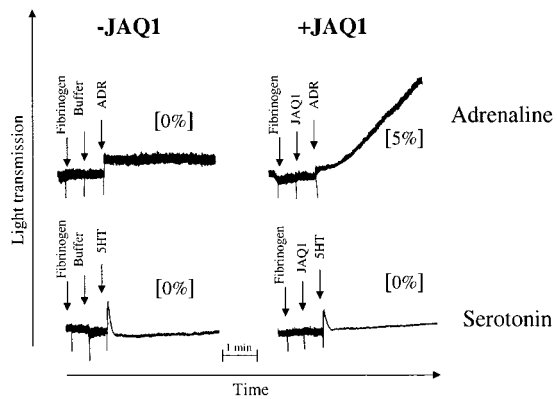

Figure 3. JAQ1 potentiates Gi-coupled, but not Gq-coupled, activation pathways. Washed platelets were activated with adrenaline (ADR) (10 μM) or serotonin (10 μM) in the presence or absence of JAQ1 (10 μg/mL). Tritiated serotonin secretion is indicated in brackets.

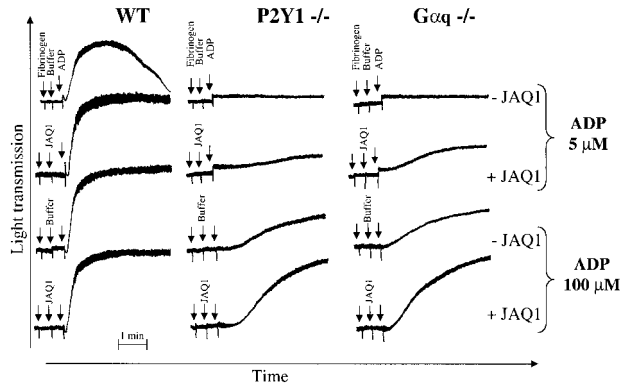

Figure 4. JAQ1-mediated potentiation of ADP-induced activation is independent of $P2Y_1$ or Gαq. Washed platelets from wild-type (WT), $P2Y_1$-deficient ($P2Y_1^{-/-}$), or Gαq-deficient ($G\alpha q^{-/-}$) mice were activated with ADP (5 or 100 μM) in the presence or absence of JAQ1 (10 μg/mL). Shape change was blocked in $P2Y_1$- and Gαq-deficient mice.

Does collagen induce clustering of GPVI?

Although collagen, even at high concentrations (5 μg/mL), only induced very limited activation of Gαq-deficient mouse platelets, cross-linking of JAQ1 produced strong and full aggregation of these platelets (Figure 5). This aggregation was accompanied by a rise in intracellular calcium concentration although at lower levels than in wild-type platelets (522 nM versus 1335 nM), which may be explained by the lack of Gαq-dependent calcium mobilization (not shown). This result demonstrated that the GPVI-dependent activation pathway was fully preserved in Gαq-deficient platelets and suggested that clustering of GPVI might not be the principal mechanism underlying collagen-induced platelet activation, at least under the experimental conditions of aggregometry at collagen concentrations up to 5 μg/mL. However, at collagen concentrations more than 30 μg/mL, partial aggregation can be induced in Gαq-deficient platelets, suggesting that such high concentrations of collagen are able to cluster GPVI (Offermanns and colleagues, manuscript in preparation).

The finding that the divalent mAb JAQ1 had similar effects on Gαq-deficient platelets as collagen (up to 30 μg/mL), raised the question of whether dimerization of GPVI by JAQ1 (or collagen) is the critical signal that can be potentiated by Gi-coupled agonists. To address this issue, we examined the effect of monovalent Fab fragments of JAQ1, which, like the intact mAb, completely block CRP (up to 100 μg/mL)-induced platelet activation (not shown and reference 27). Clearly, the Fab fragments of JAQ1 did not exert any amplifying effect in the presence of ADP or adrenaline (Figure 6). These results demonstrated that the dimeric form of the antibody was required for efficient activation of GPVI under these conditions.

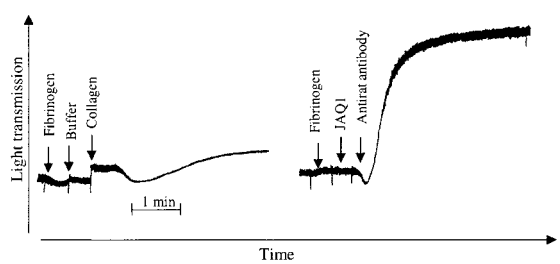

Figure 5. Normal aggregation of Gαq-deficient platelets on JAQ1 cross-linking. Washed platelets from Gαq-deficient mice were activated with collagen (5 μg/mL, left) or JAQ1 (10 μg/mL) followed by polyclonal antirat IgG antibodies (10 μg/mL).

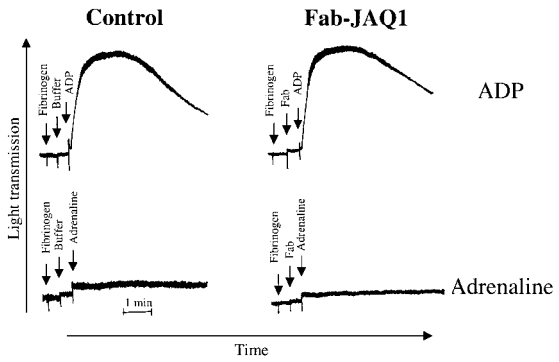

Figure 6. Monovalent Fab fragments of JAQ1 have no potentiating effect. Washed wild-type platelets were activated with ADP (5 μM) or adrenaline (10 μM) in the presence or absence of JAQ1 Fab fragments (10 μg/mL).

The combination of JAQ1 and adrenaline or ADP activates the $\alpha_{IIb}\beta_3$ integrin

To further characterize platelet activation/aggregation to JAQ1/adrenaline or JAQ1/ADP, we determined fibrinogen binding to $\alpha_{IIb}\beta_3$ by flow cytometry. As shown in Figure 7A, the combination of JAQ1 and adrenaline or ADP induced fibrinogen binding in wild-type and Gαq-deficient platelets, which was inhibited by a blocking anti-$\alpha_{IIb}\beta_3$ mAb (JON/A[31]). Consequently, aggregation induced either by adrenaline or ADP in the presence of JAQ1 was inhibited by JON/A (not shown) or an other blocking anti-$\alpha_{IIb}\beta_3$ mAb (RAM.2[21]) (Figure 7B), suggesting this process to be dependent on activation of the integrin.

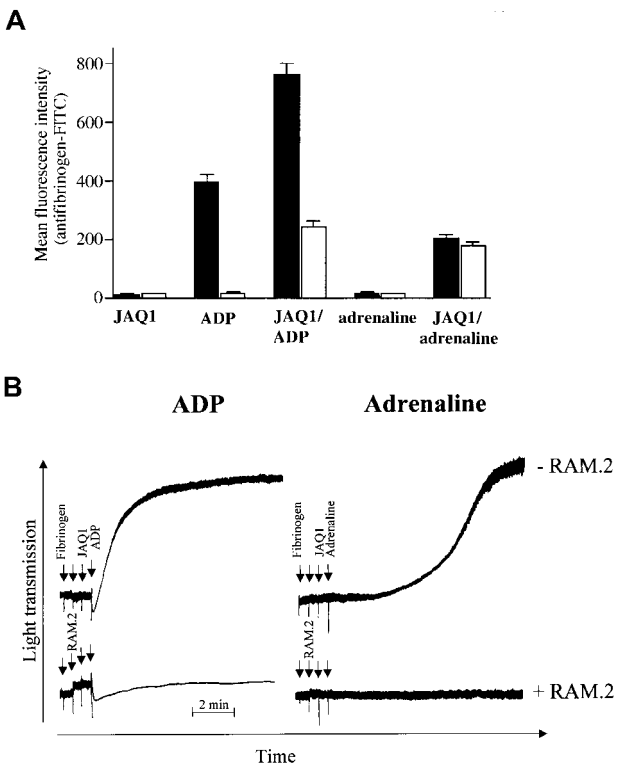

Figure 7. The combination of JAQ1 and adrenaline or ADP induces $\alpha_{IIb}\beta_3$ activation. (A) Flow cytometric quantitation of fibrinogen binding to wild-type (■) and Gαq-deficient (□) platelets stimulated with ADP (5 μM) or adrenaline (10 μM) in the presence or absence of JAQ1 (10 μg/mL). (B) Washed wild-type platelets were incubated with JAQ1 (10 μg/mL) and then activated with ADP (5 μM) or adrenaline (10 μM) in the absence or presence of the blocking antimouse $\alpha_{IIb}\beta_3$ mAb RAM.2 (1/10 dilution of ascites fluid).

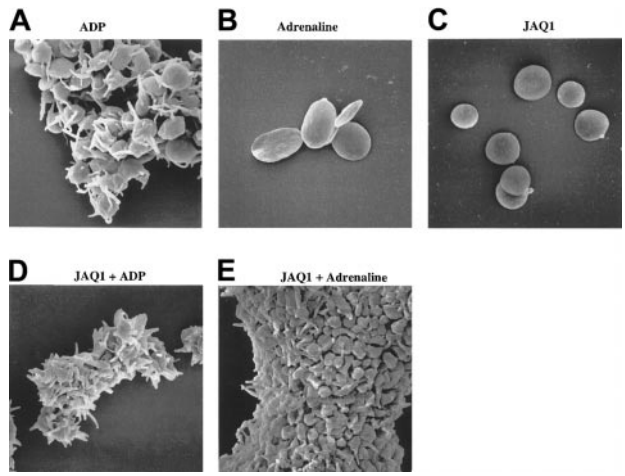

Figure 8. Synergy between dimerized GPVI and Gi-coupled signals results in aggregation without shape change. Scanning electron microscopy (original magnification × 8000) of wild-type platelets activated with ADP (5 μM) or adrenaline (10 μM) in the absence (A-B) or presence (D-E) of JAQ1 (10 μg/mL). (C) JAQ1 alone, like adrenaline, did not induce shape change or aggregation, whereas the combination of both results in large and densely packed aggregates of platelets that had only poorly changed their shape (extrusion of short pseudopods). ADP-induced aggregates increased in size and appeared more "clasped" in the presence of JAQ1.

Morphologic characterization

Scanning electron microscopy was used to characterize platelet aggregates, especially in terms of shape change, induced by the combination of JAQ1 with ADP or adrenaline. As shown in Figure 8, JAQ1 alone as well as adrenaline alone did not affect platelet morphology. Platelets, in the presence of 10 μg/mL JAQ1 or 10 μM adrenaline, displayed the characteristic discoid shape of unstimulated platelets and were not aggregated. However, the combination of JAQ1 and adrenaline had resulted in striking stacking of platelets, which had only poorly changed their shape with the extrusion of short pseudopods. In contrast, ADP (5 μM) had induced a marked shape change and the formation of loose aggregates that appeared significantly tighter in the presence of JAQ1.

Discussion

Collagen-induced platelet activation and aggregation is probably one of the most complicated and integrated processes of platelet physiology because it involves a large number of surface receptors including integrins, Ig-like receptors, G-protein–coupled receptors, and their various different signal transduction pathways. The identification of GPVI as a major receptor for activation on binding of collagen was a milestone discovery. However, other collagen receptors such as integrin $\alpha_2\beta_1$, GPIV, and 65- and 85-kd proteins[2] may also be involved in the process of platelet activation. We recently confirmed this hypothesis when we provided evidence that collagen contains 2 distinct epitopes contributing to activation of murine platelets. One of these epitopes specifically binds to GPVI and this interaction is mimicked by CRP which, therefore, is a strong GPVI-specific platelet agonist. Although JAQ1 completely blocks CRP-induced platelet aggregation, the inhibitory effect of the mAb on collagen-induced platelet aggregation is limited.[28] Therefore, different collagen receptors may contribute to the signaling processes in response to collagen, making it difficult to determine the exact role of GPVI. These observations demonstrate

that the mechanisms by which collagen activates platelets through GPVI are not fully understood despite the existence of powerful tools such as CRP and convulxin.[5,6,34] Both CRP and convulxin induce profound clustering of GPVI, resulting in strong platelet aggregation that is insensitive to aspirin or ADP scavengers (B.N. et al, unpublished observation, August 2000). Cross-linking of surface-bound JAQ1 has identical effects (reference 27 and Figure 1) even in the absence of Gαq (Figure 5). This result confirms that the defect in collagen-induced aggregation of Gαq-deficient platelets is not related to defects in GPVI signaling and indicates that collagen might activate platelets by mechanisms other than clustering of GPVI. This hypothesis is supported by the fact that collagen-induced platelet activation, in contrast to platelet activation by GPVI clustering, is very sensitive to aspirin and to ADP antagonists or inhibitors. On the other hand, it is clear that GPVI/FcRγ plays a pivotal role in collagen-induced platelet activation, but the receptor may act synergistically with other receptors and signaling pathways. We found such a synergy between GPVI and Gi-coupled receptors when GPVI was stimulated with JAQ1. JAQ1 is the only divalent GPVI-specific reagent described to date and was by itself, despite its potentiating effects on platelet responses to other agonists such as ADP (Figure 2), unable to induce shape change, aggregation, or any calcium signal (Figure 1). Very recently, we demonstrated that JAQ1 induces subliminal signaling through GPVI, resulting in threshold phosphorylation of a variety of proteins known to be involved in GPVI signaling, including FcRγ chain, Syk, the adapters LAT and SLP-76, and phospholipase Cγ2 (PLCγ2). In contrast, Fab fragments of JAQ1 had no such effect, suggesting that JAQ1-induced signaling is based on receptor dimerization.[28] In the present study, Fab fragments of JAQ1 did not exert any potentiating effect on platelet activation induced by ADP or adrenaline (Figure 6). Thus, it appears that GPVI dimerization by JAQ1 may account for its ability to potentiate platelet responses to ADP or adrenaline. We show that this effect is selective for Gi-coupled receptor agonists (Figures 2 and 3) because it did not occur with serotonin, which is a Gq-coupled receptor agonist in platelets and did not involve the $P2Y_1$ component of platelet activation by ADP, which is also coupled to Gq (Figure 2).[35] Furthermore, the amplifying effect of JAQ1 was conserved in Gαq and in $P2Y_1$-deficient platelets demonstrating that cross-talk between GPVI and Gi-coupled receptors is sufficient to promote platelet aggregation. Such a cross-talk between FcγRIIa and the Gi pathway has been found to be critical for platelet activation by sera from patients with heparin-induced thrombocytopenia.[36] Furthermore, similar mechanisms may also account for the recently reported observation that a mAb against the newly identified Ig-like platelet receptor, termed F11, potentiates platelet responses to other agonists.[37] These observations indicate that dimerization of Ig-like platelet receptors may generally provide a signal that acts synergistically with Gi-coupled agonists.

Striking is the fact that both JAQ1 alone (Figure 1) and adrenaline alone (reference 31 and Figure 8) are unable to promote any detectable activation, shape change, or calcium movement. The combination of JAQ1 and adrenaline, however, resulted in $\alpha_{IIb}\beta_3$-dependent platelet aggregation without detectable calcium signaling (Figures 3 and 4). The morphologic examination revealed striking stacking of platelets activated by JAQ1 plus adrenaline although the cells had only poorly changed their shape (Figure 8). Aggregation without calcium signaling and without shape change has previously been observed in $P2Y_1$-deficient as well as in Gαq-deficient mouse platelets activated by high concentration of ADP (100 μM).[21,24] In the present study, this effect was obtained by the combination of GPVI dimerization by JAQ1 and usual concentrations of adrenaline (10 μM) in wild-type mice or JAQ1 and usual concentrations of ADP (5 μM) or adrenaline in $P2Y_1$ and in Gαq-deficient mice.

Altogether, the reported data provide a new model of platelet activation by collagen where GPVI might dimerize on binding of collagen (mimicked by divalent JAQ1), which allows synergism with low amounts of ADP released in the vicinity of the vessel lesion. This hypothesis is strongly supported by the recent finding of Ohlmann and coworkers,[21] who reported that the impaired aggregation of Gαq-deficient platelets is fully restored by exogenously added ADP, suggesting that collagen-mediated activation of GPVI is not sufficient to induce activation of $\alpha_{IIb}\beta_3$ but requires a cosignal from other, presumably Gi-coupled, receptors. The transduction mechanisms underlying this cross-talk between GPVI and Gi-coupled receptors remain to be established.

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Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation

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