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Letter to the Editors-in-Chief

Adenylate cyclase inhibition is required for normal redistribution of platelet surface GPIb in response to PAR1 activation☆

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1. Introduction

Thrombin exerts its cellular effects primarily through a family of G protein coupled protease-activated receptors (PARs): PAR1 and PAR4 [1]. These receptors are activated by a unique mechanism in which the protease creates a new N-terminal sequence that can act as a tethered ligand.

Early studies demonstrated that platelet surface expression of GPIb is down-regulated in response to thrombin or to synthetic PAR receptor activating peptides [2]. The mobility of GPIb from the platelet surface into the cell was shown to be reversible and to derive from a re-organization of components of the contractile and microtubular systems [3]. We have previously reported that PAR1-induced redistribution of GPIb appeared to be reduced in platelets from patients with platelet dense granules defects (or δ-storage pool disease; δ-SPD), indicating that impairment of GPIb mobilization might contribute to additional platelet phenotype [4,5]. Nonetheless, whether down-regulation of platelet surface GPIb is dependent on δ-granules secretion is still unclear.

δ-granules contain serotonin, calcium and adenine nucleotides (ADP and ATP). Although ADP, per se, is a weak agonist, it has a pivotal role in hemostasis by playing its action as a key cofactor of platelet activation [6]. Human platelets express two distinct receptors for ADP: the Gq-coupled $P2Y_1$ receptor and the Gi-coupled $P2Y_{12}$ receptor [7]. It is now well established that the $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets [8], whereas the $P2Y_{12}$ receptor leads to the inhibition of adenylyl cyclase. However, full platelet aggregation in response to ADP requires both $P2Y_1$ and $P2Y_{12}$ activation [9].

A role of ADP to potentiate platelet function initiated by thrombin has been also established [10], but the selective contribution of P2Y receptor subtypes on redistribution of GPIb from the platelet surface have not been determined. Here, we investigated how ADP plays a role as a cofactor in GPIb movement in response to specific PAR receptor activating peptides and whether a specific signaling pathway is modulated by released ADP.

2. Materials and methods

2.1. Preparation of platelets

Blood was collected by venipuncture into trisodium citrate (0.105 M) from healthy volunteers who had provided informed consent. This study has been carried out in accordance with the Declaration of Helsinki. Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 1200 rpm for 10 min at room temperature.

2.2. Platelet stimulation

Platelets were pretreated with vehicle alone (PBS), apyrase grade VII (Sigma-Aldrich, Saint-Louis, Missouri, US), AR-C69931MX (Medecines Company, Parsippany-Troy Hills, New-Jersey, US), A3P5P (Sigma-Aldrich, Saint-Louis, Missouri, US) or wortmannin (Valbiotech, Paris, France) before their exposure to PAR peptides. Platelets were incubated for 5 min (except where noted) with 300 μM TRAP-14 (BACHEM, Bubendorf, Switzerland) or 500 μM PAR4-AP (Biosyntan, Berlin). All incubations were at room temperature (RT) or 37 °C without stirring.

2.3. Flow cytometry analysis

A volume (10 μL) of PRP (250 G/L) was incubated for 15 min at room temperature with SZ2 (Beckman Coulter, Brea, California), a mAb directed against GPIbα and secondly labeled with FITC-conjugated goat antimouse IgG for 15 min in the dark. Cells were analyzed using a FC500 flow cytometer (Beckman Coulter, Brea, California) and results were expressed, when appropriate, as a percent of the mean fluorescence intensity (MFI) corresponding to SZ2 binding to unstimulated platelets.

☆ Declarations of interest: none.

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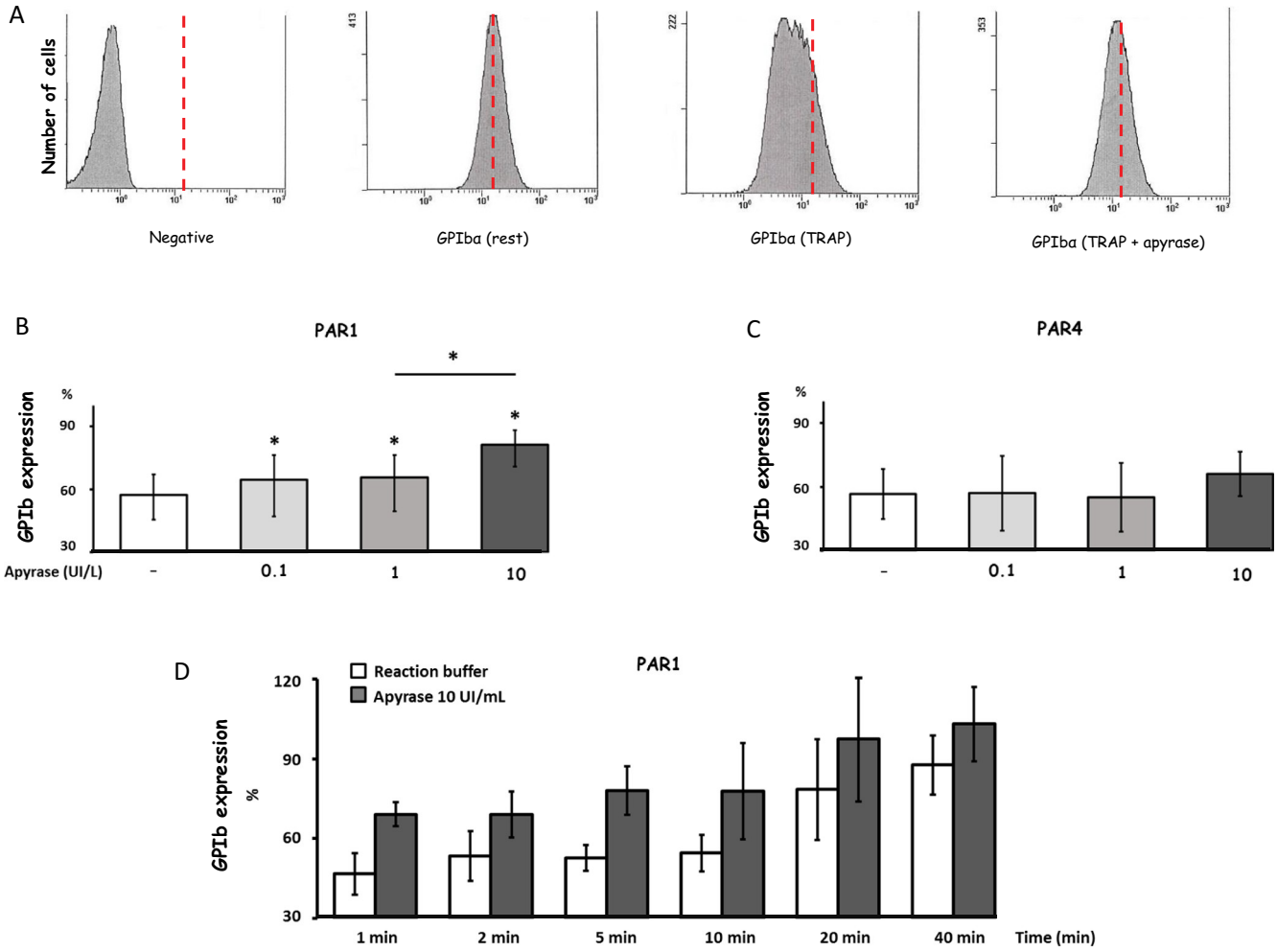

Fig. 1. A – A representative histogram of the flow cytometry analysis indicating the overall GPIb-positivity of resting platelets with subsequent decrease in MFIs after platelet activation by TRAP-14 with the presence, or not, of apyrase 10 UI/L.
B and C - Apyrase impaired GPIb redistribution of TRAP-stimulated human platelets. PRP were incubated with reaction buffer or different concentrations of apyrase and then stimulated by 300 μM TRAP-14 (B) or 500 μM PAR4-AP (C) at RT. Aliquots were incubated with SZ2, a mAb against GPIbα. Bound SZ2 was revealed by an FITC-conjugated second antibody. Samples were analyzed by flow cytometry and results expressed as a percent of the MFI obtained for unstimulated platelets. Data points are the mean ± SD of 6 experiments. Statistical significance was analyzed by the Wilcoxon signed-rank test and an asterisk indicates $p < 0.05$.
D- Kinetic analysis of platelet surface GPIb redistribution after TRAP-14 stimulation in presence of apyrase. PRP were incubated with reaction buffer (white histogram) or with apyrase 10 UI/mL (grey histogram) at RT for 5 min before stimulation by TRAP-14 as described above. Results are from a minimum of 4 experiments.

2.4. Statistical analysis

Results were expressed as percent ± standard deviation (SD). Statistical analysis was performed using the Wilcoxon signed-rank test, which is a non-parametric statistical test used when comparing two related samples. Values were considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. Apyrase impaired GPIb redistribution of TRAP-stimulated human platelets

We first tested whether ADP hydrolysis might cause impairment of TRAP-14 or PAR4-induced GPIb redistribution. To do that, we used apyrase VII to hydrolyze secreted ADP after platelet activation. PRP from healthy volunteers were activated with TRAP-14 or PAR4-AP in the presence of different final concentrations of apyrase (0.1, 1.0, or 10.0 UI/L) and platelet surface expression of GPIb was measured by flow cytometry (Fig. 1A).

Results showed that stimulation through PAR1 or PAR4 induced GPIb redistribution at a rate of 57 ± 12% (Fig. 1B). These data were in accordance with those previously published [2]. Results also showed that all concentrations of apyrase induced impaired GPIb internalization by TRAP and that the higher dose of apyrase was associated with much reduced redistribution (Fig. 1B).

By contrast, as shown in Fig. 1C, we demonstrated that incubation of normal platelets with apyrase had no effect on GPIb surface redistribution after activation with 500 μM of PAR4-AP.

Altogether, these data indicated that treatment of platelets with apyrase led to a change in GPIb redistribution induced by TRAP-14 agonist, whereas activation by PAR4-AP was not affected.

3.2. Kinetic analysis of platelet surface GPIb redistribution after TRAP-14 stimulation in presence of apyrase

Platelet activation by PAR1-AP induces a rapid and significant reduction of platelet surface GPIb. Usually, this phenomenon is transient,

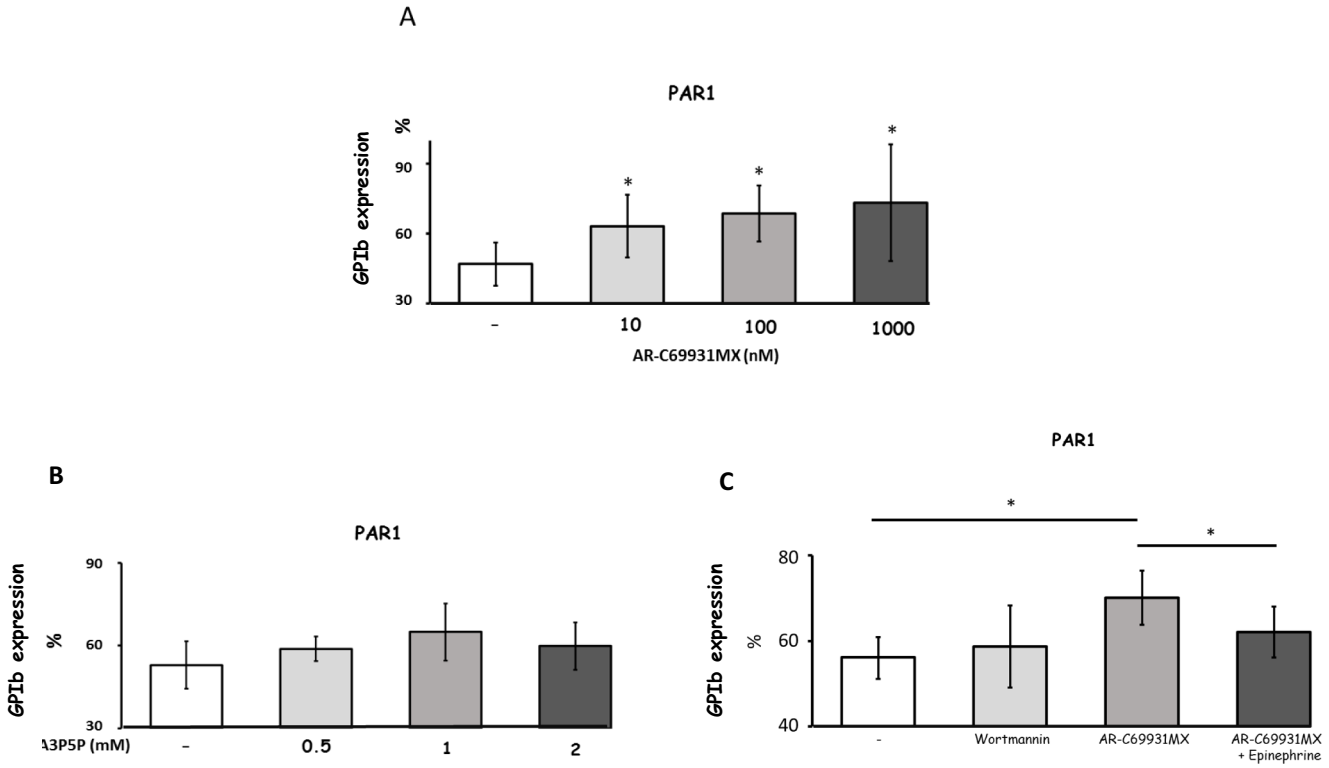

Fig. 2. A and B - Effect of selective ADP receptor antagonists on TRAP-induced platelet GPIb redistribution. PRP were incubated with reaction buffer, AR-C69931MX or A3P5P at 37 °C for 5 min before stimulation by TRAP-14. Aliquots were incubated with SZ2, a mAb against GPIbα. Bound SZ2 was revealed by an FITC-conjugated second antibody. Samples were analyzed by flow cytometry and results expressed as a percent of the MFI obtained for unstimulated platelets. Data points are the mean ± SD of 6 distinct experiments. Statistical significance was analyzed by the Wilcoxon signed-rank test and an asterisk indicates $p < 0.05$.
C - PI3K antagonism does not affect GPIb movement and α2A-adrenergic receptor activation overcomes AR-C69931MX mediated GPIb inhibition. PRP were incubated with reaction buffer, wortmannin (50 nM for 15 min), or AR-C69931MX (1 μM for 5 min) at 37 °C before stimulation by TRAP (300 μM) ± epinephrine (10 μM) for 5 min. Each data point is the mean ± SD of 6 different experiments.

peaking at 1–2 min and returning to basal levels after 60 min [2].

Here, we tested whether ADP hydrolysis causes impairment of TRAP-induced GPIb redistribution after different time points of incubation (1, 2, 5, 10, 20 and 40 min). Without apyrase, we indeed observed a decrease of surface GPIb after 1 min incubation and a return to near basal level after 40 min. However, in presence of a high dose of apyrase, GPIb internalization was decreased after 1 min incubation, while kinetic profile of redistribution was similar to the control condition (Fig. 1D).

These data suggest that absence of ADP release reduces the capacity of GPIb to be immediately internalized rather than accelerating its return to basal level.

3.3. Effect of selective ADP receptor antagonists on TRAP-induced platelet GPIb redistribution

The relative contribution of the different platelet purinergic ADP receptors to GPIb redistribution was then investigated using selective pharmacologic antagonists. A3P5P, a $P2Y_1$-selective antagonist, was used to block ADP signaling through its Gq-coupled receptor. The second approach utilized AR-C69931MX, a selective antagonist of the Gi-coupled $P2Y_{12}$ receptor.

Fig. 2A shows that AR-C69931MX can block TRAP-induced platelet GPIb redistribution in a manner similar to that of apyrase, whereas the selective $P2Y_1$ antagonist A3P5P do not (Fig. 2B), whatever the concentration used. Thus, these results strongly suggest that the cofactor role of ADP in GPIb clearance may be due to its P2 receptor coupled to Gi-signaling.

3.4. α2A-adrenergic receptor triggering overcomes AR-C69931MX mediated GPIb inhibition in platelets stimulated by TRAP-14

The $P2Y_{12}$ receptor couples to members of the G_i family, mediating inhibition of adenylyl cyclase and activation of PI3K [10]. As PI3K functions downstream of $P2Y_{12}$, we checked the effect of its inhibition by wortmannin (50 nM) which irreversibly inhibits PI3K in the nanomolar range [2]. However, addition of wortmannin to platelets during 15 min at 37 °C had no significant effect on TRAP-induced GPIb movements (Fig. 2C).

We next wanted to confirm the specific role of adenylyl cyclase inhibition in regulating GPIb at the platelet surface after activation by TRAP-14. In platelets, epinephrine has the ability to bind to the α2A-adrenergic receptor and to activate the Gz pathway leading to inhibition of adenylyl cyclase activity. Then, we determined the effect of epinephrine (10 μM), in the presence of $P2Y_{12}$ blockade, on the capacity of platelets to internalize GPIb. Pretreatment of platelets with AR-C69931MX inhibits potency of GPIb to be cleared, but this effect was reversed by the addition of epinephrine, indicating that this phenomenon is mediated by inhibition of adenylyl cyclase (Fig. 2C).

4. Conclusion

In summary, we have further characterized the role of individual PAR and ADP receptor subtypes in agonist-induced platelet GPIb clearance. Our results show that PAR1 stimulation induces a transient decrease of surface GPIb which involves $P2Y_{12}$ dependent signaling. In contrast, the lack of inhibitory effect of apyrase on platelet GPIb distribution triggered by specific activation of PAR4, indicates that PAR4

signal is fully activated without the requirement of ADP secretion and binding to platelets.

However, surface platelet receptors can be also cleared by a proteolytic process called “ectodomain shedding” which involves disintegrin and metalloproteinases enzymes that trigger receptor cleavage after ligand binding. This mechanism allows rapid downregulation of receptor expression leading to modulation of platelet activation. It has been previously shown that the majority of GPIbα shed from human platelets is mediated by ADAM17 (A-Disintegrin-And-Metalloproteinase 17), also called TACE [11], when engagement of the GPIb-IX-V complex by von Willebrand factor occurs in flowing blood, or in *in vitro* conditions, e.g. during storage of platelets; a process that we cannot exclude in our case.

Finally, the fact that ADP plays a role in GPIb redistribution might explain why it represents an additional feature involved in the platelet phenotype dysfunction of SPD patients. The significance and clinical relevance of our findings are yet difficult to determine. Lowering GPIb receptor density could reduce the adhesivity of platelets, thus controlling platelet activation. Conversely, in the context of storage pool disease, GPIb mobilization defect might confer a pro-adhesive phenotype limiting bleeding diathesis. However, this remains to be investigated.

Statement of conflict of interest

The authors declare no conflict of interest.

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Thomas Pouget[a], Xavier Pillois[b], Mathieu Fiore[a,b,*]
[a] *Laboratoire d'Hématologie, Centre Hospitalier Universitaire de Bordeaux, Pessac, France*
[b] *Centre de Référence des Pathologies Plaquettaires, Centre Hospitalier Universitaire de Bordeaux, Pessac, France*
E-mail address: mathieu.fiore@chu-bordeaux.fr (M. Fiore).

* Corresponding author at: Laboratoire d'Hématologie, Hôpital Cardiologique, Avenue Magellan, 33604 Pessac, France.