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The ATP-Gated $P2X_1$ Ion Channel Acts as a Positive Regulator of Platelet Responses to Collagen

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Keywords

Platelet receptor, ATP, collagen, shape change, aggregation

Summary

ATP is a potent agonist of the $P2X_1$ ion channel, mediating a rapid, quickly desensitized influx of Ca^{2+}. In hirudinized PRP, containing apyrase, the two stable selective $P2X_1$ agonists, α,β-methylene ATP, and L-β,γ-methylene ATP induced extracellular Ca^{2+}-dependent fast and reversible platelet shape change, leading to desensitization of the $P2X_1$ ion channel. Preincubation with HPLC-purified ADP potently antagonized the subsequent α,β-methylene ATP- and L-β,γ-methylene ATP-evoked platelet shape change. Accordingly, upon heterologous expression of $P2X_1$ in *Xenopus* oocytes, HPLC-purified ADP acted as an antagonist of the ATP-induced current, but was inactive itself. Since ATP and ADP are co-released from dense granules during platelet activation, we investigated whether the $P2X_1$ ion channel is involved in the response of platelets to collagen. We found that platelet shape change and aggregation induced by low concentrations of collagen were strongly inhibited after selective desensitization of $P2X_1$ with its agonists or by pretreating the platelets with a low concentration of ADP (0.5 μM), that antagonizes the $P2X_1$ channel without desensitizing the $P2Y_1$ receptor. Our data suggest that, during collagen-initiated platelet activation, the early secretion of ATP results in the activation of the $P2X_1$ ion channel, which plays a role as a positive regulator of further platelet responses.

Introduction

Present at very high concentrations in the platelet dense granules (1), both ADP and ATP are secreted during platelet activation (2, 3). ADP has long been recognized as an important activator of platelets, playing an essential role in enhancing secretion (4), and stabilizing platelet aggregation induced by other agonists (5-7). According to present knowledge, the activation of platelets by ADP involves two receptors (reviewed in 8): $P2Y_1$, coupled to a Gq protein, is responsible for shape change through mobilization of Ca^{2+} from intracellular stores; the recently identified $P2Y_{12}$ receptor (previously denominated $P2Y_{CYC}$ or $P2T_{AC}$) (9), target for specific antithrombotic drugs, leads to adenylate cyclase inhibition through a Gi protein and promotes the completion and amplification of platelet responses to ADP. The use of $P2Y_1$ and $P2Y_{12}$ receptor selective antagonists (8), as well as the generation of $P2Y_1$-null mice (10, 11), have shown that both $P2Y_1$ and $P2Y_{12}$ receptors are required for normal ADP-induced platelet aggregation.

Platelets also express the ionotropic $P2X_1$ receptor, which mediates a rapid influx of Ca^{2+} (12). Owing to the lack of selective P2X-type receptor antagonists and because of quick receptor desensitization, its role in platelet activation and aggregation has been difficult to determine so far (13-16). Although previously considered as a third ADP receptor, a recent study (17) proposed that this receptor is an ATP-gated ion channel at which ADP is not an agonist. The authors showed that the agonist activity of commercial ADP was solely due to contaminating ATP, leading to previously reported artefactual results. ATP is commonly regarded as a platelet inhibitor *via* its antagonistic action at the platelet ADP receptors, $P2Y_1$ and $P2Y_{12}$ (2, 18, 19). The recent findings (17), however, question the purely antagonistic view of ATP in haemostasis. The authors proposed a revised scheme for physiological activation of P2 receptors in human platelets: ATP stimulates $P2X_1$, whereas ADP is a selective agonist at $P2Y_1$ and $P2Y_{12}$ receptors. Evidence for a contribution of the $P2X_1$ ion channel to platelet activation was recently provided by the ability of its selective agonist, α,β-methylene ATP, to produce reversible platelet shape change (20).

The aim of our study was to investigate the role of the $P2X_1$ ion channel during platelet activation evoked by collagen, major player in haemostasis (21). Apyrase-treated hirudinized PRP was used in order to retain maximal $P2X_1$ functionality for platelet shape change and aggregation analyses. An exclusive role for ATP *versus* ADP was emphasized by the observation that HPLC-purified ADP acted as an antagonist of platelet shape change produced by the $P2X_1$ agonists, α,β-meATP or L-β,γ-meATP, and of the ATP-induced current in voltage-clamped $P2X_1$-expressing *Xenopus* oocytes. To determine the role of $P2X_1$ during collagen-induced platelet shape change and aggregation, we took advantage of the fact that $P2X_1$ can be quickly and selectively desensitized by its two stable agonists, α,β-meATP and L-β,γ-meATP, and antagonized by ADP prior to platelet activation with collagen. We conclude that the $P2X_1$ receptor plays a positive role in the response of platelets to collagen.

Materials and Methods

Materials and Nucleotide Purification on HPLC

Adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene adenosine 5'-triphosphate (α,β-meATP), L-β,γ-methylene ATP (L-β,γ-meATP), and serotonin were purchased from Sigma. Collagen (collagen reagent horm) was from Nycomed (Munich). ADP, ATP, L-β,γ-meATP, and α,β-meATP were purified by HPLC on a Adsorbosphere HS C18, 7 μm, 250 × 4.6 mm, column (Alltech). Nucleotides were eluted at a flow rate of 1.5 ml min^{-1} with a linear gradient composed of buffer A (0.02 M KH_2PO_4, 0.05 M tetrabutylammonium phos-

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phate, pH 5.0:methanol [95:5]) and B (methanol). Samples were lyophilized and the pH was adjusted to 7.0 with potassium phosphate buffer. The purified products were kept at –80° C and were found to be stable during the experimental procedures. The buffer was demonstrated to have no effect on platelet shape change or aggregation, induced by various agonists.

Platelet Aggregations and Shape Change Analyses

Blood, freshly drawn from healthy donors, was anticoagulated with 20 μg/ml hirudin (Lepirudin, Hoechst, Germany) to maintain physiological Ca^{2+} levels. Apyrase (Grade I, Sigma; final concentrations 0.125, 0.25, 0.5, 1, or 5 U/ml, as indicated) was added to the blood before collecting the platelet rich plasma (PRP) by centrifugation at 150 g for 15 min. Platelet count was adjusted to 250,000 platelets/μl with autologous platelet poor plasma (PPP). The apyrase treatment, degrading nucleotides released during platelet preparation, was found to be required to retain platelet sensitivity to the $P2X_1$ selective agonists while also maintaining platelet responsiveness to ADP (see results). Light transmission during aggregation was recorded on a Chrono-Log and shape change analysis was performed on an ELVI 840 aggregometer using a 5-fold amplification of the signals. During analysis of platelet shape change, platelet aggregation was blocked by use of the neutralizing monoclonal anti-GPIIb/IIIa antibody MA-16N7C2 (50 μg/ml) (22). In each case, at least 3 independent experiments were performed on different individuals.

Heterologous Expression of $P2X_1$ in Xenopus Oocytes and Electrophysiological Recordings

The pcDNA3.1-$P2X_1$ expression vector (23) was used for T7 promoter driven *in vitro* transcription; 10 ng cRNA were microinjected into *Xenopus* oocytes. Current measurements were performed using a conventional two-electrode voltage clamp technique as previously described (23). The external solution contained ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.5); the pipettes were filled with 3M KCl. Oocytes were maintained at –60 mV holding potential in a continuously perfused small volume (40 μl) chamber; the agonists and antagonists were applied from a pipette above the oocyte. The effect of ADP on the ATP-induced current was analyzed by using three different protocols: 1) simultaneous addition of ADP with ATP, 2) preincubation with ADP for 1 min prior to ATP application, or 3) preincubation with ADP for 1 min, followed by a 1 min-perfusion with ND96 buffer before ATP application. Signals were recorded with the PCLAMP 5.0 software. The data are represented as the mean ± SEM. Statistical analysis of the data was made using non-paired Student's t-test.

Results

Pharmacology of the $P2X_1$ Ion Channel Expressed in Xenopus Oocytes

Recent reports have stressed the importance of using purified nucleotides to correctly evaluate P2 purinoceptor function (17-19). Notably, it was shown that ATP but not ADP is an agonist of the $P2X_1$ ion channel (17). Commercial nucleotides used in this work were therefore purified by HPLC; HPLC profiles are shown for purified ADP and ATP in Fig. 1A. Since ADP and ATP are coreleased during platelet activation, we have investigated the effects of ADP on the ATP-induced $P2X_1$ activation. For this purpose, the $P2X_1$ receptor was

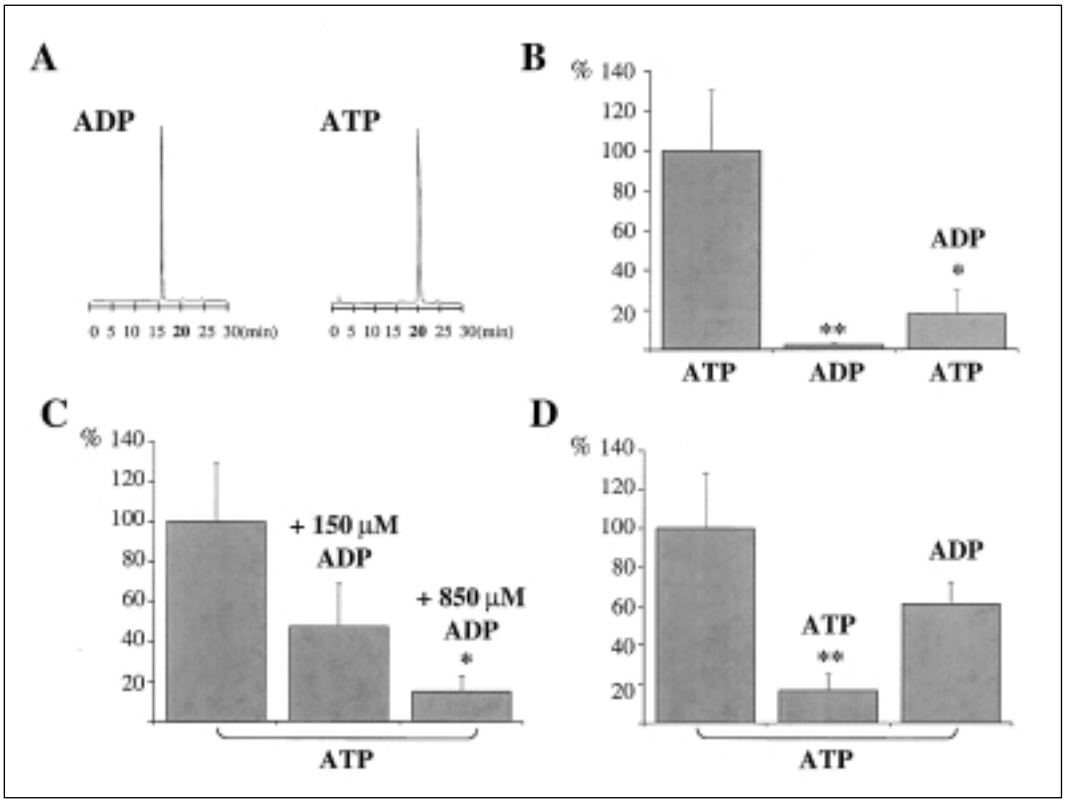

Fig. 1 ADP acts as an antagonist at the $P2X_1$ channel expressed in *Xenopus* oocytes. A. HPLC profiles of the purified adenine nucleotides, ADP and ATP. B. Averaged peak currents measured in $P2X_1$-expressing *Xenopus* oocytes stimulated with ATP (100 μM, n = 17) and/or ADP (100 μM, n = 20); during inactivation studies, 100 μM ADP (n = 8) was applied 1 min prior to addition of ATP (100 μM). C. ADP (150 μM, n = 9, or 850 μM, n = 9) was added simultaneously with 100 μM ATP. D. Oocytes were stimulated with ATP (100 μM) (n = 7) or ADP (100 μM) (n = 7) followed by perfusion of the chamber for 1 min before a (second) application of ATP (100 μM). The values are shown as percentages of the peak current induced by a single application of ATP within the same batch of oocytes (*: $p < 0.05$; **: $p < 0.01$)

heterologously expressed in *Xenopus* oocytes. As previously described (17, 23, 24), 100 μM ATP produced a robust inward current (mean peak current = 1.34 ± 0.4 μA, n = 17), more potent than that induced by 100 μM α,β-meATP (mean peak current = 0.73 ± 0.212 μA, n = 18, not shown), while 100 μM ADP was unable to induce any significant current (mean peak current = 0.053 ± 0.02 μA, n = 20) (Fig. 1B). Preincubation of the oocytes with 100 μM ADP for 1 min reduced the ATP-induced current to 17.9 ± 12.2% of its initial value (n = 8) (Fig. 1B), indicating that ADP pretreatment potently inhibits further $P2X_1$ activity. Fig. 1C shows the result of simultaneous addition of 150 μM or 850 μM ADP together with 100 μM ATP leading to dose-dependent reduction of the ATP-induced current to 47.3 ± 21.3% (n = 7) and 14.9 ± 7.4% (n = 9), respectively. These data indicate that ADP acts as an antagonist for ATP at $P2X_1$, antagonist which is more effective when preincubated for 1 min (compare Fig. 1B with Fig. 1C). Currents evoked by ATP at $P2X_1$ channels undergo marked desensitization within a few hundred milliseconds, the recovery of the peak amplitude being related to the time interval between applications (25). $P2X_1$ desensitization is illustrated in Fig. 1D, showing that the peak amplitude of the current evoked by a second ATP application was reduced to 16.8 ± 8.9% (n = 7) (Fig. 1D). To further specify whether ADP application resulted in $P2X_1$ channel desensitization, oocytes were similarly pretreated with ADP, perfused for 1 min with buffer, prior to ATP application. In these conditions, the peak current induced by ATP was only reduced to 61.3 ± 10.9% (n = 8) (Fig. 1D), indicating that the inhibition of $P2X_1$ channel activity by preincubation with ADP (Fig. 1B) is not the result of channel desensitization but reflects the ADP antagonism. These data thus confirmed that ADP behaves as a potent antagonist at $P2X_1$, at which it is inactive itself.

Functionality of the Platelet $P2X_1$ Receptor in Hirudinized PRP: the $P2X_1$-mediated Platelet Shape Change

Recently, the selective $P2X_1$ agonist, α,β-meATP, was shown to evoke a transient Ca^{2+} increase accompanied by reversible platelet shape change, only when measures were taken to avoid spontaneous activation and desensitization of $P2X_1$ during platelet preparation (20). In order to preserve maximal $P2X_1$ responses, we used hirudinized PRP, in which physiological Ca^{2+} levels are maintained, and apyrase concentrations defined to minimize $P2X_1$ receptor desensitization. The ability of the stable $P2X_1$ specific agonists, α,β-meATP, and L-β,γ-meATP to evoke platelet shape change was analyzed dose-dependently. In the presence of 1 U/ml apyrase, α,β-meATP (Fig. 2A) and L-β,γ-meATP (Fig. 2B) caused a fast, reversible platelet shape change with maximal response at 0.5 μM, and 5 μM, respectively. The amplitude of this platelet shape change corresponded to 5-10% change of light transmission, varying between individuals. The amplitude of platelet shape change induced by α,β-meATP (Fig. 2C) or L-β,γ-meATP (not shown) increased with the concentration of apyrase added to the blood, up to 5 U/ml. Because in the presence of 5 U/ml apyrase, platelets did not aggregate in response to 2.5 μM ADP or 0.5 μg/ml collagen (not shown), for the purpose of the present study, 1 U/ml apyrase was considered to be optimal for the preservation of $P2X_1$ function. Consistent with an event requiring extracellular Ca^{2+}, the α,β-meATP- or L-β,γ-meATP-induced shape change disappeared in the presence of EGTA (Fig. 2A, B).

In agreement with the pharmacological properties of the $P2X_1$ channel (24, 25) and our findings in *Xenopus* oocytes, reapplication of α,β-meATP (up to 20 μM) at any time following the first addition (0.5 μM) was unable to induce a second shape change (Fig. 3A),

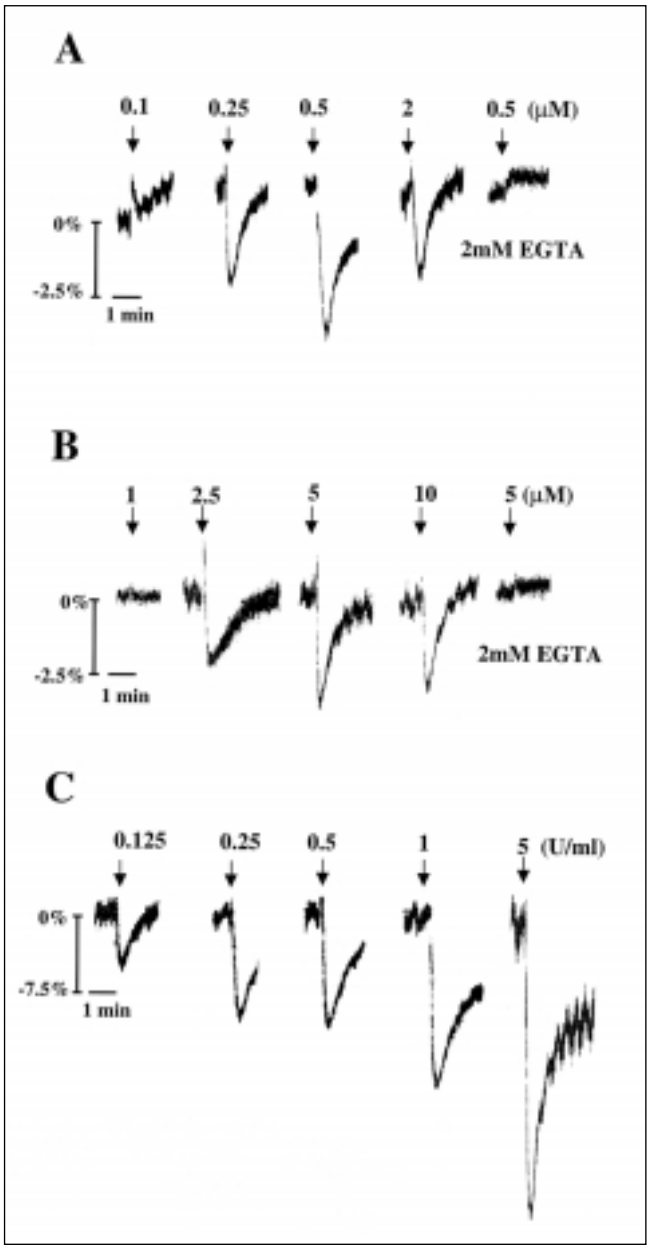

Fig. 2 α,β-meATP and L-β,γ-meATP-evoked platelet shape change. Light transmission recordings in hirudinized PRP after addition of increasing concentrations of α,β-meATP (A) or L-β,γ-meATP (B). Arrows indicate the time of addition of these agonists. For α,β-meATP (0.5 μM), shape changes are also shown as a function of the apyrase concentration (U/ml) (C). Percentages of light transmission as well as time bars are included. The curves are representative of at least 3 independent experiments performed on different individuals

reflecting fast $P2X_1$ channel desensitization in the platelets, as well as the need for a nucleotide-free environment for channel recovery. The property of ADP to antagonize the $P2X_1$ ion channel, delineated in *Xenopus* oocytes, was then analyzed during platelet shape change recordings. We found that incubation of platelets with a ADP concentration as low as 0.5 μM abolished further shape change in response to α,β-meATP (0.5-10 μM) (Fig. 3B), confirming that ADP pretreatment could potently inhibit the platelet $P2X_1$ ion channel. Yet, the α,β-meATP-induced shape change still occurred after a prior shape change induction with serotonin (Fig. 3C), excluding $P2Y_1$-mediated

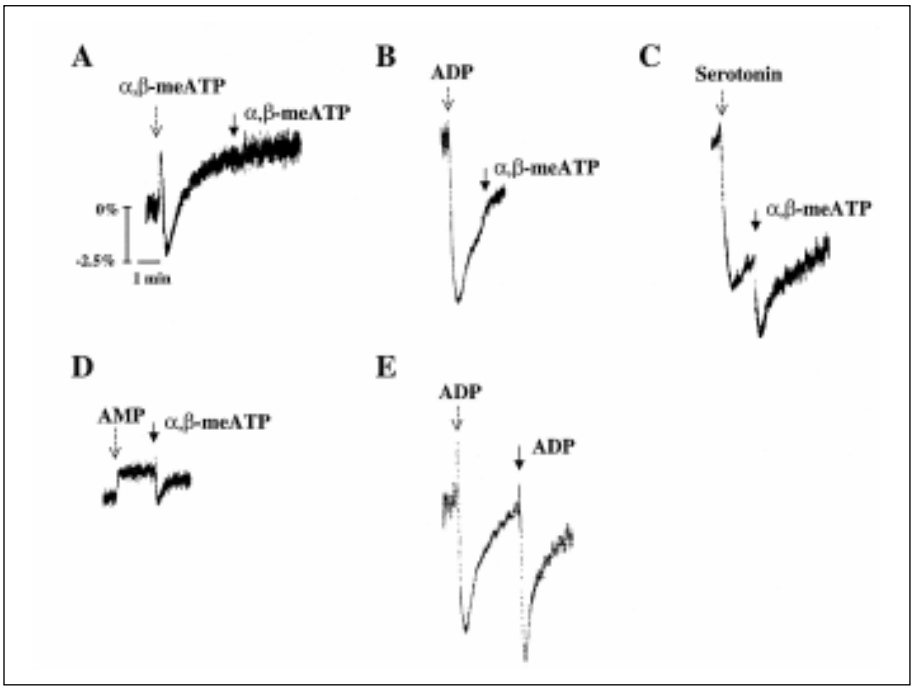

Fig. 3 Inhibition of the α,β-meATP-evoked platelet shape change. Light transmission recordings in hirudinized PRP. $P2X_1$ desensitization by two consecutive additions of α,β-meATP (0.5 μM) is represented (A). 0.5 μM ADP (B), 1 μM serotonin (C), or 0.5 μM AMP (D) were added prior to α,β-meATP (0.5 μM). E. ADP (0.5 μM) was added twice consecutively. Dashed arrows represent the pretreatments and solid arrows, the induction of subsequent shape change. A reference scale of light transmission is provided in part A

Gq protein activation or ATP release to be responsible for the observed $P2X_1$ channel inactivation by 0.5 μM ADP. The preservation of $P2X_1$ functionality requires the use of high concentrations of apyrase during the experiments (see Fig. 2C), potentially breaking down ADP into AMP and adenosine. In a previous study, Evans et al. (24) showed that AMP and adenosine produced 0-6% of the maximal current evoked by ATP. To determine whether these compounds affect the α,β-meATP-induced platelet shape change, platelets were preincubated with HPLC-purified AMP or adenosine for 1 min before α,β-meATP application. Fig. 3D indicates that pretreatment with 0.5 μM AMP partially inhibited platelet shape change evoked by 0.5 μM α,β-meATP. This inhibition was complete at 1 μM AMP (not shown). Likewise, adenosine pretreatment could prevent the α,β-meATP-induced platelet shape change (not shown). Similar results were obtained when platelet shape changes were induced with L-β,γ-meATP (not shown). These results thus indicate that the ADP degradation products, like ADP, behave as antagonists at $P2X_1$, providing that, even in the presence of apyrase, addition of ADP antagonizes $P2X_1$. In Fig. 3E, we show that 0.5 μM ADP did not prevent a second ADP-induced shape change from occurring, substantiating that, in apyrase-treated platelets, this concentration of ADP does not desensitize the $P2Y_1$ receptor, despite its potent antagonism at $P2X_1$.

Inhibition of $P2X_1$ Prevents Platelet Shape Change and Aggregation in Response to Low Concentrations of Collagen

The role of ADP as a cofactor in platelet aggregation induced by collagen is clearly described (7, 26, 27). This dependence on ADP was confirmed in apyrase-treated hirudinized PRP, as depicted by the use of the selective $P2Y_{12}$ receptor antagonist, ARC-69931MX (28), which severely affected platelet aggregations induced by collagen (data not shown). Because ATP is co-released with ADP during ongoing platelet activation, potentially activating $P2X_1$, we have investigated the role of this ion channel in platelet responses to collagen. During collagen-induced platelet activation, ATP secretion occurs very rapidly, even before onset of shape change (29). The collagen-induced platelet shape change requires PLC-γ_2-mediated intracellular Ca^{2+} mobilization which results in MLC-kinase activation (30). In order to determine whether the rapid $P2X_1$-mediated Ca^{2+} influx could be involved in modulating platelet responses to collagen through secreted ATP, we took advantage of the possibility to quickly and selectively desensitize $P2X_1$ by its selective agonists or to inhibit this channel by using ADP as an antagonist, before inducing platelet shape change with collagen. As shown in Fig. 4, preincubation of platelets with α,β-meATP (0.5 μM) (Fig. 4A) or L-β,γ-meATP (5 μM) (Fig. 4B) resulted in a delayed and reduced platelet shape change evoked by collagen (0.5-1 μg/ml). To further analyze the role for $P2X_1$ in this process, platelets were incubated with 0.5 μM ADP (a concentration not causing $P2Y_1$ receptor desensitization, Fig. 3E), prior to shape change induction with collagen (Fig. 4C). Similarly as with α,β-meATP and L-β,γ-meATP pretreatments, the preincubation of platelets with ADP resulted in a severely impaired collagen-induced shape change, indicating that $P2X_1$ acts as a positive modulator of this platelet response. Hence, this experiment with ADP, which does not cause Ca^{2+} influx, confirms that the use of α,β-meATP and L-β,γ-meATP to desensitize $P2X_1$ correctly reflects channel blockade, despite the Ca^{2+} influx that these agonists provoke.

Interestingly, we showed that preincubation with α,β-meATP (0.1-0.5 μM) (Fig. 4D) or L-β,γ-meATP (0.5-5 μM) (Fig. 4E) for 1 min strongly inhibited platelet aggregations in response to collagen (0.2-1 μg/ml), suggestive of a role for $P2X_1$ also in platelet aggregation. The inhibition did no longer occur with higher concentrations of collagen (Fig. 4F). Since ADP is a necessary cofactor for the platelet aggregation induced by collagen, it was important to rule out competitive activity by these concentrations of the ATP analogs at the level of the ADP receptors, as reported for higher concentrations of these analogs (2, 18, 19, 31, 32). For this purpose, platelets were preincubated with ADP or α,β-meATP before restimulation with ADP. In the presence of apyrase, platelet aggregations induced by ADP (5 μM) were quickly reversible, indicative of rapid degradation by this nucleotidase (Fig. 5A); restimulation with ADP (5 μM) immediately after disaggregation (1 min following the first addition) resulted in a reduced platelet aggregation (Fig. 5A), due to desensitization of the $P2Y_1$

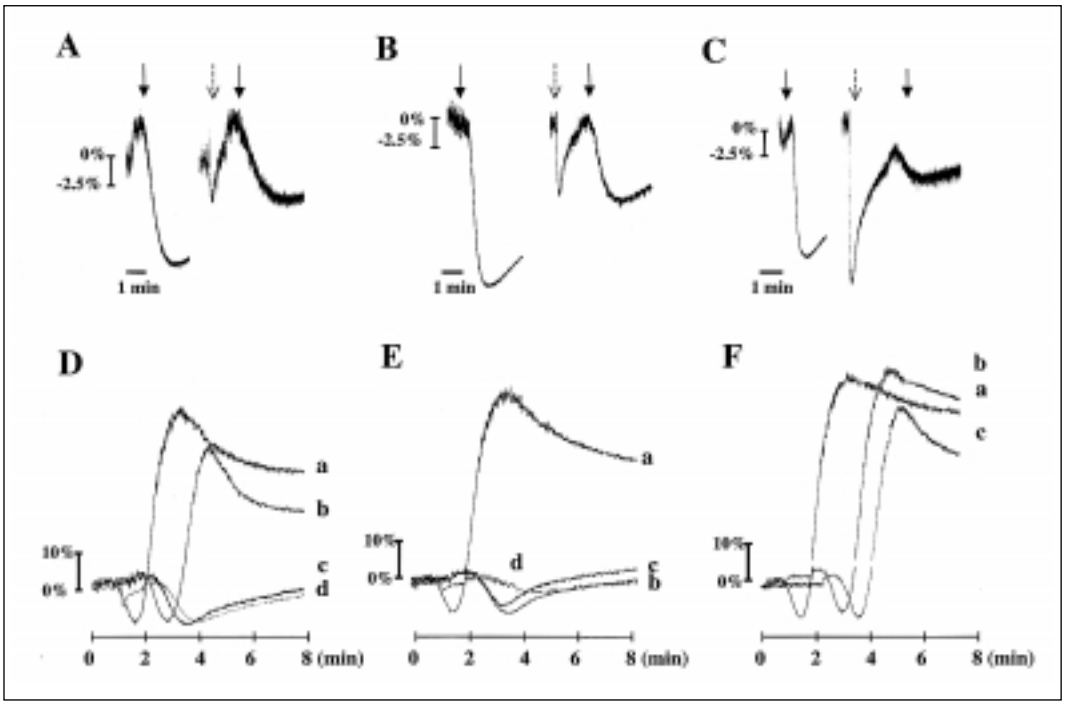

Fig. 4 Consequence of $P2X_1$ inhibition on platelet responsiveness to collagen. Light transmission (%) recordings in hirudinized PRP during platelet shape change (A-C) and aggregation (D-F) analyses. Platelet shape changes were induced with collagen (0.5 μg/ml) (solid arrows) with or without prior addition of 0.5 μM α,β-meATP (A), 5 μM L-β,γ-meATP (B), or 0.5 μM ADP (C), as indicated by the dashed arrows. D. Platelets were preincubated for 1 min with α,β-meATP (none, a; 0.05 μM, b; 0.1 μM, c; 0.5 μM, d) before induction of aggregation with collagen (1 μg/ml). E. Similar experiments (1 μg/ml collagen) performed with L-β,γ-meATP preincubations (none, a; 0.5 μM, b; 1 μM, c; 5 μM, d). F. Platelet aggregations in response to 1.5 μg/ml collagen (a) following pretreatment with 0.5 μM α,β-meATP (b), or 5 μM L-β,γ-meATP (c)

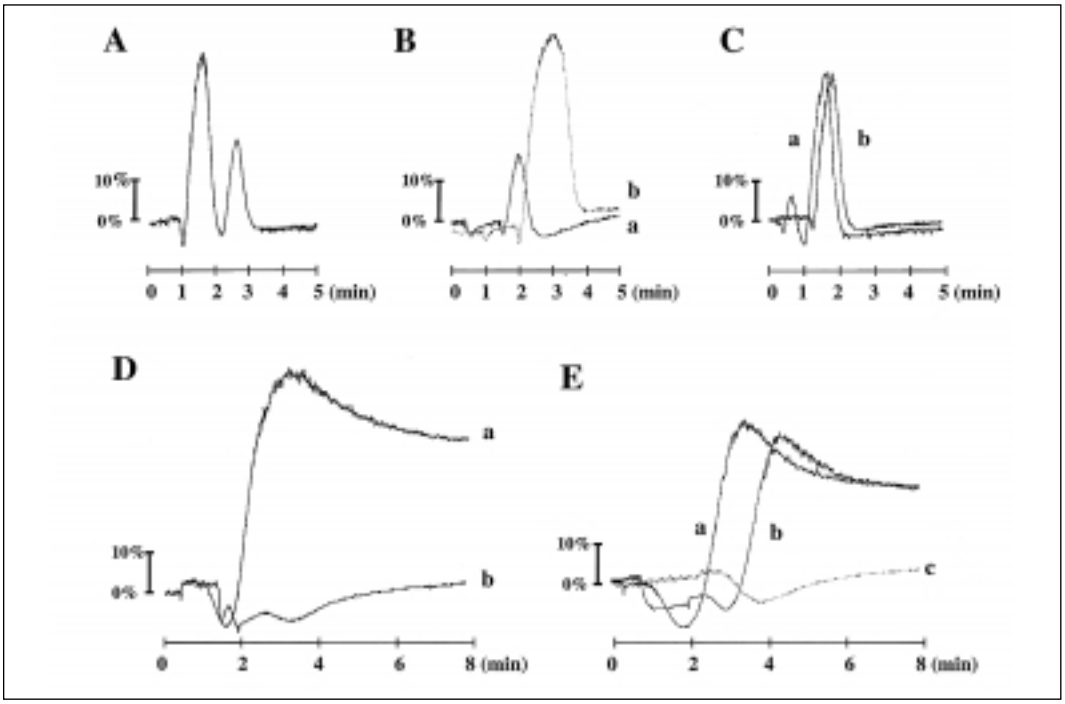

Fig. 5 Preincubation with α,β-meATP, L-β,γ-meATP, and ADP all enable $P2X_1$ receptor inhibition. A. Light transmission (%) recordings in hirudinized PRP after induction of platelet aggregation by two consecutive additions of 5 μM ADP. B. Platelets were preincubated with an excess of α,β-meATP (10 μM) for 1 min before inducing aggregation with 0.5 μM (a) or 5 μM (b) ADP. C. Platelets were stimulated with 5 μM ADP with (a) or without (b) prestimulation by 0.5 μM ADP. D. Platelet aggregation induced by 1 μg/ml collagen alone (a) or after preincubation with 0.5 μM ADP (b). E. Similar experiment where collagen (1 μg/ml) was preceded (b, c) or not (a) by serotonin (1 μM) (b) or L-β,γ-meATP (5 μM) (c) pretreatments

receptor (33). In contrast, we show that α,β-meATP (from 0.5 up to 10 μM) pretreatment did not affect the subsequent platelet aggregation in response to ADP (0.5-5 μM) (Fig. 5B), implying that α,β-meATP neither desensitized $P2Y_1$ nor competitively antagonized the $P2Y_1$ and $P2Y_{12}$ receptors for ADP in this concentration range.

Whereas not affecting the aggregation induced by 5 μM ADP (Fig. 5C a, b), we observed that preincubation of platelets with 0.5 μM ADP totally abolished aggregations in response to 1 μg/ml collagen (Fig. 5D), similarly to pretreatments with the $P2X_1$ agonists (Fig. 4D, E). On the contrary, preincubation with serotonin (1 μM), which raises intracellular Ca^{2+} levels through the 5-HT_{2A} Gq protein-coupled receptor, did not alter the collagen-induced aggregations (Fig. 5E). These results indicate that neither $P2Y_1$ receptor desensitization nor ADP-dependent Ca^{2+}-mobilization is responsible for the observed inhibition of collagen response by ADP, which rather reflects ADP antagonism at $P2X_1$.

These data thus demonstrate that the $P2X_1$ activation is involved in the positive regulation of platelet aggregations induced by low concentrations of collagen.

Discussion

In this study, we provide functional and pharmacological evidence that HPLC-purified ADP behaves as an antagonist at the platelet $P2X_1$ ion channel as well as at $P2X_1$ heterologously expressed in *Xenopus* oocytes. As recently proposed (17), the scheme of platelet activation by ADP has to be revised such that ADP acts at $P2Y_1$ and $P2Y_{12}$, whereas $P2X_1$ is activated by ATP (Fig. 6). Accordingly, the presently described antagonistic property of ADP at $P2X_1$ should be integrated with the accepted antagonistic effect of ATP at the platelet $P2Y_1$ receptor (18, 19). Thus, a model emerges in which two platelet purinoceptors, $P2Y_1$ and $P2X_1$, are controlled by two agonists, ADP and ATP, respectively, acting as antagonists for each other. These opposite actions at two distinct platelet receptors, mediating fast ($P2X_1$) and slow ($P2Y_1$) signaling, suggest mutually exclusive roles for these adenine nucleotides during platelet activation. ATP has long been described as a platelet inhibitor (2, 34) due to its competitive antagonism at the ADP receptors, albeit at much higher concentrations than those needed to activate $P2X_1$. However, considering the ATP-to-ADP ratio in platelet dense granules being approximately two (35) as well as the actual concentrations of ATP (μM) released during agonist-evoked platelet activation (36), both elements consistent with potential $P2X_1$ stimulation, the ATP dogma should be reanalyzed. This statement is strengthened by our finding in $P2X_1$-expressing *Xenopus* oocytes (Fig. 1C) showing that ATP can potently activate the channel when added simultaneously with ADP.

In agreement with the recent study of Rolf et al. (20) stressing the importance of Ca^{2+} levels as well as the use of high concentrations of an adenine nucleotide scavenger to preserve $P2X_1$ responses during platelet preparation, platelet shape change and aggregation analyses were performed in apyrase-treated hirudinized PRP. Under these conditions, we could demonstrate the ability of the two related stable $P2X_1$ agonists, α,β-meATP and L-β,γ-meATP, to evoke quickly reversible platelet shape through $P2X_1$, leading to channel desensitization. According to our data in $P2X_1$-expressing *Xenopus* oocytes showing that pre-incubation with ADP potently antagonized the peak amplitude of the current evoked by ATP, it appeared that ADP pretreatment prevented $P2X_1$-mediated platelet shape change. The fast kinetics of the $P2X_1$-mediated shape change as well as the antagonistic action of ADP on $P2X_1$ activity suggest that this shape change preceeds $P2Y_1$ response to ADP; in addition, rapidly activated Ca^{2+} entry *via* $P2X_1$ has been reported to potentiate the ADP-induced Ca^{2+} response of the $P2Y_1$ receptor (36), suggesting a positive effect of Ca^{2+} entry on internal Ca^{2+} release, possibly occurring at the $Ins(1,4,5)P_3$ receptor level.

The study of $P2X_1$ function in platelets is hampered by the lack of selective antagonists of the P2X-type receptor *versus* P2Y receptors. Here, we found that low concentrations of ADP (and its degradation

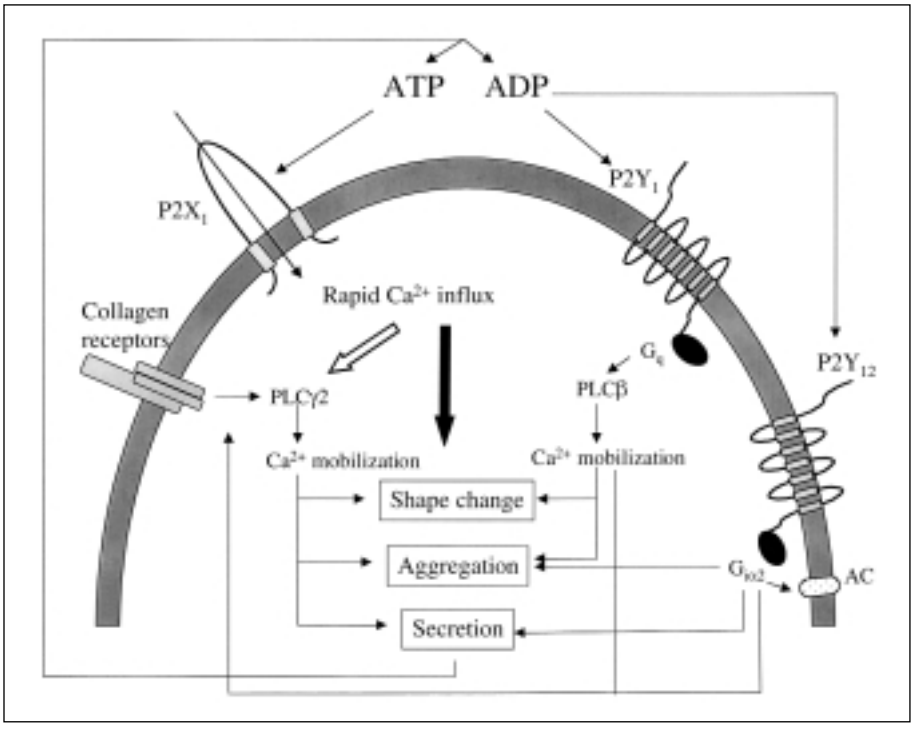

Fig. 6 Model depicting $P2X_1$ function during collagen-induced platelet activation. ATP and ADP are co-released from platelet dense granules during platelet activation evoked by collagen. ATP activates the $P2X_1$ ion channel, whereas ADP acts at $P2Y_1$ and $P2Y_{12}$ receptors. $P2X_1$ participates in the platelet shape change via Ca^{2+} influx, as indicated by the black arrow. The contribution of $P2X_1$ stimulation to collagen-induced platelet activation is shown by the open arrow. The involvement of the ADP receptors in the amplification of the platelet response is also represented

products, AMP and adenosine) were capable of potently antagonizing $P2X_1$ in apyrase-treated platelets. The preincubation of platelets with low ADP concentrations was, therefore, used to pharmacologically neutralize $P2X_1$ function, in conditions where normal responsiveness of the $P2Y_1$ receptor for ADP was maintained. Hence, the use of both $P2X_1$ agonists, leading to channel desensitization, and ADP, as an antagonist, enabled us to determine whether ATP, by acting at the $P2X_1$ ion channel, would influence platelet responses to other agonists capable of triggering dense granule release. We have investigated the contribution of $P2X_1$ to platelet activation evoked by collagen. This extracellular matrix protein plays, indeed, a primary role in hemostasis, providing an important site for adhesion of platelets during vascular damage; it also stimulates platelet activation, leading to inside-out regulation of the integrin GPIIb-IIIa, secretion from dense and α granules, generation of thromboxanes, and expression of procoagulant activity, all of which support the hemostatic process (21). Furthermore, ATP secretion has been described to be an initial response during activation of platelets with collagen, even occurring before onset of shape change (29). Here, we show that the PLCγ2-dependent collagen-induced platelet shape change was delayed and reduced when the $P2X_1$ receptor was inhibited prior to the induction of shape change. Also, substraction of $P2X_1$ function led to the inhibition of platelet aggregation in response to low concentrations of collagen. In these experiments, the competitive antagonism of the $P2X_1$ agonists at the ADP receptor level ($P2Y_1$ and $P2Y_{12}$) was ruled out by the fact that the ADP-induced aggregations were not affected by pretreatment with these agonists; moreover, the low concentrations of α,β-meATP and L-β,γ-meATP needed to desensitize $P2X_1$ were far below the concentrations able to interfere with P2Y functions. The fact that $P2X_1$ desensitization can no longer inhibit collagen-induced aggregations when platelets are activated with higher concentrations of collagen, probably reflects engagement of other pathways related to production of thromboxane, coupled to a more intense release reaction.

ADP is a necessary release product for the normal activation of platelets by collagen (7, 29, 30); especially, its Gi-coupled pathway is critically involved in a synergism with agonists targeting tyrosine kinases which lead to PLCγ2 activation (37). We propose that ATP, originating from the same sources as ADP, primarily activates the $P2X_1$ ion channel, producing a rapid Ca^{2+} influx, which together with Gi- and PLC-dependent pathways, contributes to platelet responses to collagen (Fig. 6). The presently decribed process clearly concerns agonists capable of triggering ATP release since platelet aggregations induced by weak agonists as ADP (Fig. 5B) or serotonin and epinephrine (not shown) were not altered after $P2X_1$ desensitization. Current studies aim at determining whether this phenomenon can be generalized to platelet activation by other agonists such as thrombin and thromboxane A_2. If so, ATP would play a general role complementary to that of ADP.

In conclusion, we postulate that ATP, released from platelet dense granules during platelet activation or from damaged cells during vascular injury, activates the $P2X_1$ ion channel rapidly, and that this activation participates in physiological platelet responses, especially under conditions of mild platelet stimulation.

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