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Extracellular Ca\textsuperscript{2+} modulates ADP-evoked aggregation through altered agonist degradation: implications for conditions used to study P2Y receptor activation

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Running Title

Role of ectonucleotidases in Ca\textsuperscript{2+}-dependent platelet aggregation

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Summary

ADP is considered a weak platelet agonist due to the limited aggregation responses it induces in vitro at physiological concentrations of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]_o). Lowering [Ca\(^{2+}\)]_o paradoxically enhances ADP-evoked aggregation, an effect that has been attributed to enhanced thromboxaneA\(_2\) production. We have now examined the role of ectonucleotidases in the [Ca\(^{2+}\)]_o-dependence of platelet activation. Reducing [Ca\(^{2+}\)]_o from millimolar to micromolar levels converted ADP (10 µM)-evoked platelet aggregation from a transient to a sustained response in both PRP and washed suspensions. Block of thromboxaneA\(_2\) production with aspirin had no effect on this [Ca\(^{2+}\)]_o-dependence. Prevention of ADP degradation abolished the differences between low and physiological [Ca\(^{2+}\)]_o resulting in a robust and sustained aggregation in both conditions. Measurements of extracellular ADP revealed reduced degradation in both plasma and apyrase-containing saline at micromolar compared to millimolar [Ca\(^{2+}\)]_o. As reported previously, thromboxaneA\(_2\) generation was enhanced at low [Ca\(^{2+}\)]_o, however this was independent of ectonucleotidase activity. P2Y receptor antagonists Cangrelor and MRS2179 demonstrated the necessity of P2Y\(_{12}\) receptors for sustained ADP-evoked aggregation, with a minor role for P2Y\(_1\). In conclusion, Ca\(^{2+}\)-dependent ectonucleotidase activity is a major factor determining the extent of platelet aggregation to ADP and must be controlled for in studies of P2Y receptor activation.

(200)

Keywords: Platelets, aggregation, calcium, ectonucleotidases, ADP
Introduction

ADP is an important platelet agonist during haemostasis and thrombosis, exerting its effects through two G-protein-coupled receptors P2Y\textsubscript{1} and P2Y\textsubscript{12}. P2Y\textsubscript{1} is coupled to G\textsubscript{αq}, leading to an increase in cytosolic calcium through stimulation of phospholipase C\textsubscript{β} (PLC\textsubscript{β}) (Jin, et al 1998, Savi, et al 1998), whereas P2Y\textsubscript{12} is coupled to G\textsubscript{ai}, leading to activation of phosphatidylinositol 3-Kinase (PI3-K) (Jackson, et al 2005, Trumel, et al 1999) and inhibition of adenylate cyclase. For platelet-platelet adhesion, and thus thrombus formation, activation of the fibrinogen receptor α\textsubscript{IIb}β\textsubscript{3} is required, a process that depends upon concomitant stimulation of G\textsubscript{αq} and G\textsubscript{ai} signalling pathways (Jin and Kunapuli 1998). Several platelet agonists bind to receptors coupled to G\textsubscript{αq} including thrombin, thromboxaneA\textsubscript{2} and ADP. However, ADP is the only platelet agonist that stimulates G\textsubscript{ai} signalling at physiological concentrations and is therefore an essential co-stimulus to achieve full functional responses for all known platelet agonists (Gachet 2008, Paul, et al 1999). Despite the central role of ADP in platelet aggregation and thrombogenesis, it is normally considered to be a weak platelet agonist due to the reversible nature of the aggregation response observed in vitro at physiological levels of external Ca\textsuperscript{2+} (Gachet 2008). Extremely low levels of extracellular Ca\textsuperscript{2+} abolish fibrinogen binding to α\textsubscript{IIb}β\textsubscript{3} integrin, however at micromolar extracellular calcium concentrations, ADP-evoked aggregation is enhanced compared to physiological Ca\textsuperscript{2+} levels and not readily reversible (Mustard, et al 1975, Packham, et al 1989). Although this paradoxical effect was reported more than two decades ago, the underlying basis whereby extracellular calcium modulates ADP-evoked aggregation remains unclear. It has been proposed that millimolar Ca\textsuperscript{2+} levels inhibit TXA\textsubscript{2} generation via altered ERK phosphorylation (Garcia, et al 2007), leading to loss of secondary aggregation (Mustard, et al 1975, Packham, et al 1989), however exactly how Ca\textsuperscript{2+} achieves this effect is not known.
Following stimulation of platelet P2Y receptors with ADP, the duration and amplitude of the response can be regulated by two principal mechanisms, firstly desensitization of the P2Y receptors preventing further signalling, and secondly removal of ADP by ectonucleotidases. Ectonucleotidases comprise a large family of extracellular nucleotide degrading enzymes including ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases and 5’ nucleotidase (Zimmermann 2000). ADP derived from platelets and other blood cells is thought to predominantly be metabolised by E-NTPDase1 (CD39), a membrane-bound enzyme expressed by endothelial cells, lymphocytes and macrophages (Kansas, et al 1991, Marcus, et al 1997), as well as microparticles that originate from these cell types (Atkinson, et al 2006, Banz, et al 2008). CD39 converts ADP to AMP, which is subsequently converted to adenosine, an inhibitor of platelet function, by 5’nucleotidase (CD73) expressed on endothelial cells and in plasma (Coade and Pearson 1989, Heptinstall, et al 2005, Zimmermann 2000). There is also evidence that soluble E-NPPases in plasma can degrade ADP directly to adenosine (Birk, et al 2002, Cauwenberghs, et al 2006), thus ectonucleotidases can convert prothrombotic mediators into inhibitors of platelet activation.

In this study we have investigated further the mechanism(s) underlying the differential responses to ADP at physiological compared to low (micromolar) extracellular calcium concentrations. We demonstrate that degradation of ADP by Ca²⁺-dependent ectonucleotidases is an important factor in determining the amplitude and duration of platelet aggregation. The results have consequences for understanding the effectiveness of ADP as a platelet agonist, and in the selection of experimental conditions to explore P2Y receptor activation.
Materials and Methods

Materials

CHRONO-LUME was purchased from Labmedics (Manchester, UK), Pyruvate kinase was obtained from Roche Diagnostics Limited (East Sussex, UK), GF109203X and MRS2179 were purchased from Tocris (Bristol, UK). TXB\textsubscript{2} assay kits were purchased from Cambridge Bioscience LTD (Cambridge, UK). Cangrelor (ARC-69931MX) was a kind gift from AstraZeneca (Moindal, Sweden). ADP, apyrase (gradeVII), aspirin, phosphoenol pyruvate and all other chemicals were purchased from Sigma (Poole, UK).

Platelet preparation

Blood was obtained from healthy, aspirin-free, volunteers according to a protocol approved by the local ethical committee of the University of Leicester. Blood was drawn from the forearm by venepuncture into a syringe containing acid citrate dextrose anticoagulant (ACD: in mM 85 trisodium citrate, 78 citric acid, 111 glucose) 9:1 \textit{v/v}. Platelet-rich plasma (PRP) was obtained by centrifugation at 700\textit{g} for 5 minutes. When re-calcified, 20mM CaCl\textsubscript{2} (calculated using a Nomogram (Hastings, \textit{et al} 1934)) was added to citrated PRP to achieve \([\text{Ca}^{2+}]_o\) of approximately 2mM immediately prior to each experiment. The extracellular Ca\textsuperscript{2+} in nominally Ca\textsuperscript{2+}-free saline and in similar citrated plasma: saline mixtures has been estimated to be approximately 20\textmu M and 17\textmu M, respectively (Packham, \textit{et al} 1987, Rolf, \textit{et al} 2001).

To prepare washed platelet suspensions, apyrase (0.32 U/ml), and where stated aspirin (100\textmu M or 1mM), were added to the PRP and platelets pelleted by centrifugation at 350\textit{g} for 20 min. Platelets were then resuspended in a volume of nominally Ca\textsuperscript{2+} free saline (in mM: 145 NaCl, 5 KCl, 1 MgCl\textsubscript{2} 10 HEPES, 10 glucose, fibrinogen 1mg/ml pH 7.35) equal to that
of the removed plasma. In experiments performed at physiological calcium concentrations, 2mM CaCl$_2$ was added to the platelets immediately prior to use.

**Platelet aggregation**

PRP or washed platelet suspensions with or without apyrase (0.32U/ml) (prior to substantial P2Y receptor desensitisation) were diluted (1:1) in saline and stimulated with ADP at 37°C under stirring conditions. Aggregation was measured using optical aggregometry (Model 400 lumi-aggregometer, Chronolog, Havertown, USA).

**Platelet disaggregation**

Washed, apyrase-free platelets were stimulated with ADP (10µM) at 37°C under stirring conditions in the presence of 2mM Ca$^{2+}$. After 2 minutes, apyrase (0.32U/ml), the P2Y$_1$ receptor antagonist MRS2179 (10µM), the P2Y$_{12}$ receptor antagonist AR-C69931MX (1µM) or a saline control was added to the suspension. Disaggregation was assessed 3 minutes after the addition of the P2Y receptor antagonists or apyrase and calculated as a percentage of the peak ADP-evoked aggregation.

**ADP measurement**

The concentration of extracellular ADP was assessed by luciferin: luciferase luminescence measurements after conversion to ATP via a method adapted from Heath (2004). Briefly, 2 minutes after addition of 10 µM ADP to plasma or apyrase-containing saline, with or without Ca$^{2+}$, 50µl samples were removed and added to a mixture of 420µl Tris-K acetate buffer (in mM 100 Tris-acetate, 2mM EDTA, 25 potassium acetate), 10 µl pyruvate kinase/phosphoenolpyruvate (prepared by mixing equal volumes of 10 mg/ml pyruvate kinase and 200mM phosphoenolpyruvate) and 20µl CHRONO-LUME. Luminescence was
measured using a Model 400 lumi-aggregometer (Chronolog, Havertown, USA) and
converted to ATP levels based upon a calibration curve for each batch of CHRONO-LUME.

**TXB₂ measurements**

TXB₂ synthesis was measured as an indication of TXA₂ production due to the highly labile
nature of TXA₂. Washed platelets were stimulated with ADP (10µM) at 37°C under stirring
conditions for 3 minutes in the presence and absence of apyrase (0.32U/ml), in both
physiological and nominally Ca²⁺-free conditions, and reactions terminated by snap freezing.
For analysis of TXB₂, samples were thawed and centrifuged at 3000g for 10 minutes at 4°C.
The supernatant was diluted 1:5 using the buffer supplied with the assay kit and TXB₂
determined according to the manufacturer’s instructions (Cambridge Bioscience, UK).

**Statistics**

Records of aggregation are from individual experiments, typical of ≥4 donors. Differences
between means ± s.e.m were assessed using paired student t-tests and a p value of <0.05
considered to be significant. p values indicated at levels of <0.05 (*), <0.01 (**) and <0.001
(***).
Results

Extracellular Ca\(^{2+}\) levels regulate ADP-evoked aggregation independently of thromboxaneA\(_2\) synthesis

ADP (10 µM) evoked a sustained aggregation of platelets in plasma anti-coagulated with citrate that reduces the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) to the micromolar range (Fig. 1a, e; average peak aggregation of 53.9 ± 3.4%). When the medium was recalcified to approximately 2mM free Ca\(^{2+}\), the aggregation was converted to a transient response that returned to baseline levels of transmission (-2.8 ± 2%) within approximately 2 minutes (Fig. 1a, e). Previous studies of this [Ca\(^{2+}\)]\(_o\)-dependent aggregation response have shown that production of TXA\(_2\) is enhanced at micromolar compared to millimolar [Ca\(^{2+}\)]\(_o\) levels and concluded that secondary stimulation of TXA\(_2\) receptors is responsible for the reversible nature of the ADP-evoked aggregation (Garcia, et al 2007, Mustard, et al 1975, Packham, et al 1989). However, we observed a similar effect of [Ca\(^{2+}\)]\(_o\) on aggregation when thromboxaneA\(_2\) synthesis was blocked by aspirin (Fig. 1b, e; average values 50.7 ± 3% and 0.9 ± 0.3% in low and physiological Ca\(^{2+}\) levels respectively). In platelets resuspended in a physiological saline, 10 µM ADP evoked a transient aggregation response in the presence of 2mM [Ca\(^{2+}\)]\(_o\), which was also converted to a sustained response by omission of CaCl\(_2\) from the saline (Fig. 1c, e; transmission levels 2 min after ADP of 0.6 ± 2% and 53.2 ± 5.4%, respectively), in agreement with reports by other groups (Mustard, et al 1975, Packham, et al 1989). As observed for platelets in the presence of plasma, aspirin did not block the sustained aggregation in salines with micromolar [Ca\(^{2+}\)]\(_o\) (Fig. 1d, e; average values 55.9 ± 1.3% and 7.1 ± 4.2% in low and physiological Ca\(^{2+}\) levels respectively). To ensure that TXA\(_2\) generation was completely inhibited experiments were repeated in the presence of 1mM
aspirin, which also had no significant effect on the ability of reduced \([\text{Ca}^{2+}]_o\) to enhance platelet aggregation (p>0.05 data not shown). Together, these data suggest that factor(s) other than altered TXA\(_2\) production must contribute to the ability of reduced \([\text{Ca}^{2+}]_o\) to enhance ADP-evoked aggregation.

**Prevention of ADP degradation abolishes the reversal of aggregation by calcium**

Since our washed platelet preparation contained apyrase (E-NTPDase1 isolated from potato to prevent P2Y receptor desensitization) and PRP has been reported to contain endogenous ectonucleotidases, we considered whether degradation of ADP contributed to the transient nature of the ADP-evoked aggregation at millimolar \([\text{Ca}^{2+}]_o\) levels. Aggregation evoked by the hydrolysis-resistant analogue ADP\(_{\beta S}\) was not significantly different in the presence or absence of extracellular \([\text{Ca}^{2+}]_o\) (Fig. 2a, d; aggregation at 2 minutes of 45.8±3.7% and 42.9±5.6% in normal and low \([\text{Ca}^{2+}]_o\), respectively; p>0.05). Moreover, when platelets were resuspended in the absence of apyrase, and experiments performed rapidly to limit effects of desensitization, ADP-evoked aggregation was also sustained in the presence of 2mM extracellular \([\text{Ca}^{2+}]_o\) (Fig. 2b, d; 59.3±3.8 and 61.9±2.4% in normal and low \([\text{Ca}^{2+}]_o\) respectively).

Finally, the \([\text{Ca}^{2+}]_o\)-dependence of ADP-evoked aggregation responses in plasma was abolished when platelets were resuspended in autologous heat-treated plasma (60°C, 30 min) to destroy enzymatic activity (Fig 2c, d; 41.1±1.1 and 41.5±1.9% in millimolar versus micromolar \([\text{Ca}^{2+}]_o\), respectively). Together, these observations are consistent with a role for \([\text{Ca}^{2+}]_o\)-dependent nucleotidase activity in the \([\text{Ca}^{2+}]_o\)-dependence to ADP-evoked sustained aggregation responses.

**ADP degradation is accelerated by millimolar calcium concentrations**

To directly assess the extent of ADP degradation in millimolar versus micromolar \([\text{Ca}^{2+}]_o\) concentrations, ADP (10µM) was added to apyrase-treated saline or platelet-free plasma and
the ADP concentration after 2 min measured by luminescence following conversion to ATP (see methods). The concentration of ADP remaining in nominally Ca\(^{2+}\)-free saline was 1.61 ± 0.06µM, which was significantly reduced to 0.079 ± 0.03µM (p<0.001) in the presence of 2mM Ca\(^{2+}\), indicating accelerated nucleotidase activity by physiological [Ca\(^{2+}\)]\(_{o}\) (Fig. 3a).

Similarly, ADP incubated with citrated plasma was degraded from 10µM to 2.1 ± 0.27µM by enzymes endogenous to plasma, whereas under recalcified conditions the ADP remaining was markedly lower, at 0.78 ± 0.14µM (p<0.001)(Fig. 3b). A significant effect of [Ca\(^{2+}\)]\(_{o}\) on degradation of 10µM ADP was also detected at earlier time points, as shown by measurements after only 10s (see Supplementary Fig. 1). This suggests that platelets in the presence of millimolar Ca\(^{2+}\) will be exposed to a reduced level of ADP throughout most of the experiment compared to at micromolar Ca\(^{2+}\) levels. In contrast, addition of 2mM MgCl\(_{2}\) to nominally Ca\(^{2+}\)-free saline did not significantly affect ADP degradation or lead to a transient aggregation response (see supplementary Fig. 2). Together with the data in Fig. 2, these results support the conclusion that reduced ADP degradation substantially contributes to the paradoxical amplifying effect of reducing Ca\(^{2+}\) on platelet aggregation. This is also consistent with the reported enhancement of ectonucleotidase activity at millimolar concentrations of calcium compared to its nominal absence or in the presence of a chelator such as EGTA (Christoforidis, et al 1995, Marcus, et al 1997, Strobel, et al 1996).

Reversal of degradation is due to removal of ADP, not negative feedback by adenosine.

In plasma, 5’nucleotidases convert AMP generated by the degradation of ATP and ADP to adenosine [16-17], thus we also considered whether the transient responses to ADP in plasma involved inhibition via G\(_{\alpha_{s}}\)-coupled adenosine A2a receptors. In citrated PRP, adenosine (10µM) inhibited ADP (10µM)-evoked responses, resulting in aggregation traces similar to those observed with ADP in physiological calcium concentrations (supplementary Fig. 3), an
effect which is abolished by the addition of adenosine deaminase (1U/ml). In recalcified PRP however, the addition of adenosine deaminase had no effect on ADP-evoked aggregation (Fig. 4), indicating that negative feedback by the generation of adenosine does not contribute to the reversibility of ADP mediated responses.

*Sustained aggregation to ADP requires constant P2Y₁₂ stimulation*

We next sought to determine whether reversal of aggregation by Ca²⁺-dependent nucleotidases was due to loss of activation of P2Y₁, P2Y₁₂ or both receptors. Disaggregation of ADP (10µM)-stimulated platelets was measured in response to apyrase (0.32 U/ml), the P2Y₁₂ antagonist cangrelor (1µM) or the P2Y₁ antagonist MRS2179 (10µM) (Fig. 5a).

Cangrelor reversed aggregation by 74.6 ± 5.9%, comparable to that observed with apyrase 74.9 ± 1.2% (Fig. 5b). In contrast, MRS2179 caused a more moderate reversal of aggregation (reduction of 24.0 ± 7.8%; Fig 5b). Thus, reversal of ADP induced aggregation by ectonucleotidases is largely due to loss of signalling through P2Y₁₂ receptors.

*TXA₂ generation is enhanced at low extracellular calcium concentrations independently of altered ectonucleotidase activity.*

To investigate whether reduced TXA₂ generation previously reported by others (Harfenist, et al 1987, Packham, et al 1989, Packham, et al 1987) at millimolar [Ca²⁺]₀ was a consequence of termination of ADP signalling by ectonucleotidases, the of presence of apyrase (0.32 U/ml) at micromolar and millimolar [Ca²⁺]₀ was examined on TXB₂ production from washed platelets 3 minutes after stimulation with 10 µM ADP (Fig. 6a). As reported previously (Harfenist, et al 1987, Packham, et al 1989, Packham, et al 1987), TXB₂ production from apyrase-treated platelets was markedly reduced at physiological extracellular Ca²⁺ concentrations compared to that observed in the nominal absence of Ca²⁺. However, similar results were observed in salines lacking apyrase. This indicates that although increased
[Ca^{2+}]_o reduces TXB$_2$ synthesis, this is not dependent on the effect of Ca$^{2+}$ on ectonucleotidases.

Relative contribution of ADP degradation versus desensitisation in limiting platelet responses to ADP

P2Y$_1$ and P2Y$_{12}$ receptors are both susceptible to receptor desensitization after prolonged agonist stimulation (Hardy, et al 2005, Mundell, et al 2006). To determine the relative importance of receptor desensitization versus ectonucleotidase activity in ADP-evoked aggregation, the pan-PKC inhibitor GF109203X was used to attenuate receptor desensitization and aggregation was measured in citrated PRP before and after recalcification (Fig 7a). An intermediate concentration of ADP (2 µM) was used for these experiments to unmask a clear potentiating effect of PKC inhibition, which increased the sustained aggregation at 3 min from 36.9 ± 6.5% to 60.5 ± 5.6% in micromolar [Ca$^{2+}]_o$ (Fig 7b). In contrast, after recalcification, aggregation in the presence of GF109203X or vehicle control, was not significantly different and returned to baseline levels of -3.5 ± 1.6% and 2.7 ± 1.2% respectively 3 min after stimulation (Fig 7b). Thus, ADP degradation overrides any contribution by P2Y receptor desensitisation to the reversal of aggregation in physiological external Ca$^{2+}$ concentrations.
Discussion

Reports of differential platelet responses to ADP in physiological versus nominally Ca\(^{2+}\)-free conditions emerged over 20 years ago (Mustard, et al 1975, Packham, et al 1989). These studies concluded that enhanced thromboxaneA\(_2\) production accounts for the paradoxical amplifying effect of lowering Ca\(^{2+}\) on ADP-evoked aggregation. The present study now shows that altered degradation of ADP can also contribute to this phenomenon. The known Ca\(^{2+}\)-dependence of ecto-ADPases (Marcus, et al 1997; Zimmermann 2000) provides the basis for the difference observed in millimolar versus micromolar [Ca\(^{2+}\)\(_o\)] and this conclusion is supported by direct measurements of ADP. The sustained aggregation evoked by ADP is largely due to stimulation of P2Y\(_{12}\) receptors, consistent with previous reports of the more crucial role of this G\(_i\)-coupled pathway compared to P2Y\(_1\) in amplifying responses to ADP, collagen and thrombin receptors (Cosemans 2006, et al, Dorsam and Kunapuli 2004, Hechler, et al 1998, Jackson, et al 2005, Trumel, et al 1999). The present results also highlight the importance of controlling for nucleotide breakdown in studies of P2 receptor signalling when the external Ca\(^{2+}\) concentration is modified. For example, it is common practice to include soluble apyrase to limit P2 receptor desensitisation within in vitro experiments and simply to vary the external [Ca\(^{2+}\)] to investigate the relative contribution of Ca\(^{2+}\) entry versus release pathways in nucleotide-evoked signalling events.

The limited aggregation response at normal [Ca\(^{2+}\)\(_o\)] has contributed to the view that ADP is a “weak platelet agonist”. However, when metabolism of ADP is limited, the ability of this agonist to stimulate sustained aggregation, as shown in the present study, is more consistent with the substantial reduction in platelet activation observed in P2Y\(_1\) and P2Y\(_{12}\) receptor-deficient mice (Andre, et al 2003, Fabre, et al 1999, Leon, et al 1999) and the major role of ADP in amplifying collagen and thrombin-evoked responses in vitro. It is possible that dense
granule secretion evoked by collagen and thrombin provides a more sustained source of ADP compared to the single bolus application used in standard in vitro experiments. Furthermore, ATP (and thus also ADP) remains sustained for considerable time near sites of vascular injury (Born and Kratzer, 1981), probably reflecting the continual recruitment and activation of platelets during the haemostatic process. Thus, the in vitro experimental condition that limits ADP degradation, such as micromolar [Ca\(^{2+}\)]\(_o\), may more closely represent the ability of this agonist to stimulate platelet function in vivo. Alternatively, use of a non-hydrolysable analogue such as ADP\(\beta\)S or repeated application of ADP to replace degraded agonist should be considered within in vitro studies designed to investigate mechanisms of ADP-dependent platelet activation.

Whilst ADP degradation significantly contributed to the transient nature of the responses to ADP in millimolar [Ca\(^{2+}\)]\(_o\), we agree with earlier studies (Garcia, et al 2007, Packham, et al 1989) that TXA\(_2\) generation is lower at millimolar compared to micromolar [Ca\(^{2+}\)]\(_o\). In our experiments, the marked enhancement of TXB\(_2\) generation by lowering [Ca\(^{2+}\)]\(_o\) was also observed in apyrase-free saline, indicating that the Ca\(^{2+}\)-dependent modulation of TXA\(_2\) generation can occur independently of effects on ectonucleotidase activity. It has been reported that reduced TXA\(_2\) synthesis in physiological calcium concentrations is the result of inhibited ERK phosphorylation (Garcia, et al 2007), however the process by which this is achieved is unclear, and whether these effects are downstream of an extracellular event or whether calcium influx is required, remains to be investigated. Despite there being no TXA\(_2\) generation at millimolar [Ca\(^{2+}\)]\(_o\) in the absence of apyrase, aggregation was indistinguishable from that in low [Ca\(^{2+}\)]\(_o\), suggesting that an initial application of 10 \(\mu\)M ADP can mediate sustained aggregation independently of secondary signalling. At lower concentrations of ADP, however, the release of secondary agonists is required to achieve full aggregation, therefore the effect of extracellular calcium on TXA\(_2\) may be more significant, and
modulation of ADP-evoked aggregation by \([\text{Ca}^{2+}]_o\), likely results from a combination of both altered ectonucleotidase activity and TXA2 production.

Although we did not observe any difference in aggregation within the normal physiological range of extracellular calcium concentrations (0.5-2mM) (3 donors, data not shown), results from this study demonstrate the impact of variable ectonucleotidase activity on platelet function, which may have profound implications in certain clinical conditions. It has previously been reported that in blood from patients with elevated leukocyte counts, degradation of ADP is accelerated and aggregation in response to ADP is reduced due to increased NTDPase levels (Glenn, et al 2008, Pulte, et al 2007). Moreover, in a rat model of cholestatic liver disease where plasma ectonucleotidase activity is enhanced, reduced aggregation is exhibited in response to ADP and low dose collagen (which is dependent on ADP secretion) (Witters, et al 2010). Conversely, individuals demonstrating reduced ectonucleotidase expression may have more reactive platelets and be more susceptible to thrombotic events. Such patients may benefit from therapeutic intervention with soluble forms of NTPDase1.

In conclusion, the present study shows that reduced degradation of ADP by ectonucleotidases contributes to the paradoxical amplification of ADP-evoked aggregation at micromolar compared to millimolar extracellular Ca\(^{2+}\) levels. The sustained inside-out activation of fibrinogen receptors that occurs in response to ADP at low \([\text{Ca}^{2+}]_o\) is likely to be more representative of the potential contribution of ADP to a developing thrombus in vivo, where a constant supply of this P2Y receptor agonist from activated platelets can override enzymatic clearance in the vicinity of a developing thrombus.
Figure Legends

Figure 1. Platelet responses to ADP are sustained at low extracellular calcium concentrations. Sample (a-d) or average (e) responses to 10 µM ADP without added extracellular Ca\(^{2+}\) (low Ca\(^{2+}\)) or in the presence of approximately 2mM external Ca\(^{2+}\). (a) citrated PRP; (b) citrated PRP treated with aspirin (100 µM); (c) platelets resuspended in saline containing (0.32U/ml) apyrase; (d) platelets resuspended in saline containing (0.32U/ml) apyrase and aspirin (100µM); (e) Aggregation measured at 120s.

Figure 2. Prevention of ADP degradation leads to sustained aggregation at physiological extracellular calcium concentrations. Sample (a-c) or average (d) aggregation responses without added extracellular Ca\(^{2+}\) (low Ca\(^{2+}\)) or in the presence of approximately 2mM external Ca\(^{2+}\). (a) Washed platelets (containing 0.32U/ml apyrase) stimulated with the hydrolysis-resistant analogue ADPβS (10µM); (b) platelets soon after resuspension in apyrase-free saline stimulated with ADP (10µM); (c) platelets resuspended in heat-treated citrated plasma stimulated with ADP (10µM); (d) Average aggregation measured at 120s.

Figure 3. ADP degradation by apyrase and endogenous ectonucleotidases present in plasma is reduced at micromolar calcium concentrations. Degradation of a single bolus of 10 µM ADP was assessed by measurements of the concentration of ADP remaining after 2 minutes in (a) saline containing apyrase (0.32U/ml) in the nominal absence of Ca\(^{2+}\) or in the presence of 2mM Ca\(^{2+}\); and (b) citrated plasma before and after re-calcification to 2mM.

Figure 4. Transient ADP-evoked aggregation responses resulting from Ca\(^{2+}\)-dependent ectonucleotidase activity do not involve negative feedback by adenosine. Platelet
aggregation stimulated by ADP (10µM) in nominally Ca^{2+} free conditions or in the presence of 2mM Ca^{2+} with or without adenosine deaminase (AD) 1U/ml.

**Figure 5.** Reversal of aggregation by ADP degrading enzymes is largely due to the termination of P2Y_{12} signalling. (a) Aggregation of washed platelets (apyrase-free) was stimulated with ADP (10µM) soon after resuspension in the presence of 2mM Ca^{2+} and after 2 minutes (arrow) one of the following added: apyrase (0.32U/ml), ARC-69931MX (1µM) or MRS2179 (10µM) or a vehicle control. (b) Disaggregation was measured 3 minutes after the addition of the inhibitors and calculated as a percentage of peak ADP-evoked aggregation response.

**Figure 6.** TXA\(_2\) generation is enhanced at low extracellular calcium concentrations independently of apyrase activity. ADP (10µM)-evoked thromboxaneB\(_2\) production (as a measure of TXA\(_2\) generation) in washed suspensions of platelets with and without apyrase in nominally Ca^{2+} free conditions or in the presence of 2mM extracellular Ca^{2+}. TXB\(_2\) was measured 3 minutes after addition of ADP.

**Figure 7.** ADP degradation is the predominant mechanism regulating ADP-mediated platelet aggregation at physiological extracellular Ca^{2+} concentrations. (a) Platelet aggregation evoked by 2 µM ADP in PRP before and after re-calcification, in the presence and absence of the PKC inhibitor GF 109203X (10µM). Traces are representative of 3 separate experiments. (b) Average aggregation 3 min after addition of ADP.
Online supplement 1. Reduced ADP degradation in low (micromolar) calcium concentrations is evident after 10s. (a) Degradation of ADP (10µM) by apyrase (0.32U/ml) in nominally Ca$^{2+}$ free conditions or in the presence of 2mM Ca$^{2+}$ measured 10s after ADP addition. (b) Degradation of ADP (10µM) by ectonucleotidases present in plasma was measured before and after re-calcification, 10s after ADP addition.

Online supplement 2. Millimolar concentrations of Mg$^{2+}$ do not accelerate ADP degradation. (a) Washed platelets stimulated with ADP (10µM) in nominally Ca$^{2+}$ free saline, or in the presence of 2mM Mg$^{2+}$. (b) Degradation of ADP (10µM) by apyrase (0.32U/ml) present in the platelet saline buffer measured after 2 minutes in nominally calcium free saline or in the presence of 2mM Mg$^{2+}$.

Online supplement 3. Adenosine deaminase blocks the inhibition of ADP-mediated aggregation by adenosine. Aggregation of washed platelets stimulated with ADP (10µM) in the presence of adenosine (10µM) with or without prior addition of adenosine deaminase (AD) 1U/ml.
References

Andre, P., Delaney, S.M., LaRocca, T., Vincent, D., DeGuzman, F., Jurek, M., Koller, B., Phillips, D.R. & Conley, P.B. (2003) P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest, 112, 398-406.

Atkinson, B., Dwyer, K., Enjyoji, K. & Robson, S.C. (2006) Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: Potential as therapeutic targets. Blood Cells Mol Dis, 36, 217-222.

Banz, Y., Beldi, G., Wu, Y., Atkinson, B., Usheva, A. & Robson, S.C. (2008) CD39 is incorporated into plasma microparticles where it maintains functional properties and impacts endothelial activation. Br J Haematol, 142, 627-637.

Birk, A.V., Bubman, D., Broekman, M.J., Robertson, H.D., Drosopoulos, J.H., Marcus, A.J. & Szeto, H.H. (2002) Role of a novel soluble nucleotide phospho-hydrolase from sheep plasma in inhibition of platelet reactivity: hemostasis, thrombosis, and vascular biology. J Lab Clin Med, 139, 116-124.

Born, G.V. & Kratzer, M.A. (1981) Contribution of blood platelets to the pathogenesis of myocardial infarction. Rev Med Brux, 2, 157-160.

Cauwenberghs, S., Feijge, M.A., Hageman, G., Hoylaerts, M., Akkerman, J.W., Curvers, J. & Heemskerk, J.W. (2006) Plasma ectonucleotidases prevent desensitization of purinergic receptors in stored platelets: importance for platelet activity during thrombus formation. Transfusion, 46, 1018-1028.

Christoforidis, S., Papamarcaki, T., Galaris, D., Kellner, R. & Tsolas, O. (1995) Purification and properties of human placental ATP diphosphohydrolase. Eur J Biochem, 234, 66-74.

Coade, S.B. & Pearson, J.D. (1989) Metabolism of adenine nucleotides in human blood. Circ Res, 65, 531-537.

Cosemans, J.M., Munnix, I.C., Wetzker, R., Heller, R., Jackson, S.P. & Heemskerk, J.W. (2006) Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. Blood, 108, 3045-3052.

Dorsam, R.T. & Kunapuli, S.P. (2004) Central role of the P2Y12 receptor in platelet activation. J Clin Invest, 113, 340-345.

Fabre, J.E., Nguyen, M., Latour, A., Keifer, J.A., Audoly, L.P., Coffman, T.M. & Koller, B.H. (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nat Med, 5, 1199-1202.

Gachet, C. (2008) P2 receptors, platelet function and pharmacological implications. Thromb Haemost, 99, 466-472.

Garcia, A., Shankar, H., Murugappan, S., Kim, S. & Kunapuli, S.P. (2007) Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. Biochem J, 404, 299-308.

Gibbins, J.M. & Mahaut-Smith, M.P. (eds.) (2004) Platelets and Megakaryocytes. Humana Press.
Glenn, J.R., White, A.E., Johnson, A.J., Fox, S.C., Myers, B. & Heptinstall, S. (2008) Raised levels of CD39 in leucocytosis result in marked inhibition of ADP-induced platelet aggregation via rapid ADP hydrolysis. *Platelets*, 19, 59-69.

Hardy, A.R., Conley, P.B., Luo, J., Benovic, J.L., Poole, A.W. & Mundell, S.J. (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood*, 105, 3552-3560.

Harfenist, E.J., Packham, M.A., Kinlough-Rathbone, R.L., Cattaneo, M. & Mustard, J.F. (1987) Effect of calcium ion concentration on the ability of fibrinogen and von Willebrand factor to support the ADP-induced aggregation of human platelets. *Blood*, 70, 827-831.

Hastings, A.B., Mclean, F., Eichelberger, L., Lowell Hall, J. & Da Costa, E. (1934) The ionization of calcium, magnesium, and strong citrates. *The Journal of Biological Chemistry*, 107, 351-370.

Heath, M.F. (2004). Secretion from dense granules: luminescence method for adenine nucleotides *Methods Mol Biol* 272 89-93.

Hechler, B., Cattaneo, M. & Gachet, C. (2005) The P2 receptors in platelet function. *Semin Thromb Hemost*, 31, 150-161.

Heptinstall, S., Johnson, A., Glenn, J.R. & White, A.E. (2005) Adenine nucleotide metabolism in human blood--important roles for leukocytes and erythrocytes. *J Thromb Haemost*, 3, 2331-2339.

Jackson, S.P., Schoenwaelder, S.M., Goncalves, I., Nesbitt, W.S., Yap, C.L., Wright, C.E., Kenche, V., Anderson, K.E., Dopheide, S.M., Yuan, Y., Sturgeon, S.A., Prabaharan, H., Thompson, P.E., Smith, G.D., Shepherd, P.R., Daniele, N., Kulkarni, S., Abbott, B., Saylik, D., Jones, C., Lu, L., Giuliano, S., Hughan, S.C., Angus, J.A., Robertson, A.D. & Salem, H.H. (2005) PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat Med*, 11, 507-514.

Jin, J., Daniel, J.L. & Kunapuli, S.P. (1998) Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem*, 273, 2030-2034.

Jin, J. & Kunapuli, S.P. (1998) Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A*, 95, 8070-8074.

Jin, J., Quinton, T.M., Zhang, J., Rittenhouse, S.E. & Kunapuli, S.P. (2002) Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. *Blood*, 99, 193-198.

Kansas, G.S., Wood, G.S. & Tedder, T.F. (1991) Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes. *J Immunol*, 146, 2235-2244.

Leon, C., Hechler, B., Freund, M., Eckly, A., Vial, C., Ohlmann, P., Dierich, A., LeMeur, M., Cazenave, J.P. & Gachet, C. (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest*, 104, 1731-1737.

Marcus, A.J., Broekman, M.J., Drosopoulou, J.H., Islam, N., Alyonycheva, T.N., Safier, L.B., Hajjar, K.A., Posnnett, D.N., Schoenborn, M.A., Schooley, K.A., Gayle, R.B. & Maliszewski, C.R. (1997) The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest*, 99, 1351-1360.

Mundell, S.J., Jones, M.L., Hardy, A.R., Barton, J.F., Beaucourt, S.M., Conley, P.B. & Poole, A.W. (2006) Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. *Mol Pharmacol*, 70, 1132-1142.
Mustard, J.F., Perry, D.W., Kinlough-Rathbone, R.L. & Packham, M.A. (1975) Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol*, 228, 1757-1765.

Packham, M.A., Bryant, N.L., Guccione, M.A., Kinlough-Rathbone, R.L. & Mustard, J.F. (1989) Effect of the concentration of Ca2+ in the suspending medium on the responses of human and rabbit platelets to aggregating agents. *Thromb Haemost*, 62, 968-976.

Packham, M.A., Kinlough-Rathbone, R.L. & Mustard, J.F. (1987) Thromboxane A2 causes feedback amplification involving extensive thromboxane A2 formation on close contact of human platelets in media with a low concentration of ionized calcium. *Blood*, 70, 647-651.

Paul, B.Z., Daniel, J.L. & Kunapuli, S.P. (1999) Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem*, 274, 28293-28300.

Pulte, D., Olson, K.E., Broekman, M.J., Islam, N., Ballard, H.S., Furman, R.R., Olson, A.E. & Marcus, A.J. (2007) CD39 activity correlates with stage and inhibits platelet reactivity in chronic lymphocytic leukemia. *J Transl Med.*, 5, 23.

Rolf, M.G., Brearley, C.A. & Mahaut-Smith, M.P. (2001) Platelet shape change evoked by selective activation of P2X1 purinoceptors with alpha,beta-methylene ATP. *Thromb Haemost*, 85, 303-308.

Savi, P., Beauverger, P., Labouret, C., Delfaud, M., Salel, V., Kaghad, M. & Herbert, J.M. (1998) Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett.*, 422, 291-295.

Strobel, R.S., Nagy, A.K., Knowles, A.F., Buegel, J. & Rosenberg, M.D. (1996) Chicken oviductal ecto-ATP-diphosphohydrolase. Purification and characterization. *J Biol Chem.*, 271, 16323-16331.

Trumel, C., Payrastre, B., Plantavid, M., Hechler, B., Viala, C., Presek, P., Martinson, E.A., Cazenave, J.P., Chap, H. & Gachet, C. (1999) A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood*, 94, 4156-4165.

Witters, P., Hoylaerts, M., Freson, K., de Vos, R., van Pelt, J., Nevens, F., van Geet, C. & Cassiman, D. ADP-degrading enzymes inhibit platelet activation in bile duct-ligated rats. (2010) *J Thromb Haemost*, 8, 360-368.

Zimmermann, H. (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol.*, 362, 299-309.
Figure 1.
Figure 2.
Figure 3.

For Peer Review

Low Ca\(^{2+}\) 2mM Ca\(^{2+}\)

Saline (0.32U/ml apyrase)

b)

Citrated plasma

[ADP] at 2 min (μM)

[ADP] at 2 min (μM)

Low Ca\(^{2+}\) 2mM Ca\(^{2+}\)
Figure 4.
Figure 5.
Figure 6.
Figure 7.
**Supplementary Figure 1.**

(a) Saline (0.32U/ml apyrase)

(b) Citrated plasma

[D diarrhoea Patients (μM) at 10s (μM)]

Low Ca^{2+} 2mM Ca^{2+}

Low Ca^{2+} 2mM Ca^{2+}
Supplementary Figure 2.
Supplementary Figure 3.