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Monitoring modulators of platelet aggregation in a microtiter plate assay

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Abstract

Platelets play a central role in maintaining biological hemostasis. Inappropriate platelet activation is responsible for thrombotic diseases such as myocardial infarction and stroke. Therefore, novel agents that can inhibit platelet activation are necessary. However, assays that monitor platelet aggregation are generally time-consuming and require high volumes of blood and specialized equipment. Therefore, a medium- to high-throughput assay that can monitor platelet aggregation would be considered useful. Such an assay should be sensitive, comparable to the "gold standard" assay of platelet aggregometry, and able to monitor multiple samples simultaneously but with low assay volumes. We have developed such a microtiter assay. It can assay an average of 60 independent treatments per 60 ml blood donation and demonstrates greater sensitivity than the current gold standard assay, namely platelet aggregation in stirring conditions in a platelet aggregometer. The microtiter plate (MTP) assay can detect known inhibitors of platelet function such as indomethacin, aspirin, and ReoPro. It is highly reproducible when using standard doses of agonists such as thrombin receptor-activating peptide (20 μM) and collagen (0.19 mg/ml). Finally, the MTP assay is rapid and sensitive and can detect unknown platelet-modulating agents from a library of compounds.

Keywords: Platelet; Aggregation; Peptide; Inhibitors; Microtiter assay

Platelets have a fundamental role in vivo in mediating hemostasis and thrombosis [1]. They play a vital role in the pathogenesis of atherosclerosis, angina, myocardial infarction, stroke, and ischemic heart disease. It is well documented that antiplatelet agents are effective in preventing the recurrence of infarction/stroke and restenosis [2,3]. However, the recent failure of oral antiplatelet glycoprotein IIb/IIIa (GPIIb/IIIa)[1] antagonists in clinical trials has highlighted the need for newer and better antiplatelet agents [4–7]. Many new targets for developing such anti-thrombotic drugs are emerging [8–12], and the progress in developing newer antiplatelet drugs with better efficacy will be facilitated by improved large-scale functional screening methods.

A myriad of techniques to study platelet function ex vivo have been developed [13–17]. Currently, the most commonly used method to detect platelet activation by a given agonist is aggregometry despite the fact that the technique required for this is time-consuming and requires relatively large volumes of blood, skilled staff, and special equipment. However, standard aggregometry presents a difficulty in comparing samples tested at early and later times following blood preparation due to the lessening of platelet responses with storage even for times as short as 3 h. Two to eight channel aggregometers have been developed for the detection of platelet activation in an attempt to overcome these limitations of time, but even this allows assay of only relatively small numbers of samples simultaneously [18–20].

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[1] *Abbreviations used:* GPIIb/IIIa, glycoprotein IIb/IIIa; TRAP, thrombin receptor-activating peptide (SFLLRN in single-letter amino acid code); MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; ACD, acid–citrate–dextrose; Tris, tris(hydroxymethyl)aminomethane; MTP assay, microtiter plate assay; DSP, disappearance of single platelet; PAP4, platelet aggregation profiler; COX, cyclooxygenase.

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Previously, we reported the dissection of the molecular mechanisms of integrin activation using novel cell-permeable peptides corresponding to a highly conserved, membrane-adjacent sequence known to regulate integrin activation [21]. Similarly, we reported novel cell-permeable peptide-based platelet antagonists that targeted membrane-adjacent conserved sequences in GpIbβ [22] and a channel regulatory protein [23]. To further explore novel targets for antiplatelet agents, we required the availability of a high-throughput, reproducible platelet assay. Therefore, we sought to establish a rapid, efficient, and reproducible method for testing platelet function in a large number of samples simultaneously to eliminate the effect of time on platelet function assays. Moreover, we were equally interested in bioactive agents that promote platelet activation, either alone or in combination with a platelet agonist, because these will provide insights into the molecular mechanisms of cellular signaling induced by specific proteins. Therefore, the aim of the current study was to develop and validate a simple and rapid method to characterize the response of platelets to standard agonists and investigational peptides in a microtiter plate reader. Our new assay was compared with the "gold standard" platelet aggregation assay and a second sensitive assay of platelet microaggregates. The utility of this approach was then confirmed by screening a number of known platelet agonists and antagonists.

Materials and methods

Materials

Collagen type 1 was purchased from BioData (Horsham, PA, USA). Thrombin was purchased from Sigma–Aldrich (Dublin, Ireland). Peptides and the platelet agonist thrombin receptor-activating peptide (TRAP, SFLLRN) were synthesized by the the Center for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland. Peptides with the sequence palmityl-KVGFFKR, identified as having platelet agonist activity [21], and palmityl-RRERRDLFTE, identified as being an inhibitor of thrombin-induced aggregation, were synthesized on an Applied Biosystems automated peptide synthesizer (model 433A, Foster City, CA, USA) by standard solid-phase peptide synthesis [24,25] according to the Fmoc/*t*-Bu procedure [26] with a C-terminal amidation and an N-terminal palmitylation as described previously [21] because we and others [27,28] have shown previously that this method delivers peptides into platelets. Using reverse-phase HPLC, purity of all synthesized peptides was determined to be approximately 95%. Peptide masses were confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry.

Platelet preparation

Platelets were drawn from healthy volunteers, free from any medications for 2 weeks, into 15% (v/v) acid–citrate–dextrose (ACD: 38 mM citric acid, 75 mM sodium citrate, 124 mM dextrose). Washed platelets were prepared as described previously [21,22]. Platelets were resuspended in buffer A (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 3 mM KCl, 10 mM Tris [tris(hydroxymethyl)aminomethane], pH 7.4), adjusted to 3×10^5/μl, and supplemented with 1.8 mM $CaCl_2$.

Platelet aggregation in microtiter plates

To optimize the microtiter plate (MTP) assay, variable concentrations of platelets and their response to thrombin were evaluated. Washed platelets were prepared and resuspended at concentrations of 1×10^5/μl, 3×10^5/μl (as used in standard aggregation), and 6×10^5/μl in buffer A and 1.8 mM $CaCl_2$ added just prior to assay. Aliquots (90 μl) of platelets were dispensed into 96-well, flat-bottomed, microtiter black isoplates with buffer, peptides, or inhibitors, and buffer A was added to bring the final volume to 98 μl. During the run time, the plate was incubated at 37 °C and was shaken vigorously in an orbital shaking mode at the maximal speed available. This provided rotational shaking equivalent to 1000 rpm. Measurements of the optical density were performed using a Victor^2 V Multilabel Counter (PerkinElmer, Wellesley, MA, USA). Readings were taken at the indicated times up to 12 min. The agonists, collagen or TRAP (2 μl, 1–50 μM) and collagen (type 1, 2 μl, 38–285 ng/μl), were added after the 6-min reading was taken. Platelet modulators included the known inhibitory pharmacological agents indomethacin (10 μM), aspirin (20 μM), apyrase (1 U/ml), ReoPro (20 μg/ml), and peptides (Pal-KVGFFKR and Pal-RRERRDLFTE). The technique was compared with standard dose–response curves of the same platelet agonists and a sensitive assay to measure microaggregates by quantitating the disappearance of single platelets (DSPs). Platelet aggregation was calculated by subtracting the final reading from the initial reading of the same well. This was then normalized by comparing it with a maximal response, defined as that induced by 20 μM TRAP.

Standard platelet aggregation

Platelets were resuspended in buffer A to a concentration of 3×10^5/μl and were allowed to rest for 15 min prior to use. Calcium (1.8 mM) was added just prior to assay. Platelet aggregations were performed at 37 °C using a BioData PAP4 aggregometer (platelet aggregation profiler, Horsham, PA, USA) as described previously using 250 μl platelet suspension per assay. Samples were preincubated at 37 °C for 3 min in the presence of buffer, peptides, or antagonists. Aggregation was monitored for 3 min after the addition of agonist. Data were expressed as percentages maximal response normalized to 100%.

DSP assay

Washed platelets were prepared as described above and resuspended at 3×10^5/μl. Single platelets were measured by analyzing the samples on a Sysmex KX-21N Cell Counter (Kobe, Japan). Platelet aggregation studies were performed as above, but samples were fixed at the end of the assay by the addition of 25 μl of 1% formaldehyde. An aliquot of platelets was then analyzed for the presence of single platelets and was compared with the initial sample volume. The agonist-induced DSPs value was calculated by expressing the final count as a percentage of the initial count.

Results

To develop a novel assay for the assessment of platelet function, we explored the use of a colorimetric measure of platelet behavior following activation in a 96-well plate. This assay format has the advantage of allowing testing of multiple samples over an identical short time period and thus eliminates the factor of platelet aging over the course of an experiment. Following treatment with a strong platelet agonist such as thrombin (0.2–1 U/ml), a significant decrease in absorbance was observed at either 405 or 490 nm relative to the absorbance at time 0 (Fig. 1). However, the magnitude of the decrease observed (0.16 ± 0.03 AU) when using washed platelets at a concentration of 3×10^5/μl or less was not consistent with an assay that could have a large dynamic range. To address this, the platelet concentration was doubled to 6×10^5/μl. The resultant change in absorbance observed at 405 nm yielded a difference of 0.31 ± 0.12 AU, suggestive of an assay that could be used to measure small or subtle changes in platelet responsiveness. To obtain this platelet concentration, we routinely collected 60 ml of blood from volunteer donors and obtained sufficient washed platelets for 55 to 70 assay wells. As a simple rule of thumb, when planning assays, we assumed one assay well per milliliter of whole blood donated. In addition, for all assays, we included sample wells that had no agonist added and sample wells that contained vehicles corresponding to the diluent for any pharmacological agent.

Other groups have reported the use of different wavelengths to observe agonist-induced changes in light transmission through platelet suspensions [29,30]. We compared data obtained using a 405-nm wavelength to detect platelet activation with the results obtained using a 490-nm wavelength and found them to be equivalent (Fig. 1B). Maximal response to thrombin was observed at both wavelengths at 4 min. Because no substantial difference was observed

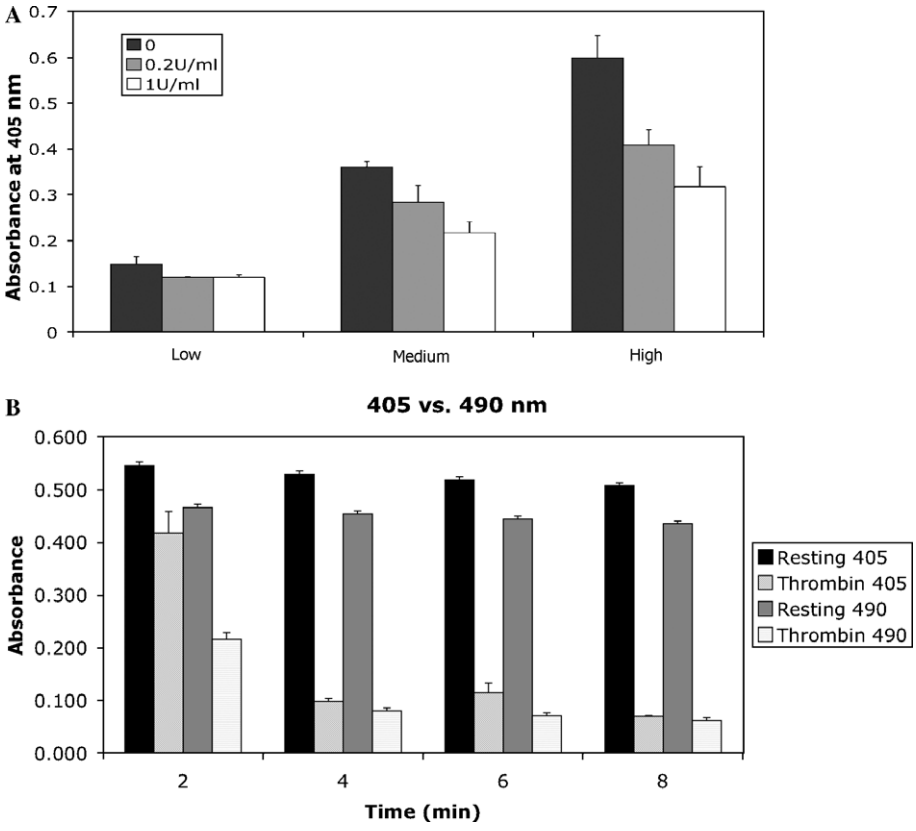


Fig. 1. Effect of platelet concentration on dynamic range of assay. (A) Platelets were resuspended to 1.5×10^5/μl (low), 3.0×10^5/μl (medium), or 6.0×10^5/μl (high) concentrations and incubated with buffer alone (black bars), low-dose thrombin (0.2 U/ml, gray bars), or high-dose thrombin (1 U/ml, white bars) for 6 min at 37 °C with shaking. Changes in absorbance were monitored at 405 nm in a Victor^2 V Multilabel Counter. Data represent means ± SEM ($n = 4$). (B) Platelets (6.0×10^5/μl) were incubated with buffer alone (resting, dark bars) or thrombin (0.2 U/ml, light bars) for the indicated times. Changes in absorbance were monitored at both 405 and 490 nm. Data represent means ± SEM ($n = 4$).

between the assays measured at 405 and 490 nm except at the 2-min time point, we opted to use the 405-nm wavelength throughout our subsequent studies. All further assays were performed at 405 nm, and readings were taken at 3, 6, 9, and 12 min.

To test the potential power of this assay to register changes in platelet responsiveness in comparison with established assays of platelet function, we performed parallel assays of platelet function using this high-throughput (MTP) 96-well assay and two other platelet function assays. Standard platelet aggregation with a BioData (PAP4) four-channel platelet aggregometer was used as the gold standard assay for comparison purposes. Furthermore, an additional assay that measures the DSPs from a stirred suspension was also used.

All three assays were performed in parallel to examine the effects of increasing doses of two different potent platelet agonists, TRAP (1–50 μM) and collagen (0.01–2.8 mg/ml), on platelet activation. Fig. 2 shows the comparative dose–response curves. In all three assays, it is clear that the maximal response happens at the same doses of agonist. However, the MTP assay is better at recording responses in the low-agonist range. Thus, at concentrations as low as 1 or 3 μM TRAP, responses are apparent in the MTP assay but not in either the PAP4 assay or the DSP assay. As the dose of TRAP is increased, it is evident that the MTP assay is more sensitive than either of the other two assays despite the presence of approximately a 10% response (10.9 ± 2.4%) even in the absence of any agonist. This reflects a level of platelet activation induced by 6 min of stirring alone. This level of activation appears to be high but probably indicates either the sensitivity of the MTP assay to low levels of activation or an enhanced effect of secreted ADP in this assay. Because the platelet density in these assays is double that observed in normal blood or in the DSP and PAP4 assays, it can be expected that the agonist effects of secreted ADP will be greater here. The doses of agonist for which a half-maximal response is obtained (EC_{50}) are approximately 2 μM for TRAP and 0.06 mg/ml for collagen in this assay. In contrast, the apparent EC_{50} values are 5 μM for TRAP-induced responses and 0.13 mg/ml for collagen assays.

To ascertain whether the assay also shows sensitivity to standard platelet inhibitors, we tested indomethacin, aspirin, apyrase, and ReoPro in all three assays in parallel (Fig. 3). Low-dose collagen (100 μg/ml) is a weak platelet agonist, and its actions are potently inhibited by inhibitors of prostaglandin synthesis (aspirin and indomethacin) as

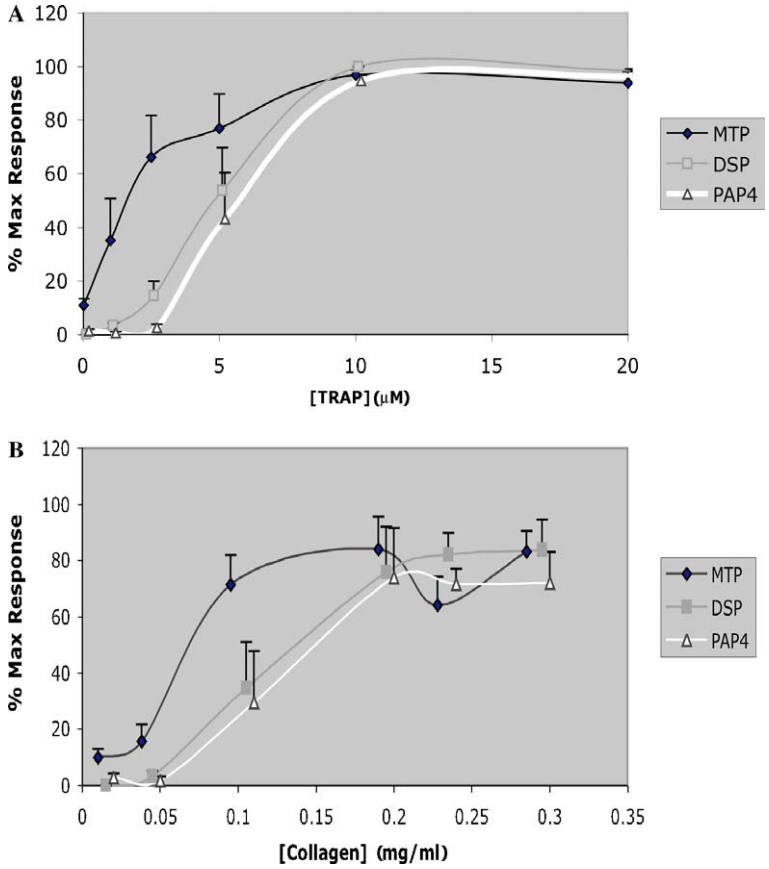


Fig. 2. The MTP assay has greater sensitivity than the standard aggregometry and DSP assays. TRAP (A) and collagen (B) cause dose-dependent activation of platelets in the MTP assay (black line), the standard aggregometry assay (PAP4, white line), and the DSP assay (gray line). Platelets from five independent donors were assayed simultaneously in all three assays as described in Materials and Methods. Data are expressed as percentages maximal (Max) response to TRAP (20 μM). Baseline measurements for each assay at time 0 are subtracted from the measurement at the end of the assay. Data represent means ± SEM ($n = 5$).

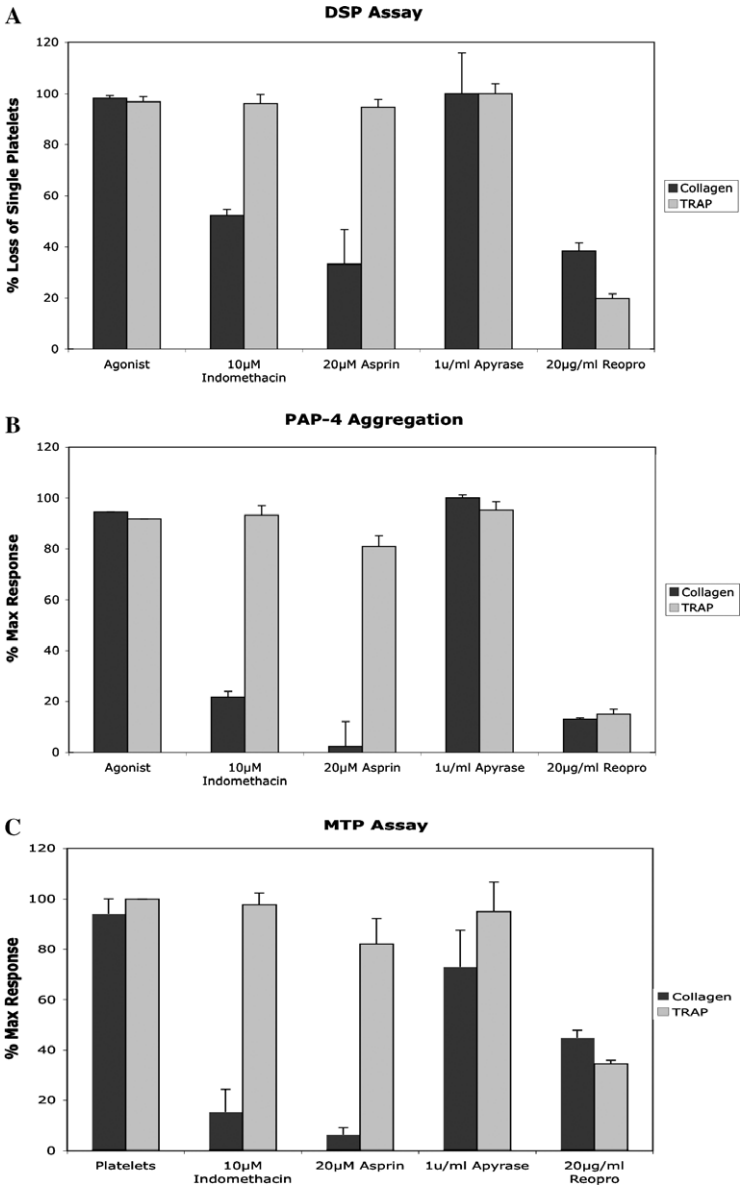


Fig. 3. All assays are equivalent in detecting inhibition of platelet aggregation by standard agents. Platelets were preincubated with indomethacin (10 μM), aspirin (20 μM), apyrase (1 U/ml), or ReoPro (20 mg/ml) for 3 min before the addition of TRAP (10 μM, dark bars) or collagen (0.1 mg/ml, light bars) for an additional 3 min. Platelet aggregation was monitored in the DSP assay (A), the standard PAP4 platelet aggregation assay (B), and the MTP assay (C). Data represent means ± SEM ($n = 4$ for DSP and PAP4 assays, $n = 5$ for MTP assay).

measured in all three platelet assays. TRAP is a potent agonist and is, as expected, not significantly affected by cyclooxygenase (COX) inhibitors but is, like all platelet agonists, inhibited by the GPIIb/IIIa receptor blocker ReoPro. It is commonly observed in standard platelet aggregation methods that a residual level of platelet aggregation (~10–15%) remains despite maximal inhibition with ReoPro or other GPIIb/IIIa antagonists. This residual aggregation is more obvious in both the DSP and MTP assays. There was no significant inhibition by apyrase of responses in any of the assays. However, the increased variability reflected in the larger error bars suggests that ADP release may play some slight role in modulating platelet aggregation responses to high doses of agonists.

For an assay such as the presented MTP assay to be useful for the screening of pharmacological modulators of platelet function, it is important to ascertain its reproducibility from day to day and from donor to donor. We analyzed the responses from 11 separate healthy donors to a single high dose of TRAP (20 μM). Data are plotted as a scatter graph, where each donor is represented by a single spot on the graph (Fig. 4). It is clear from this graph that the data obtained are highly reproducible. The 11 donors behaved identically at all time points. To illustrate the small variability in this assay, the mean results with standard errors are plotted in Fig. 4B.

Finally, we tested the ability of the assay to reflect what was observed in standard aggregometry (PAP4) assays.

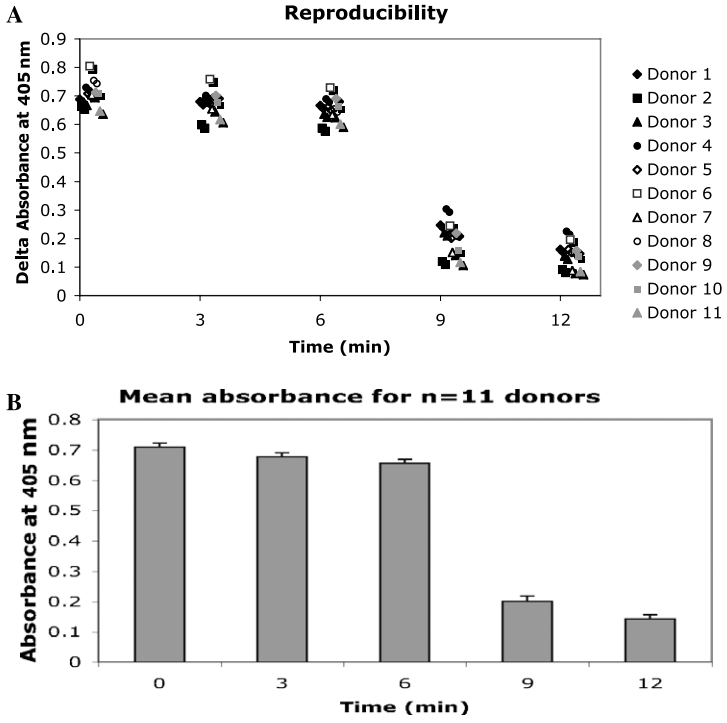


Fig. 4. The response to thrombin stimulation is highly reproducible. Platelet responses were evaluated in 11 separate donors in the MTP assay. Platelets were incubated at 37 °C with buffer alone and constant shaking. Absorbance values were monitored at 0, 3, and 6-min time points. Immediately after the 6-min reading, 0.2 U/ml thrombin was added and absorbances were monitored at 9 and 12 min. (A) Data from each donor are plotted separately. (B) Mean data (± SEM) from 11 donors are presented.

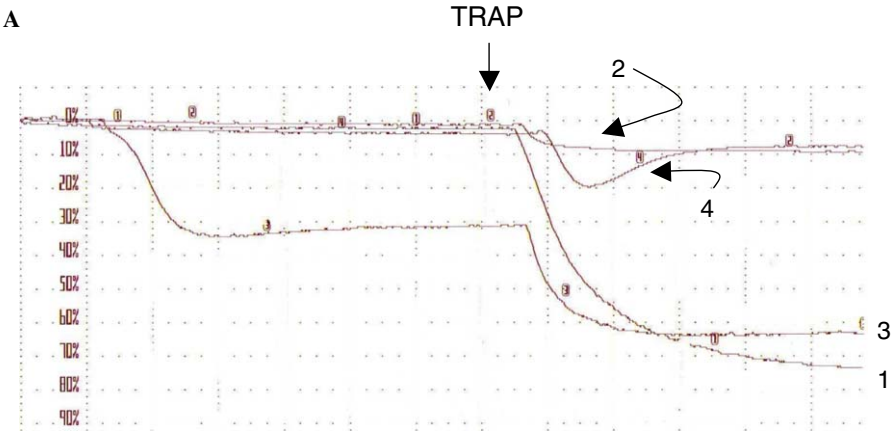
Here aggregation tracings in standard PAP4 aggregometry were recorded in parallel with absorbance values in the MTP assay in the presence of buffer, ReoPro, agonist peptide Pal-KVGFFKR, or antagonist peptide Pal-RRERRDLFTE and then subjected to an additional treatment with 20 μM TRAP. In this way, the assay can be used to obtain information on both the potential agonist and antagonist potencies of unknown pharmacological agents. Responses are recorded in real time in standard PAP4 aggregometry and at 0, 3, 6, 9, and 12 min in the MTP assay. TRAP is added to both assays at the 6-min time point. Fig. 5 demonstrates that both assays reflect the same trend in results. However, relative differences in sensitivity are apparent. Both assays demonstrate that ReoPro can act as an antagonist and inhibit agonist-induced changes. However, standard aggregometry is more able to detect the agonist activity of low doses of the known peptide agonist, KVGFFKR [21]. In contrast, the MTP assay is more sensitive for the detection of residual activity in the presence of antagonists. This highlights an additional strength of this assay in that it allows the detection in a single 96-well plate of modulators of platelet function regardless of whether such modulators have agonist or antagonist actions. Thus, agents that promote platelet aggregation, such as Pal-KVGFFKR, cause alterations in the baseline reading at 3 and 6 min. Antagonist agents that inhibit TRAP-induced platelet aggregation are detected as an alteration in the readings at 9 and 12 min. Therefore, this assay is ideal for monitoring biomimetic agents from a library of compounds.

Discussion

We have developed a simple MTP platelet assay that can be employed in any laboratory without the need for specialist equipment. It is rapid and requires only 90 min from blood draw to result. It can assay an average of 60 independent treatments per 60 ml blood donation and demonstrates greater sensitivity than the current gold standard assay, namely platelet aggregation in stirring conditions in a platelet aggregometer (PAP4). The MTP assay can detect known inhibitors of platelet function such as indomethacin, aspirin, apyrase, and ReoPro. In addition, it is more sensitive than the related DSP assay that also proposes to be amenable to all laboratories and does not require specialist equipment. As expected, indomethacin inhibits the response to low-dose collagen but has no effect on the response to 10 μM TRAP. This reflects the known role for TXA_2 synthesis in the platelet response to low-dose collagen. ReoPro inhibits responses to both collagen and TRAP in the MTP assay to an extent equal to the inhibition observed in standard aggregometry. It is highly reproducible when using standard doses of agonists such as TRAP (20 μM) and collagen (0.19 mg/ml). At lower agonist doses, it is more sensitive than either DSP or PAP4 aggregometry.

Platelet aggregation response measured by standard methods using stirred platelets is nonlinear [21]. This is because a minimum threshold level of platelet activation and aggregate size is required before any response can be observed. Thereafter, increasing doses of agonist tend to

A



B

	Time (min)			
	3	6	9	12
Buffer	1	4	54	60
ReoPro	4	6	22	20
KVGFFKR	11	13	47	54
RRERRDLFTE	3	4	33	30

Fig. 5. Comparison of novel agents in MTP and PAP4 assays. (A) Platelet aggregation tracings obtained from a PAP4 BioData aggregometer are shown. Platelets (3.0×10^5/μl) are allowed to aggregate for 6 min in the presence of buffer (1), ReoPro (2, 10 μg/ml), or palmitylated peptides KVGFFKR (3, 20 μM) and RRERRDLFTE (4, 50 μM) before the addition of TRAP (10 μM, arrow) for an additional 6 min. (B) Platelets (6.0×10^5/μl) were incubated under parallel conditions in the MTP assay. Readings taken at 3 and 6 min represent responses to the buffer, ReoPro, or peptides alone, and readings taken at 9 and 12 min demonstrate responses following the addition of TRAP (10 μM). Data are presented as percentage changes from the baseline reading (taken at time 0).

produce a maximal response. Consequently, the dose range for a standard agonist, required to give a 20 to 80% response, always is less than the 1.8 log units routinely observed in biological assays. Thus, the utility of a rapid and effective method of screening platelet function in response to investigational reagents, such as the MTP assay presented here, seems obvious. The additional advantages of the MTP assay over the current gold standard of platelet aggregometry include its availability to all laboratories and its capacity to record multiple conditions or treatments simultaneously without the usual changes encountered over the time course of a standard aggregometry assay. Moreover, we demonstrated the capacity of this assay to measure either the agonist or antagonist potency of unknown pharmacological agents in a single assay system. For this purpose, unknown agents are incubated with the washed platelet sample in the assay plate, and responses are recorded at 3- and 6-min time points. An agonist such as TRAP is then dispensed into all wells of the assay plate, and responses are recorded for a further 6 min. In this way, agonist agents can be determined by changes in activity from buffer alone at 3 and 6 min, whereas antagonist activity can be inferred from a reduced response to TRAP (or other agonist) at 9- and 12-min time points. Finally, the MTP assay is sensitive and reproducible.

In summary, the use of a microplate reader to examine platelet aggregation rapidly with less sample volume and high reproducibly offers a significant advantage over standard platelet aggregation methods. It allows the simultaneous analysis of responses to a range of different agonists and eliminates the issue of variability over time that is encountered in sequentially performed single aggregations. Therefore, this assay will allow, under reproducible conditions, the determination of bioactive agents from a library of compounds such as peptides to identify new therapeutic targets for antiplatelet therapies. In addition, this MTP assay has the capacity to detect both pro- and anti-aggregatory agents from a library of unknown compounds in a single assay. Moreover, its high sensitivity for detecting platelet aggregation over a low-agonist dose range suggests that this assay will also be of interest to those monitoring relative platelet function in large populations.

Acknowledgments

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