

# Efficient platelet $\delta$ -granule release induced by $[Ca^{2+}]_i$ elevation is modulated by GPIIb/IIIa

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**Abstract.** Intracellular  $Ca^{2+}$  elevation generates a cascade of events that leads to platelet activation and degranulation. The GPIIb/IIIa-ligand molecular complex plays a central role in several aspects of platelet activation. Taking advantage of the flow cytometric simultaneous analysis of surface GPIIb/IIIa expression and intracellular serotonin content, we demonstrate here that the functional inhibition of GPIIb/IIIa generates an impairment of  $\delta$ -granule release even upon maximal intracellular  $Ca^{2+}$  elevation. In healthy subjects, the GPIIb/IIIa inhibitor tirofiban impairs platelet  $\delta$ -granule release. Analogously, Glanzmann thrombasthenia patients show an impairment of  $\delta$ -granule release that is proportional to their residual expression of platelet GPIIb/IIIa. These data show that platelet surface expression of functional GPIIb/IIIa is required for a fully efficient secretion of  $\delta$ -granules and serotonin release. The implications of our findings are discussed in the light of the complex interplay between vesicle release and ligand-receptor triggering during platelet activation.

## Introduction

Platelets initiate repair to damaged vessel by adhering to the exposed subendothelium matrix. Consequent cellular activation induces platelet spreading, activation and granule secretion. GPIIb/IIIa is the more abundant receptor at the membrane of normal platelets (1,2). It is also present as intracellular stores

at the surface of the canalicular system and  $\alpha$ -granules (3,4). This intraplatelet pool can be rapidly recruited to the cell surface upon activation. Platelet agonists, that commonly induce an increase of  $[Ca^{2+}]_i$ , lead to the conversion of GPIIb/IIIa from a low affinity/avidity to a high affinity/avidity receptor state (5), a phenomenon commonly indicated as inside-out signalling (6,7), that implies conformational changes of GPIIb/IIIa and requires at least the integrity of specific cytoplasmic domains of the  $\beta_3$  integrin subunit (reviewed in ref. 8). Activation-induced inside-out signalling, therefore, greatly enhances GPIIb/IIIa affinity for fibrinogen. Release of platelet granule content activates more platelets, while efficient plasmonic fibrinogen binding bridges platelets to each other into the forming thrombus. Finally, by interaction of GPIIb/IIIa with cytoskeleton, tense forces are generated on platelet pseudopodia, causing clot retraction that facilitates wound closure. Fibrinogen binding to its receptor initiates a cascade of events that leads to release of procoagulant vesicles and platelet cytoskeleton reorganization. These outside-in signalling events are mediated by the activation of several intracellular signalling pathways (reviewed in refs. 7 and 8) that include tyrosine kinase, PI3K and MAP kinase phosphorylation.

A large body of evidence supports a general role for  $Ca^{2+}$  in granule secretion. A rise in  $[Ca^{2+}]_i$  accompanies platelet granule secretion that can be triggered by  $Ca^{2+}$ -ionophore treatment. Although the  $Ca^{2+}$ -binding proteins that mediate this effect have not been fully elucidated yet, calmodulin and calyculin have recently received particular attention (9,10). The process of exocytosis itself is highly regulated, and implies preparatory lipid bilayer conformational changes and a final step of lipid membrane fusion for which a role for phospholipid signaling molecules such as PIP2 and PA has been suggested (reviewed in ref. 11).

Therefore, although it is now clear that a rise in  $[Ca^{2+}]_i$  induces on one side the passage to the high affinity state of GPIIb/IIIa and, on the other side, the cytoskeletal rearrangements required for efficient granule secretion, the functional

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Table I. Characteristics of the Glanzmann thrombasthenia patients.

Patients	Sex	Age	Aggregometry results			Clot retraction	Bleeding time (min)	GPIIb/IIIa expression (mean fluorescence of the flow cytometric peak)
			ADP	Collagen	Ristocetin			
1	M	23	Absent	Absent	Normal	Absent	>10	5.6
2	F	32	Absent	Absent	Normal	Absent	>10	1.0
3	F	32	Absent	Absent	Normal	Absent	>10	0.9
Controls (n=3)	F=1 M=2	32 43	Present	Present	Normal	Present	<9	10.8

role of GPIIb/IIIa in the modulation of serotonin-containing  $\delta$ -granule secretion has not yet been elucidated.

We have developed a flow cytometric method to detect platelet  $\delta$ -granule release in association with platelet surface phenotype (12). We demonstrate here that, upon  $[Ca^{2+}]_i$  elevation, the functional expression of GPIIb/IIIa is necessary for a fully efficient platelet  $\delta$ -granule release; receptor occupancy by RGDS or GPIIb/IIIa defects, such as in Glanzmann thrombasthenia (GT) patients, decreases platelet  $\delta$ -granule release. In GT patients we found a defect of platelet  $\delta$ -granule release upon activation that is proportional to the expression of platelet surface GPIIb/IIIa.

The implications of our findings are discussed in the light of the complex interplay between vesicle release and ligand-receptor triggering during platelet activation.

## Materials and methods

**Preparation of platelet-rich plasma (PRP) and platelet activation.** PRP from 10 healthy blood donors (0 group) and 3 Glanzmann thrombasthenia patients (Table I) were prepared after informed consent. Blood was drawn into sodium citrate vacuum tubes (3.8% final concentration; BD Vacutainer, Becton Dickinson, San Jose, CA) and centrifuged at 500 g for 5 min at room temperature. The PRP was collected in a new tube and the platelet concentration was measured by an haemocytometer (Beckman Coulter, Miami, FL, USA). The PRP concentration was then adjusted at  $300 \times 10^3$  platelets/ $\mu$ l with phosphate-buffered saline (PBS). PRP was immediately used for subsequent analysis.

Aliquots of 70  $\mu$ l of PRP were incubated with  $Ca^{2+}$ -ionophore A23187 (3  $\mu$ M final concentration; Sigma, St. Louis, MO, USA) for 20 min at room temperature, as previously described (12). A23187 was dissolved in DMSO.

**Phenotype of activated platelets.** An aliquot of each sample was used for platelet phenotyping and activation analysis. Samples were washed in PBS and incubated for 20 min at room temperature with 10  $\mu$ l of a Cy-Chrome (Cy-Ch)-conjugated anti-CD41a monoclonal antibody (mAb) (Becton Dickinson) directed against the GPIIb/IIIa and with fluorescein isothiocyanate (FITC) conjugate Annexin V (Actiplate, Valter Occhiena, Torino, Italy), following manufacturer's protocol. The affinity of anti-CD41a mAb for GPIIb/IIIa decreases with platelet activation (13,14), and we have

previously demonstrated that, after activation, the platelet population that releases  $\delta$ -granules has a low affinity for anti-CD41a (12). In some experiments (n=3), PRP were preincubated with different concentrations (3, 6, 60, 280  $\mu$ M) of tirofiban [a nonpeptide tyrosine derivative that blocks Arg-Gly-Asp (RGDS) binding sites of GPIIb/IIIa] or abciximab (5, 10, 100, 1 mM) or eptifibatide (3, 6, 60, 280  $\mu$ M) to inhibit fibrinogen binding. All samples were analysed by flow cytometry.

**Platelet  $\delta$ -granule release.** Another aliquot of activated platelets from blood donors (either pre-treated or not with tirofiban) and from GT patients, was processed for the functional analysis of granule release, as previously described (12). Briefly, samples were treated with IntraPrep™ Permeabilization Reagent (Immunotech, Marseille, France), following manufacturer's protocol: 100  $\mu$ l of Reagent 1 (fixation reagent) was added to all the samples before washing. After 20 min of incubation at room temperature, the samples were divided in two aliquots and washed; one aliquot was incubated with 100  $\mu$ l of Reagent 2 (permeabilization reagent) and 1  $\mu$ l of rabbit anti-serotonin antiserum (Sigma) antibody working dilutions were pre-optimised, data not shown; the other aliquot was incubated with 100  $\mu$ l of Reagent 2 and 1  $\mu$ l of irrelevant rabbit IgG (2 mg/ml; Sigma) and 10  $\mu$ l of anti-CD41a CyChrome for 2 h at room temperature in the dark. After a washing step, 50  $\mu$ l of Reagent 2 and 1  $\mu$ l of R-phycoerythrin (RPE) conjugate goat anti-rabbit IgG (Valter) were added to all the samples and incubated for 30 min at room temperature in the dark. All samples were analysed on an Epics XL flow cytometer (Beckman Coulter). Data were finally analysed with Expo ADC (Beckman Coulter) and Excel 2000.

Data are given as mean  $\pm$  standard deviation from three (n=3) independently performed experiments. Linear regression was used when appropriate for data correlation.

## Results

We studied the functional role of GPIIb/IIIa expression in  $\delta$ -granule secretion from  $Ca^{2+}$ -ionophore activated platelets.

**Phenotype of activated platelets.** Upon treatment with A23187, donor platelets bound Annexin V, while CD41a staining decreased, as expected (Fig. 1). Pretreatment with tirofiban

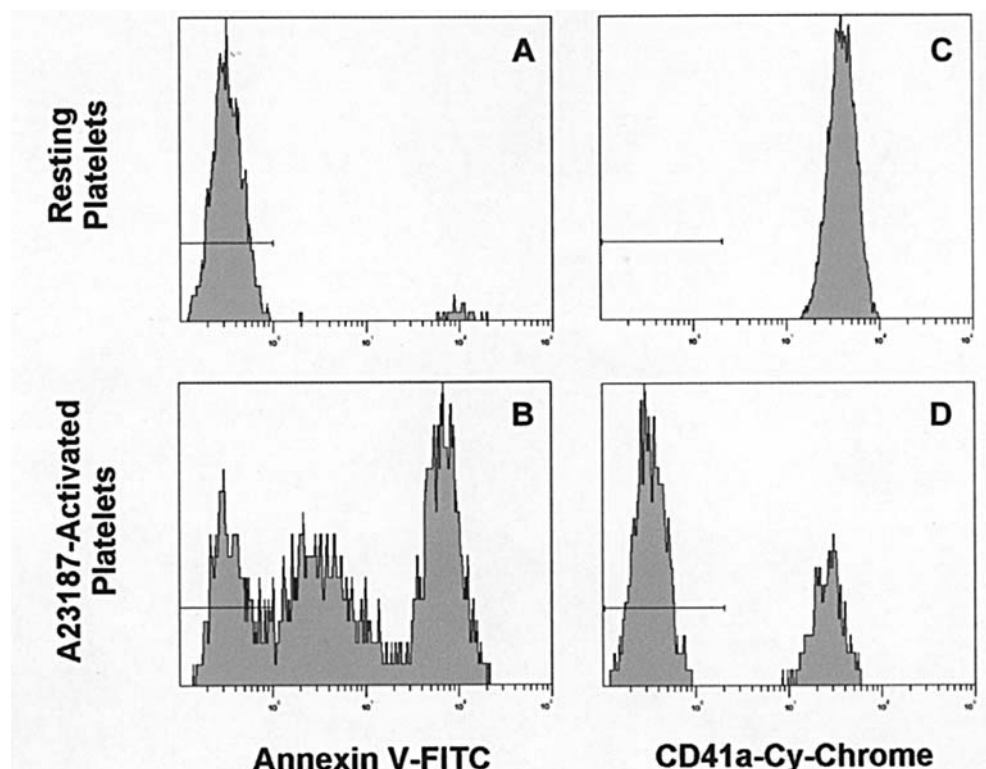


Figure 1. Anti-CD41a and Annexin V staining of the resting and activated donor platelets. Activated platelets express Annexin V (A and B), while the binding of anti-CD41a monoclonal antibody decreases upon platelet activation (C and D). A representative of 10 independent experiments is shown.

did not significantly affect the phosphatidylserine expression and consequent Annexin V binding to activated platelets (not shown).

***δ-granule release from activated platelets.*** Platelets from healthy donors were permeabilized to stain the serotonin stored in δ-granules. Upon activation with A23187, and in parallel to the decrease of anti-CD41a binding to GPIIb/IIIa, platelets released δ-granules (Fig. 2, Control).

On the contrary, when donor platelets were pre-treated with tirofiban, δ-granule release was significantly inhibited (Fig. 2, bars A-D) in a dose-dependent manner. Inhibition of δ-granule release from donor platelets pre-treated with abciximab or eptifibatide at different concentrations gave similar results (Fig. 2, bars A-D).

***Platelets from GT patients show an impairment of δ-granule release.*** To independently study the role of GPIIb/IIIa in δ-granule release in a different model system, in the absence of pharmacological inhibitors, we selected a group of 3 GT patients with different levels of GPIIb/IIIa surface expression. Intracellular staining of serotonin, combined with surface expression of CD41a was then used to detect δ-granule release from GT platelets after A23187 activation. Fig. 3 shows the simultaneous analysis of serotonin content and CD41a expression in GT patients as compared to healthy subjects. Resting platelets from GT patients and healthy donors had a similar serotonin content (Fig. 3, left panels). However, when activated, platelets from GT patients showed a decreased δ-granule release as compared to healthy controls (Fig. 3, right panels). The impairment of δ-granule release in these patients

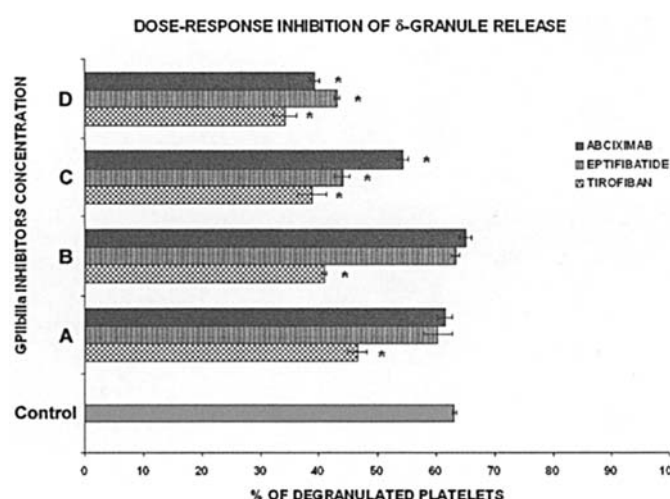


Figure 2. Serotonin content of A23187-activated donor platelets. Effect of different concentration of tirofiban, eptifibatide or abciximab on δ-granule release. Control: untreated platelets; (A) 3 μM (tirofiban or eptifibatide), 5 nM (abciximab); (B) 6 μM (tirofiban or eptifibatide), 10 nM (abciximab); (C) 60 μM (tirofiban or eptifibatide), 100 nM (abciximab); (D) 280 μM (tirofiban or eptifibatide), 1 mM (abciximab). Upon activation, most untreated platelets release their serotonin content (Control). Pretreatment of platelets with different doses of tirofiban, eptifibatide or abciximab proportionally decreases δ-granule release (Bars A-D). Data are expressed as means ± SD from 3 independent experiments; \*p<0.001.

was proportional to the surface CD41a expression on their platelets (Fig. 4). Patient 1, that had only a slight decrease of GPIIb/IIIa expression, showed a  $71.5 \pm 7.7\%$  decrease of serotonin fluorescence as compared to the controls. On the contrary, patients 2 and 3, that expressed GPIIb/IIIa at a lower



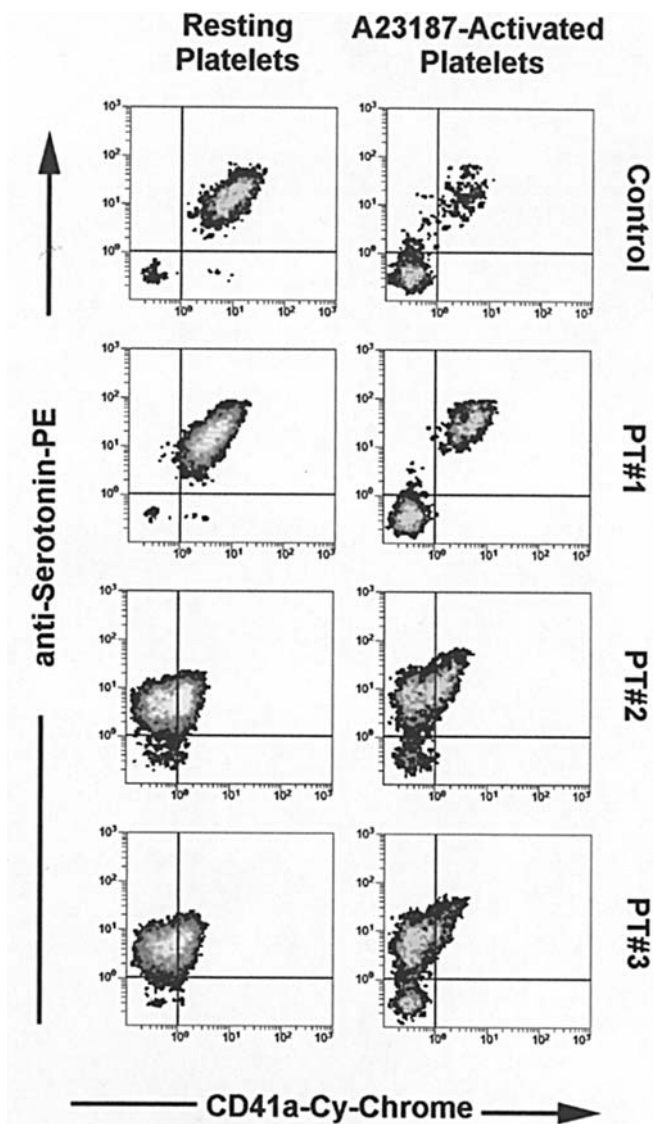


Figure 3. Serotonin content and anti-CD41a expression in resting and activated platelets from GT patients. The impairment of  $\delta$ -granule release upon activation is proportional to GPIIb/IIIa expression before activation. Patient 1 had only a slight decrease of GPIIb/IIIa expression, while patients 2 and 3 had a lower expression of GPIIb/IIIa and a more serious impairment of  $\delta$ -granule release.

level than patient 1, showed a  $49.7 \pm 3.5\%$  decrease of serotonin fluorescence with respect to controls. Patients 2 and 3 are twins, they show a similar decrease of both GPIIb/IIIa expression and  $\delta$ -granule release.

## Discussion

Platelet aggregation is essential for hemostasis, and is dependent on the integrin  $\alpha$ IIb $\beta$ 3. This molecular complex, GPIIb/IIIa, recognizes at least four ligands, fibrinogen, fibronectin, vitronectin and von Willebrand factor (15,16). GPIIb/IIIa receptor has the capacity to shift through conformational changes from a low affinity state, essentially unable to bind macromolecular ligands, to a high affinity state on activated platelets (5). Glanzmann thrombasthenia is an inherited bleeding disorder due to either the reduction/absence of GPIIb/IIIa on platelet surface or, in rare cases, to mutations

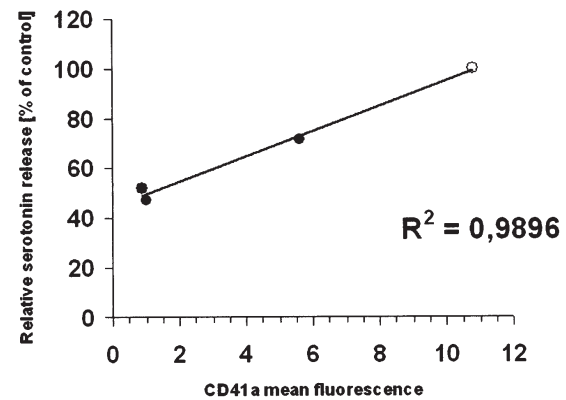


Figure 4. Correlation between GPIIb/IIIa (CD41a) and  $\delta$ -granule release (relative serotonin release) was calculated by linear regression ( $R^2=0.9896$ ). Open circle, control; black circle, GT patients.

or deletions that impair its molecular function (reviewed in ref. 17).

Platelet activation is a very complex process that implies cross-talk of several signalling pathways not fully elucidated yet. However, it is generally agreed that, upon activation: i) platelets undergo membrane phospholipid turnover, to prepare for vesicle fusion and granule exocytosis; ii) GPIIb/IIIa shifts from the low affinity to the high affinity state (inside-out signalling); iii) GPIIb/IIIa binds plasmatc fibrinogen that bridges activated platelets to one another and generates a number of both activatory and inhibitory intracellular signalling pathways (essentially via the cytoplasmic portion of the  $\beta_3$  subunit) that complete the process of platelet activation.

In this study, we demonstrate that an even maximal rise of intracellular  $Ca^{2+}$  concentration generates only a partial release of platelet  $\delta$ -granules when surface GPIIb/IIIa is functionally inhibited or its expression is reduced. Activated platelets expose phosphatidylserine on the external surface of plasma membrane, which is a classical event in platelet activation (18-21). Starting from our observation that a GT patient showed a clear impairment of platelet  $\delta$ -granule exocytosis, we asked if this functional defect might be related to the decreased expression of GPIIb/IIIa typical of GT. We therefore recruited 3 GT patients, with different levels of GPIIb/IIIa expression, to test their platelet  $\delta$ -granule release. Our data show that  $\delta$ -granule exocytosis upon  $[Ca^{2+}]_i$  elevation is impaired in GT patients. Moreover, in the patients studied the degree of  $\delta$ -granule release defect appears proportional to the impairment of GPIIb/IIIa expression on platelet surface. These observations suggested that a functional GPIIb/IIIa might be essential to reach the maximal  $\delta$ -granule release from platelets even upon pharmacologically-induced maximal intracellular  $Ca^{2+}$  elevation. To exclude that our observations in GT patients could be due to other molecular defects in the signalling response to  $Ca^{2+}$  elevation, we tested  $\delta$ -granule release in normal donors upon functional inhibition of GPIIb/IIIa. RGDS are able to block the binding of fibrinogen to GPIIb/IIIa without generating signalling downstream of the intracellular domain of the receptor (reviewed in refs. 22 and 23). Our results show that RGDS significantly decreased  $\delta$ -granule secretion from the platelets of normal subjects, thus



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1g, from the secretory point of view, the impairment in GT patients. Other aspects of normal platelet activation, such as exposure of surface phosphatidylserine, were not altered by GPIIb/IIIa inhibition, suggesting a specific role of the signalling generated by fibrinogen binding in the achievement of fully functional secretory activity. Our observations are in agreement with the accumulating evidence of the functional correlation linking activated GPIIb/IIIa and cytoskeletal reorganization (24). Previous studies correlate fibrinogen binding to activated GPIIb/IIIa to the cytoskeletal reorganization of the platelet necessary for clot retraction. We demonstrate for the first time that GPIIb/IIIa is functionally relevant for the complete degranulation of the platelet. It is well known that the binding of HIP-8 mAb (anti-CD41a) to GPIIb/IIIa decreases with platelet activation, and we have demonstrated previously that, upon  $[Ca^{2+}]_i$  elevation, degranulation takes place only in the CD41a negative platelet population (12). These results suggest that the decrease of CD41a staining might not be only an independent marker for activated platelets, but rather functionally link the activation of GPIIb/IIIa to  $\delta$ -granule release, that significantly depends on the expression of functional GPIIb/IIIa, even in the presence of a maximal  $[Ca^{2+}]_i$  elevation induced by A23187. GT patients have a  $\delta$ -granule secretion that responds to  $Ca^{2+}$  elevation proportionally to their level of GPIIb/IIIa expression.

We therefore suggest that in the full secretory response of platelets to  $Ca^{2+}$  elevation, GPIIb/IIIa represents a functionally relevant molecular intermediate playing a central role in several platelet functions, ranging from full platelet degranulation to clot retraction.

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