The $P2Y_{12}$ receptor induces platelet aggregation through weak activation of the $\alpha_{IIb}\beta_3$ integrin – a phosphoinositide 3-kinase-dependent mechanism

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Abstract High concentrations of adenosine-5′-diphosphate ADP are able to induce partial aggregation without shape change of $P2Y_1$ receptor-deficient mouse platelets through activation of the $P2Y_{12}$ receptor. In the present work we studied the transduction pathways selectively involved in this phenomenon. Flow cytometric analyses using R-phycoerythrin-conjugated JON/A antibody (JON/A-PE), an antibody which recognizes activated mouse $\alpha_{IIb}\beta_3$ integrin, revealed a low level activation of $\alpha_{IIb}\beta_3$ in $P2Y_1$ receptor-deficient platelets in response to 100 μM ADP or 1 μM 2MeS-ADP. Adrenaline induced no such activation but strongly potentiated the effect of ADP in a dose-dependent manner. Global phosphorylation of ^{32}P-labeled platelets showed that $P2Y_{12}$-mediated aggregation was not accompanied by an increase in the phosphorylation of myosin light chain (P_{20}) or pleckstrin (P_{47}) and was not affected by the protein kinase C (PKC) inhibitor staurosporine. On the other hand, two unrelated phosphoinositide 3-kinase inhibitors, wortmannin and LY294002, inhibited this aggregation. Our results indicate that (i) the $P2Y_{12}$ receptor is able to trigger a $P2Y_1$ receptor-independent inside-out signal leading to $\alpha_{IIb}\beta_3$ integrin activation and platelet aggregation, (ii) ADP and adrenaline use different signaling pathways which synergize to activate the $\alpha_{IIb}\beta_3$ integrin, and (iii) the transduction pathway triggered by the $P2Y_{12}$ receptor is independent of PKC but dependent on phosphoinositide 3-kinase. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Platelet; ADP; $P2Y_{12}$ receptor; Phosphoinositide 3-kinase; $\alpha_{IIb}\beta_3$ integrin

1. Introduction

ADP is one of the main agonists of platelet activation and aggregation. After its release from dense granules in response to secretory agents like thrombin or collagen, ADP acts as a cofactor by contributing to enhancing platelet activation and stabilizing aggregates [1]. Two G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$, are involved in platelet aggregation in response to ADP. The $P2Y_1$ receptor, coupled to calcium mobilization, is necessary for ADP-induced platelet shape change and the initiation of aggregation [2,3]. The recently cloned $P2Y_{12}$ receptor [4,5], coupled to inhibition of adenylyl cyclase (AC) through a pertussis toxin-sensitive Gi_2 protein [5–7], is responsible for completion and amplification of the platelet response to ADP and other aggregating agents [8–10].

The generation of $P2Y_1$ receptor-deficient mice has shown that platelet aggregation in response to a usual concentration of ADP (5 μM) is totally lost when this receptor is absent [11,12], whereas a higher concentration of ADP (100 μM) is able to induce partial aggregation through activation of the $P2Y_{12}$ receptor [12]. This aggregation occurs without calcium movement or platelet shape change and leads to the formation of small aggregates of discoid resting-like platelets [12]. Interestingly, a similar aggregation without shape change in response to a high concentration of ADP occurs in Gαq-deficient mouse platelets [10] which confirms the involvement of the $P2Y_{12}$/Gi protein signaling pathway in this partial response.

In the present work, as we wished to determine the specific transduction pathways involved in $P2Y_{12}$ receptor-dependent platelet aggregation, we used $P2Y_1$ receptor-deficient mouse platelets in order to study $P2Y_{12}$ receptor signaling without $P2Y_1$ receptor transduction. Our results show that (i) ADP-induced $P2Y_{12}$ receptor activation triggers an inside-out signal leading to activation of the $\alpha_{IIb}\beta_3$ integrin and platelet aggregation, (ii) adrenaline, which per se does not induce platelet aggregation, although leading to inhibition of AC, strongly potentiates this response indicating that ADP may trigger some other transduction events, and (iii) the signaling pathway leading to aggregation of $P2Y_1$ receptor-deficient mouse platelets clearly involves activation of a phosphoinositide 3-kinase (PI 3-kinase) but is independent of protein kinase C (PKC).

2. Materials and methods

2.1. Materials

The ATP analogue AR-C69931MX was a generous gift from Astra Zeneca (Loughborough, UK). ADP, 2MeS-ADP, thrombin, adrenaline, prostaglandin E_1 (PGE_1), staurosporine, EGTA and human serum albumin (HSA) were from Sigma-Aldrich (Saint Quentin-Fallavier, France). LY294002 and wortmannin were from Calbiochem (Meudon, France). Human fibrinogen was from Kabi (Stockholm, Sweden) and the BCA protein assay kit from Pierce (Rockford, IL, USA). The cAMP assay kit and carrier-free orthophosphate (^{32}P) were from Amersham Pharmacia Biotech (Les Ulis, France). Apyrase was purified from potatoes as previously described [13].

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Abbreviations: AC, adenylyl cyclase; MLC, myosin light chain; PGE_1, prostaglandin E_1; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; SEM, scanning electron microscopy

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2.2. Animals

Mutant mice deficient in the $P2Y_1$ receptor were produced as described by Léon et al. [12] and both wild-type (WT) and knockout mice were of 129/Sv×C57BL/6 genetic background.

2.3. Platelet aggregation

Washed mouse platelets were prepared from blood (nine volumes) drawn from the abdominal aorta of anesthetized mice into a plastic syringe containing acid citrate dextrose anticoagulant (one volume). Pooled blood (8 ml) was centrifuged at 1570×*g* for 80 s at 37°C and platelet-rich plasma was removed and centrifuged at 1570×*g* for 8 min at 37°C. The platelet pellet was washed twice in Tyrode's 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% HSA 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 [14]. 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 (0.25 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.

2.4. cAMP measurement

A 450 μl aliquot of washed platelet suspension was stirred at 1100 rpm in an aggregometer cuvette and reagents were added at 30 s intervals. One minute after addition of the last agonist the reaction was stopped by adding 50 μl of ice-cold 6 N perchloric acid (PCA). cAMP was isolated from the supernatant by extraction with a mixture of trioctylamine and freon (28/22, v/v). The upper aqueous phase was lyophilized and the dry residue dissolved in the buffer provided with the commercial cAMP radioimmunoassay kit. cAMP was measured according to the kit instruction as previously described [3].

2.5. Scanning electron microscopy (SEM)

A 450 μl aliquot of platelet suspension was fixed at the maximum of ADP-induced platelet aggregation (2 min after addition of ADP 100 μM for WT platelets, 6 min for $P2Y_1$ receptor knockout mouse platelets) by addition of an equal volume of fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose, 305 mOsm, pH 7.3) previously warmed to 37°C. After 5 min at 37°C, the platelets were centrifuged at 12000×*g* for 20 s and the pellet was resuspended in fixative solution. The fixed platelets were allowed to adhere for 45 min to coverslips preincubated with 10 μg/ml poly-L-lysine. The coverslips were then washed three 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) S-800 scanning electron microscope (5 kV) as previously described [10].

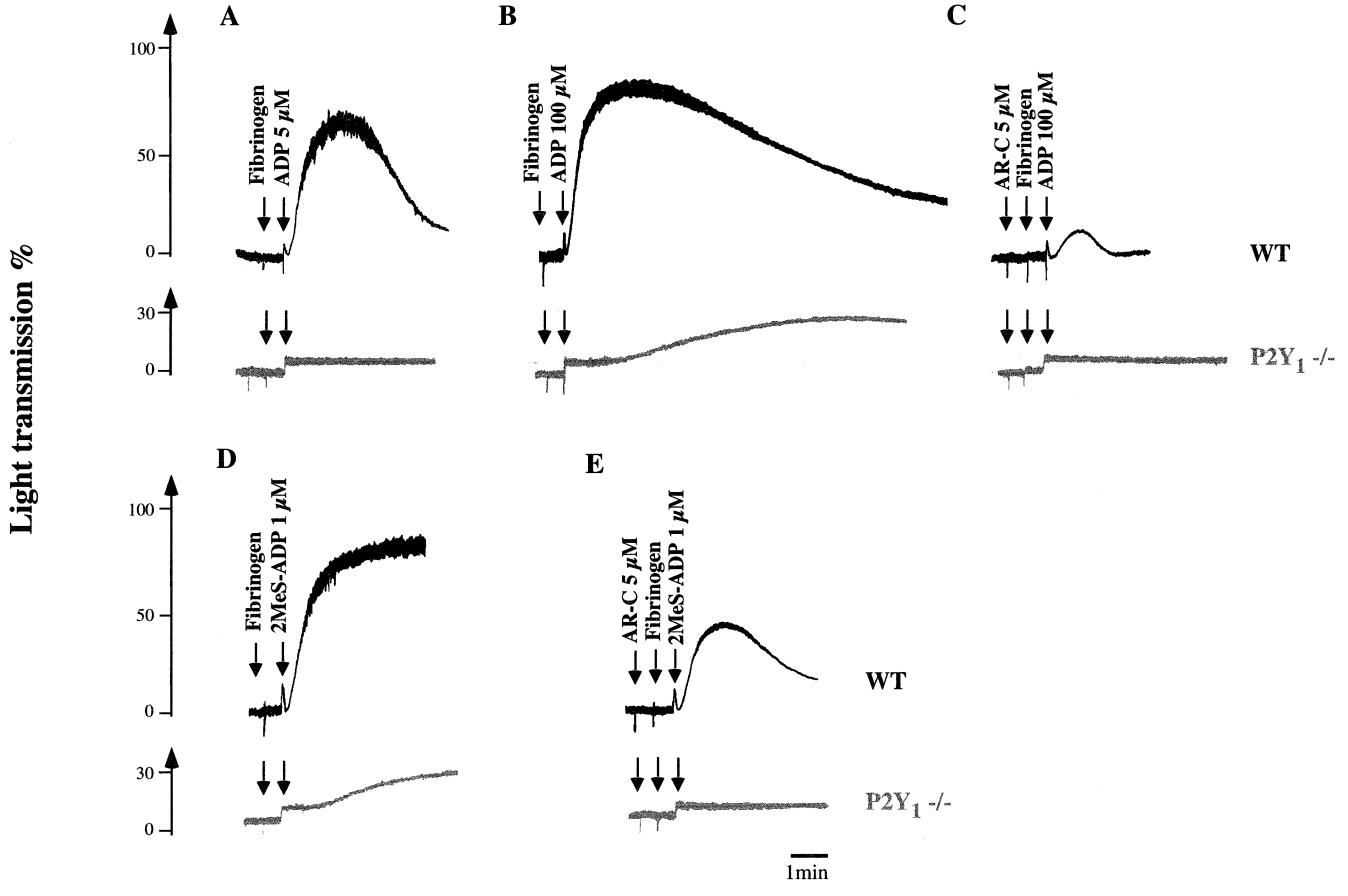

Fig. 1. Partial aggregation of $P2Y_1$ receptor-deficient mouse platelets induced by ADP or 2MeS-ADP is mediated by the $P2Y_{12}$ receptor. Aggregation of wild-type (WT, black curves) and $P2Y_1$ receptor-deficient mouse platelets ($P2Y_1-/-$, gray curves) was measured as described in Section 2.3 and the addition of reagents is indicated by arrows. A: Shape change and aggregation in response to 5 μM ADP is lost in $P2Y_1-/-$ as compared to WT platelets. B: ADP 100 μM induces partial aggregation of $P2Y_1-/-$ mouse platelets relative to the control. C: The selective $P2Y_{12}$ receptor antagonist AR-C69931MX (5 μM) inhibits aggregation of WT platelets and abolishes the partial aggregation induced by ADP 100 μM on $P2Y_1-/-$ mouse platelets. D: 2MeS-ADP 1 μM induces the same partial aggregation of $P2Y_1-/-$ platelets. E: AR-C69931MX (5 μM) inhibits aggregation in response to 2MeS-ADP 1 μM. The tracings are representative of three independent experiments giving identical results.

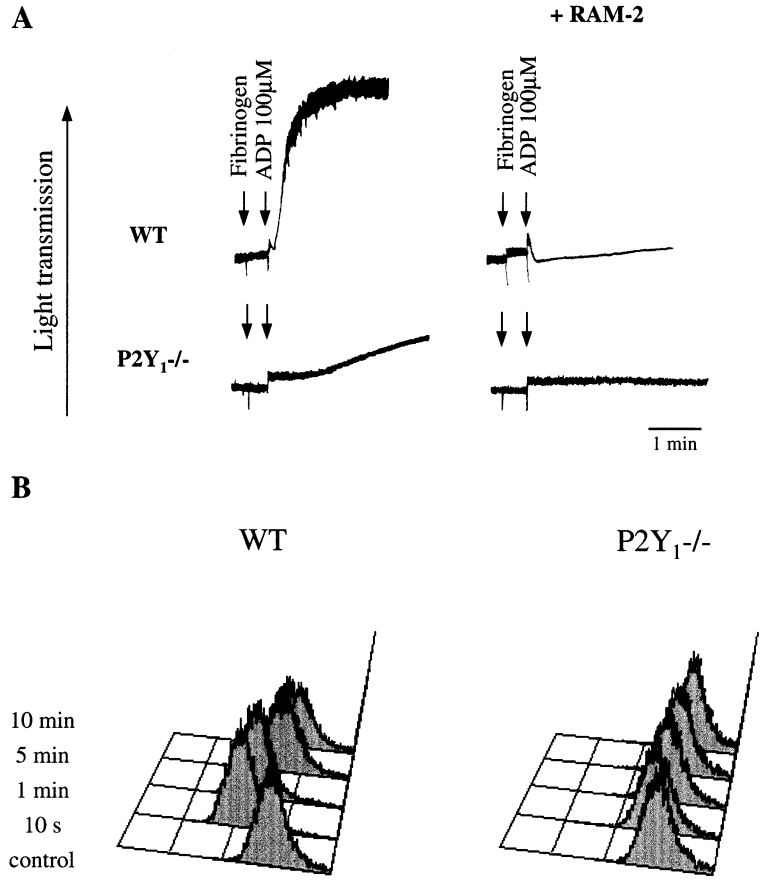

Fig. 2. $P2Y_{12}$ receptor-mediated aggregation of $P2Y_1-/-$ platelets occurs through activation of the $\alpha_{IIb}\beta_3$ integrin. A: RAM-2 antibody (1 μg/ml) against mouse $\alpha_{IIb}\beta_3$ integrin blocks aggregation of $P2Y_1-/-$ or WT platelets induced by 100 μM ADP. B: JON/A-PE, which preferentially binds to the active form of the integrin, was used to evaluate $\alpha_{IIb}\beta_3$ integrin activation. As indicated on the left, JON/A-PE was added to activated platelets at different time points after addition of ADP (100 μM) and samples were incubated for 10 min at 37°C. JON/A-PE binding to WT and $P2Y_1-/-$ platelets was analyzed on a FACScalibur.

2.6. Flow cytometric analysis of activation of the $\alpha_{IIb}\beta_3$ integrin

The activation of washed platelets was assessed with R-phycoerythrin-conjugated JON/A antibody (JON/A-PE) which preferentially binds to the active but not the resting form of the integrin (Bergmeier et al., in preparation). Washed platelets (5×10^7/ml in Tyrode–HEPES/1 mM $CaCl_2$) were stimulated with 100 μM ADP in the presence or absence of 5 μM adrenaline. JON/A-PE (5 μg/ml) was added to the platelets at the indicated time points after stimulation and suspensions were incubated for 10 min at 37°C. Data were analyzed using Cell Quest Software (FACScalibur 4C, Becton Dickinson, Pont de Claix, France).

2.7. Measurement of protein phosphorylation

To study the phosphorylation of myosin light chain (MLC) and pleckstrin during aggregation, platelets (8×10^5/μl) were first labeled for 90 min at 37°C with ^{32}P (10.5 MBq/10^9 platelets), in Tyrode's buffer without phosphate containing 0.35% HSA and apyrase. The platelets were then washed in Tyrode's buffer containing 0.35% HSA and finally resuspended at a density of 5×10^5 platelets/μl in Tyrode's buffer containing 0.1% HSA. Platelet aggregation was stopped at different time points by addition of 0.3 N ice-cold PCA. The precipitated proteins were centrifuged at $12\,000\times g$ for 4 min at 4°C and the pellet was washed in 500 μl of Tyrode's buffer without glucose, calcium or magnesium. After a second centrifugation at $12\,000\times g$ for 2 min, the proteins were dissolved in 50 μl of solubilization buffer (50 mM Tris pH 6.8, 2 mM *N*-ethylmaleimide, 3% glycerol, 2% SDS, 5% 2-mercaptoethanol) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM aPMSF). Protein concentration was determined by BCA, and aliquots (50 μg) were separated on polyacrylamide gel gradients (7.5–12%) in migration buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.35). The gels were stained with Coomassie blue, dried and labeled proteins were located by autoradiography on β-max film (Amersham Pharmacia Biotech, Les Ulis, France). Band densities were quantified with Quantity One Software (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Partial aggregation of $P2Y_1$ receptor-deficient mouse platelets in response to a high concentration of ADP is mediated by the $P2Y_{12}$ receptor

As previously reported [12] $P2Y_1$ receptor-deficient ($P2Y_1-/-$) mouse platelets were unable to aggregate in response to 5 μM ADP (Fig. 1A). In contrast, stimulation with 100 μM ADP (Fig. 1B) or 1 μM 2MeS-ADP (Fig. 1D) resulted in partial aggregation which reached 29.0 ± 2.7% for ADP and 28.4 ± 3.5% for 2MeS-ADP, with no turbidimetrically measurable effect on shape change ($n = 3$). Control aggregation of WT platelets reached 77.9 ± 3.1% (Fig. 1B). The time course of this partial aggregation was slow and attained its maximum only after 6–7 min, whereas aggregation of WT platelets was maximal at 2 min (Fig. 1B). Aggregation of $P2Y_1-/-$ mouse platelets in response to ADP or 2MeS-ADP (100 μM and 1 μM respectively) was abolished by 5 μM AR-C69931MX (Fig. 1C,E), a selective $P2Y_{12}$ receptor antagonist [15], indicating its dependence on the $P2Y_{12}$ receptor.

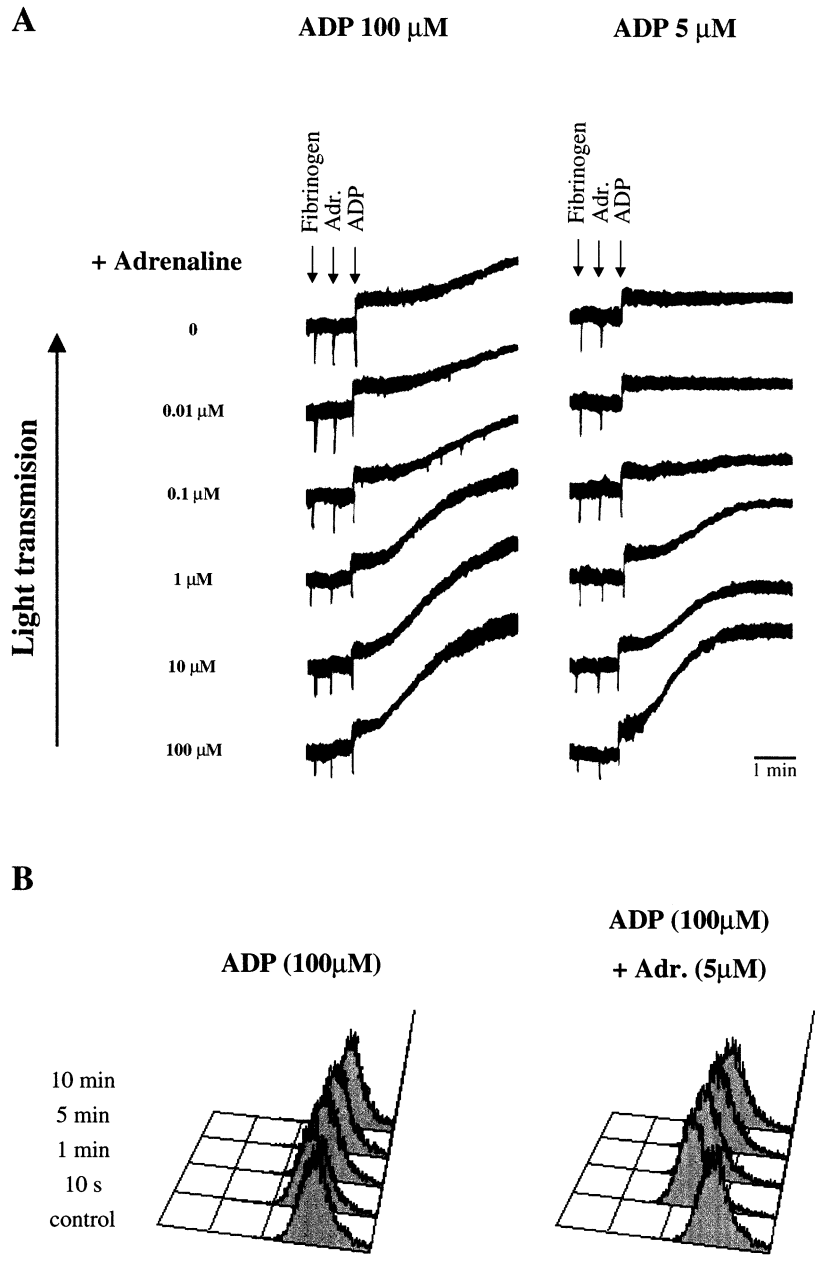

Fig. 3. Adrenaline potentiates the responses of $P2Y_1-/-$ platelets to ADP. A: $P2Y_1-/-$ platelets were stimulated with ADP (5 or 100 μM) in the presence of increasing concentrations of adrenaline. Adrenaline potentiates the aggregation induced by 100 μM ADP in a dose-dependent manner (left). Addition of adrenaline (>0.1 μM) restores aggregation in response to 5 μM ADP (right). The tracings are representative of two separate experiments giving identical results. B: Adrenaline (5 μM) enhances the binding of JON/A-PE to ADP-activated mouse $\alpha_{IIb}\beta_3$ integrin on $P2Y_1-/-$ platelets. At the indicated time points, JON/A-PE was added to $P2Y_1-/-$ platelets activated with 100 μM ADP (left panel) or 100 μM ADP in combination with 5 μM adrenaline (right panel). Samples were incubated for 10 min at 37°C and analyzed directly. C: WT (a) and $P2Y_1-/-$ (b–f) mouse platelets were prepared for SEM as described in Section 2.5. ADP (5 μM) induces shape change and aggregation in WT platelets (a), whereas $P2Y_1-/-$ platelets remain single and discoid after stimulation with 5 μM ADP (b) or 100 μM adrenaline alone (c). ADP (100 μM) induces small aggregates of discoid $P2Y_1-/-$ platelets (d) and the size of these aggregates is increased in the presence of 100 μM adrenaline (f). Adrenaline (1 μM) restores aggregation of $P2Y_1-/-$ platelets stimulated with 5 μM ADP (e). Magnification: ×6000.

3.2. *$P2Y_{12}$ receptor activation leads to exposure of the $\alpha_{IIb}\beta_3$ integrin*

RAM.2, a rat polyclonal antibody against mouse $\alpha_{IIb}\beta_3$ integrin known to inhibit mouse platelet aggregation [10], was added to the platelet suspension 1 min before ADP. It prevented aggregation of WT or $P2Y_1-/-$ platelets induced by 100 μM ADP (Fig. 2A), showing that $P2Y_{12}$ receptor-dependent aggregation occurred through $\alpha_{IIb}\beta_3$ activation. The kinetics of $\alpha_{IIb}\beta_3$ integrin activation on WT and $P2Y_1-/-$ platelets were assessed by flow cytometric analysis of binding of JON/A-PE, a rat monoclonal antibody derivative which preferentially recognizes activated mouse $\alpha_{IIb}\beta_3$ integrin (Bergmeier et al., in preparation). In WT platelets, $\alpha_{IIb}\beta_3$ integrin activation occurred rapidly and was reversible, reaching its maximum 10 s after stimulation with ADP (100 μM) (Fig. 2B). In comparison, $\alpha_{IIb}\beta_3$ integrin activation of stimulated

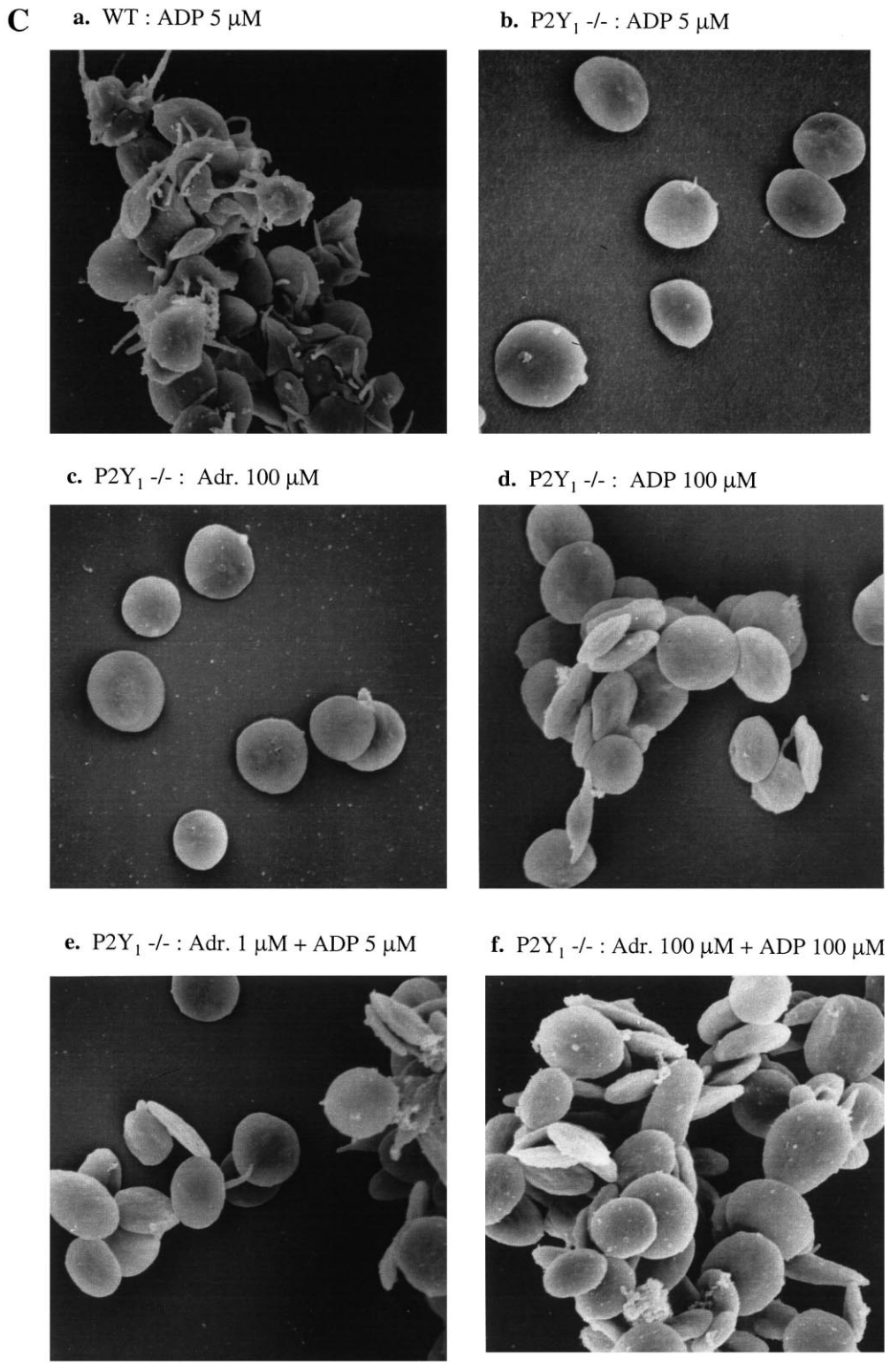

Fig. 3 (*continued*).

$P2Y_1-/-$ platelets was strongly reduced. Although activation was complete at 10 s as in the WT, it remained irreversible during the 10 min of observation which correlates with the aggregation tracings (Fig. 2B). The apparent discrepancy between the slow onset of aggregation and $\alpha_{IIb}\beta_3$ integrin activation is mainly due to a lack of sensitivity of the turbidimetric method which fails to detect small aggregates. In order to address this issue, single platelet counting was used to evaluate platelet aggregation to 100 μM ADP. A 7% decrease in the platelet count was observed as soon as 40 s after addition of

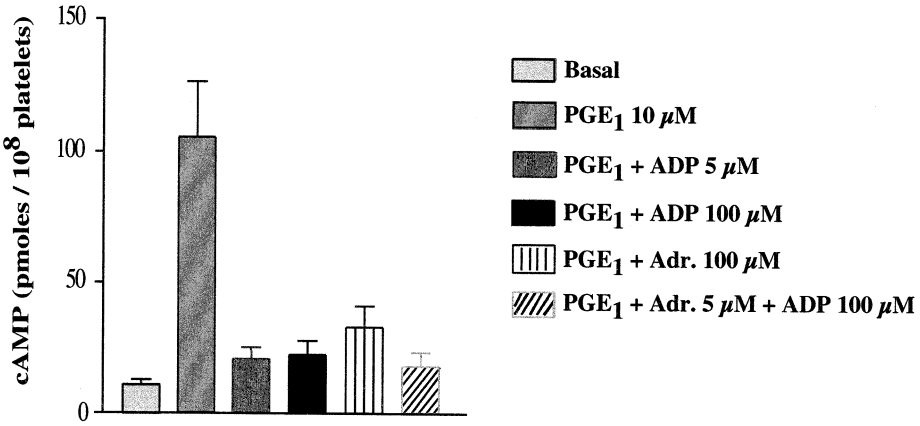

Fig. 4. Inhibition of AC by ADP and adrenaline in $P2Y_1$ receptor-deficient mouse platelets. Inhibition of AC activity was measured in platelets prestimulated with PGE_1 10 μM as described in Section 2. ADP (5 or 100 μM), adrenaline (100 μM), or a combination of these two agonists decreased cAMP levels in prestimulated platelets to the same extent. Data are the mean ± S.E.M. from three separate experiments. $^*P < 0.05$.

ADP, reaching 52% at 10 min. These experiments have been repeated two times and gave identical results.

3.3. *Potentiation of $P2Y_{12}$ receptor-mediated platelet aggregation by adrenaline*

Adrenaline is not in itself an aggregating agent [16,17] and at concentrations of up to 1 mM was not able to induce aggregation of either WT or $P2Y_1-/-$ mouse platelets (data not shown). On the other hand, adrenaline potentiated $P2Y_1-/-$ mouse platelet aggregation in response to either ADP 100 μM (Fig. 3A, left panel) or 2MeS-ADP 1 μM (data not shown) in a dose-dependent manner from 1 to 100 μM. Moreover, addition of adrenaline at a concentration as low as 1 μM allowed the aggregation of $P2Y_1-/-$ mouse platelets in response to 5 μM ADP (Fig. 3A, right panel). These results suggest that ADP and adrenaline activate signaling pathways which potentiate each other, whereas adrenaline alone is not able to trigger an inside-out signal leading to platelet aggregation like ADP does. Flow cytometric analysis further showed that 5 μM adrenaline potentiated $\alpha_{IIb}\beta_3$ integrin activation on $P2Y_1-/-$ platelets stimulated with 100 μM ADP (Fig. 3B) or 1 μM 2MeS-ADP (data not

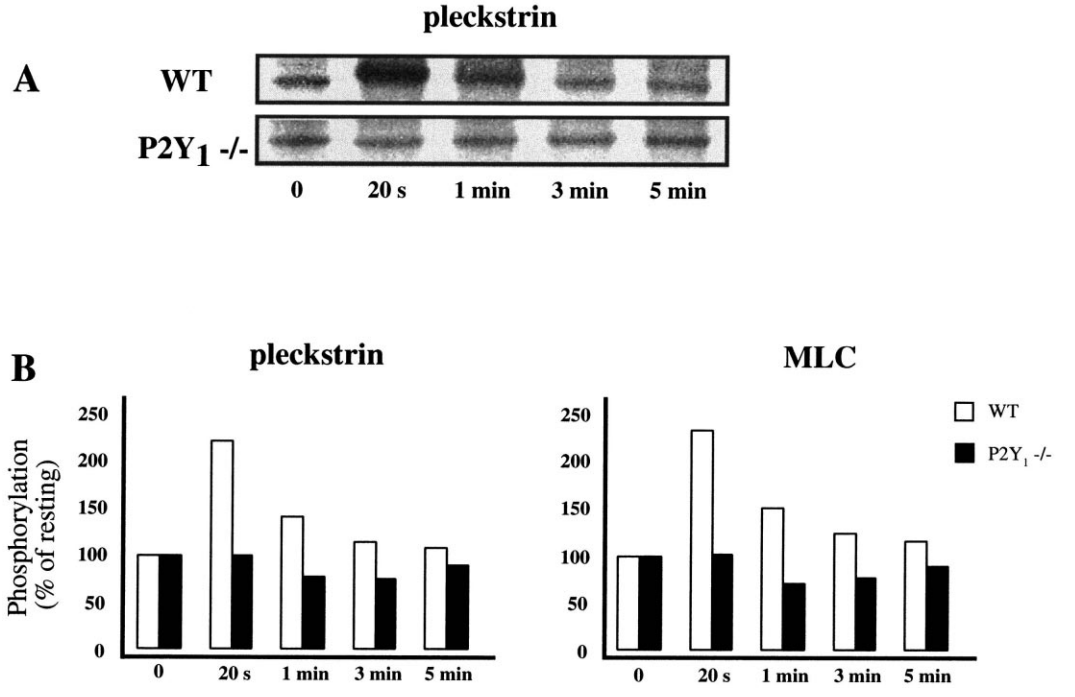

Fig. 5. $P2Y_{12}$ receptor-mediated aggregation of $P2Y_1-/-$ platelets occurs without phosphorylation of pleckstrin or MLC. The time course of the phosphorylation of pleckstrin and MLC was determined in WT and $P2Y_1-/-$ platelets activated with 100 μM ADP. A: Representative autoradiography showing that 100 μM ADP induces early phosphorylation of pleckstrin in WT platelets (upper panel) but not in $P2Y_1-/-$ platelets (lower panel). B: WT (open bars) and $P2Y_1-/-$ platelets (black bars) were stimulated with 100 μM ADP. At the indicated times, the reaction was stopped and phosphorylated proteins were isolated and separated by SDS–PAGE (7.5–12%). The labeled proteins were located by autoradiography and quantified as described in Section 2.7. Curves are representative of two independent experiments giving similar results.

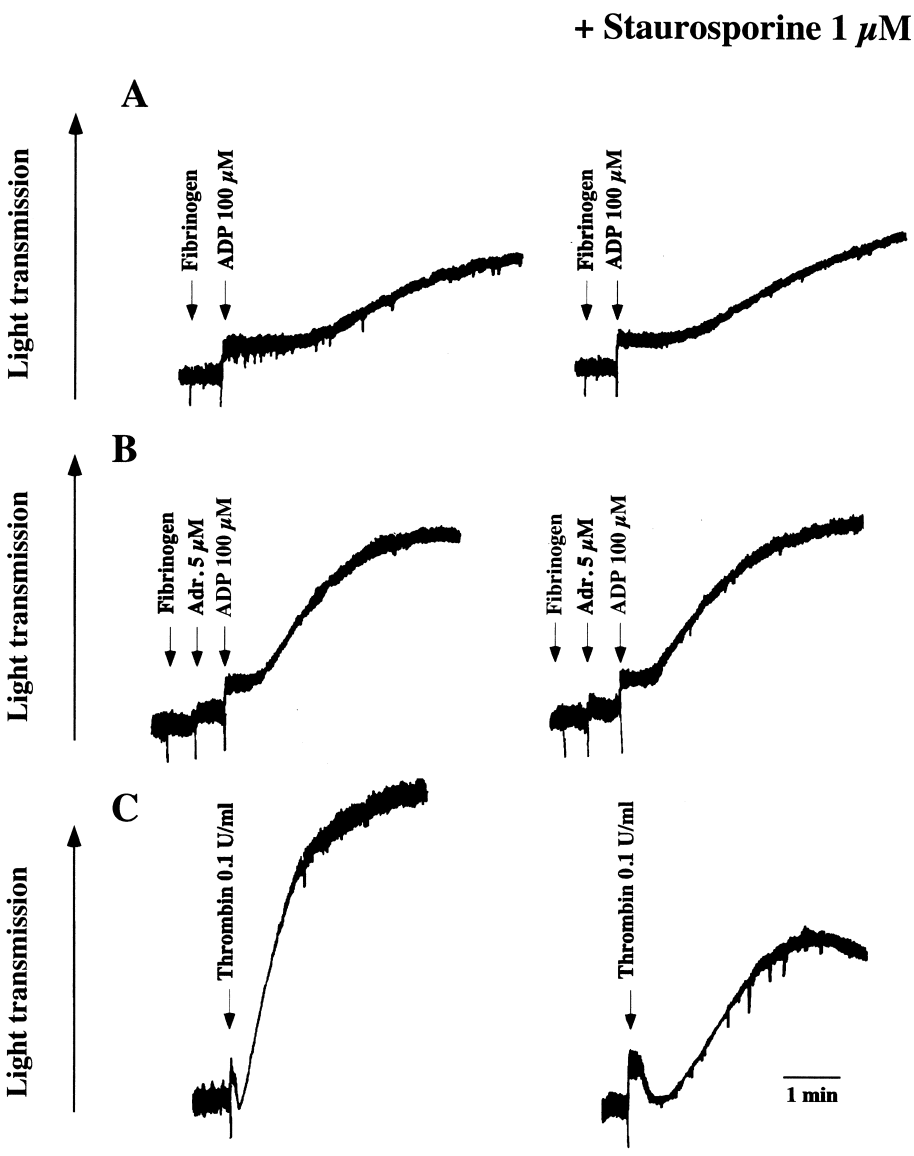

Fig. 6. Staurosporine does not affect $P2Y_{12}$ receptor-mediated platelet aggregation. Aggregation of $P2Y_1-/-$ mouse platelets was induced by addition of 100 μM ADP (A), 5 μM adrenaline and 100 μM ADP (B) or 0.1 U/ml thrombin (C). The platelets were previously treated with either vehicle or 1 μM staurosporine for 30 min. A, B: Staurosporine does not modify the response of $P2Y_1-/-$ platelets to 100 μM ADP, alone or in the presence of 5 μM adrenaline. C: $P2Y_1-/-$ platelet aggregation induced by 0.1 U/ml thrombin is decreased by staurosporine. The tracings are representative of two independent experiments giving identical results.

shown). Hence, the potentiating effect of adrenaline on platelet aggregation results in stronger activation of the $\alpha_{IIb}\beta_3$ integrin.

3.4. *$P2Y_{12}$ receptor-dependent aggregation occurs without platelet shape change*

SEM showed that $P2Y_1-/-$ platelets stimulated with 5 μM ADP (Fig. 3Cb) or 100 μM adrenaline (Fig. 3Cc) conserved the typical morphology of discoid resting platelets, in contrast to the large aggregates of packed cells with membrane extrusions formed by WT platelets activated with 5 μM ADP (Fig. 3Ca). In $P2Y_1-/-$ platelets, 100 μM ADP induced the formation of small aggregates of platelets which have not changed their shape (Fig. 3Cd). In the presence of 1 μM adrenaline, 5 μM ADP induced some similar small aggregates of discoid platelets (Fig. 3Ce). The association of 100 μM adrenaline and 100 μM ADP induced the formation of larger aggregates (Fig. 3Cf). The potentiating effect of adrenaline on ADP-induced platelet aggregation was not accompanied by shape change in $P2Y_1-/-$ mouse platelets.

3.5. *Inhibition of AC by ADP cannot explain $P2Y_{12}$ receptor-mediated aggregation*

ADP (5 or 100 μM) or adrenaline (up to 100 μM) decreased cAMP accumulation in PGE_1-prestimulated platelets to the same extent (Fig. 4), suggesting that a lowering of cAMP levels is not sufficient to explain the $P2Y_{12}$ receptor-mediated aggregation induced by 100 μM ADP in accordance with results obtained in Gαq-deficient mouse platelets [10]. A combination of 5 μM adrenaline and 100 μM ADP likewise did not inhibit cAMP levels significantly more than either agonist alone. Thus, adrenaline must potentiate the effect of ADP

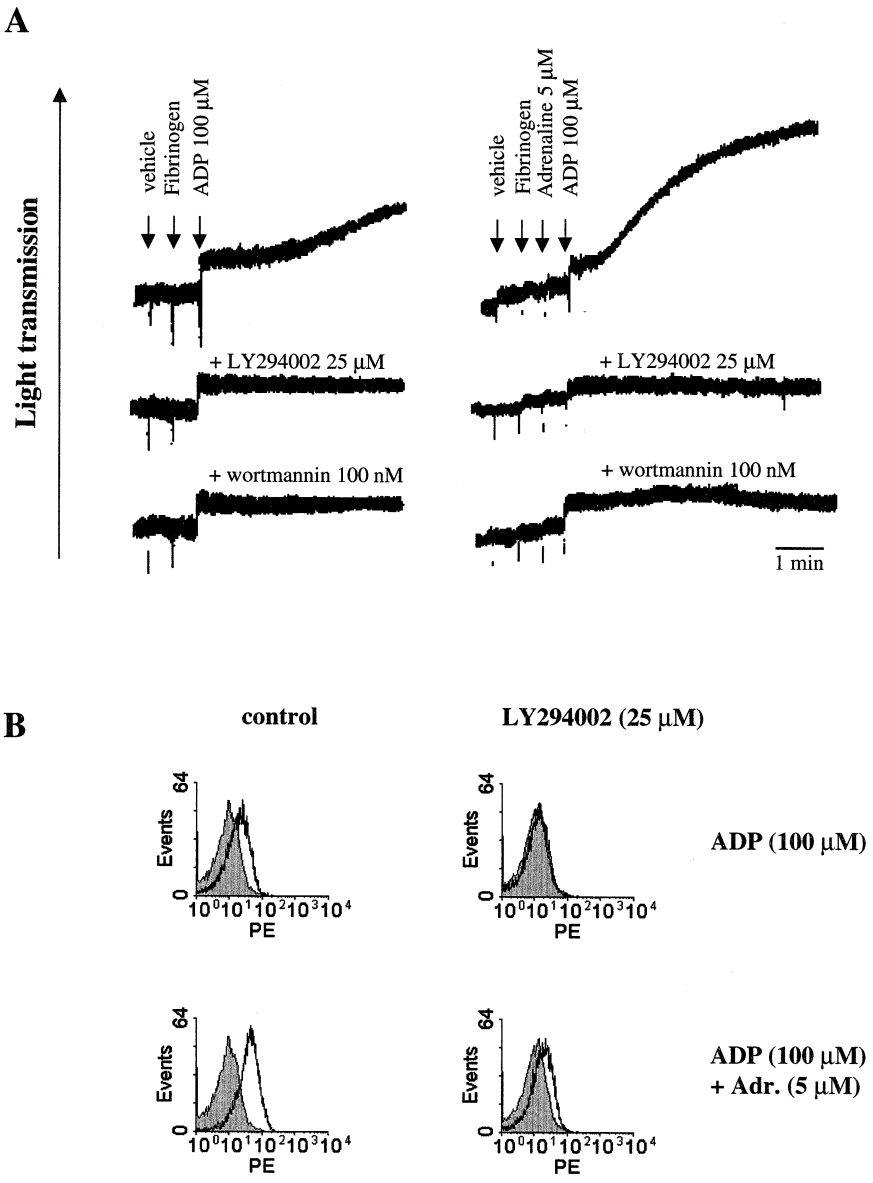

Fig. 7. $P2Y_{12}$ receptor-mediated platelet aggregation involves activation of PI 3-kinase. A: $P2Y_1-/-$ platelets treated for 1 min with either vehicle (upper tracing), 25 μM LY294002 (middle tracing) or 100 nM wortmannin (lower tracing) were stimulated with 100 μM ADP (left panel) or with 5 μM ADP and 5 μM adrenaline (right panel). Partial aggregation in response to 100 μM ADP was abolished by both inhibitors of PI 3-kinase. The tracings are representative of four separate experiments. B: Flow cytometric analysis of the inhibitory effect of LY294002 (25 μM) on JON/A-PE binding to $P2Y_1-/-$ platelets stimulated with 100 μM ADP in the presence or absence of 5 μM adrenaline. JON/A-PE was added to activated platelets 10 s after addition of agonist(s) and samples were incubated for 10 min at 37°C. Mean log fluorescence intensity of JON/A-PE bound to resting (gray curve) and activated (solid line) platelets was measured in the absence (left panel) or presence (right panel) of 25 μM LY294002.

through some transduction pathway different from AC inhibition.

3.6. $P2Y_{12}$ receptor-mediated platelet aggregation is not accompanied by phosphorylation of MLC and pleckstrin

The global phosphorylation of platelet proteins during aggregation showed that aggregation of WT platelets in response to 100 μM ADP was accompanied by phosphorylation of pleckstrin and MLC (Fig. 5). This phosphorylation was reversible and reached a maximum at 20 s. In contrast, no phosphorylation of these two proteins was observed during $P2Y_{12}$ receptor-mediated aggregation. Moreover, despite its potentiating effect on $P2Y_{12}$ receptor-mediated aggregation (Fig. 3), 5 μM adrenaline in combination with 100 μM ADP did not induce MLC or pleckstrin phosphorylation in $P2Y_1-/-$ mouse platelets (data not shown). The phosphorylation of MLC and pleckstrin therefore does not seem to be involved in the signaling pathway leading to $P2Y_{12}$ receptor-mediated aggregation or its potentiation by adrenaline.

3.7. $P2Y_{12}$ receptor-mediated platelet aggregation is independent of PKC activation

To test the possible involvement of PKC in $P2Y_{12}$ receptor-mediated aggregation, we used the PKC inhibitor staurosporine. In $P2Y_1-/-$ platelets, 1 μM staurosporine had no effect on the partial aggregation induced by 100 μM ADP (Fig. 6A),

or on its potentiation by adrenaline (Fig. 6B), but diminished thrombin-induced aggregation in WT mouse platelets (Fig. 6C). Hence $P2Y_{12}$-mediated platelet aggregation does not depend on PKC activation.

3.8. $P2Y_{12}$-mediated platelet aggregation involves PI 3-kinase activation

The two unrelated PI 3-kinase inhibitors, LY294002 (25 μM) and wortmannin (100 nM), both abolished aggregation induced by 100 μM ADP alone (Fig. 7A, left panel) or by 5 μM ADP in the presence of 5 μM adrenaline (Fig. 7A, right panel) in $P2Y_1-/-$ platelets. Flow cytometric analysis of $P2Y_1$ receptor-deficient platelets showed that preincubation with the PI 3-kinase inhibitor LY294002 (25 μM) abolished $\alpha_{IIb}\beta_3$ integrin activation induced by 100 μM ADP and strongly diminished its activation induced by 100 μM ADP in combination with 5 μM adrenaline (Fig. 7B). These results clearly demonstrate that PI 3-kinase activation is involved in $P2Y_{12}$ receptor-mediated inside-out signaling.

4. Discussion

The aim of the present work was to study the transduction pathways selectively involved in $P2Y_{12}$ receptor-mediated aggregation in platelets lacking the $P2Y_1$ receptor. In a previous report, we showed that aggregation of $P2Y_1$ receptor-deficient mouse platelets in response to 100 μM ADP occurred in the absence of calcium movement and platelet shape change [12]. This aggregation has a profile very different from that of the WT platelet aggregation induced by ADP. It is of low amplitude and slow, reaching its maximum only after 6–7 min (Fig. 1B). However, it is dependent on $\alpha_{IIb}\beta_3$ integrin activation, as shown by flow cytometric monitoring of the JON/A-PE antibody recognizing the activated integrin. Activation of the $\alpha_{IIb}\beta_3$ integrin following ADP stimulation was low in $P2Y_1-/-$ as compared to WT platelets, which correlates with the weak profile of $P2Y_{12}$ receptor-mediated aggregation.

Adrenaline is known to potentiate platelet functions and downregulate AC activity in a similar manner to ADP [16]. ADP 5 or 100 μM inhibited AC to the same extent (Fig. 4), while 100 μM but not 5 μM ADP induced $P2Y_{12}$ receptor-mediated platelet aggregation in $P2Y_1$-deficient platelets. These results indicate that inhibition of AC by itself is not sufficient to trigger aggregation of $P2Y_1$-deficient platelets in response to ADP and are in agreement with earlier studies [18,19]. These results also suggest that activation of the $P2Y_{12}$ receptor triggers some additional events distinct from those induced by α_2-adrenergic receptor activation. Combination of 5 μM adrenaline and 100 μM ADP did not further decrease cAMP levels as compared to 100 μM ADP alone but greatly potentiated aggregation suggesting that the potentiating effect of adrenaline on $P2Y_{12}$ receptor-mediated aggregation is not due to inhibition of AC. It was recently reported [20] that specific disruption of the Gαz subunit gene resulted in severe impairment of the ability of adrenaline to potentiate aggregation and inhibit AC, suggesting that the α_2-adrenergic receptor prefers Gz to other members of the Gi family expressed in platelets. Concerning the $P2Y_{12}$ receptor, it would appear to couple mainly to a Gi_2 protein. This was first shown by specific 4-azidoanilido-[α-^{32}]GTP photolabeling of Gi_2 in platelets stimulated with ADP [6] and has since been confirmed in $G\alpha i_2$-deficient mice, which display impairment of ADP-induced platelet aggregation and AC inhibition [7]. The overall findings therefore suggest that the $P2Y_{12}$ receptor associates preferentially with a Gi_2 protein to trigger $\alpha_{IIb}\beta_3$ integrin activation and aggregation, whereas the α_2-adrenergic receptor associates more readily with a Gz subtype which cannot transmit such a message but could be responsible for its potentiation. Another possibility would be that the $P2Y_{12}$ receptor is linked to some other signaling pathway in addition to Gi_2.

Two polypeptides of 20 kDa (MLC) and 45–47 kDa (pleckstrin) display increased phosphorylation after stimulation of WT mouse platelets with ADP (Fig. 5). In contrast, increased phosphorylation of these two proteins was not observed in $P2Y_1-/-$ mouse platelets stimulated by ADP (Fig. 5), thus MLC and pleckstrin phosphorylations are not involved in $P2Y_{12}$-dependent platelet aggregation. MLC is involved in platelet shape change [21] and its absence of increased phosphorylation is in accordance with the absence of shape change during $P2Y_{12}$ receptor-mediated aggregation. Since pleckstrin was likewise not phosphorylated during $P2Y_{12}$-dependent aggregation, it appears that the $P2Y_1$ receptor is responsible for this effect. This result was confirmed by the use of the selective $P2Y_1$ receptor antagonist, MRS2179 [22], which totally inhibited pleckstrin phosphorylation induced by ADP in WT mouse platelets while AR-C69931MX, the selective $P2Y_{12}$ receptor antagonist [15], did not decrease it significantly (data not shown). Moreover, the PKC inhibitor staurosporine did not affect $P2Y_{12}$-dependent aggregation. These results suggest that PKC does not participate in $P2Y_{12}$ receptor signaling. In addition, the potentiating effect of adrenaline on $P2Y_{12}$-mediated aggregation was neither accompanied by an increase in pleckstrin phosphorylation (data not shown) nor inhibited by staurosporine (Fig. 6), indicating that PKC is not involved in potentiation of platelet functions through the α_2-adrenergic receptor.

Activation of PI 3-kinase following its translocation to the cytoskeleton has been shown to stabilize platelet aggregates [23,24]. Trumel et al. [9] have clearly demonstrated that the $P2Y_{12}$ receptor itself is responsible for stabilization of platelet aggregates induced by PAR-1 thrombin receptor activation in a PI 3-kinase-dependent manner. This stabilization seems to be linked to the late production of phosphatidylinositol 3,4-bisphosphate. Our results using LY294002 and wortmannin (Fig. 7) show that PI 3-kinase activity is required for $P2Y_{12}$ receptor-mediated platelet aggregation. Interestingly PI 3-kinase inhibitors abolished both $P2Y_{12}$-dependent platelet aggregation and $\alpha_{IIb}\beta_3$ integrin activation in response to ADP 100 μM (Fig. 7) suggesting that PI 3-kinase activity is required upstream of integrin activation. Since LY294002 and wortmannin do not show selectivity upon PI 3-K subtypes the question remains as to which subtype(s) of enzyme is (are) involved in this activation. Several studies have described a platelet PI 3-kinase activity which is regulated by Gβγ oligomers [25]. The monomeric p110γ PI 3-kinase [26] has been shown to be directly activated by Gβγ oligomers in vitro and could thus play a role in $P2Y_{12}$ receptor signaling. Selheim et al. showed recently that adrenaline was able to sustain SFRLLN-induced human platelet aggregation in an ADP and PI 3-K-independent manner. However, adrenaline could potentiate phosphatidylinositol 3,4-bisphosphate formation, but this effect was dependent on secreted ADP [27]. All these data suggest that in $P2Y_{12}$ receptor-dependent platelet aggregation

adrenaline potentiates a PI 3-kinase activity that has been triggered by ADP. Analysis of the PI 3-kinase products, namely phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, in the course of ADP-dependent aggregation of $P2Y_1$-deficient mouse platelets will be helpful in order to determine which PI 3-kinase subtype is involved downstream of $P2Y_{12}$ receptor activation.

In conclusion, the present results demonstrate that the $P2Y_{12}$ receptor is able to trigger a PI 3-kinase-dependent inside-out signal leading to partial aggregation of $P2Y_1$ receptor-deficient mouse platelets.

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References

[1] Cattaneo, M. and Gachet, C. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2281–2285.
[2] Jin, J., Daniel, J.L. and Kunapuli, S.P. (1998) J. Biol. Chem. 273, 2030–2034.
[3] Hechler, B., Leon, C., Vial, C., Vigne, P., Frelin, C., Cazenave, J.P. and Gachet, C. (1998) Blood 92, 152–159.
[4] Zhang, F.L., Luo, L., Gustafson, E., Lachowicz, J., Smith, M., Qiao, X., Liu, Y.H., Chen, G., Pramanik, B., Laz, T.M., Palmer, K., Bayne, M. and Monsma, F. (2000) J. Biol. Chem. 276, 8608–8615.
[5] Hollopeter, G., Jantzen, H.M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.B., Nurden, P., Nurden, A., Julius, D. and Conley, P.B. (2001) Nature 409, 202–207.
[6] Ohlmann, P., Laugwitz, K.L., Nurnberg, B., Spicher, K., Schultz, G., Cazenave, J.P. and Gachet, C. (1995) Biochem. J. 312, 775–779.
[7] Jantzen, H.M., Milstone, D.S., Gousset, L. and Conley, P.B. (2000) Haematologica 85 (The Platelet ADP Receptors Supplement), 85.
[8] Hechler, B., Eckly, A., Ohlmann, P., Cazenave, J.P. and Gachet, C. (1998) Br. J. Haematol. 103, 858–866.
[9] Trumel, C., Payrastre, B., Plantavid, M., Hechler, B., Viala, C., Presek, P., Martinson, E.A., Cazenave, J.P., Chap, H. and Gachet, C. (1999) Blood 94, 1–11.
[10] Ohlmann, P., Eckly, A., Freund, M., Cazenave, J.P., Offermanns, S. and Gachet, C. (2000) Blood 96, 2134–2139.
[11] Fabre, J.E., Nguyen, M.T., Latour, A., Kiefer, J.A., Audoly, L.P., Coffman, T.M. and Koller, B.H. (1999) Nature Med. 5, 1199–1202.
[12] Léon, C., Hechler, B., Feund, M., Eckly, A., Vial, C., Ohlmann, P., Dierich, A., LeMeur, M., Cazenave, J.P. and Gachet, C. (1999) J. Clin. Invest. 104, 1731–1737.
[13] Cazenave, J.P., Hemmedinger, S., Beretz, A., Sutter-Bay, A. and Launay, J. (1983) Ann. Biol. Clin. 41, 167–179.
[14] Baurand, A., Eckly, A., Bari, N., Léon, C., Hechler, B., Cazenave, J.P. and Gachet, C. (2000) Thromb. Haemost. 84, 484–491.
[15] Ingall, A.H., Dixon, J., Bailey, A., Coombs, M.E., Cox, D., McInally, J.I., Hunt, S.F., Kindon, N.D., Teobald, B.J., Willis, P.A., Humphries, R.G., Leff, P., Clegg, J.A. and Tomlinson, W. (1999) J. Med. Chem. 42, 213–220.
[16] Lanza, F., Beretz, A., Stierlé, A., Hanau, D., Kubina, M. and Cazenave, J.P. (1988) Am. J. Physiol. 255 (6 Pt 2), H1276–1288.
[17] Steen, V.M., Holmsen, H. and Aarbakke, G. (1993) Thromb. Haemost. 70, 506–513.
[18] Haslam, R.J., Davidson, M.M. and Desjardins, J.V. (1978) Biochem. J. 176, 83–95.
[19] Savi, P., Pflieger, A.M. and Herbert, J.M. (1996) Blood Coagul. Fibrinolysis 7, 249–252.
[20] Yang, J., Wu, J., Kowalska, M.A., Dalvi, A., Prevost, N., O'Brien, P.J., Manning, D., Poncz, M., Lucki, I., Blendy, J.A. and Brass, L.F. (2000) Proc. Natl. Acad. Sci. USA 97, 9984–9989.
[21] Daniel, J.L., Molish, I.R., Rigmaiden, M. and Stewart, G. (1984) J. Biol. Chem. 259, 9826–9831.
[22] Boyer, J.L., Mohanram, A., Camaioni, E., Jacobson, K.A. and Kendall, Harden,T. (1998) Br. J. Pharmacol. 124, 1–3.
[23] Gachet, C., Payrastre, B., Guinebault, C., Trumel, C., Ohlmann, P., Mauco, G., Cazenave, J.P., Plantavid, M. and Chap, H. (1997) J. Biol. Chem. 272, 4850–4854.
[24] Kovacsovics, T.J., Bachelot, C., Toker, A., Vlahos, C.J., Duckworth, B., Cantley, L.C. and Hartwig, J.H. (1995) J. Biol. Chem. 270, 11358–11366.
[25] Thomason, P.A., James, S.R., Casey, P.J. and Downes, C.P. (1994) J. Biol. Chem. 269, 16525–16528.
[26] Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanov, S., Vanhaesebroeck, B., Dhand, R., Nürnberg, B., Gierschik, P., Seedorf, K., Justin Hsuan, J., Waterfield, M.D. and Wetzker, R. (1995) Science 269, 690–693.
[27] Selheim, F., Froyset, A.K., Strand, I., Vassbotn, F.S. and Holmsen, H. (2000) FEBS Lett. 485, 62–66.