Immobilization of Nonactivated Unfixed Platelets for Real-Time Single-Cell Analysis

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Abstract

Existing methods for measuring the response of individual platelets to stimulation are limited. They either measure each platelet at one discrete time-point (flow cytometry) or rely on adhesive ligands to immobilize platelets that concomitantly generate activation signals (microscopy). Such methods of immobilization make it impossible to assess resting platelets, the changes that occur as platelets transition from resting to active states, or the signals generated by soluble agonists, such as ADP and thrombin, or by mechanical stimulus, independently from those generated by the adhesive ligand. Here we describe a microscopy method that allows the immobilization of platelets to a glass cover slip without triggering platelet activation. This method makes use of specific antibodies that bind platelet PECAM-1 without activating it. Platelets can therefore be immobilized to PECAM-1 antibody coated biochips without causing activation and perfused with agonists or inhibitors. Using this method, platelets can be stimulated by an array of soluble agonists at any concentration or combination, in the presence or absence of inhibitors or shear forces. This chapter describes in detail this PECAM-1 mediated immobilized platelet method and its use for measuring changes in Ca2+ signaling in individual platelets under a number of different conditions. While we focus on the measurement of Ca2+ dynamics in this chapter, it is important to consider that the basic method we describe will easily lend its self to other measures of platelet activation (integrin activation, shape change, actin dynamics, degranulation), and may, therefore, be used to measure almost any facet of platelet activation.

Key words Immobilized Platelet, Calcium, ADP, Thrombin, Microscopy, Biochip

Introduction

It is desirable, in many cases, to monitor the cellular processes in individual platelets rather than a population of platelets. This is relatively straight forward and can be done either by flow cytometry or by microscopy. Flow cytometry enables analysis of both resting platelets and platelets activated by an array of agonists either individually or in combination, at fixed time points or in real-time assays, but with the limitation of only measuring each platelet at one discrete time-point [1–3]. Microscopy assays, by contrast, allow changes in individual platelets to be followed over time but 2 with the limitation that platelets must be adhered to coverslips coated with adhesive receptor ligands such as fibrinogen, collagen, von Willebrand factor, or synthetic peptides [4 – 7]. In this approach activation signals are, however, generated by the adhesive ligand making it impossible to assess resting platelets or the changes that occur as platelets transition from resting to active states. It also makes it impossible to assess signals generated by soluble agonists, such as ADP or thrombin, or by mechanical stimulus, independently from those generated by the adhesive ligand. With these limitations in mind we have developed a micros - copy method that allows the immobilization of platelets to a glass cover slip without triggering platelet activation. This approach allows the measurement of calcium transients in individual resting platelets and the changes in platelets in response to agonist- or shear-induced mechanical stimulation.

The method we outline in detail below is carried out in five stages using biochips which provide a convenient small capillary channel onto which platelets can be immobilized and through which agonists or inhibitors can be infused over the immobilized platelets (Fig. 1a). The biochips are initially coated in PECAM-1 (WM59) antibodies (Fig. 1b—Stage 1). Excess antibody is removed and the channel is blocked with 2% BSA to prevent artifactual platelet activation (Fig. 1b—Stage 2). Platelets are gently loaded into the flow channel, allowed to bind to the immobilized anti - body and nonadherent

platelets are removed leaving just immobi - lized nonactivated platelets (Fig. 1b—Stages 3 and 4). Image acquisition of the immobilized platelets is started and concomi - tantly agonists are perfused, or shear is increased to cause platelet activation which can be imaged (Fig. 1b—Stage 5). This method makes use of the ability of certain antibodies to bind platelet PECAM-1 without activating it. On the platelet sur - face PECAM-1 has been estimated to be expressed at between 5000 and 8800 copies per cell [8 –10]. Although the level of platelet PECAM-1 varies widely within the human population with levels up to 20,000 molecules per platelet seen in around 20% of the population [11]. PECAM-1 is made up of a 574-amino acid resi - due extracellular portion organized into six immunoglobulin (Ig)-like homology domains (Fig. 1c), a 19-amino acid transmembrane domain and a cytoplasmic domain which includes an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) ((L/V/ I/S/T)XYXX(L/V)) and an Immunoreceptor Tyrosine-based Switch Motif (ITSM) (TxYxx(V/I)) contained in a lipid-interacting α-helical segment [12–14] . The principal ligand for PECAM-1 is PECAM-1 itself through homophilic interaction between immu - noglobulin (Ig) domains 1 and 2 of the molecules on nearby plate - lets [15].

In laboratory studies activation of PECAM-1 is most usually achieved through the binding of selected antibodies that bind to membrane proximal Ig domain 6 of PECAM-1 [16–18]. These antibodies bind to PECAM-1 but importantly leave Ig domains 1 and 2 free to undergo homophilic interaction that is enhanced when cross-linking secondary antibodies are used to cluster the primary antibody and hence PECAM-1 [16–19]. In response to this direct stimulation of PECAM-1 tyrosine phosphorylation and signaling occurs leading to an array of inhibitory (and some activatory) responses [16–18, 20, 21]. In the method described below we use antibodies specifically directed against the Ig domain 1 and 2 of PECAM-1 which inhibit the activation of PECAM-1 by blocking homophilic ligation that is dependent on these domains (Fig. 1c) [15, 16]. Thus detailed knowledge of PECAM-1 physiology and the availability of well characterized specific reagents have allowed us to design a method that provides a surface onto which platelets can attach, via an abundant receptor, and become immobilized without becoming overtly activated.

This chapter describes in detail the PECAM-1 mediated immobilized platelet method developed in our laboratory, and its use for measuring changes in Ca2+ signaling in individual platelets under a number of different conditions. We endeavour to provide all of the important technical details needed to successfully use this method. While we focus on the measurement of Ca2+ dynamics in this chapter it is important to bear in mind that the basic method we describe (the capture and immobilization of nonactivated platelets on a PECAM-1 antibody surface in capillary channels) will easily lend its self to other measures of platelet activation (integrin activation, shape change, actin dynamics, degranulation), and may, therefore, be used to measure almost any facet of platelet activation.

Materials

Reagents

1. Acid Citrate Dextrose (ACD): 85 mM sodium citrate 111 mM glucose, and 78 mM citric acid [pH 6.4].
2. 1 μM ADP, 1 μM TRAP-6, or 10 μg/ml CRP-XL (collagenrelated peptide-cross-linked, monomeric sequence GCI[GPO]10GCOG) was prepared as described previously [22] (see Note 1, Fig. 2).
3. 500 μM Fluo-4 AM in DMSO (see Notes 2–5).

Flow Chip Preparation and Imaging

1. Tyrode's-HEPES buffer—134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM N-2- hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5 mM glucose and 1 mM MgCl2, pH 7.3—filter using 0.2 μm, 32 mm Syringe Filters.
2. Monoclonal mouse anti-human antibody against the first or second Ig domain of PECAM-1 (CD31), clone: WM59 (Serotec, UK). The antibody binds to PECAM-1 but prevents its activation [15, 16].
3. 2% bovine serum albumin (BSA) in Tyrode's-HEPES buffer filtered using 0.2 μm, 32 mm Syringe Filters.
4. Glass-bottom biochips which are precast disposable perfusion chambers comprising channels, each 1.6 mm wide, 0.16 mm high, and 28 mm long, with a volume of 0.8 μl.
5. Nanopump to enable precision microfluidic flow at variable shear rates.
6. Confocal microscope with Resonant Scanner housed in an environmental hood to maintain the temperature of the biochips, platelets, and any buffer or agonist perfused over the immobilized platelets and a constant 37 °C.
7. ImageJ 1.47v52 using the Time Series Analyzer V2.0 plugin.

Method

The method outlined below is for imaging of Ca2+ transients in single immobilized platelets. The method of preparing and immobilizing platelets to a glass biochip without causing their activation is, however, adaptable to many experimental designs of which this is one example.

Biochip Preparation

1. Coat glass-bottom biochips with monoclonal mouse antihuman PECAM-1 (CD31, WM59) antibody by pipetting 1 μl (containing 1 μg) of antibody into each channel and incubating at 37 °C for 1 h.
2. Remove excess antibody by very gently flushing each channel by directly pipetting 10 μl of Tyrode's-HEPES buffer into the channels. The buffer should be prewarmed to 37 °C.
3. To prevent glass-induced platelet activation block each channel by gently directly pipetting 10 μl of 2% BSA, prewarmed to 37 °C, into each channel. Then incubate for 1 h at 37 °C.
4. Remove excess BSA by gently flushing each channel by directly pipetting 50 μl of Tyrode's-HEPES buffer (prewarmed to 37 °C) into each channel (see Note 6).
5. Biochips are now ready to use and can be kept at 37 °C or stored in the fridge at 4 °C overnight. If storing in at 4 °C overnight make sure that the biochips are brought up to 37 °C prior to use.

Blood Collection and platelet preparation

1. Obtain human blood (see Note 7) from consenting healthy volunteers who have not taken anti-platelet medication (for example aspirin or ibuprofen) in the previous 10 days, via venesection of the antecubital vein. Collect the blood into 3.8% (w/v) sodium citrate at a ratio of 9 parts blood to 1 part sodium citrate, adding acid citrate dextrose (ACD) to a final concentration of 12.5% (v/v).
2. Transfer the blood into 12 × 75 mm polystyrene test tubes and prepare platelet rich plasma (PRP) by centrifugation at 100 × g for 20 min using the slowest centrifuge braking setting.
3. Transfer PRP slowly using a wide bore pipette tip to avoid artifactual shear-induced activation and maintain at 30 °C.

Dye Loading

1. Load PRP with 2 μM Fluo-4 for 1 h at 30 °C.

2. Wash the platelets by centrifugation at 350 × g for 20 min and resuspension in Tyrode's-HEPEs buffer prewarmed to 30 °C (see Note 8). Adjust platelet concentration to 4 × 107 cells/ml to ensure that platelets are spatially separated on the biochip, thereby minimizing artifactual platelet–platelet interaction.
3. Rest platelet for 10 min at 30 °C prior to introducing them into the biochips.

Immobilisation of Platelets on Biochips

1. Immediately prior to each experiment (see Note 9), introduce the Fluo-4 loaded washed platelets into the biochip channel by very gently pipetting 1 µl of Fluo-4 loaded washed platelets directly into the channel ensuring that the platelets are not exposed to shear stress (see Note 10).
2. Incubate at 37 °C for 10 min with occasional very gentle shaking.
3. The biochip can then be mounted on the microscope stage making sure that the environmental chamber and the stage are at 37 °C.
4. Using the pump slowly (<5 dyne/cm3) draw Tyrode's-HEPEs buffer prewarmed to 37C through the channel to wash off any nonimmobilized platelets.

Imaging

1. Set up the confocal microscope to monitor platelet calcium signaling (see Note 11). Observe fluorescence with excitation at 488 nm and emission at 525 nm with a 60× magnification lens.
2. Record fluorescence in unstimulated platelets for 5 min prior to stimulation. The frequency of image acquisition should be at least 2 FPS (see Note 12)

Inhibiting or Stimulating Platelets

1. Pharmacological reagents may be introduced at a very low shear rate (<200 s-1) prior to stimulation.
2. When the microscope is set up and fluorescence has been monitored in unstimulated platelets for 5 min, perfuse agonists through the channels at low shear rate (<400 s-1) (see Note 10) whilst continuously recording the calcium signal for 5 min (Fig. 2)

Image Analysis

1. Captured time-series images are analyzed on ImageJ 1.47v52 using the Time Series Analyzer V2.0 plugin, to obtain the Ca2+ traces (Fig. 2).
2. Changes in Ca2+ concentration can be plotted as F/F0, where F is the fluorescence intensity (at t = x s) minus background and F0 is the fluorescence intensity (at t = 0 s) minus background.

NOTES

1. Using this method, platelets can be stimulated by an array of soluble agonists at any concentration or combination, in the presence or absence of inhibitors or shear stress. Figure 2 provides typical Ca2+ traces obtained when stimulating with 1 µM ADP, 1 µM TRAP-6, or 10 µg/ml CRP-XL. 2.
2. Both Fluo-3 and Fura-2 AM may be used with this method if the confocal microscope is fitted with appropriate lasers.
3. Fluo-4 AM is generally preferable to Fluo-3. Because of its greater absorption near 488 nm. Fluo-4 emits significantly brighter fluorescence than Fluo-3, enabling it to be used at lower

intracellular concentrations, both reducing the cost of experimentation and the amount of invasive dye loading [23].

4. Fura-2 AM: This ratiometric dye allows the measurement of Ca2+-dependent fluorescence at 340 nm and 380 nm generating 340/380 ratios to represent intracellular Ca2+ concentrations. The main advantage of using Fura-2 is that variables such as differences in dye loading, cell thickness or potential cell movement can be eliminated. These variables could generate artifacts when nonratiometric dyes are used to measure intracellular Ca2+ concentrations. The standard procedure of Fura-2 dye loading involves the treatment of PRP with 2 μM Fura-2 AM for 45 min at 37 °C, with gentle inversions throughout the incubation period to allow mixing.
5. Ensure that platelets are not exposed to light during and after the dye loading steps to avoid photobleaching of Fluo-4.
6. When removing excess antibody or BSA care must be taken not to flush too vigorously, otherwise the coating will be disrupted.
7. The technique may be used to study platelets from nonhuman species, but dye-loading conditions are likely to differ from those described and may require optimization.
8. Include 0.32 U/ml apyrase in the buffer used to resuspend platelets and in the external buffer, if P2X1 receptors are being studied under shear. This will prevent P2X1 channels from desensitization by ATP which may be found in the extracellular milieu [24]. Apyrase can be omitted from these steps depending on the objectives of the study.
9. In our experience it is advisable to immobilize and then stimulate the platelets in one channel at a time to obtain optimal results.
10. Human platelets have been reported to express several shear stress-operated mechanosensitive ion channels including Piezo1 [25–28], whose contribution to function in singly attached platelets can be studied using this protocol. For further details on how this method can be adapted to monitor fluid-shear induced mechanosensitive Ca2+ entry in singly attached platelets, see Ilkan et al. [28]. Fluid shear stress can cause mechanosensitive ion channel activation by inducing tensions within the lipid bilayer of the membrane, which in turn operate mechanosensitive ion channels permitting ionic fluxes from the cell exterior [29, 30]. Evidence indicates that this mechanism is independent of any links to the cytoskeleton [31], and thus shear stress is directly sensed and transmitted to the ion channels by the membrane. Using the in vitro approach described in this chapter, normal or pathological venous or arterial flow rates (in ml/min) can be applied to immobilized platelets in biochips by drawing physiological saline through the biochip channels. The conversion between flow rate (ml/min) and shear rate (s−1) can be performed using the following formula, taking into account the dimensions of the biochip channels:

$$Q = \frac{wh^2 t}{6\mu}$$

where Q = flow rate (ml/s), w = microslide lumen width in cm for biochip (0.08); h = microslide lumen height in cm biochip (0.008); t = shear stress (Pa); μ = viscosity of water (0.001002 Pa/s). Finally, to calculate the shear rate (s−1), t is divided by μ.

11. It is essential that everything in the system is at a stable 37 °C. It is therefore advisable to turn the microscope incubator on early (1–2 h prior to the start of the experiment) to ensure that the stage reaches a uniform 37 °C.
12. If shear-induced Ca2+ responses are being studied (such as mechanosensitive cation channel responses), image capturing frequency can be adjusted to optimally to capture Ca2+ responses. Higher frequencies will allow the recording of very brief Ca2+ elevations which would not be captured using lower frequencies.

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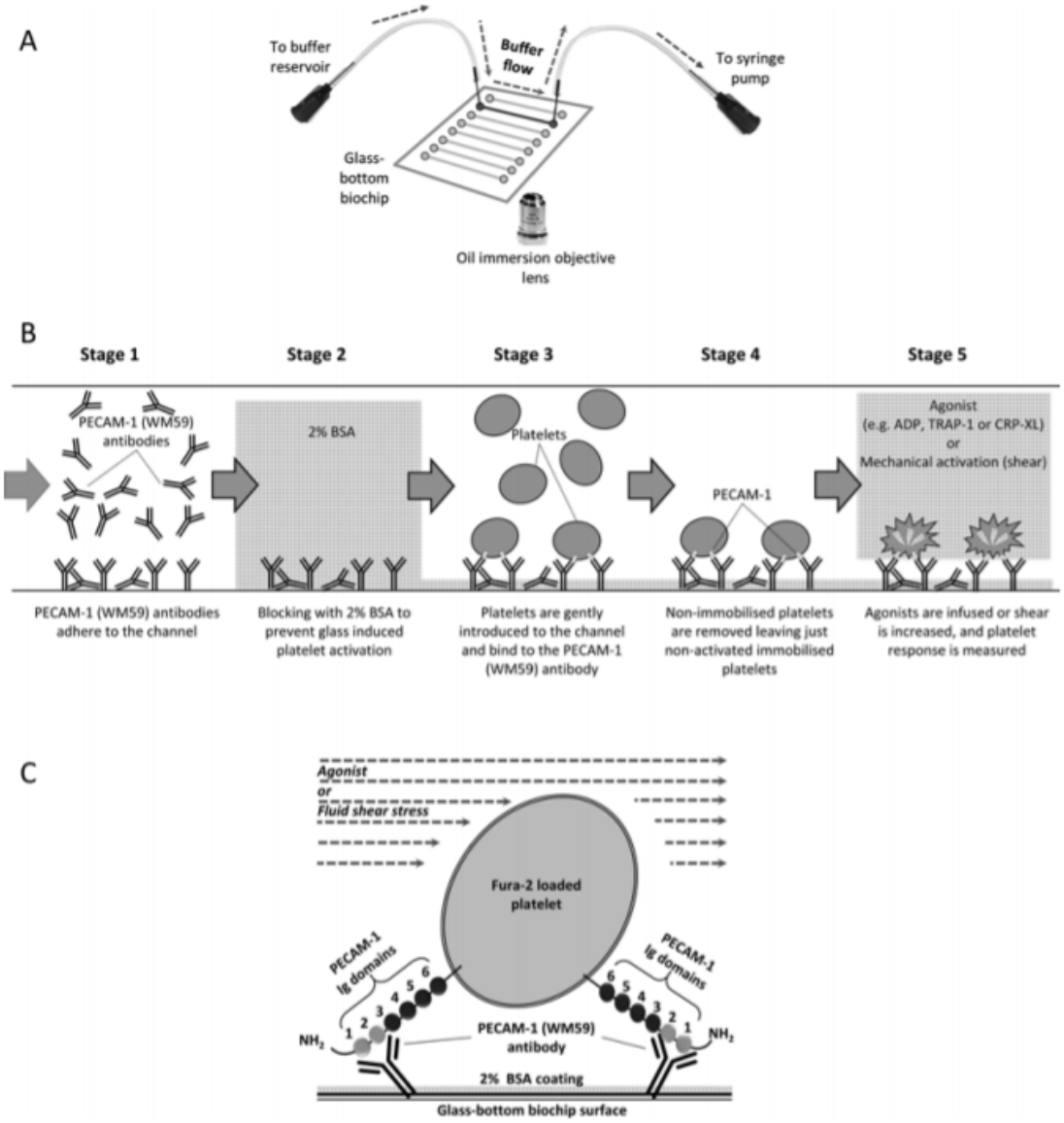

Fig. 1 PECAM-1 immobilized platelet method. Cartoon representations of the PECAM-1 immobilized platelet method. (a) Nonactivated platelets are immobilized on to glass-bottom biochips allowing for agonists, inhibitors, or buffered to be flowed over them at a range of shear stress while continuously imaging changes in platelet activation (e.g. calcium flux). (b) The five stages of the method for immobilizing platelets. Stage 1—Biochips are coated in PECAM-1 (WM59) antibodies. Stage 2—Exposed glass is coated with 2% BSA to prevent artifactual platelet activation. Stage 3—Platelets are loaded into the flow channel and allowed to bind to the immobilized antibody. Stage 4—Nonadherent platelets are removed leaving just immobilized nonactivated platelets. At which point image acquisition can start. Stage 5—Agonists are infused or shear is increased to cause platelet activation which can be measured. (c) Closer image of Stage 4, showing a platelet immobilized by PECAM-1 tethers. The PECAM-1 (WM59) antibody binds to Ig domains 1 or 2 of the PECAM-1 molecule which prevents its activation, thereby tethering the platelets without inducing their activation or inhibitory PECAM-1 signaling [15, 16]

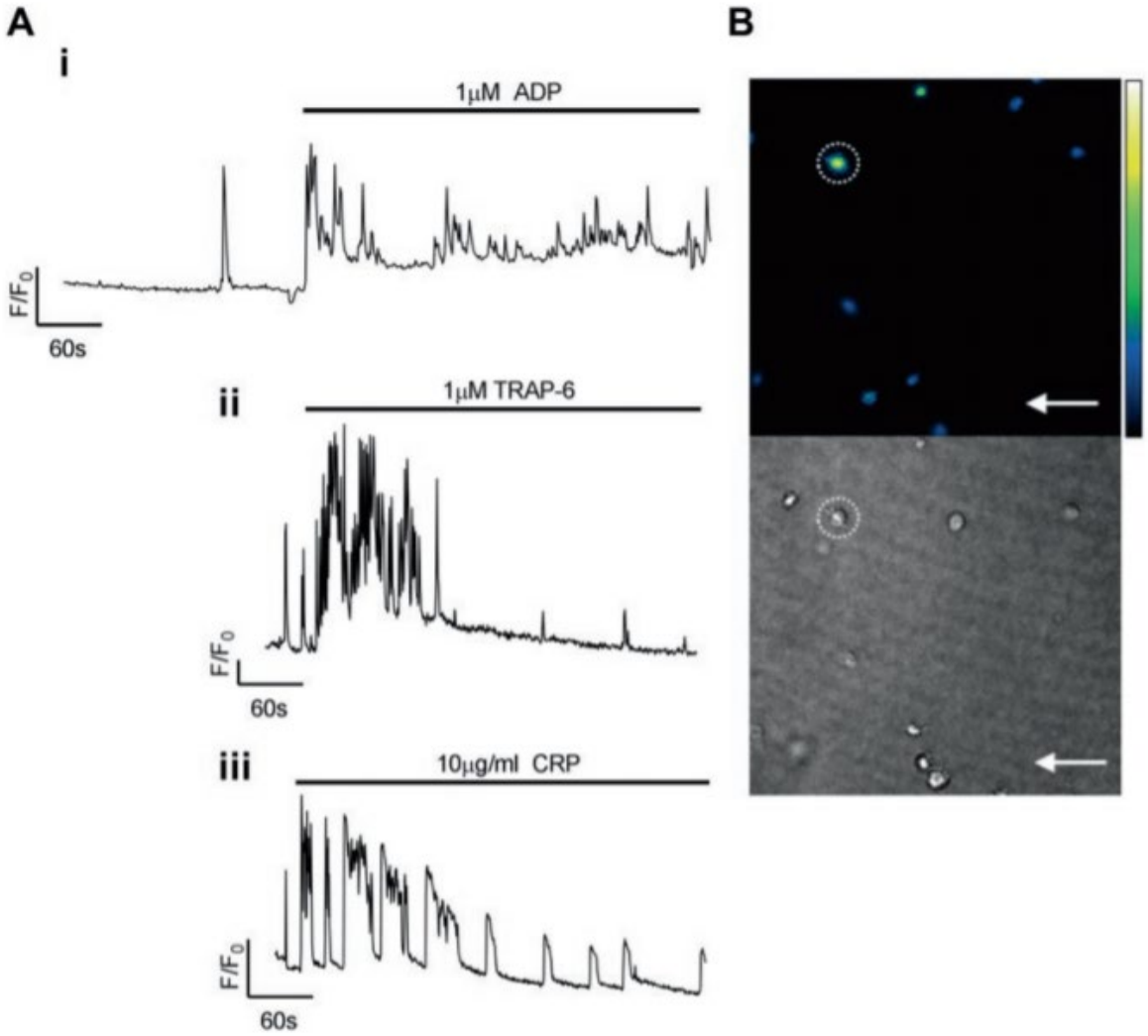

Fig. 2 Intracellular Ca2+ measured in platelets adhered to anti-PECAM-1 coated flow chambers during perfusion with agonists. Washed human platelets incubated with Fluo-4 AM for 1 h at 30 °C were adhered to a microfluidic flow cell chamber coated with anti-PECAM-1 antibody for 30 min and then perfused with agonist. (a) Traces are pseudoratios (F/F0) of Fluo-4 fluorescence measured in single platelets (indicated in images by dashed circle) during perfusion with (i) 1 μM ADP, (ii) 1 μM TRAP-6, or (iii) 10 μg/ml CRP-XL for 7 min. (b) Fluo-4 fluorescence image (top) and brightfield image (bottom) of platelets during perfusion with ADP (white arrow indicates direction of flow)