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IN THE PRESENCE OF STRONG P2Y₁₂ RECEPTOR BLOCKADE, ASPIRIN PROVIDES LITTLE ADDITIONAL INHIBITION OF PLATELET AGGREGATION

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Abstract

Background—Aspirin and antagonists of platelet ADP P2Y₁₂ receptors are often co-prescribed for protection against thrombotic events. However, blockade of platelet P2Y₁₂ receptors can inhibit thromboxane A₂ (TXA₂) dependent pathways of platelet activation independently of aspirin.

Objectives—To assess *in vitro* whether aspirin adds additional anti-aggregatory effects to strong P2Y₁₂ receptor blockade.

Method—Using platelet-rich plasma from healthy volunteers, determinations were made in 96-well plates of platelet aggregation, TXA₂ production and ADP/ATP release caused by ADP, arachidonic acid, collagen, epinephrine, TRAP-6 amide and U46619 (six concentrations of each) in the presence of prasugrel active metabolite (PAM; 0.1–10 μmol L⁻¹), aspirin (30 μmol L⁻¹), PAM+aspirin or vehicle.

Results—PAM concentration-dependently inhibited aggregation; e.g. aggregations to all concentrations of ADP and U46619 were inhibited 95% by PAM >3 μmol L⁻¹. In further tests of PAM (3 μmol L⁻¹), aspirin (30 μmol L⁻¹) and PAM+aspirin, aspirin generally failed to produce more inhibition than PAM or additional inhibition to that caused by PAM. The anti-aggregatory effects of PAM were associated with reductions in the platelet releases of both TXA₂ and ATP +ADP. Similar effects were found when using either citrate or lepirudin as anti-coagulants, and when conducting traditional light transmission aggregometry at low stirring speeds.

Conclusions—P2Y₁₂ receptors are critical to the generation of irreversible aggregation through the TXA₂-dependent pathway. As a result, strong P2Y₁₂ receptor blockade alone causes inhibition of platelet aggregation that is little enhanced by aspirin. The clinical relevance of these observations remains to be determined.

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Addendum: PCJA, PL, MVC and NSK conducted research, analysed and interpreted data, and assisted in preparation of the manuscript. JAJ, JAM and TDW contributed to the concept and/or design of the research, interpretation of the data and preparation of the manuscript.

Keywords

P2Y₁₂ receptors; TP receptors; thrombosis; thromboxane; prasugrel

Introduction

Aspirin and antagonists of platelet ADP P2Y₁₂ receptors, such as clopidogrel and prasugrel, are often prescribed together as secondary protection against thrombotic vascular events [1, 2, 3]. This “dual anti-platelet therapy” approach in part reflects the assumption that aspirin and P2Y₁₂ antagonists will independently inhibit the thromboxane A₂ (TXA₂)-dependent and P2Y₁₂ receptor-dependent pathways of platelet activation and thus have additive effects on inhibition of platelet function [4, 5, 6, 7]. However, antagonism of platelet P2Y₁₂ receptors can inhibit platelet activation and aggregation mediated by TXA₂ pathways, both by reducing platelet production of TXA₂ and by inhibiting responses following TP receptor activation [8, 9, 10, 11]. To explore these interactions further we conducted a study to examine *in vitro* the individual and combined effects of aspirin and P2Y₁₂ receptor antagonism, employing prasugrel’s active metabolite (PAM), R-138727 [5], as a pharmacological tool. A particular aim of our study was to assess if aspirin adds additional anti-aggregatory effects in the face of strong P2Y₁₂ receptor antagonism.

Methods

Blood Collection

Blood was collected from healthy volunteers (St Thomas’s Hospital Research Ethics Committee, reference 07 / Q0702 / 24), who had abstained from NSAID consumption for the preceding 14 days, by venepuncture into tri-sodium citrate (3.2%, 1:9 v/v; Sigma, Poole, Dorset, U.K.) or lepirudin (Refludan™; 25 µg mL⁻¹). Platelet-rich plasma (PRP) was obtained by centrifugation at 175 × g for 15 min at 25°C. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 15000 × g for 2 min. Platelet counts were made to confirm normal platelet number, 2–4 × 10⁸ cells mL⁻¹, but samples were not adjusted. All experiments were completed within 2h of blood collection.

96-Well Plate Light Transmission Aggregometry

To assess the aggregation of platelets in 96-well plates, a modified light transmission method was used [12]. Briefly, 100 µL samples of PRP were pre-incubated for 30 min at 37°C with PAM (0.1–10 µmol L⁻¹), and/or aspirin (30 µmol L⁻¹), or vehicle (0.1% DMSO) before being placed into the individual wells of a 96-well microtitre plate (Nunc, Lutterworth, Leicestershire, U.K.) containing 10 µL of vehicle or agonist: adenosine diphosphate (ADP; 0.1–30 µmol L⁻¹; LabMedics, Salford, Manchester, U.K.), arachidonic acid (AA; 0.03–1 mmol L⁻¹; Sigma, Poole, U.K.), Horm collagen (0.1–30 µg mL⁻¹; Nycomed, Linz, Austria), epinephrine (0.001–100 µmol L⁻¹; LabMedics), the PAR-1 activation peptide TRAP6 amide (SFLLRNamide; 0.1–30 µmol L⁻¹; Bachem, Bubendorf, Switzerland), and the stable TXA₂-mimetic U46619 (0.1–30 µmol L⁻¹; Cayman Chemical Company, Ann Arbor, MI, U.S.A.). These agonists were chosen as together they represent the range of platelet agonists most widely used in *in vitro* platelet testing [13]. The plate was then placed into a 96-well plate reader (Tecan Sunrise) at 37°C, and absorbance was measured at 595 nm every 15 s for 16 minutes with vigorous shaking between readings. Percentage aggregation was calculated with reference to the absorbance of PPP as a surrogate for 100% aggregation. Graphs shown are for aggregation responses at 16 min.

Light Transmission Aggregometry

Briefly, 225 μL samples of PRP were pre-incubated for 30 min at 37°C with PAM (3 $\mu\text{mol L}^{-1}$), and/or aspirin (30 $\mu\text{mol L}^{-1}$), or vehicle (0.1% DMSO) before being placed into individual cuvettes and incubated for 90 s at 37°C without or with stirring (100, 300 or 1200 rpm) in a PAP-8E aggregometer (Alpha Laboratories, Eastleigh, UK). 25 μL Horm collagen (3 $\mu\text{g mL}^{-1}$; final) was then added and aggregation responses followed for 5 min without or with stirring (100, 300, 1200rpm). The percentage aggregation was calculated with reference to the absorbance of PPP as a surrogate for 100% aggregation. Graphs shown are for aggregation responses at 5 min.

Total ATP and ADP release

To determine the ATP and ATP+ADP release from the platelets, a modified luminometric method was used [14, 15]. Briefly, 100 μL of PRP was placed in each well of a white clear bottomed 96-well plate (Nunc) containing 10 μL Horm collagen (0.1-30 $\mu\text{g mL}^{-1}$), TRAP6 amide (0.1-30 $\mu\text{mol L}^{-1}$), or U46619 (0.1-30 $\mu\text{mol L}^{-1}$) and placed in the plate reader as for platelet aggregation above. After five minutes the plate was removed from the plate reader. To measure ATP release CHRONO-LUME reagent (1:5 v/v, LabMedics) was then added to individual wells; while to measure ATP+ADP release 50 μL of substrate containing phosphocreatine (1.5 mmol L^{-1} , Sigma), creatine phosphokinase (3000 U mL^{-1} , Sigma), and CHRONO-LUME reagent (1:5 v/v) was added to wells. The plate was shaken at 350 rpm for two minutes at 37°C and then luminescence determined on a 1450 Microbeta Trilux (Perkin Elmer, Waltham, MA, USA). The luminescence of a 20 nmol L^{-1} ATP standard (LabMedics) in PPP was used to calculate the concentrations of ATP and ATP+ADP released.

Thromboxane B₂ ELISA

At the end of the PRP aggregation monitoring (i.e. 16min for 96-well plate aggregometry and 5 min for traditional light transmission aggregometry), cyclooxygenase activity was halted by the addition of 1 mmol L^{-1} diclofenac (Sigma), the samples were centrifuged at 1300 g for 10 minutes at 5°C, and the supernatants removed and frozen. TXB₂ levels in the supernatant, as a measure of TXA₂ formation, were determined using selective ELISA (Assay Designs, Cambridge Bioscience, Cambridge, U.K.). In some experiments, to determine the direct effects of aspirin and PAM upon platelet cyclooxygenase activity, 100 μL samples of PRP were pre-incubated for 30 min at 37°C with PAM (0.1-10 $\mu\text{mol L}^{-1}$), aspirin (30 $\mu\text{mol L}^{-1}$), or vehicle (0.1% DMSO) before being placed into the individual wells of a 96-well microtitre plate (Nunc) and then rapidly frozen, to lyse the platelets. Plates were then rapidly thawed and incubated (37°C) for 16min with shaking in the presence of arachidonic acid (1 mM). At the end of this period the 96-well plates were processed for the assessment of TXA₂ production in the same way as for 96-well plates containing intact platelets.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Agonist concentration response curves were plotted and analysed according to the four parameter logistic equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$$

Concentration-response curves were compared by two way ANOVA and Bonferroni post-tests with $p < 0.05$ being taken as significant. Other analyses are stated in text as appropriate.

Results

Influence of PAM on platelet aggregation induced by ADP, collagen or U46619 by 96-well plate aggregometry using citrated PRP

PAM caused concentration-dependent inhibitions of aggregation induced by ADP (0.1–30 $\mu\text{mol L}^{-1}$; Figure 1A), such that aggregation to all ADP concentrations were inhibited 95% by PAM at concentrations greater than 3 $\mu\text{mol L}^{-1}$. Aggregation induced by low concentrations of collagen, <3 $\mu\text{g mL}^{-1}$, were inhibited more strongly by PAM than those induced by high concentrations (Figure 1B). Notably, PAM also produced insurmountable blockade of aggregation induced by U46619 (0.1–30 $\mu\text{mol L}^{-1}$; Figure 1C) with very similar IC_{50} values to those for ADP: e.g. PAM $\log \text{IC}_{50}$ ($\pm$ s.e.m.) values against 1 $\mu\text{mol L}^{-1}$ of ADP and U46619 were -6.6 ± 0.5 and -6.6 ± 0.3 , respectively; the $\log \text{IC}_{50}$ values against 10 $\mu\text{mol L}^{-1}$ of ADP and U46619 were -6.2 ± 0.1 and -5.8 ± 0.1 , respectively (Figures 1A and 1C).

Individual and combined effects of PAM and aspirin on platelet aggregation in response to arachidonic acid, ADP, collagen, epinephrine, TRAP6 or U46619 by 96-well plate aggregometry using citrated PRP

Across the range of platelet agonists used, aspirin (30 $\mu\text{mol L}^{-1}$) did not produce either more inhibition than PAM (3 $\mu\text{mol L}^{-1}$) or additional inhibition to that caused by PAM alone, except weakly for collagen at the two highest concentrations tested (two ANOVA; Figure 2).

Individual and combined effects of PAM and aspirin on platelet TXA_2 production in response to arachidonic acid, collagen, or epinephrine using citrated PRP by 96-well plate aggregometry

In control experiments, we measured the production of TXA_2 to all platelet agonists (data not shown). From these studies, we selected arachidonic acid, collagen and epinephrine as the most reliable agonists to use in further investigations of the effects of PAM and aspirin. Aspirin (30 $\mu\text{mol L}^{-1}$) alone caused complete inhibition of the production of TXA_2 in response to arachidonic acid (Figure 3A), collagen (Figure 3B) and epinephrine (Figure 3C). Whilst PAM (3 $\mu\text{mol L}^{-1}$) also reduced TXA_2 production to the same three platelet activators, for arachidonic acid and collagen it was to a lesser extent than that found for aspirin. Addition of aspirin on top of PAM significantly (two way ANOVA; $p < 0.01$) inhibited any remaining TXA_2 formation in the case of arachidonic acid and collagen, while for epinephrine aspirin added no significant extra effect to that of PAM. In broken platelet preparations, the formation of TXA_2 following incubation with arachidonic acid (control: 435 ± 17 ng/ml TXB_2 ; mean $\pm$ s.e.m., $n=24$) was markedly inhibited by aspirin (10, 30 and 100 $\mu\text{mol L}^{-1}$ caused inhibitions of $46 \pm 11\%$, $79 \pm 4\%$ and $90 \pm 3\%$, respectively; mean $\pm$ s.e.m., $n=4$ for each; $p < 0.05$) whereas PAM was without effect (1, 3 and 10 $\mu\text{mol L}^{-1}$ caused inhibitions of $2 \pm 8\%$, $16 \pm 5\%$ and $4 \pm 11\%$, respectively; mean $\pm$ s.e.m., $n=4$ for each; n.s.).

Individual and combined effects of PAM and aspirin on the release of ATP and ATP+ADP from platelets stimulated with collagen, TRAP6 or U46619 by 96-well plate aggregometry using citrated PRP

In control experiments, we measured the release of ATP and ATP+ADP to all platelet agonists (data not shown). From these studies we selected collagen, TRAP-6 and U46619 as the most reliable agonists to use in further investigations of the effects of PAM and aspirin. Platelet release of ATP (data not shown) and ATP+ADP stimulated by collagen was similarly inhibited by PAM (3 $\mu\text{mol L}^{-1}$) and aspirin (30 $\mu\text{mol L}^{-1}$), with no significant

additive effect (two way ANOVA; Figure 4A). Release of adenine nucleotides in response to TRAP6 was inhibited by PAM but not by aspirin, with no additive effect (two way ANOVA; Figure 4B). Release of adenine nucleotides in response to U46619 was >90% inhibited by PAM but unaffected by aspirin; there was no additive effect of aspirin to that of PAM (two way ANOVA; Figure 4C).

Individual and combined effects of PAM and aspirin on platelet aggregation in response to arachidonic acid, ADP, collagen, epinephrine, TRAP6 or U46619 by 96-well plate aggregometry using lepirudinized PRP

In lepirudinized PRP, PAM ($3 \mu\text{mol L}^{-1}$) and aspirin ($30 \mu\text{mol L}^{-1}$) produced the same pattern of inhibitory effects upon platelet aggregation induced by the range of platelet agonists as was found in citrated PRP. In particular, aspirin did not produce either more inhibition than PAM or additional inhibition to that caused by PAM alone (Figure 5).

Individual and combined effects of PAM and aspirin on platelet TXA₂ production in response to arachidonic acid or collagen using lepirudinized PRP by 96-well plate aggregometry

The pattern of TXA₂ production in lepirudinized PRP was similar to that in citrated PRP, although the amounts produced were approximately halved (Figure 6). Aspirin ($30 \mu\text{mol L}^{-1}$) alone caused complete inhibition of the production of TXA₂ in response to arachidonic acid and collagen, while PAM caused partial inhibitions (Figure 6). Addition of aspirin on top of PAM inhibited any remaining TXA₂ formation. Our assays were not sufficiently sensitive to provide robust data regarding the production of TXA₂ in response to epinephrine in lepirudinized PRP and the effects of PAM and aspirin (data not shown).

Individual and combined effects of PAM and aspirin on platelet aggregation in response to collagen in traditional LTA using citrated PRP

When conducting LTA at different stir speeds (100, 300, and 1200 rpm), we noted no significant differences in the maximal responses to collagen ($3 \mu\text{g mL}^{-1}$; Figure 7A) although the AUC was less at 100 rpm than at 300 rpm or 1200 rpm due to the slower development of the response (Figure 7B). PAM ($3 \mu\text{mol L}^{-1}$) inhibited the collagen-induced aggregations with a very notable sensitivity to stirring speed, such that at 100 rpm PAM inhibited the maximum aggregation by $70 \pm 7\%$ whereas at 1200rpm it inhibited by only $15 \pm 4\%$ ($n=4$; Figure 7C); the associated values for AUC were $67 \pm 8\%$ and $15 \pm 4\%$ ($n=4$; Figure 7D). Aspirin produced much greater additive effects at 1200 rpm than at 100 rpm, such that there were no significant differences between the responses to collagen in the presence of PAM+aspirin at any speed (100, 300 and 1200 rpm; Figures 7E and 7F).

Individual and combined effects of PAM and aspirin on platelet TXA₂ production in response to collagen in traditional LTA using citrated PRP

There was a clear association between stir speed of PRP and the production of TXA₂ in response to collagen (Figure 8). Aspirin ($30 \mu\text{mol L}^{-1}$) either alone or in combination with PAM ($3 \mu\text{mol L}^{-1}$) inhibited TXA₂ production by more than 98% in all cases ($n=32$). PAM alone inhibited TXA₂ production at 100 rpm by $19 \pm 11\%$, at 300rpm by $36 \pm 5\%$, and at 1200rpm by $30 \pm 6\%$ ($n=4$ for each).

Discussion

In experiments using multiple platelet agonists across wide concentration ranges, we found that aspirin, at a concentration that completely inhibited TXA₂ formation, neither produced more inhibition of platelet aggregation than PAM nor substantially increased inhibition of

platelet aggregation when added to PAM, as assessed by either 96-well plate aggregometry or traditional light transmission aggregometry at low speed. Furthermore, our data suggest that the failure of aspirin to produce additional anti-aggregatory effects in the presence of strong P2Y₁₂ blockade reflects blockade by PAM of TXA₂ pathways of platelet activation due to (i) a reduction in the formation of TXA₂ and (ii) an inhibition of TXA₂-driven aggregation mediated by TP receptor stimulation. These observations indicate, at least *in vitro*, that under certain conditions the platelet inhibition resulting from combined aspirin and P2Y₁₂ receptor blockade is not appreciably greater than that produced by high levels of P2Y₁₂ receptor blockade alone.

In the studies presented here, we initially characterised the effects of PAM upon platelet aggregation induced by ADP, to confirm the ability of our 96-well plate aggregation assay to characterise concentration-dependent P2Y₁₂ receptor blockade [5, 16, 17, 18, 19, 20]. Then, consistent with the roles of platelet-derived ADP in collagen-induced aggregation, we demonstrated PAM to be inhibitory with more activity against low than high concentrations of collagen [5, 18]. We next showed that PAM blocked platelet aggregations induced by the TP receptor agonist, U46619, at concentrations very similar to those that blocked responses to ADP. This is consistent with reports that the second, irreversible wave of platelet aggregation following TP receptor activation is dependent upon platelet-derived ADP acting upon platelet P2Y₁₂ receptors [8, 9, 16]. We also found that aggregation in response to ADP was associated with increased production of thromboxane A₂ (e.g. TXB₂ levels in presence of ADP 10 μ M: control, 9.9 $\pm$ 1.9 ng/ml; aspirin, 2.6 $\pm$ 0.8 ng/ml; p <0.05, two-way ANOVA plus Dunnett's test, n =8 for each: full curves not shown), even though we found only a weak anti-aggregatory effect of aspirin (significant at ADP 3 μ M; p <0.05; 2-way ANOVA; Figure 2B). This weak inhibition was not seen in experiments using lepirudinized PRP, which may well be explained by the enhancement of TXA₂-dependent responses in the presence of low extracellular calcium (as discussed below) [21, 22, 23, 24, 25]. From these experiments we selected concentrations of PAM (3 μ mol L⁻¹) and aspirin (30 μ mol L⁻¹) for further study, consistent with the effective peak plasma concentrations of these drugs in clinical use [5, 10, 11, 12]. These studies showed clearly that, with the exception of a weak additive effect for aggregation induced by collagen, there were no circumstances in which aspirin produced either more inhibition of platelet aggregation than PAM or additional inhibition of platelet aggregation when added to PAM. PAM also blocked platelet aggregation induced by arachidonic acid, a response which is well characterised as being TXA₂ dependent and widely used as an *ex vivo* test for the effects of aspirin [26]. We also found that PAM caused reductions in the production of TXA₂ following stimulation of platelets with arachidonic acid, collagen or epinephrine, but that this effect was lost in broken platelets confirming that PAM, unlike aspirin, is not a direct inhibitor of cyclooxygenase. These observations are in agreement with our previous report that treatment with standard dose of clopidogrel for one week reduces the *ex vivo* production of TXA₂ by platelets [10], and demonstrates that this production is partially dependent upon activation of P2Y₁₂ receptors, consistent with previous reports [25]. Finally, we also found that PAM had generally greater effects upon ADP and ATP release than aspirin, and for aspirin to have only weak, or no, additive effects to PAM. We are aware that the effects we report here from studies employing 96-well plate aggregometry differ in some important regards to those previously reported using traditional light transmission aggregometry. In particular, studies using hirudin as an anti-coagulant and/or employing stirred platelet preparations have shown PAM, and other P2Y₁₂ receptor blockers, to have relatively weak effects against aggregation induced by collagen with a much more important additive effect of aspirin [19, 20]. One reason for the difference in results could accord with the understanding that analysis of platelet aggregation in the presence of citrate may over emphasise the role of TXA₂. This phenomenon occurs because the production of TXA₂ in response to a range of platelet agonists becomes enhanced when the concentration of extracellular calcium is decreased, as can be seen in washed platelet

preparations using calcium-free buffers and similarly in citrated PRP [21, 22, 23, 24, 25]. However, in studies using lepirudinized PRP, we found the same pattern of results as in citrated PRP, although with an approximate 50% reduction in the associated production of TXA₂. Indeed, the response to collagen actually became less sensitive to the effects of aspirin, with a proportionately greater difference with regard to the effects of PAM. As the differences between our results and those previously published were not explainable by differences in the anti-coagulant we considered they must be explained by the methods of mixing in 96-well plate versus traditional light transmission aggregometry. In particular, we noted that PAM was considerably more active against collagen-induced aggregation in 96-well plates than previously reported in traditional light transmission aggregometry [19, 20]. To explore the reasons for this, we repeated our studies of aggregation responses to collagen using a traditional (PAP-8E) light aggregometer, and paid particular attention to the speed of the stirrer bar, since we hypothesised that the shaking provided by the 96-well plate reader would be better mimicked at low stirrer bar speeds. We found that the effects of PAM and aspirin against collagen-induced aggregation at 1200 rpm were as reported previously, i.e. that PAM was only poorly effective with a marked additional effect of aspirin [19, 20]. However, as we reduced the speed we found that the effects of PAM become relatively larger, such that at 100 rpm it inhibited collagen-induced aggregation by around 70%. Interestingly, despite these marked differences in sensitivity to PAM, aggregatory responses to collagen were little different between 100 rpm and 1200 rpm. These data show that the effects of PAM against collagen-induced aggregation are strongly influenced by physical conditions and offer a good explanation for differences between our results and those reported previously.

Of course *in vitro* systems are of value if they assist in understanding how systems work *in vivo*, and so consideration must be given to determining if aggregometry conducted in 96-well plates has any usefulness in this regard. One way to address this issue is to assess whether the mechanistic *in vitro* data we present here accords with mechanistic *in vivo* data; i.e. does aspirin fail to add to the anti-thrombotic effects of strong P2Y₁₂ receptor blockade *in vivo*. A number of studies suggest that our mechanistic *in vitro* data is indeed in accord. For example, thrombotic occlusion of injured canine coronary arteries can be abolished by strong P2Y₁₂ receptor blockade without the need for aspirin [27, 28], and the same observation has been made for platelet deposition in a porcine denuded arterial shunt [29]. Furthermore, in wild type mice, FeCl₃ damage of mesenteric arteries has been reported to result in occlusion within about 15 min, whereas in P2Y₁₂ mice the same was only seen in 1 out of 9 animals [30]. This result led the authors to conclude that 'more aggressive strategies of P2Y₁₂ antagonism will be anti-thrombotic without the requirement of aspirin co-therapy'. Of course this does not deny that aspirin can add anti-thrombotic effects when P2Y₁₂ receptor blockers are used at doses producing lower levels of P2Y₁₂ receptor blockade, even a potent P2Y₁₂ blocker such as prasugrel [31, 32].

In conclusion, we show that P2Y₁₂ receptors underpin key components of the TXA₂ pathway of platelet activation and appear to be critical to the generation of irreversible aggregation following TP receptor activation by TXA₂, especially at lower speeds of platelet mixing which could accord with low shear areas of thrombus formation *in vivo* [33]. As a result strong P2Y₁₂ receptor blockade alone produces inhibition of platelet aggregation to a broad range of platelet agonists that is not further enhanced by addition of aspirin, an observation that is consistent with data from some pre-clinical *in vivo* models. The clinical relevance of these observations remains to be determined in appropriately designed clinical investigations. However, in a highly speculative manner, one could postulate that addition of aspirin to individuals receiving strong P2Y₁₂ receptor blockers has the potential to produce side effects secondary to inhibition of cyclooxygenase at non-platelet sites, as has recently become apparent for non-steroidal anti-inflammatory drugs [34, 35, 36, 37].

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Figure 1.

Platelet aggregations induced by ADP ($0.1\text{--}30\ \mu\text{mol L}^{-1}$; panel A), collagen ($0.1\text{--}30\ \mu\text{g mL}^{-1}$; panel B), or U46619 ($0.1\text{--}30\ \mu\text{mol L}^{-1}$; panel C) were inhibited in a concentration-dependent manner by PAM ($p < 0.05$ for all; two way ANOVA). Curves for $0.1\ \mu\text{mol L}^{-1}$ and $0.3\ \mu\text{mol L}^{-1}$ ADP are superimposed upon one another (panel A), as are curves for $0.1\ \mu\text{mol L}^{-1}$ and $0.3\ \mu\text{mol L}^{-1}$ U46619 (panel B). Data shown are mean $\pm$ s.e.m. of responses measured by 96-well plate aggregometry in citrated PRP prepared from 6 different individuals.

Figure 2.

Platelet aggregation induced by arachidonic acid ($0.03\text{--}1\text{ mmol L}^{-1}$; panel A), ADP ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel B), collagen ($0.1\text{--}30\text{ }\mu\text{g mL}^{-1}$; panel C), epinephrine ($0.001\text{--}100\text{ }\mu\text{mol L}^{-1}$; panel D), TRAP6 ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel E) and U46619 ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel F) in the presence of aspirin ($30\text{ }\mu\text{mol L}^{-1}$) and/or PAM ($3\text{ }\mu\text{mol L}^{-1}$). Data shown are mean $\pm$ s.e.m. of responses measured by 96-well plate aggregometry in citrated PRP prepared from 4 different individuals. * shows $p < 0.05$ difference from vehicle by 2-way ANOVA plus Bonferroni post test; † shows $p < 0.05$ difference between PAM and PAM+aspirin. Symbols at end of lines signify difference in set; symbols at individual points signify particular differences.

Figure 3.

Platelet production of TXA₂ induced by arachidonic acid (0.03-1 mmol L⁻¹; panel A), collagen (0.1-30 μg mL⁻¹; panel B), and epinephrine (0.1-30 μmol L⁻¹; panel C) in the presence of PAM (3 μmol L⁻¹), and/or aspirin (30 μmol L⁻¹), or vehicle (control, 0.1% DMSO). Data shown are mean ± s.e.m. of responses collected from 96-well plate aggregometry in citrated PRP prepared from 4 different individuals. * shows p<0.05 difference from vehicle by 2-way ANOVA plus Bonferroni post test; † shows p<0.05 difference between PAM and PAM+aspirin. Symbols at end of lines signify difference in set; symbols at individual points signify particular differences.

Figure 4.

The release of ATP+ADP from platelets stimulated with collagen ($0.1\text{--}30\ \mu\text{g mL}^{-1}$; panel A), TRAP6 ($0.1\text{--}30\ \mu\text{mol L}^{-1}$; panel B) and U46619 ($0.1\text{--}30\ \mu\text{mol L}^{-1}$; panel C) in the presence of PAM ($3\ \mu\text{mol L}^{-1}$), and/or aspirin ($30\ \mu\text{mol L}^{-1}$), or vehicle (control, 0.1% DMSO). Data shown are mean $\pm$ s.e.m. of responses measured by 96-well plate aggregometry in citrated PRP prepared from 5 different individuals. * shows $p < 0.05$ difference from vehicle by 2-way ANOVA plus Bonferroni post test. Symbols at end of lines signify difference in set; symbols at individual points signify particular differences.

Figure 5.

Platelet aggregation induced by arachidonic acid ($0.03\text{--}1\text{ mmol L}^{-1}$; panel A), ADP ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel B), collagen ($0.1\text{--}30\text{ }\mu\text{g mL}^{-1}$; panel C), epinephrine ($0.001\text{--}100\text{ }\mu\text{mol L}^{-1}$; panel D), TRAP6 ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel E) and U46619 ($0.1\text{--}30\text{ }\mu\text{mol L}^{-1}$; panel F) in the presence of aspirin ($30\text{ }\mu\text{mol L}^{-1}$) and/or PAM ($3\text{ }\mu\text{mol L}^{-1}$). Data shown are mean $\pm$ s.e.m. of responses measured by 96-well plate aggregometry in lepirudinized PRP prepared from 4 different individuals. * shows $p < 0.05$ difference from vehicle by 2-way ANOVA plus Bonferroni post test. Symbols at end of lines signify difference in set; symbols at individual points signify particular differences.

Figure 6.

Platelet production of TXA₂ induced by arachidonic acid (0.03-1 mmol L⁻¹; panel A) and collagen (0.1-30 μg mL⁻¹; panel B) in the presence of PAM (3 μmol L⁻¹), and/or aspirin (30 μmol L⁻¹), or vehicle (control, 0.1% DMSO). Data shown are mean ± s.e.m. of responses collected from 96-well plate aggregometry in lepirudinized PRP prepared from 4 different individuals. * shows p<0.05 difference from vehicle by 2-way ANOVA plus Bonferroni post test; † shows p<0.05 difference between PAM and PAM+aspirin. Symbols at end of lines signify difference in set; symbols at individual points signify particular differences.

Figure 7.

Platelet aggregation induced by collagen ($3 \mu\text{g mL}^{-1}$) as measured by traditional light transmission aggregometry at stir speeds of 100, 300 or 1200 rpm using citrated PRP. Responses are shown as % maximum aggregation (panels A, C and E), and AUC of aggregation (panels B, D and F), as calculated over a 5 min aggregation period. Control responses shown in panels A and B; responses in the presence of PAM ($3 \mu\text{mol L}^{-1}$) in panels C and D; responses in the presence of PAM ($3 \mu\text{mol L}^{-1}$) + aspirin ($30 \mu\text{mol L}^{-1}$) in panels E and F. Data shown are mean $\pm$ s.e.m. of responses in PRP prepared from 4 different individuals; * $p < 0.05$, one-way ANOVA.

Figure 8.

Platelet production of TXA₂ induced by collagen (3 μg mL⁻¹) in the presence of PAM (3 μmol L⁻¹), and/or aspirin (30 μmol L⁻¹), or vehicle (control, 0.1% DMSO). Data shown are mean ± s.e.m. of samples collected from traditional light transmission aggregometry in citrated PRP prepared from 4 different individuals.