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ORIGINAL ARTICLE

Reference curves for aggregation and ATP secretion to aid diagnose of platelet-based bleeding disorders: Effect of inhibition of ADP and thromboxane A_2 pathways

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Abstract
Platelet aggregation is widely used in clinical laboratories to evaluate patients with bleeding disorders of suspected platelet aetiology. Simultaneous monitoring of ATP release as a measure of dense granule secretion provides additional information to aid diagnosis. There is, however, no standard way of performing or interpreting these tests. The present study has evaluated aggregation and ATP secretion to eight platelet agonists in healthy donors and has evaluated the reproducibility of response for a number of variables, including platelet number and time after donation. The effect of inhibition of the two major platelet feedback mediators, ADP and thromboxane A_2 (TxA_2), was investigated using the $P2Y_1$ and $P2Y_{12}$ receptor antagonists, MRS2179 and AR-C67085, and the cyclooxygenase inhibitor, indomethacin. The results demonstrate that, if used within certain boundaries, the investigation of platelet aggregation and secretion is a powerful way to discriminate between differing pathways of platelet activation. The present data-set are an invaluable resource to the clinical laboratory to aid evaluation of patients with suspected platelet-based bleeding disorders.

Keywords: *Patient testing, platelets aggregation, dense granule secretion, ADP receptors, thromboxane A_2*

Introduction

Platelets are critical elements in the maintenance of normal haemostasis. Abnormalities in platelet number or platelet function may result in excessive bleeding. Platelet function testing is routinely used in the clinic to diagnose patients with suspected platelet-based bleeding disorders. One of the most widely used tests for assessing platelet reactivity is platelet aggregation in platelet-rich plasma (PRP). However, there is no accepted way to perform or interpret these studies [1], with practices varying between laboratories as recently documented by a review of 46 North American Clinical Laboratories [2]. Variables include factors such as sample collection, platelet preparation, platelet number, agonist selection and agonist concentrations. Furthermore, the full value of aggregometry is seldom achieved because of the use of a limited number of agonists and concentrations. Indeed, platelet aggregation methodology remained largely unchanged since the late 1980s despite important advances in our understanding of platelet activation. For example, the guidelines in the UK for investigation of patients with suspected platelet-based bleeding disorders, including information on platelet aggregation studies, were drawn up by British Society for Haematology Task Force in 1988 [3]. A more recent report on behalf of the Rare Haemostatic Disorders Working Party of the UK Haemophilia Centre Doctors Organisation (UKHCDO) summarizes heritable platelet disorders and gives guidelines on their analysis and clinical management [4]. This report emphasizes the importance of aggregation testing in clinical diagnosis but does not give experimental details on how to perform these studies. Further, development of expertise in platelet function testing is hampered by the small number of clinical patients who require testing and

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by the even smaller number of patients with defined, platelet-based disorders.

The interpretation of aggregation concentration response curves in patients is highly specialized because of the complexity of interacting mechanisms that coordinate the aggregation response. For example, a defect in response to more than one platelet agonist could be due to a generalized defect in platelet activation, to impairment in release of the major feedback mediators, ADP and TxA_2, or to a defect in the $P2Y_{12}$ receptor for ADP or in the thromboxane receptor. For example, a defect in the $P2Y_{12}$ ADP receptor would result in abnormal responses to low concentrations of nearly all platelet agonists, including arachidonic acid, which is frequently used to investigate possible defects in cyclooxygenase activity. On the other hand, a defect in the $P2Y_1$ ADP receptor would have a more selective effect because it generates a relatively weak signal and its activation is masked by receptors which signal through the same pathway, such as the thromboxane receptor and the two thrombin receptors, PAR_1 and PAR_4 [5]. The interpretation of aggregation traces is also influenced by the natural variation in responses that exist within the population, thereby making it essential for each laboratory to have standard reference curves for comparison to patients' responses. For example, a recent study on aggregation responses in 359 healthy individuals identified a subset of donors with hyperresponsive platelets which was maintained over independent experiments [6].

Several laboratories have published their own methods for monitoring platelet aggregation [1], but none has used a full range of platelet agonists and evaluated responses in the presence of inhibitors of the two major feedback inhibitors, ADP and TxA_2. Further, few laboratories have extended their analysis to the simultaneous monitoring of ATP secretion from dense granules, despite the availability of lumi-aggregometers for more than 25 years and recognition of the value in monitoring ATP secretion in diagnosis [7].

The aim of the present study was to investigate aggregation and dense granule secretion to low, intermediate and high concentrations of a range of platelet agonists and to investigate the extent to which these responses vary between healthy donors and factors such as time after donation and platelet number. Importantly, several of the agonists are not routinely used in clinical testing, even though their inclusion would aid diagnosis of what are anticipated to be among the more common causes of mild platelet-based disorders. These include the thromboxane analogue, U46619, PAR_1 and PAR_4 thrombin receptor-specific peptides, and the GPVI collagen receptor agonist, collagen-related peptide (CRP). The effect of inhibition of the major platelet feedback agonists, TxA_2 and ADP, on responses to these agonists has also been evaluated. The results provide a valuable resource of data to aid patient testing and suggest that, if used within certain boundaries, the simultaneous investigation of platelet aggregation and ATP secretion is a powerful way to aid diagnosis of patients with suspected platelet-based bleeding disorders.

Materials and methods

Materials

Trisodium citrate, ADP, adrenaline, U46619, arachidonic acid, indomethacin and MRS2179 were purchased from Sigma (Poole, UK). Horm collagen was purchased from Nycomed Austria (Linz, Austria). The PAR_1 peptide SFLLRN was purchased from Alta Bioscience Laboratory (University of Birmingham, UK). The PAR_4 peptide AYPGKF and CRP was purchased from Dr Richard Farndale (Cambridge University, UK). AR-C67085 was a gift from Astrazeneca (Loughborough, UK). Luciferin Luciferase reagent (Chrono-lume) was purchased from Chrono-log Corporation (Havertown, PA, USA). Platelet aggregation and secretion was performed on a Dual Channel Lumi-Aggregometer (model 460VS, Chronolog). ADP (10 mM), adrenaline (100 mM), PAR_1-peptide (10 mM), PAR_4-peptide (500 mM), MRS2179 (10 mM) and AR-C67085 (10 mM) were dissolved in phosphate-buffered saline (PBS) and stored as frozen aliquots at the concentrations shown and diluted in PBS on the day of the experiment. CRP (1.4 mg/ml stock) was stored at 4°C and diluted in 0.01 M acetic acid in PBS containing 0.1% fattyacid-free bovine serum albumin on the day of the experiment. Collagen was stored as a concentrated stock at 1mg/ml as supplied by the manufacturer at 4°C. Arachidonic acid (1 M) and indomethacin (10 mM) were stored and diluted in dimethyl sulphoxide (DMSO). U46619 (1 mM) was stored in DMSO and diluted in PBS on the day of the experiments. Agents were added in dilutions of 1: 100, with the exception of arachidonic acid and indomethacin which were added at a dilution of 1: 300 to keep the DMSO concentration to 0.33%.

Preparation of platelet rich plasma

All donors gave informed consent and answered negatively to routine deferral questions such as ingestion of aspirin and other agents that may influence platelet function. Whole blood was anticoagulated with 4% trisodium citrate (trisodium citrate/whole blood ratio, 1:9), unless stated, by collecting 50 ml blood using a 21 G needle into a plastic polypropylene syringe containing anticoagulant. The blood was transferred to 5 ml polypropylene tubes, 75 × 12 mm, for centrifugation. PRP was prepared by centrifugation of anticoagulated blood at

170 *g* for 10 min at room temperature. The PRP (upper layer) was gently pipetted into a 50 ml polypropylene tube using a plastic pipette. The blood was recentrifuged at 190 *g* for 10 min at room temperature and further PRP collected in the same way and pooled. The use of two centrifugation steps increases the yield of PRP. The platelet number was measured using a Coulter Z_2 analyser, but was not adjusted prior to experimentation. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 1000 *g* for 10 min at room temperature.

Platelet aggregation and secretion studies

The PRP and PPP samples were used to set the scale prior to the onset of the aggregation recording. The difference between the light transmission of the PRP and PPP samples is taken as 100%, with the initial starting trace representing 0%. Samples (396 µl) were prewarmed at 37°C for 120 s and stirred at 1200 rpm for 60 s before agonist addition. Antagonists, indomethacin and luciferin-luciferase (diluted according to the manusfacturer's instructions) were given 120 s before experimentation. Platelet aggregation was determined by measurement of the change in optical density (i.e. light transmission) after agonist addition. Each sample was compared relative to the PPP control. Platelet aggregation was monitored for up to 5 min.

Analysis of Data

The degree of aggregation at 90 s or 180 s was measured and reported as a percentage of the difference in light transmission between the PRP and PPP samples. It should be noted that in cases of transient aggregation, the response at 90 s can be larger than that at 180 s. Results are shown as mean ± S.D. from 3–20 experiments. ATP secretion was calculated by addition of a known concentration of ATP (4 nMol) and normalized to 1×10^8/ platelets. Concentration-response curves were fitted to a three variable logistic equation, with EC_{50}, Hill slope and maximal response, as variables using Graphpad Prism software. Statistical indications were made using Student's *t*-test, with $P < 0.05$ taken as the level of significance.

Results

Standardization of aggregation studies

An initial series of experiments were designed to establish the extent of variation in platelet aggregation to a range of receptor agonists within a population of healthy volunteers who had denied having recently taken medication that is known to alter platelet function. Trisodium citrate was used as the anticoagulant at a concentration of 4%, although similar results were obtained with a lower concentration of 3.2% on a more limited number of assays (data not shown). The latter concentration is routinely used in a clinical setting. The dependency of aggregation on sex, age, platelet number and time after donation was investigated, along with investigation of the reproducibility of aggregation within a small number of donors over the course of one year. These initial studies were performed with the following platelet agonists, ADP, adrenaline, collagen, PAR_1 peptide, arachidonic acid and U46619.

The first issue to be considered was the extent to which aggregation deteriorates with time after donation, including the time taken in preparing the PRP. This is a crucial question as a full examination of the aggregation and secretion response of platelets to low, intermediate and high concentrations of a range of platelet agonists can take several hours. We have found that storage of blood in the presence of trisodium citrate (4%) at room temperature for up to 6 hours prior to platelet preparation had a minimal effect on responses to all agonists, with the exception of ADP where a reduction in aggregation of up to 20% was seen. This is most likely mediated through partial desensitisation of the $P2Y_1$ ADP receptor, as this occurs rapidly on exposure to low concentrations of the nucleotide that may have occurred during storage. Importantly, when samples were kept in PRP at room temperature for up to 6 hours, platelet responses to all agonists, including ADP, were maintained (not shown). Subsequent studies were performed on PRP samples that were prepared within 60 minutes of blood donation and analysed within 6 hours.

Concentration response curves for platelet aggregation to ADP, adrenaline, collagen, PAR_1 peptide, arachidonic acid and U46619 were determined in 10 male and 10 female donors aged between 21 and 46 years. A further set of concentration response curves for aggregation were later determined to the synthetic collagen, CRP, and the PAR_4 peptide in a smaller number of male and female donors. In all cases, aggregation was measured at 90 s. There was a good level of reproducibility in response to all agonists, with nearly all standard deviation values falling within 20% of the mean. Further, no apparent variation in response according to age, sex or platelet number over the range of $150–400 \times 10^9$/litre (which corresponds to that found in healthy volunteers) was observed. Although these studies do not prove that the concentration response curve to each agonist is not influenced by these parameters, the results demonstrate that they do not have a major influence on the position of the curves. Thus, it is therefore appropriate to pool aggregation data from this group of healthy volunteers in order to

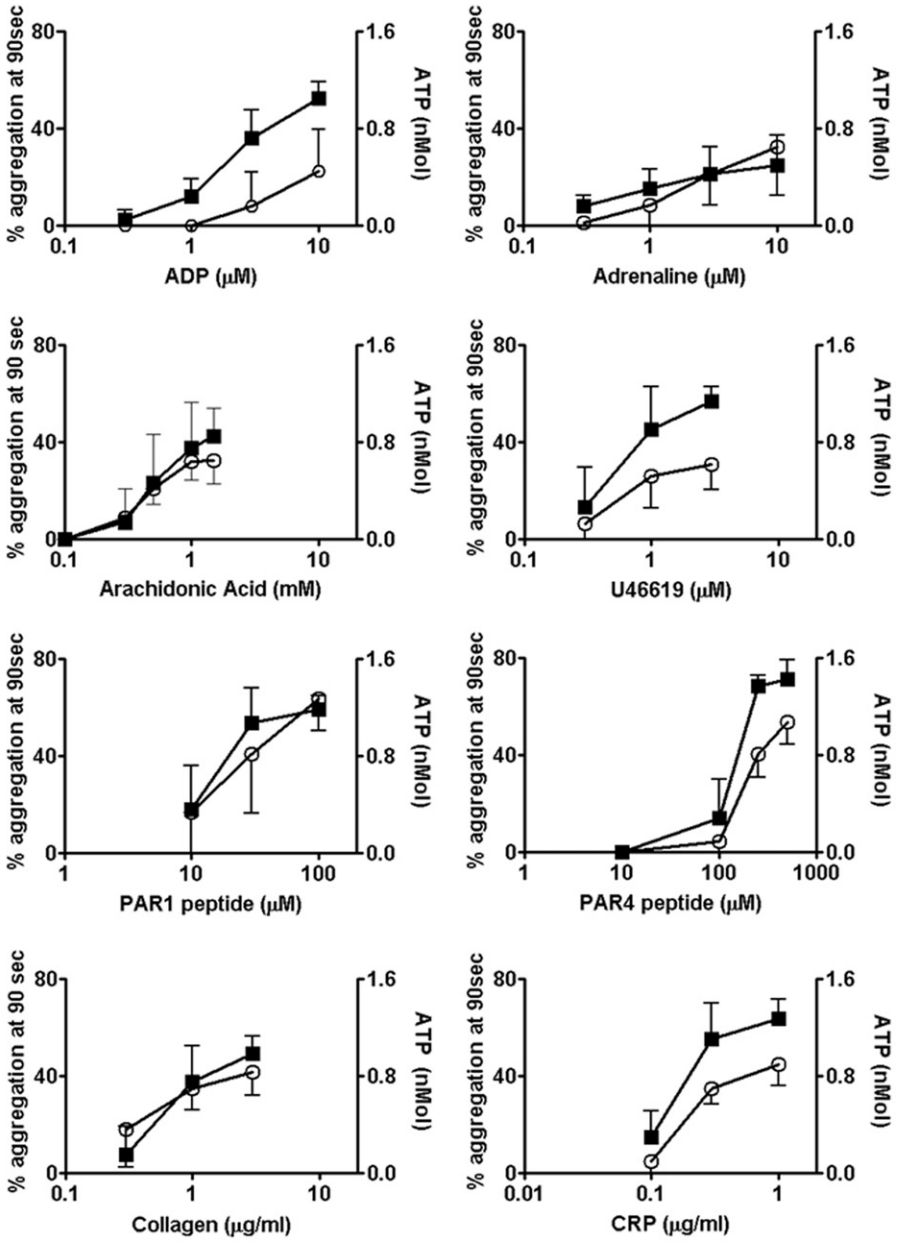

Figure 1. Concentration response curves for aggregation and ATP secretion. Aggregation and ATP secretion in platelets obtained from healthy volunteers were measured in a Born Lumi-Aggregometer in platelet rich plasma as described in the methods. Aggregation (■) results are shown as% increase in light transmission. ATP secretion (◯) was measured using luciferin-luciferase reagents and normalised to 1×10^8 platelets. Aggregation and secretion were measured 90 s and 180 s after agonist addition, respectively. Results are shown as mean ± s.d. from between 8–20 donors.

generate a series of reference curves as shown in Figure 1.

Despite the high level of reproducibility, small differences between donors in the concentration response curves for specific platelet agonists were observed, with the most marked difference being seen for ADP. Further, differences in the position of agonist response curves were maintained between donors over the course of one year as illustrated by the responses to ADP in two example donors in Figure 2. In this Figure, it can be seen that donor 1 has a greater sensitivity to ADP than donor 2. This is particularly apparent at 1 or 3 μM ADP which generate reversible or biphasic irreversible aggregation respectively, in donor 1, compared with shape change or reversible aggregation respectively, in donor 2. Further, at the higher concentration of 10 μM ADP, the response in donor 2 can clearly be seen to be biphasic whereas only a single, rapid aggregation response is observed in donor 1. This variation in response between donors emphasizes the importance of generating reference curves within a population of healthy volunteers, which ideally should be made up of responses from 10 or more individuals.

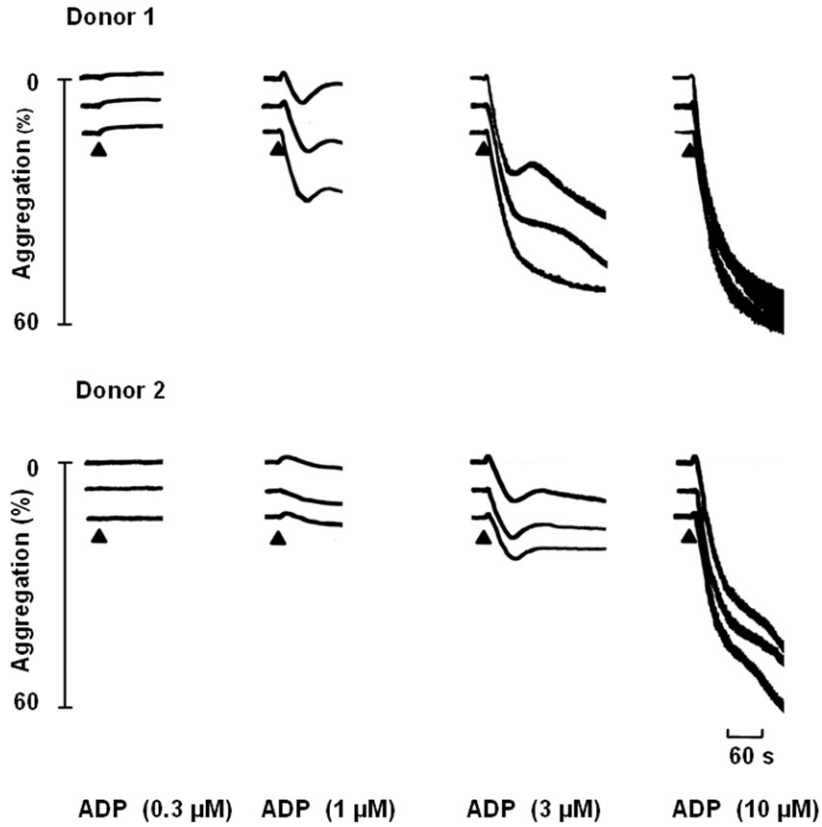

Figure 2. Reproducibility of concentration response curves for platelet aggregation to ADP in two donors. Platelet aggregation to ADP was measured in two individuals on three occasions at intervals of 10–12 weeks. Experimental conditions are described in Figure 1.

Standardization of secretion studies

The measurement of ATP as a monitor of dense granule secretion was not undertaken during the above series of aggregation studies. Nevertheless, bearing in mind that aggregation to low concentrations of agonists is dependent on dense granule secretion, the results indicate that secretion is also likely to be well maintained between donors. To confirm this, secretion of ATP was measured in healthy volunteers using luciferin-luciferase alongside aggregation to the above agonists. ATP is secreted from dense granules in a constant ratio to ADP of 2:3 [3] and so can be used as a measurement of dense granule release. Concentration-response curves for ATP secretion in response to the above agonists are shown for eight donors in Figure 1, with data normalized to the level of secretion per 1×10^8 platelets. Normalization of the data in this way was found to be essential because of the variation in platelet counts between donors. The location of the concentration response curves for ATP secretion corresponded to those for aggregation for all agonists with the exception of ADP, where the concentration response curve was located three-fold to the right of that for aggregation (Figure 1). The standard deviation for ATP secretion was nearly always within 20% of the mean, thereby confirming a good level of reproducibility of response between donors. The PAR_1 peptide stimulated the largest level of secretion of ATP, although maximal responses to all agonists fell within 50% of this response (Figure 1). Thus, these results indicate a high level of reproducibility in ATP secretion, thereby supporting previous studies that have indicated ATP secretion as a valuable marker of platelet activation.

Analysis of aggregation and dense granule secretion to platelet agonists in the presence of inhibitors of ADP and TxA_2

ADP and TxA_2 play a critical feedback role in mediating platelet activation. A defect in their release or in the function of their receptors has a widespread inhibitory effect on platelet activation. However, the phenotype of the defect differs according to whether secretion is disrupted or receptor function is impaired. To characterize this in full, we have investigated aggregation and dense granule secretion induced by low, intermediate and high concentrations of platelet agonists in the absence and presence of antagonists of the two ADP receptors, $P2Y_1$ and $P2Y_{12}$, and the cyclooxygenase inhibitor, indomethacin, which blocks the liberation of TxA_2. All of the inhibitors were used at maximally-effective concentrations, as shown by the observation that a 10-fold higher concentration of each inhibitor did not have

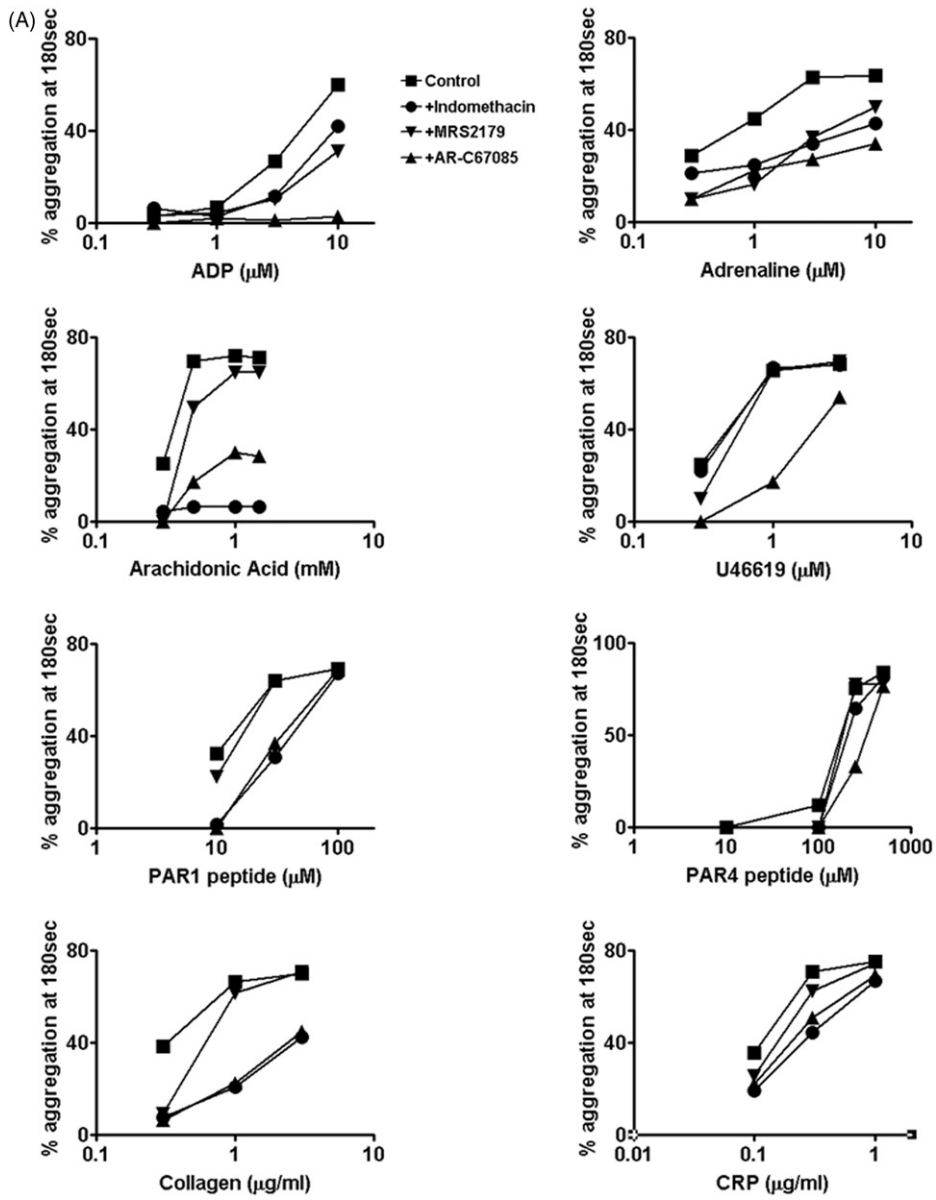

Figure 3. The effect of inhibition of ADP receptors and cyclooxygenase activity on the concentration response curves for aggregation and ATP secretion to platelet agonists. The concentration response relationships for (A) platelet aggregation and (B) ATP secretion were measured in response to eight different platelet agonists in the absence and presence of the cyclooxygenase inhibitor indomethacin (10 μM), the $P2Y_1$ receptor antagonist MRS2179 (10 μM) and the $P2Y_{12}$ antagonist AR-C67085 (1 μM). Inhibitors were given 3 min prior to the agonists. Responses were measured 180 s after agonist addition. Results represent the mean ± s.d. of a minimum of three experiments (n = 3).

an additional effect on platelet activation to any of the agonists that have been tested (data not shown).

The effect of inhibition of the two ADP receptor antagonists or cyclooxygenase on aggregation and dense granule secretion to ADP, adrenaline, PAR_1 and PAR_4 peptides, collagen, CRP, U46619 and arachidonic acid was investigated in a minimum of three donors for each agonist (Figure 3A and B). All responses were monitored at 180 s. Each aggregation response curve was fitted to a three variable logistic equation using Graphpad Prism software to calculate the EC_{50} and 95% confidence levels as shown in Supplementary Table I. A similar analysis was not performed for ATP secretion because of uncertainty in the maximal response. Example traces showing the effect of inhibitors on the response to low, intermediate and high concentrations of platelets agonists are shown in Figures 4–8. The effect of inhibitors on aggregation and dense granule secretion induced by each agonist is discussed below.

ADP

ADP stimulates platelet aggregation through a synergy between $P2Y_1$ and $P2Y_{12}$ receptors which

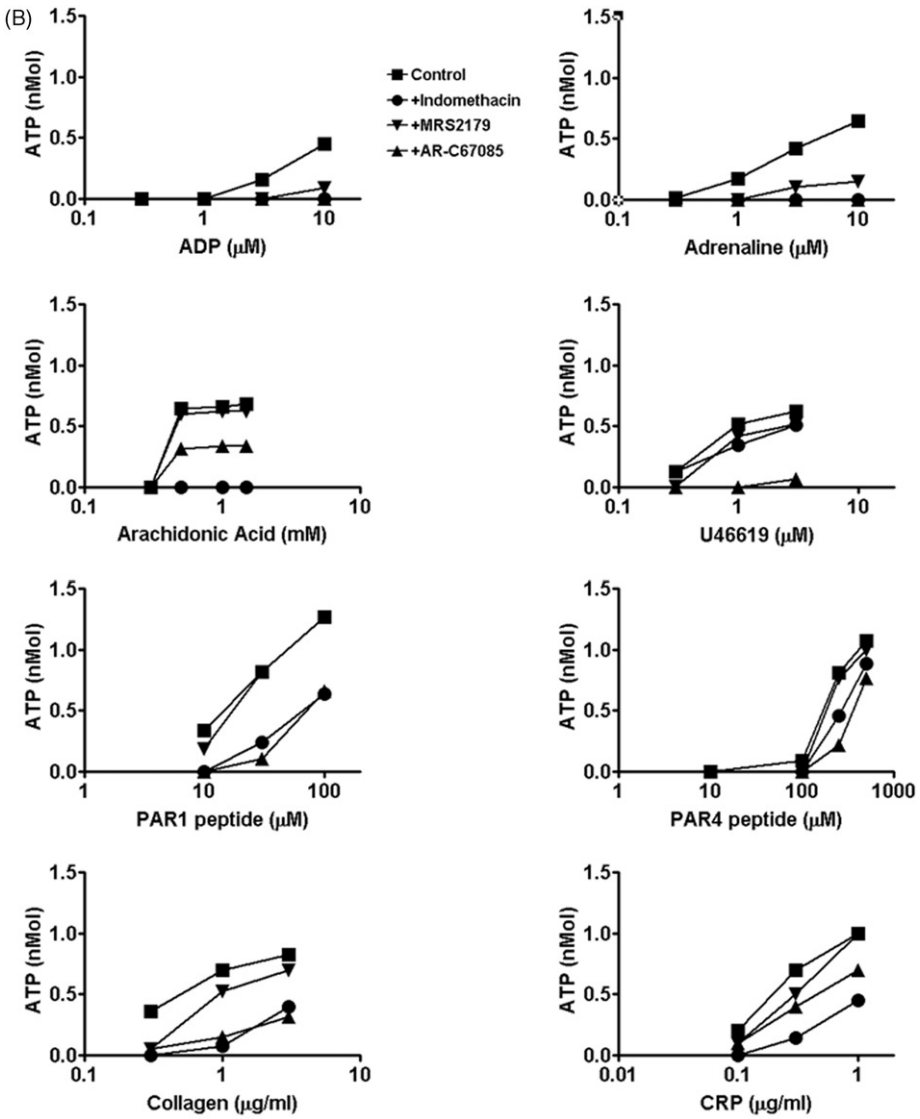

Figure 3. Continued.

are coupled to the G_q and G_{12} families, and the G_i family of heterotrimeric G proteins, respectively. The $P2Y_{12}$ receptor is recognized to undergo a marked synergy with receptors that signal through G_q and G_{12} to mediate powerful aggregation and secretion. An intermediate concentration of ADP (3 μM) induces biphasic aggregation as shown by an initial rapid response followed by a delayed second phase which is seen in most but not all donors (see traces in Figure 4). The second phase is absent at a lower concentration of ADP (1 μM) and the two phases are less distinct at a higher concentration of ADP (10 μM). The second phase of aggregation is dependent on release of thromboxane, as illustrated by conversion to a primary wave response to ADP (3 μM) in the presence of indomethacin (Figure 4). Indomethacin also causes a complete inhibition of dense granule secretion to ADP (Figure 4), although this does not contribute to the loss of the second wave of aggregation, as ADP is able to generate a full aggregation response in platelets from Hermansky-Pudlak patients which lack dense granules [8].

Strikingly, blockade of the two P2Y ADP receptors causes a qualitatively distinct pattern of inhibition to that seen with indomethacin. The $P2Y_1$ receptor antagonist, MRS2179, completely inhibits shape change (i.e. the initial transient increase in optical density) to all concentrations of ADP and also markedly reduces the extent of aggregation and dense granule secretion (Figure 4). Significantly, however, a low level of aggregation to ADP in the presence of MRS2179 is sustained and dense granule secretion is induced by a high concentration of ADP (Figure 4). On the other hand, the $P2Y_{12}$ receptor antagonist, AR-C67085, has no effect on shape change but converts aggregation to a transient response that returns to the base-line within 2 min (Figure 4). In addition, dense granule secretion is abolished to all concentrations of ADP in the presence of AR-C67085 (Figure 4). The results

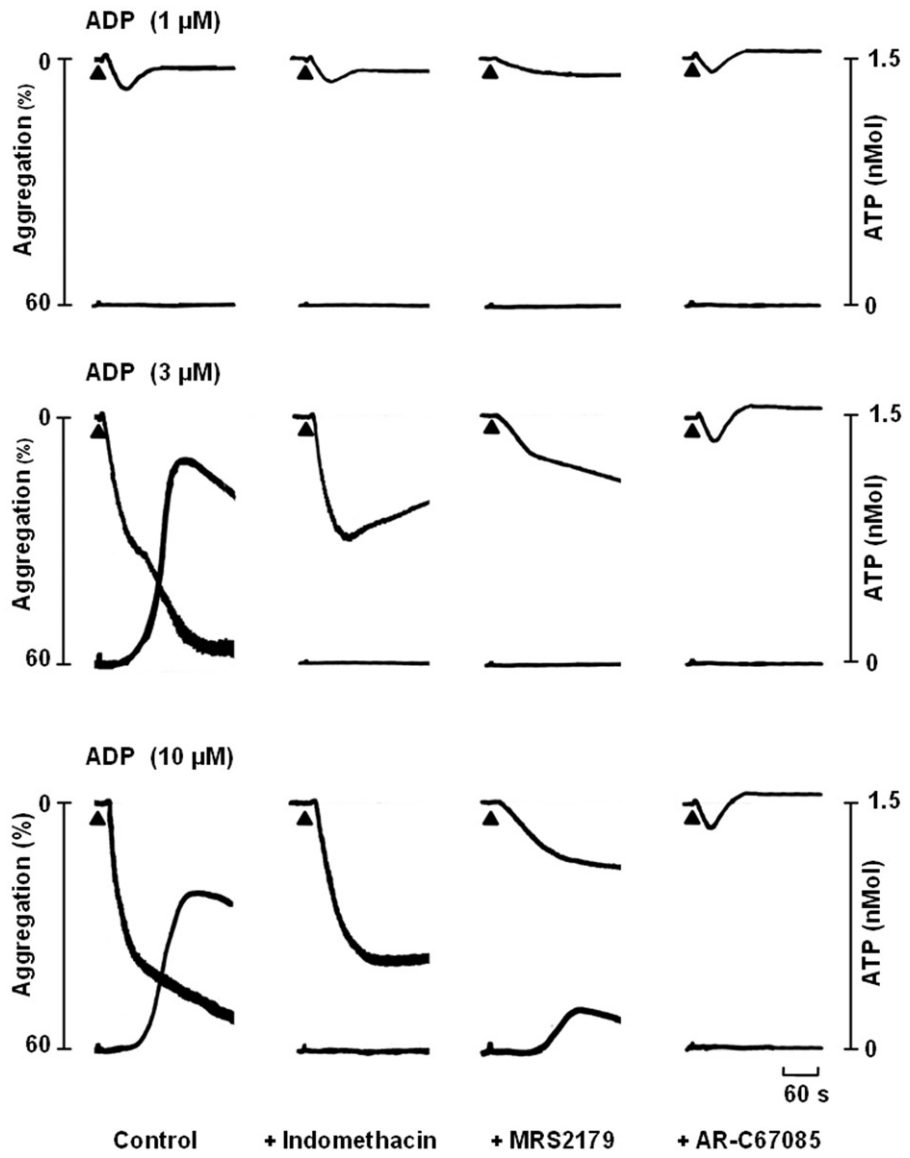

Figure 4. The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to ADP. Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of ADP in the absence or presence of cyclooxygenase inhibitor indomethacin (10 μM), the $P2Y_1$ receptor antagonist MRS2179 (10 μM) and the $P2Y_{12}$ antagonist AR-C67085 (1 μM). Traces are representative of a minimum of three experiments.

demonstrate a critical role for the $P2Y_1$ receptor in mediating shape change and in synergising with the $P2Y_{12}$ ADP receptor to mediate aggregation and secretion. The inhibitory effect of AR-C67085 demonstrates the critical role of the $P2Y_{12}$ ADP receptor in initiating and sustaining aggregation, as well as in mediating secretion.

Thus, these results demonstrate that blockade of the two ADP receptors or cyclooxygenase has distinct effects on aggregation to ADP.

Adrenaline

Adrenaline stimulates aggregation through the α_{2A}-adrenoceptor, which is coupled to the G_i family of heterotrimeric G proteins. Since this is the same family of G proteins that is regulated by the $P2Y_{12}$ ADP receptor, it is therefore important to compare responses to adrenaline and ADP in patients that have a defect in one or both of these agonists. Further, recent evidence from the α_{2A}-adrenoceptor knockout mouse suggests this adrenoceptor contributes to normal haemostasis [9].

The response to adrenaline is characterized by the absence of shape change and a biphasic aggregation response in which the second phase associated with release of dense granules as indicated by release of ATP (Figure 5). Importantly, this phase of aggregation is completely blocked in the presence of indomethacin or AR-C67085, along with the

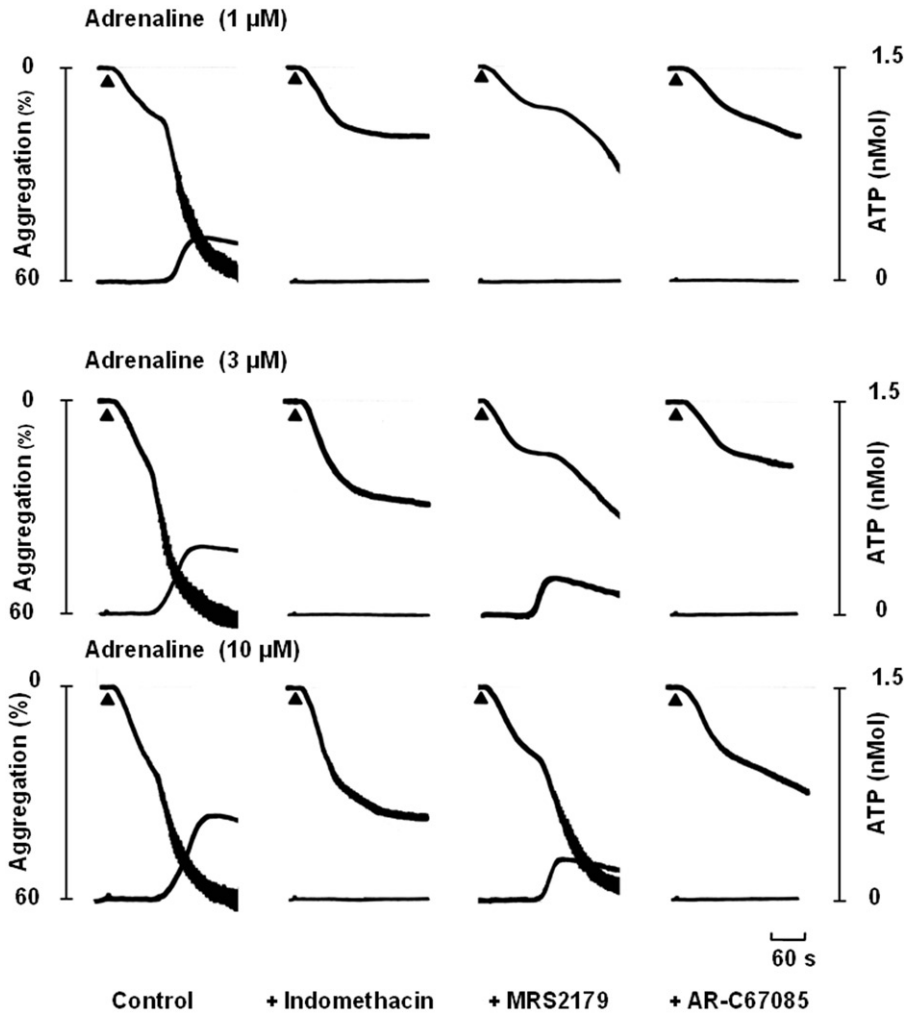

Figure 5. The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to adrenaline. Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of adrenaline in the absence or presence of cyclooxygenase inhibitor indomethacin (1 μM), the $P2Y_1$ receptor antagonist MRS2179 (10 μM) and the $P2Y_{12}$ antagonist AR-C67085 (10 μM). Traces are representative of a minimum of three experiments.

abolition of dense granule secretion (Figure 5). In contrast, blockade of the $P2Y_1$ ADP receptor has a relatively mild effect on aggregation and secretion to adrenaline that is manifest as a small shift to the right in the concentration response curves (Figure 5).

It is important to emphasize that biphasic aggregation to adrenaline was observed in all 20 donors that were investigated in this study in view of reports in the literature that a proportion of the population exhibit only primary wave aggregation. For example, Weiss and Lages observed secondary aggregation to adrenaline in 117 out 130 donors i.e. 90% [10]. The explanation as to why all of the donors monitored in the present study underwent secondary aggregation to adrenaline is unclear, although it should be emphasized that great care was taken to ensure that all volunteer donors had not taken non-steroidal anti-inflammatory drugs prior to testing.

Arachidonic acid and the thromboxane mimetic U46619

Arachidonic acid is converted to TxA_2 by platelet cyclooxygenase, which induces platelet activation through the G_q and G_{12}-coupled thromboxane receptor. TxA_2 has a very short half-life and so the stable analogue, U46619, is used to induce activation platelet activation through the thromboxane receptor under *in vitro* conditions. In addition, it is now realized that arachidonic acid induces weak platelet activation independent of thromboxane formation as demonstrated by a recent study in 700 patients which used maximally-effective concentrations of either aspirin or indomethacin to block cyclooxygenase [11].

In the present study, arachidonic acid was observed to induce biphasic aggregation and robust secretion as shown in Figure 6. In addition, arachidonic acid induces a residual shape change response but not secretion in the presence of 10 μM

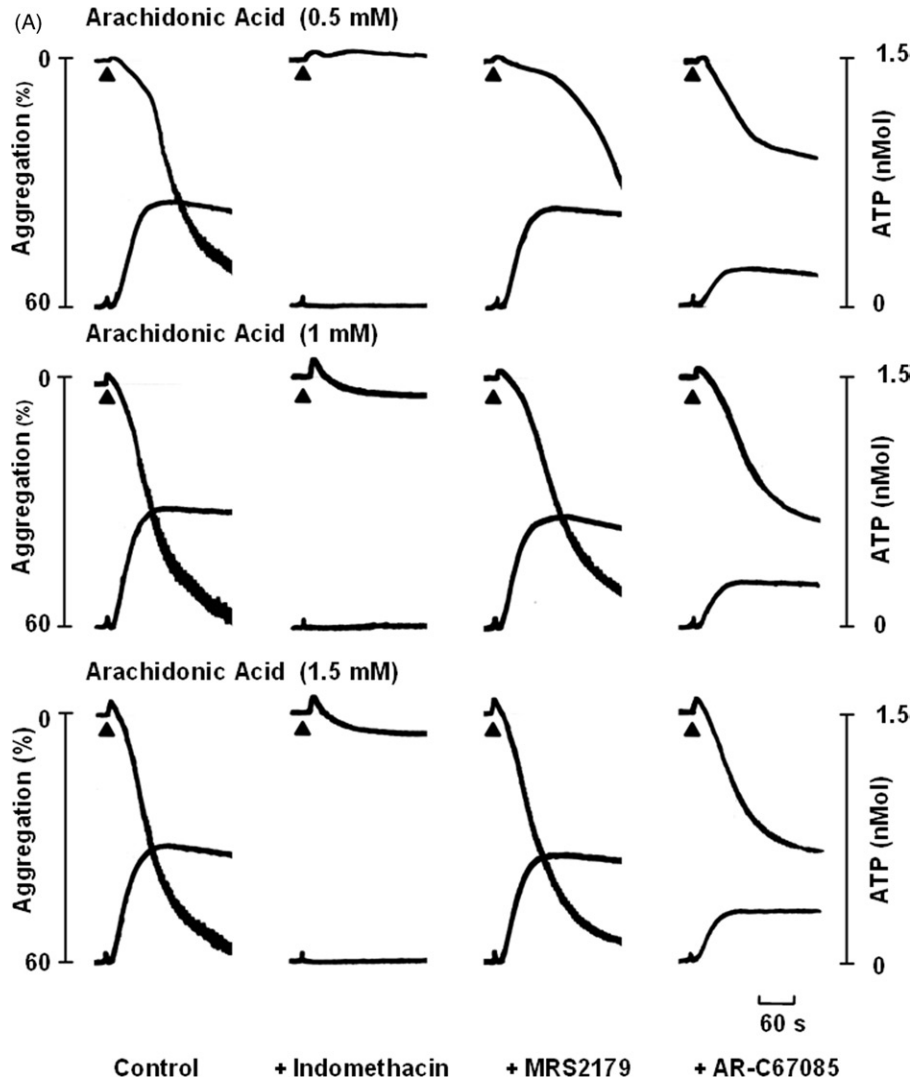

Figure 6. The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to arachidonic acid and U46619. Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of (A) arachidonic acid and (B) U46619 in the absence or presence of cyclooxygenase inhibitor indomethacin (10 μM), the $P2Y_1$ receptor antagonist MRS2179 (100 μM) and the $P2Y_{12}$ antagonist AR-C67085 (1 μM). Traces are representative of a minimum of three experiments.

(Figure 6A) and 100 μM indomethacin (data not shown) demonstrating its ability to induce weak activation independent of thromboxane formation, consistent with the results of [11]. In comparison, the $P2Y_1$ ADP antagonist, MRS2179, has a minor inhibitory effect on aggregation and ATP secretion to low but not high concentrations of arachidonic acid, while the $P2Y_{12}$ ADP antagonist, AR-C67085, inhibits the second component of aggregation and partially inhibits ATP secretion (Figure 6A). Thus, arachidonic acid mediates full aggregation and secretion through a synergy between the thromboxane receptor and the $P2Y_{12}$ ADP receptor. In addition, arachidonic acid stimulates shape change independent of thromboxane formation.

The effect of the above inhibitors on the response to U46619, a stable analogue of TxA_2, is distinct from that observed against arachidonic acid (Figure 6B). In particular, aggregation and secretion induced by U46619 are not altered in the presence of indomethacin, whereas both responses are inhibited in response to arachidonic acid (Figure 6B). In addition, U46619 is unable to induce sustained aggregation in the presence of the $P2Y_{12}$ receptor antagonist, AR-C67085. This is readily seen for an intermediate concentration of U46619 (1 μM) but is also seen with a higher concentration (10 μM) when the trace is allowed to proceed for several minutes (Figure 6B and not shown). AR-C67085 also partially inhibits secretion to U46619. The $P2Y_1$ receptor antagonist, MRS2179, has a very minor effect on the response to low but not higher concentrations of U46619 (Figure 6B), as is the case with arachidonic acid.

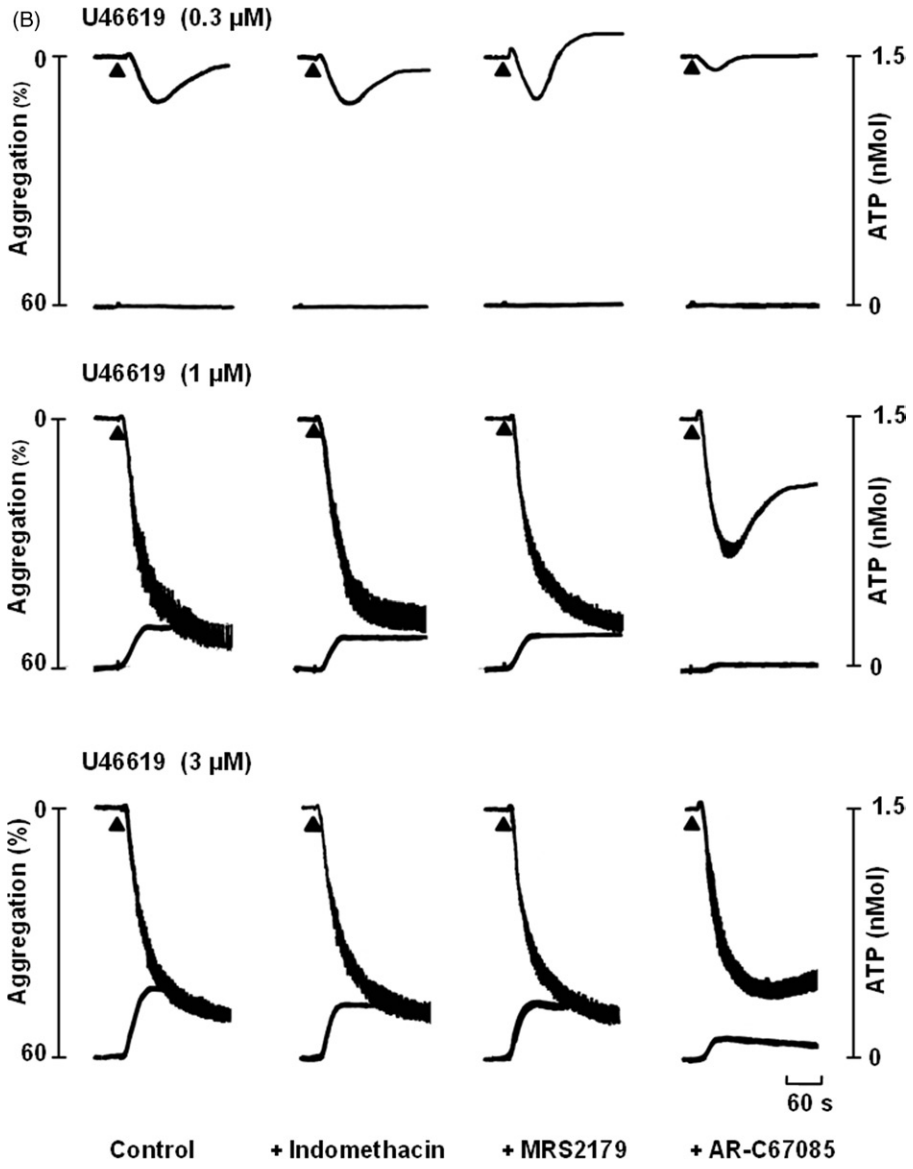

Figure 6. Continued.

Thus, comparison of responses to arachidonic acid and U46619 allows differentiation between a defect at the level of platelet cyclooxygenase or the platelet thromboxane receptor. Specifically, platelet activation by arachidonic acid is inhibited in the presence of the cyclooxygenase inhibitor indomethacin, whereas the response to U46619 is not. On the other hand, the response to both stimuli would be inhibited by a defect in the thromboxane receptor.

PAR_1 and PAR_4 peptides

The ability of the thrombin receptors, PAR_1 and PAR_4, to mediate platelet activation can be monitored using thrombin receptor activating peptides (TRAPs) that are specific for each receptor [12]. In the present study, we have used SFLLRN and AYPGKF to activate PAR_1 and PAR_4, respectively. Both thrombin receptors are coupled to G_q and G_{12} heterotrimeric G proteins.

The two PAR_1 and PAR_4-specific peptides induce similar patterns of aggregation and secretion and their responses are altered in a similar way in the presence of indomethacin and the $P2Y_{12}$ receptor antagonist. For both peptides, the $P2Y_1$ receptor does not appear to contribute to aggregation or secretion (Figure 7A and B). On the other hand, the sustained aggregation induced by intermediate concentrations of the PAR_1 and PAR_4-specific peptides is converted to a transient response in the presence of indomethacin and the $P2Y_{12}$ receptor antagonist, AR-C67085, with a corresponding decrease in dense granule secretion (Figure 7A and B). The magnitude of the effect of the two inhibitors is similar for the two thrombin receptor peptides (Figures 3 and 7). Higher concentrations of the two peptides induce sustained, maximal aggregation in the presence of

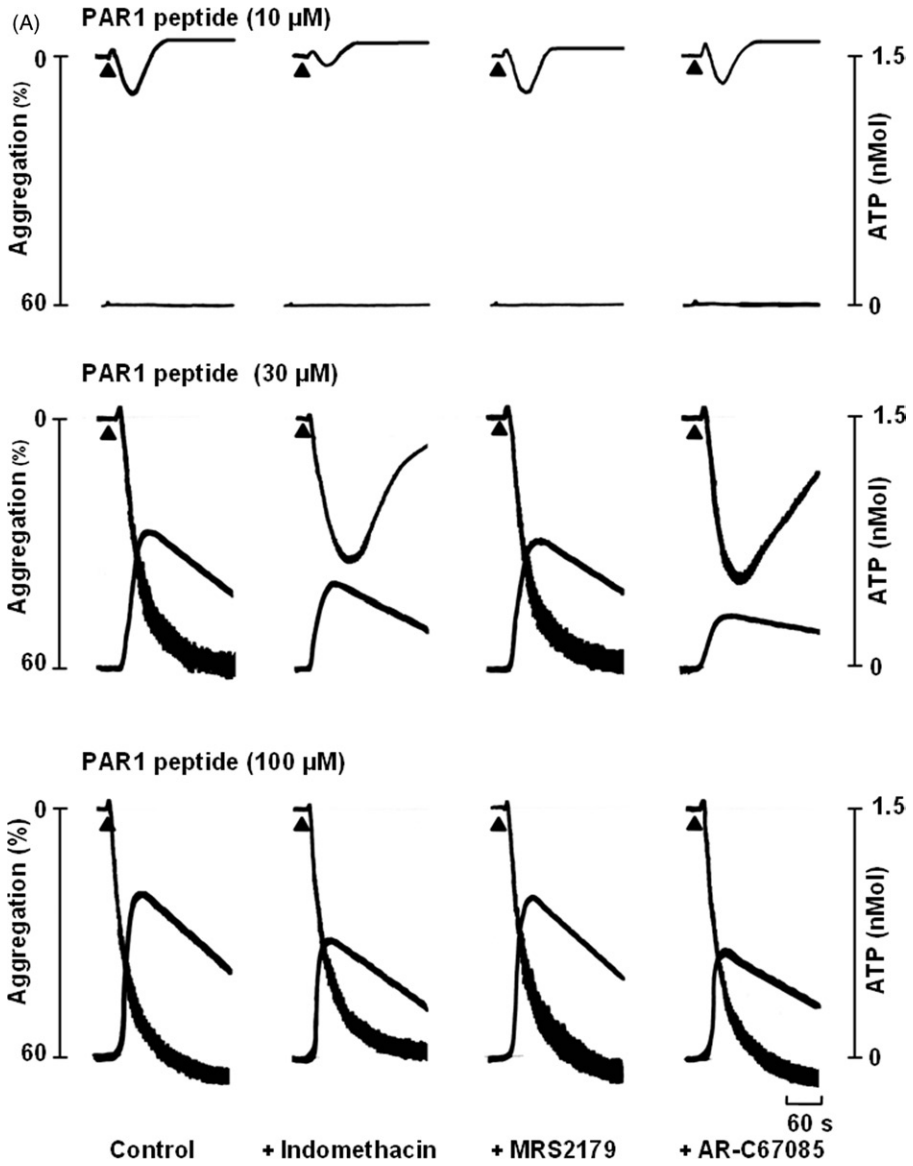

Figure 7. The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to PAR_1- and PAR_4-specific peptides. Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of (A) PAR_1-peptide and (B) PAR_4-peptide in the absence or presence of cyclooxygenase inhibitor indomethacin (10 µM), the $P2Y_1$ receptor antagonist MRS2179 (100 µM) and the $P2Y_{12}$ antagonist AR-C67085 (1 µM). Traces are representative of a minimum of three experiments.

indomethacin and AR-C67085, although dense granule secretion is reduced. Thus, these results demonstrate that platelet activation by the PAR_1 and PAR_4-specific peptides is reinforced by release of TxA_2 and secretion of ADP, and that this is most readily seen at intermediate concentration of the two peptides.

Collagen and CRP

Collagen initiates platelet activation through the two glycoprotein receptors, GPVI and integrin $\alpha2\beta1$ [13]. Crosslinking of GPVI generates powerful tyrosine kinase-dependent signals that mediate activation. In addition, the interaction of collagen with GPVI is reinforced by binding to integrin $\alpha2\beta1$. Integrin $\alpha2\beta1$ also generates weak intracellular signals, although the significance of these is not known. The synthetic collagen, CRP, induces platelet activation independent of integrin $\alpha2\beta1$ and can therefore be used to distinguish between defects in GPVI and $\alpha2\beta1$.

The ability of a low concentration of collagen to induce full aggregation and dense granule secretion is markedly inhibited in the presence of indomethacin and by the two ADP receptor antagonists, MRS2179 and AR-C67085 (Figure 8A). This demonstrates the critical role of the two ADP

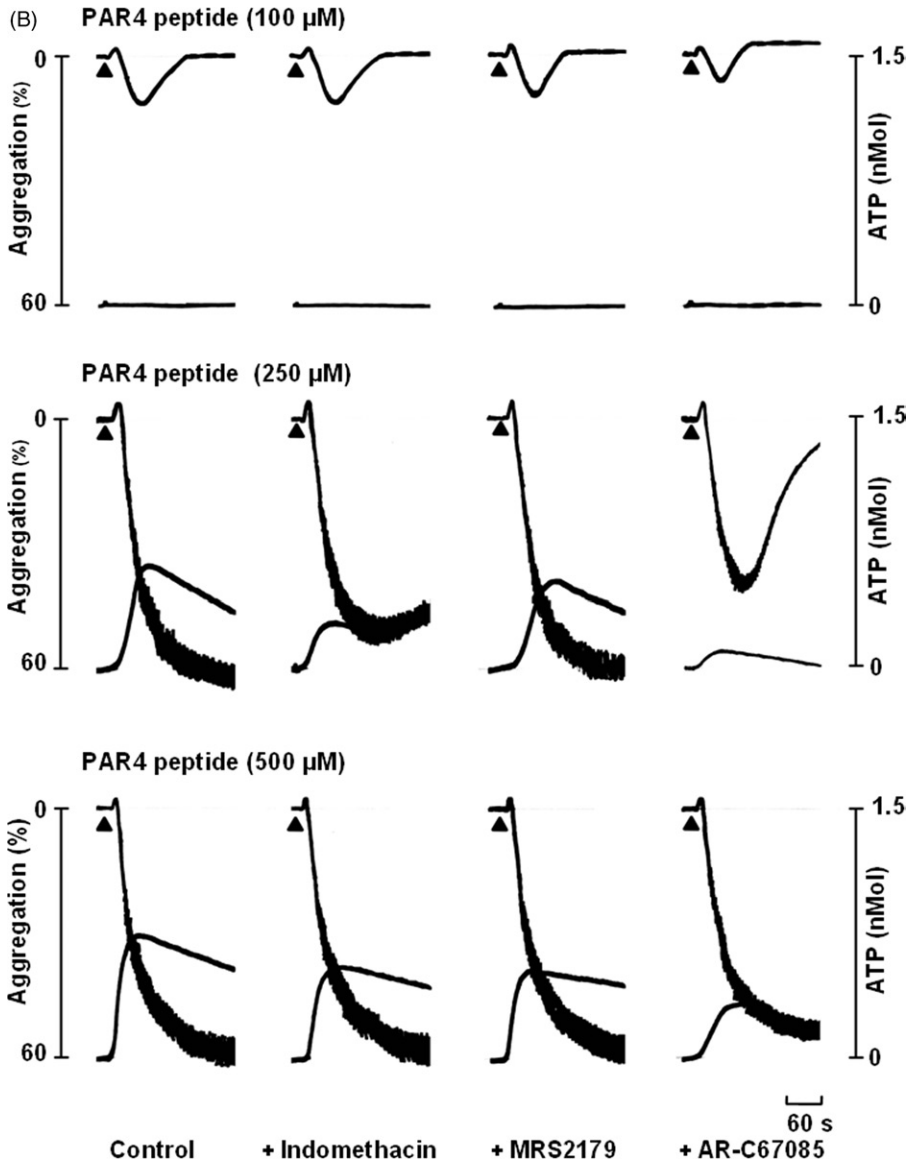

Figure 7. Continued.

receptors and thromboxane formation in mediating platelet activation to low concentrations of collagen. In comparison, the $P2Y_1$ receptor does not contribute to activation by higher concentrations of collagen (Figure 8A). On the other hand, aggregation and ATP secretion to higher concentrations of collagen is powerfully inhibited in the presence of indomethacin, whereas blockade of the $P2Y_{12}$ receptor has a less marked inhibitory effect (Figure 8A). Thus, these results demonstrate that platelet activation to collagen is critically dependent on generation of thromboxanes and, to a lesser extent, the $P2Y_{12}$ receptor.

In comparison, responses to all concentrations of CRP are independent of the $P2Y_1$ receptor and have only a partial dependency on liberation of thromboxanes and the $P2Y_{12}$ receptor (Figure 8B). Thus, a high concentration of CRP is able to induce full aggregation in the presence of indomethacin and AR-C67085, although dense granule secretion is partially reduced. Indomethacin and AR-C67085 causes a greater level of inhibition of aggregation and ATP secretion to an intermediate concentration of CRP (Figure 8B).

Thus, responses to CRP and collagen can be distinguished in terms of their dependency on thromboxanes and the $P2Y_{12}$ ADP receptor. The ability of CRP to induce powerful activation that is largely independent of secondary mediators is important in the context of testing whether there is a generalized defect in platelet responses or a defect that is restricted to activation mediated through G protein-coupled receptors or dense granule secretion. Further, the use of CRP and collagen is important in distinguishing between patients with defects in the two collagen receptors, GPVI and integrin $\alpha 2\beta 1$.

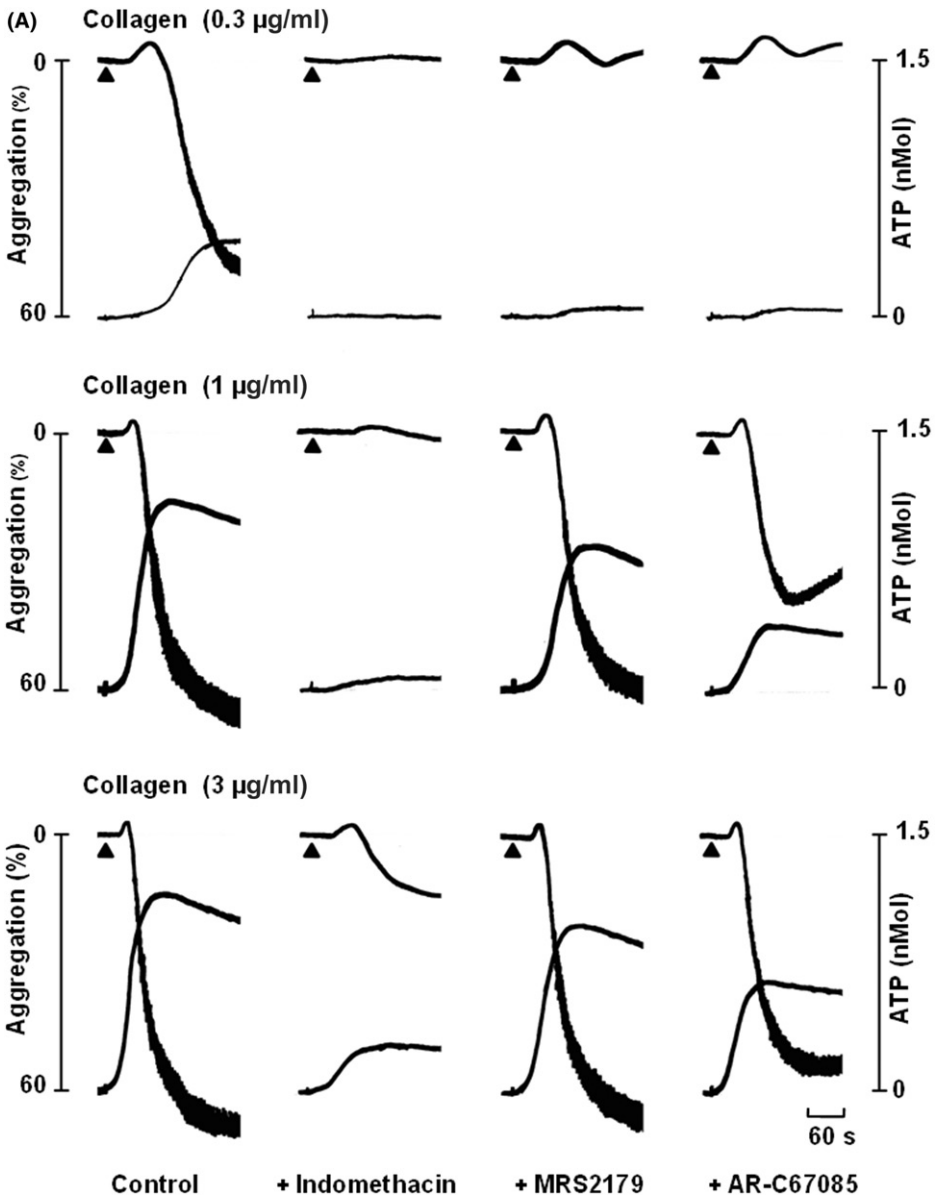

Figure 8. The effect of inhibition of ADP receptors and cyclooxygenase inhibition on platelet aggregation and ATP secretion to collagen and CRP. Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of (A) collagen and (B) CRP in the absence or presence of cyclooxygenase inhibitor indomethacin (10 µM), the $P2Y_1$ receptor antagonist MRS2179 (100 µM) and the $P2Y_{12}$ antagonist AR-C67085 (1 µM). Traces are representative of a minimum of three experiments.

Discussion

Born-aggregometry is the most widely used test in clinical research for investigating patients with suspected platelet-based bleeding disorders. Despite this, there are no established guidelines on how to perform these studies, with practices varying widely both within and between countries [1, 2]. Further, the interpretation of aggregation traces is complex and hampered by the relatively few patients that are sent for testing. There have also been surprisingly few changes in recent years in the way that aggregation testing is performed in the clinic, especially in light of our increased understanding of the mechanisms that underlie platelet activation. As a consequence, the majority of clinical laboratories are unclear on many aspects of Born-aggregometry, such as agonist selection, agonist concentration and whether to correct for platelet number etc. This has naturally led many clinical laboratories to consider whether other tests are more appropriate in analysing platelet function and to question the usefulness of aggregation in identifying conditions other than severe defects in platelet function such as those seen with Glanzmann's thrombasthenia or Bernard Soulier Syndrome.

The goal of the present study was to investigate the reproducibility of aggregation responses to platelet

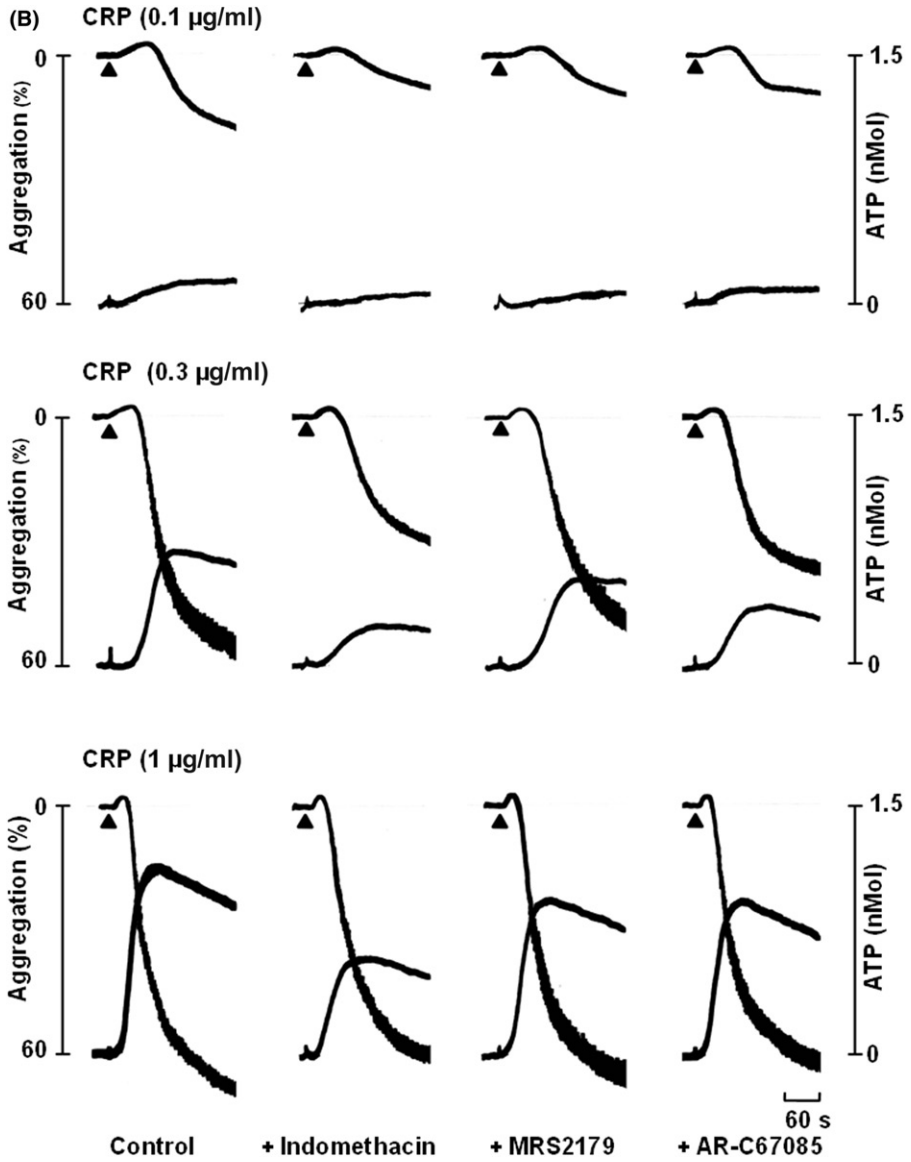

Figure 8. Continued.

agonists within a population of healthy volunteers and to establish the dependency of response on factors such as platelet number, time after donation and individual donor variation. In parallel, the present study has carried out simultaneous measurement of ATP secretion from dense granules using a lumi-aggregometer and demonstrated that measurement of both parameters provides valuable information on the effect of inhibition of the two major feedback mediators, ADP and TxA_2, on platelet activation. The data generated during this study are an important resource of information to aid clinical laboratories in testing of platelet samples.

The present study demonstrates that aggregation and secretion responses are relatively robust within a population of healthy volunteers over the range of platelet concentrations that are found within healthy volunteers ($150–400 \times 10^9$ platelets/litre) and that there is no major variation in response curves with sex, age or platelet number. The study also demonstrates that platelet activation is maintained over several hours when PRP is stored at room temperature. In comparison, many clinical testing laboratories in the UK dilute the PRP to a constant platelet concentration, usually 200×10^9/litre, and attempt to complete the study within 2 hours in line with guidelines of the British Society of Haematology Task Force [3]. Not only does this provide important constraints on the number of agonists that can be tested, there is now evidence that dilution of samples with PPP can modify aggregation [14]. However, in the case of thrombocytopenic donors, where the platelet count is 150×10^9 platelets/litre or less, aggregation is impaired and so there is a need to either dilute the PRP from the control donor to the

same count or to use washed platelets in order to increase the platelet concentration.

Although the present standard curves are a powerful resource of information, it should be stressed that each clinical laboratory should generate their own standard concentration response curves from within a population of healthy individuals because of the potential impact of subtle differences in methodology. It is also recommended that studies are performed on a healthy volunteer alongside those on a patient to control for any untoward experimental difficulties. Inherent in the use of a control volunteer in this way, however, is the possibility that the volunteer's platelets may not appear normal, possibly because of unknown ingestion of a platelet-modifying agent or because of donor-specific differences in the concentration-response relationships. For example, the present study has described donor-specific concentration response differences for ADP which are maintained over time. Nevertheless, the availability of reference curves such as those generated in this study provides confidence on the response within healthy donors for a particular agonist.

The experiments with the $P2Y_1$ and $P2Y_{12}$ ADP receptor antagonists and the cyclooxygenase inhibitor indomethacin provide important information on the distinct roles of these pathways in mediating platelet aggregation and secretion to different platelet agonists. This is important in helping to diagnose a patient with platelets which exhibit a defect in response to more than one of the conventional platelet agonists. Significantly, in many cases, the effect of inhibition of $P2Y_1$ and $P2Y_{12}$ ADP receptors or cyclooxygenase is only revealed by analysis of responses to low/intermediate concentrations of agonists, thereby emphasizing the importance of studying multiple agonist concentrations. This is pertinent in the context that testing in some but not all clinical laboratories is restricted to a single concentration of an agonist. Further, it is anticipated that these reference profiles will facilitate identification of patients with defects in the two $P2Y_1$ and $P2Y_{12}$ ADP receptors from those with a 'secretion disorder', as only the former are characterized by a loss of response to ADP [8].

The present study has investigated the concentration response relationships for a number of platelet agonists, several of which are not routinely used in clinical laboratories, namely the stable thromboxane mimetic, U46619, the synthetic collagen, CRP, and PAR_1 and PAR_4 specific peptides. Investigation of the effect of CRP provides important information on whether a loss of response to collagen is mediated at the level of GPVI or integrin $\alpha2\beta1$. A partial defect in GPVI would cause a small reduction in response to collagen because of the presence of integrin $\alpha2\beta1$, whereas it would have a much more dramatic effect on the response to CRP as shown by studies on mice platelets that express a reduced level of GPVI [15]. The use of U46619 provides important information on whether a diminished response to arachidonic acid is mediated at the level of cyclooxygenase or through the thromboxane receptor. The PAR-specific peptides would identify defects in the two thrombin receptors, PAR_1 and PAR_4. Indeed, the fact that these two PAR-specific peptides are not routinely tested in most clinical laboratories may explain why, as yet, no patients with defects in PAR_1 and PAR_4 have been described.

In conclusion, the present observations serve to demonstrate the robustness of platelet aggregation and secretion over a range of parameters and also demonstrate the known benefit of simultaneous monitoring of ATP secretion as a marker of dense granule release. The generated response relationships and patterns of aggregation are an important resource for the clinical laboratory in the testing of patients. The results also demonstrate the importance of using additional agonists, such as the two PAR-specific peptides, in the routine testing of patients, especially those with a clinical history suggestive of a platelet disorder but where a defect has not been found. The further development and refinement in the use of aggregation testing and co-analysis of ATP secretion is likely to increase the diagnosis of patients with mild, platelet-based bleeding disorders. This increase in testing will require extra resources, but in the long term will benefit both the patient and the clinic.

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Supplementary Table I. The effect of inhibition of cyclooxygenase activity and ADP receptors on the EC50 and 95% confidence limits for platelet aggregation to various agonists. The effects of indomethacin, MRS2179 and AR-C67085 on platelet aggregation were calculated from the aggregation traces generated in the experiments as described for Figure 3A. Each aggregation response curve was fitted to a three variable logistic equation using Graphpad Prism software to calculate the EC50 and 95% confidence levels. Results are representative of three experiments. N/A refers to cases where de-aggregation prohibited calculation of maximal aggregation and therefore EC50.

Agonist	**Control**	**+Indomethacin**	**+MRS2179**	**+AR-C67085**	
ADP (μM)	2.0	3.3	5.9		Lower
	3.2	**6.3**	**9.6**	**N/A**	**EC50**
	5.0	12	15		Upper
Adrenaline (μM)	0.17	1.0	1.4		Lower
	0.38	**2.1**	**2.5**	**N/A**	**EC50**
	0.85	4.1	4.2		Upper
Arachidonic Acid (mM)	0.32		0.12		Lower
	0.32	**N/A**	**0.47**	**N/A**	**EC50**
	0.33		1.9		Upper
U46619 (μM)	0.35	0.30	0.27	1.0	Lower
	0.36	**0.37**	**0.46**	**1.7**	**EC50**
	0.38	0.46	0.79	2.7	Upper
PAR1 peptide (μM)	9.6	31	12	26	Lower
	10	**32**	**13**	**29**	**EC50**
	11	33	13	32	Upper
PAR4 peptide (μM)	147	151	18	267	Lower
	151	**208**	**190**	**279**	**EC50**
	155	287	2070	293	Upper
Collagen (μg/ml)	0.24	1.5	0.38	1.5	Lower
	0.27	**2.1**	**0.54**	**1.9**	**EC50**
	0.31	2.9	0.77	2.3	Upper
CRP (μg/ml)	0.10	0.16	0.12	0.18	Lower
	0.10	**0.22**	**0.14**	**0.18**	**EC50**
	0.11	0.31	0.16	0.18	Upper

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