Interaction between adenosine diphosphate receptors and protein-kinase C isoforms in platelet adhesion under flow condition

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ABSTRACT

Adenosine diphosphate (ADP) receptors and protein-kinase C (PKC) isoforms play different role in platelet activity. In the present study, whole blood platelet adhesion at 200 - 1800 s^{-1} shear rates was investigated by Impact-R system, measuring percent of surface coverage (SC) by platelets. Gradual heightened shear rate paralleled increase of platelet adhesion. At relatively low shear (200 and 1000 s^{-1}) blockade of neither $P2Y_1$ receptor nor $P2Y_{12}$ receptor (by A2P5P and 2MeSAMP, respectively) affected SC. At high shear rate (1800 s^{-1}) reduction of SC was observed by 2MeSAMP. Treatment of blood with PKCδ inhibitor (rottlerin) but not PKCα,β inhibitor (Gö6976) diminished platelet adhesion. Among all the agents, only combination of 2MeSAMP and rottlerin used at subthreshold concentrations was able to inhibit platelet adhesion under high shear condition. We suggest that platelet agonist-induced $P2Y_{12}$ and PKCδ signaling essentially stimulates platelet adhesion under flow condition, the important initiating step of thrombin formation.

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INTRODUCTION

Adenosine diphosphate (ADP) activates human platelets via concomitant engagement of G-protein-coupled $P2Y_1$ and $P2Y_{12}$ receptors.[1,2] The $P2Y_1$ receptor triggers the mobilization of calcium from internal stores, which results in platelet shape change and weak, transient aggregation in response to ADP.[3] The $P2Y_{12}$ receptor plays a central role in platelet activation, agonist-induced dense granule release and pro-coagulant activity.[4] Accordingly, $P2Y_{12}$ receptor antagonists were reported to be clinically effective in the prevention of myocardial infarction, ischemic stroke, and vascular death,[5,6]

Downstream of these receptors activation, various signal transduction pathways are involved, among them an important role play protein kinases C (PKC). PKC isoforms are divided to classic and novel groups.[7] Human platelets possess both classic (PKCα, PKCβ) and novel PKCδ isoforms.[8] PKC isoforms play different roles in ADP signaling.[9,10] The role of PKC isoforms in ADP-induced platelet function and vice versa is not fully understood.

Many studies regarding platelet agonists and inhibitors were performed with washed platelets or platelet-rich plasma (PRP) under static conditions. The known limitations of these methods can be overcome by using whole blood and engagement of flow.

In the present study, we investigated the interaction between ADP receptors and different PKC isoforms in platelet deposition under flow conditions.

MATERIALS AND METHODS

Study population and blood preparation

Our study included 46 healthy volunteers who had not taken medications known to affect platelet function for at least 10 days before blood sampling. Peripheral vein blood was collected in polypropylene tubes using a 21G butterfly needle and a vacutainer system. The first 2 ml of blood were discarded, and the second portion of 4.5 ml blood was drawn into tubes containing 3.2% sodium citrate in an anticoagulant/blood ratio of 1:9. PRP was separated from pack cells following centrifugation of native blood for 10 min at 160g. The following ADP receptor antagonists were used: A2P5P (20-200 μM) against $P2Y_1$ and 2MeSAMP (20-160 μM) against $P2Y_{12}$.[3,11] PBS served as a vehicle in experiments with ADP inhibitors. The following PKC inhibitors were used: 0.2-1 μM Gö6976, 1-5 μM rottlerin and 2-10 μM D-erythro-sphingosine against PKCα,β, PKCδ, and pan-PKC, respectively.[12-15] DMSO was the vehicle for PKC isoform antagonists, as they do not dissolve in PBS. PRP samples were pre-incubated with all inhibitors for 10 min. Whole blood reconstitution was performed adding PRP to pack cells in a ratio equal to their volumes following blood centrifugation.

Impact-R test

This method is based on the use of polystyrene surface for platelet deposition under shear stress (DiaMed, Cressier, Switzerland).[16] Recently, it has been shown that fibrinogen and von Willebrand factor are precipitated from blood and thereby serve as platform for subsequent platelet adhesion onto polystyrene.[17] Reconstituted blood samples (130 ml) were placed into polystyrene wells for two min and subjected to flow at defined shear rates. The wells were then thoroughly washed, stained with May-Grünwald stain, and analyzed by the Impact-R image analysis system.

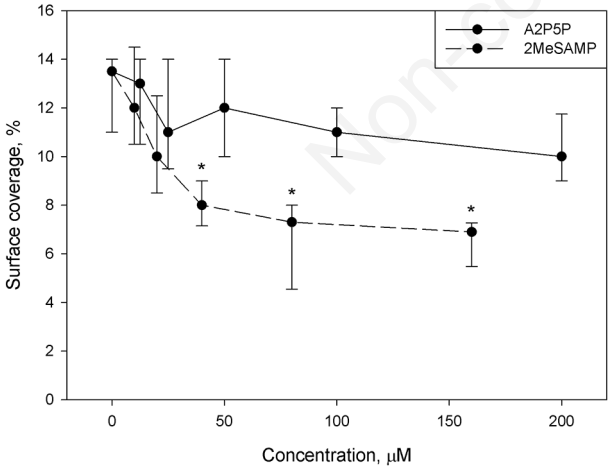

Figure 1. Dose-depending effect of A2P5P and 2MeSAMP on platelet deposition under flow conditions PRP was pre-incubated with PBS (control, white columns) and increasing concentrations of A2P5P (solid lines) and 2MeSAMP (dashed lines) for 10 min, then mixed with autologous packed cells to reconstitute whole blood and subjected to Impact-R test for 2 min at 1800 s^{-1} shear rate. The median surface coverage (SC) (25^{th}-75^{th} percentile) is presented. *P<0.01 *vs.* control (n=8).

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics (version 23.0; IBM Corp., USA). The data of SC by adhered platelets were presented as median and inter-quartile range (IQR). Multiple group comparisons were performed by Kruskal–Wallis ANOVA followed by post hoc Dunn's *Q* test. Two-tailed *P* values of less than 0.05 were considered statistically significant.

RESULTS

Dose-dependent effect of A2P5P and 2MeSAMP on platelet adhesion

Preliminary experiments in this study showed that platelet adhesion intensity was just the same using native and reconstituted blood, suggesting that even if red blood cells release some amount of ADP during blood centrifugation and reconstitution of whole blood, this does not affect platelet adhesion. Platelet adhesion at shear rate of 1800 s^{-1} was chosen according to previously found as optimal reflecting arterial blood flow in our model.[15] Incubation of platelet-rich plasma (PRP) with A2P5P ($P2Y_1$ antagonist) at concentrations from 20 mM up to 200 mM did not change the rate of platelet deposition in reconstituted blood (Figure 1). In contrast, 2MeSAMP significantly decreased SC compared to blood without inhibitors [from 11.5% (10.0-13.8%) to 8.0 (7.9-8.7%, P<0.5) at 40 mM and 7.2% (6.8-7.7%, P<0.01) at 80 mM 2MeSAMP]. This effect did not change at 160 mM compared to 80 mM. A2P5P at 100 mM and 2MeSAMP at 80 mM were chosen for subsequent experiments.

Effect of A2P5P and 2MeSAMP on platelet adhesion at different shear rate

At static conditions limited number of platelets adhered (Figure 2). Gradual increase of platelet adhesion was observed at shear rates of 200, 1000 and 1800 s^{-1}. However, no inhibitor was able to affect platelet deposition at low (200 s^{-1}) and intermediate (1000 s^{-1}) shear rate. In contrast, substantial reduction of platelet deposition was observed by 2MeSAMP at1800 s^{-1} (SC 7.2% (6.8-7.7%). Despite the lack of inhibitory effect of A2P5P alone, the combination of both drugs had an additive effect on platelet adhesion, further decreasing SC to 5.5% (5.0-6.0% compared to 2MeSAMP alone, P<0.01).

Role of PKC isotypes in platelet adhesion

At high shear (1800^{-1}) among the PKC inhibitors, D-erythro-sphingosine and rottlerin reduced SC from 11.0% (10.0-13.0%) in control to 4.2% (3.7-7.1%, P<0.001) and 7.3% (5.7-7.6%, P<0.01), respectively, whereas Gö6976 did not affect platelet adhesion (Figure 3A). In the following experiments, interaction between the ADP and PKC antagonists was assayed. For this purpose, the agents were used at subthreshold concentrations, just at 20% of the concentrations used above. As expected, the inhibitors used separately did not affect platelet adhesion (Figure 3B). In contrast, the combination of 2MeSAMP with D-erythro-sphingosine reduced SC to 6.0% *vs*. D-erythro-sphingosine alone (9.6%, P<0.01), and that of 2MeSAMP with rottlerin reduced SC to 7.4%, *vs*. rottlerin alone (10.2%, P<0.05). Platelet adhesion was not changed by concomitant blood treatment with 2MeSAMP and Gö6976.

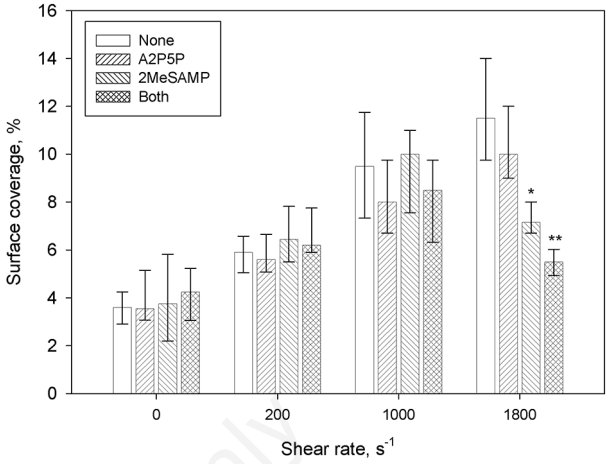

Figure 2. Shear rate-dependent effect of A2P5P and 2MeSAMP on platelet adhesion PRP was pre-incubated with PBS (control, white columns), 100 mM A2P5P, 80 mM 2MeSAMP or both inhibitors in combination for 10 min, then mixed with autologous packed cells to reconstitute whole blood and subjected to the Impact-R test at the indicated shear rates for 2 min. The median surface coverage (SC) (25^{th}-75^{th} percentile) is presented. *P<0.01, **P<0.001 represents difference *vs.* PBS-treated samples (n=8).

DISCUSSION AND CONCLUSIONS

The aim of this study was to evaluate the role of $P2Y_1$ and $P2Y_{12}$ ADP receptors as well as PKC$\alpha\beta_1$ and PKCδ isoforms in platelet adhesion under shear flow conditions. The study employed the Impact-R device measuring whole blood platelet adhesion to a polystyrene surface, where fib-

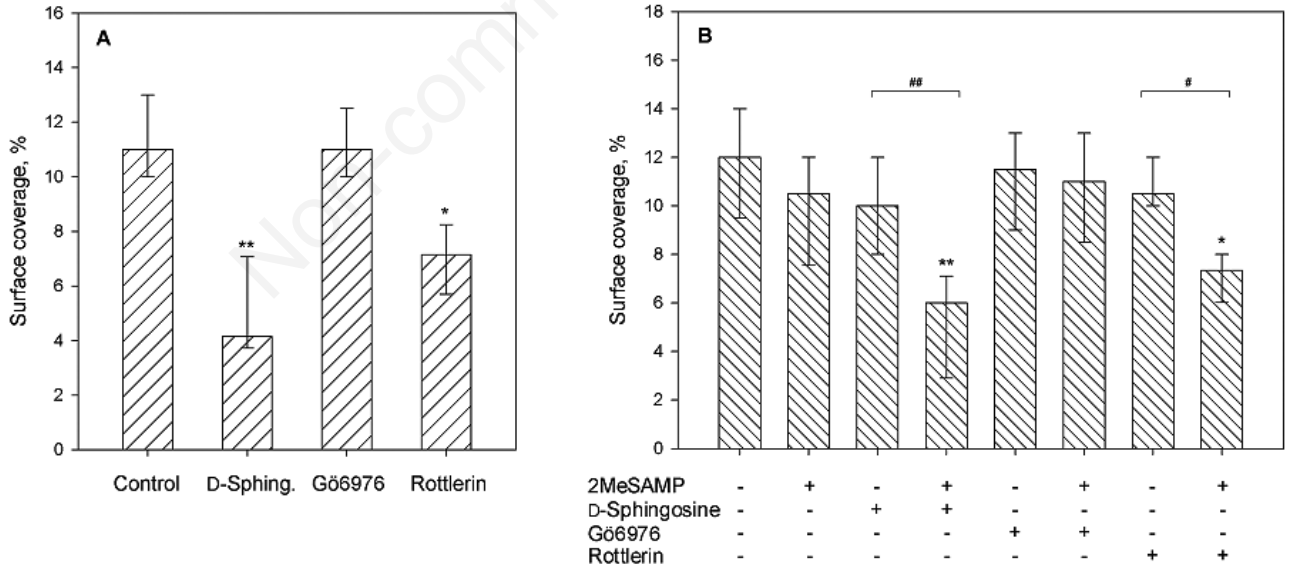

Figure 3. Role of PKC isotypes in platelet adhesion under flow. A) Modulation of platelet deposition by inhibitors of PKC isotypes. PRP samples were pre-incubated for 10 min with a vehicle (0.33% DMSO), 10mM D-erythro-sphingosine (D-Sphing.), 5 µM rottlerin and 1 µM Gö6976.[14-18] Whole blood reconstitution was performed, and the samples were subjected to the Impact-R test for 2 min at 1800 s^{-1} shear rate. The median surface coverage (SC) is presented. *P<0.05 and **P<0.01 *vs.* control (n=10). B) Combined effect of subthreshold concentrations (20% of those used above) of $P2Y_{12}$ and of PKC isotype antagonists on platelet deposition. PRP was pre-incubated for 10 min with 16 µM 2MeSAMP in the presence or absence of 2 µM D-erythro-sphingosine, 1µM rottlerin, and 0.2 µM Gö6976. Whole blood reconstitution was performed, and samples were subjected to the Impact-R test for 2 min at 1800 s-1 shear rate. The SC median (25^{th}-75^{th} percentile) is presented. *P<0.05, **P<0.01 *vs.* control, $^{\#}$P<0.01 and $^{\#\#}$P<0.001 *vs.* 2MeSAMP (n=10).

rinogen and von Willebrand factor were bound to polystyrene preceeding platelet adhesion.[17] We found that $P2Y_1$ and $P2Y_{12}$ inhibitors (A2P5P and 2MeSAMP, respectively) failed to affect platelet deposition at both static and relatively low shear conditions (200 and 1000 s^{-1}). In contrast, at high shear rate (1800 s^{-1}) inhibition of $P2Y_{12}$ but not $P2Y_1$ was followed by reduction of platelet adhesion. Despite the lack of the effect of A2P5P alone, synergistic inhibition has been achieved by combining A2P5P with 2MeSAMP. These data show that among the two ADP receptors, the main player promoting platelet deposition is the $P2Y_{12}$ receptor. The results of this study agree with the data that both $P2Y_1$ and $P2Y_{12}$ inhibitors retarded blood clotting induced by collagen-related peptide.[18] This effect was most pronounced with the $P2Y_{12}$ inhibitor. Turner and collaborators reported that both $P2Y_1$ and $P2Y_{12}$ antagonists inhibited platelet adhesion onto von Willebrand factor-collagen surface under shear rates of 750 and 1500 s^{-1}.[19] We have shown that blockade of $P2Y_{12}$ was also more effective than $P2Y_1$. The applied concentrations of ADP antagonists in our study were higher than in other studies. However, it must be taken into consideration that the effective concentrations of such agents depend on the different experimental models including static or flow conditions, as well as on the use of washed platelets, platelet-rich plasma or whole blood, and on the extent of shear rate.

It is well known that protein kinase C isoforms play an important role in platelet granule secretion, activation, aggregation, and procoagulant activity. In the present study we used inhibitors of PKC$\alpha\beta_1$ (Gö6976), PKCδ (rottlerin), as well as the pan-PKC inhibitor (D-erythro-sphingosine) to explore their role in platelet adhesion under flow conditions. We showed that PKCδ, but not PKCαβ is responsible for the reduction of platelet deposition. This is consistent with the data that PKCδ signaling is required for platelet aggregation.[20] Furthermore, novel but not conventional PKC isoforms regulate $P2Y_{12}$ function in experiments in which PKC was directly activated by phorbol ester 12-myristate 13-acetate.[10] We show here that at subthreshold concentrations (20% of those used above), combination of $P2Y_{12}$ blocker with either PKCδ or pan-PKC antagonists synergistically diminished platelet adhesion. We suggest that applying both inhibitors at low concentrations rather than separately at relatively high concentrations, may be useful in experimental or even in clinical studies. The results of our study show that $P2Y_{12}$ and PKCδ play an important role in shear stress-induced platelet adhesion, the initial step of thrombin formation.

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