Rapid Report

Reversible and irreversible intracellular Ca^{2+} spiking in single isolated human platelets

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1. We have developed conditions that permit long duration recordings of $[Ca^{2+}]_i$ in single, isolated human platelets and studied the reversibility of Ca_i^{2+} spiking following activation by physiological and artificial stimuli.

2. Fura-2-loaded platelets were immobilized at the tip of a saline-filled glass pipette using gentle suction. 'Contact' activation of Ca_i^{2+} spiking was observed in a proportion (11 %) of platelets, which continued for the duration of each recording (range 8–45 min).

3. Platelets that displayed constant, resting Ca_i^{2+} levels were used to test the effects of agonists. ADP (10 μM) increased $[Ca^{2+}]_i$ in the form of either one to two spikes followed by an elevated plateau level (60 % of cells) or multiple Ca^{2+} spikes of irregular amplitude (40 % of cells). ADP-induced Ca_i^{2+} mobilization was completely reversible and repeatable.

4. Thrombin (1 u ml^{-1}) evoked Ca_i^{2+} spiking in the majority (88 %) of platelets tested, which was not inhibited by perfusion of agonist-free saline throughout the recording period (range 8–67 min).

5. The clear difference in the reversibility of activation by different stimuli may reflect the distinct roles of individual agonists in haemostasis and have important consequences in the design of treatments for thrombosis.

Platelets are discoid, anuclear cell fragments approximately 3 μm in diameter and 1 μm thick that play an essential role in haemostasis (for review see Siess, 1989). Upon activation, they lose their discoid shape and become relatively spherical with pseudopods that increase the surface area for cell–cell contact (Born, 1962; O'Brien & Heywood, 1966). Other platelet responses include secretion, adhesion and aggregation leading to a 'haemostatic plug' that repairs an injured vessel and arrests blood loss. However, in disease, uncontrolled platelet aggregation can result in thromboembolism, ischaemia, heart failure and stroke (Packham, 1994). *In vitro*, platelet activation can be induced by a variety of agents acting via specific surface receptors (Siess, 1989). Although certain agonists such as thrombin are clearly more potent than others (e.g. 5-HT), the relative physiological and pathophysiological importance of individual agents remains unclear. This uncertainty stems in part from the existence of several mechanisms for positive feedback during platelet activation, including secretion of agonists from platelet granules, generation of membrane-permeant eicosanoids and expression of procoagulant activity at the platelet membrane surface (Siess, 1989; Bevers *et al.* 1991). Since the vast majority of platelet studies are carried out in cell suspension, such feedback pathways have complicated the study of responses to individual agonists and the reversibility of platelet activation.

Ca_i^{2+} is an important second messenger during platelet activation (Siess, 1989; Rink & Sage, 1990) and an increase in $[Ca^{2+}]_i$ is one platelet response that can be studied at the single cell level using fluorescent indicators. Hallam *et al.* (1986) were the first group to suggest that Ca_i^{2+} elevations in individual human platelets can occur in the form of oscillations rather than slow sustained increases. Other groups have since shown Ca_i^{2+} spiking in single mammalian platelets stimulated by ADP (Heemskerk *et al.* 1992; Ariyoshi & Salzman, 1995), thrombin (Ozaki *et al.* 1992; Heemskerk *et al.* 1993) and 5-HT (Nishio *et al.* 1991). These previous studies used video imaging to measure Ca_i^{2+} changes in platelets adhered to glass coverslips coated by, for example, fibrinogen. This approach causes platelet spreading over the glass surface and the reversibility of single platelet Ca_i^{2+} responses has not been studied. It is also worth noting that previous work at the single platelet level has monitored $[Ca^{2+}]_i$ in fields of cells and therefore the responses to an individual agonist may have involved paracrine activation by secreted agonists (Heemskerk *et al.* 1993). In the present study, we have used fluorescence photometry to measure $[Ca^{2+}]_i$ in single, isolated human platelets immobilized by

gentle suction at the tip of a glass pipette. This approach has enabled the recording of Ca_i^{2+} spiking in a single platelet for periods of more than an hour in response to contact or agonist activation and permitted investigations into the reversibility of Ca_i^{2+} spiking in these cell fragments. In this initial study using this technique, we have looked at the responses to the physiological agonists ADP and thrombin. ADP is released from damaged endothelium and thus may be one of the earliest activators of platelets; furthermore it is secreted from platelet dense granules and is likely to be an important paracrine agonist (Siess, 1989). Thrombin is generated from inactive circulating prothrombin during haemostasis and was selected since it is recognized as the most potent platelet agonist known (Seiss, 1989). A brief report of this work has appeared elsewhere (Hussain *et al.* 1997).

METHODS

Blood was drawn from healthy subjects with their informed consent and platelet-rich plasma (PRP) prepared as described previously (Mahaut-Smith, 1995). Aspirin (100 μM) and apyrase (20 μg ml^{-1}) were added to the PRP to reduce spontaneous activation of the platelets and PRP was stored at room temperature for up to 8 h on a rotator. For fura-2 loading, 1 ml aliquots of PRP were incubated at 37 °C for 45 min with fura-2 acetoxymethyl ester (fura-2 AM; 3–5 μM). The platelets were then spun at 'low speed' in a MSE Micro Centaur centrifuge (Fisons, UK) and resuspended in normal platelet saline (NPS) containing apyrase (20 μg ml^{-1}). Experiments were conducted at room temperature (22–25 °C) and platelets were used within 1 h of fura-2 loading. NPS contained (mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 10 Hepes (pH 7·35, adjusted with NaOH). Fura-2 AM was obtained from Molecular Probes and *N,N*-dimethyltrimethylsilylamine (Silane) was obtained from Fluka. All other reagents were from Sigma Chemical Co.

The glass pipettes used for isolating platelets (holding pipettes) were pulled from filamented borosilicate medium-walled tubing (GC150F-10, Clark Electromedical Instruments, Pangbourne, UK) using a two-step puller (Type PP-83, Narishige, Tokyo) and had external tip diameters of < 1 μm. The glass coverslips that formed the base of the recording chamber were silanized to reduce adhesion of platelets to the glass surface. *N,N*-Dimethyltrimethylsilylamine (5 μl) was added to coverslips preheated to 220 °C, which were left to dry for 20 min and then washed thoroughly prior to use. Platelet suspension (20–50 μl) was added to the recording chamber filled with approximately 500 μl NPS, the platelets allowed to settle for about 1 min and the chamber perfused with NPS. Perfusion preferentially removed platelets from the upper part of the chamber and was continued until a thin layer of floating platelets remained immediately above the coverslip. The holding pipettes were filled with nominally Ca^{2+}-free saline containing apyrase (20 μg ml^{-1}) and bovine albumin (0·1 %, w/v) in a further attempt to reduce spontaneous platelet activation. The pipette was lowered into the recording chamber whilst applying a slight positive intra-pipette pressure. The positive pressure was released when the pipette tip was approximately 5 μm from a selected platelet and gentle suction used to draw the platelet onto the tip. The chamber was then perfused to remove remaining platelets.

Fura-2 fluorescence was monitored by single cell photometry using a Cairn Spectrophotometer system (Cairn Research Ltd, Faversham, Kent, UK), coupled to a Nikon Diaphot 200 inverted microscope (for full details see Mahaut-Smith, 1995, 1998). Excitation wavelengths were 340 and 380 nm. Emitted light of 430–600 nm was selected by two dichroic filters and further filtered by a 480 nm long-pass filter. Cells were also illuminated by infrared light (780 nm long-pass filter, Comar Instruments, Cambridge, UK) which was detected by a standard CCD camera. A custom infrared light-transmitting variable aperture was used to select a rectangular area slightly larger than the platelet for fluorescence measurement, yet permit visualization of the entire field of view (Mahaut-Smith, 1998). This system allowed positioning of agonist injection pipettes close to the cell without interruption to the fluorescence recording. The 340 and 380 nm fluorescence signals were corrected for background and 340/380 ratios calculated at a rate of 15–60 Hz. The fluorescence signal from non-fura-2-loaded platelets was indistinguishable from that of the saline and microscope components. Thus, the background (non-fura-2) fluorescence was measured by removing the platelet from the region of interest. Origin (Microcal Software Inc., Northampton, MA, USA) software was used to further analyse data, including 9 or 11 point smoothing by a Savitzky-Golay filter method.

ADP and thrombin were pressure injected from a glass pipette under the control of a PLI-100 Picolitre Injector (Medical Systems, Greenvale, NY, USA). Apyrase (20 μg ml^{-1}) and bovine albumin (0·1 %, w/v) were also present in the thrombin injectate. The tip of the agonist pipette was approximately 10 μm from the immobilized platelet.

RESULTS

Contact activation of Ca_i^{2+} spiking

Platelets possessing morphological features consistent with a non-activated state, that is a discoid shape and smooth membrane surface, were isolated by gentle suction onto the tip of a saline-filled glass pipette. A proportion (4/35) of these isolated platelets displayed spontaneous elevations of $[Ca^{2+}]_i$ (Fig. 1). The isolation procedure was carried out in the complete absence of agonists; furthermore, surrounding cells were removed by continuous perfusion of the chamber following platelet immobilization. Therefore, the spontaneous Ca_i^{2+} response was not the result of agonists released from nearby platelets. Glass surfaces have been reported to evoke platelet shape change and aggregation (O'Brien & Heywood, 1966) and spontaneous Ca_i^{2+} spikes have been observed during fluorescence imaging of single platelets exposed to fibrinogen-coated glass surfaces (Heemskerk *et al.* 1992). Thus, the most likely cause of the Ca^{2+} spikes observed in our studies in the absence of exogenously applied agonist is the direct contact of the platelet membrane with the glass surface of the holding pipette and we shall refer to this response as 'contact' activation. Figure 1 shows an example of such contact-induced Ca_i^{2+} spiking immediately after commencing the fluorescence recording. The background-corrected single wavelength fluorescence signals are displayed in the upper graph and the 340 nm/380 nm ratio, which can be used as a linear indicator of $[Ca^{2+}]_i$ over the physiological range (Floto *et al.* 1995), is displayed in the lower graph. The 340 and 380 nm signals both decreased with time due to dye leakage and/or photobleach; however, this decline was largely eliminated within the ratio trace and sufficient

signal remained to allow $[Ca^{2+}]_i$ to be continuously monitored for the duration of each recording, which was more than an hour in some experiments (see studies of thrombin activation, below). The lower graph in Fig. 1 shows that repetitive Ca_i^{2+} spikes occurred with an irregular frequency and amplitude and Ca_i^{2+} levels frequently returned to basal levels between discrete spikes. The contact activation of Ca_i^{2+} spiking was not inhibited by saline perfusion and continued without run-down for the duration of the recording (range 8–45 min, $n = 4$).

Activation of single platelet Ca_i^{2+} events by ADP

The majority (31/35) of platelets isolated in NPS displayed a low, constant 340 nm/380 nm ratio following immobilization onto the tip of a glass pipette. No contact-induced elevations of $[Ca^{2+}]_i$ were observed in these platelets throughout the duration of control recordings (up to 7 min). This implies that contact activation occurs prior to the start of the fluorescence recording in the isolated platelet. At least one burst of Ca_i^{2+} spikes was observed every minute following contact activation ($n = 4$; e.g. Fig. 1), and therefore a control period of more than 1 min was used to check that no contact activation had occurred prior to investigating the effects of exogenously applied agonists. Application of ADP (10 μM) induced either one to two Ca_i^{2+} spikes followed by a raised plateau level (Fig. 2*A*; $n = 6$) or repetitive Ca_i^{2+} spiking (Fig. 2*B*; $n = 4$). Following removal of ADP, $[Ca^{2+}]_i$ returned to the resting level where it remained throughout the wash period. A second exposure to ADP produced a $[Ca^{2+}]_i$ response similar in pattern to that generated by the first application, that is either one to two Ca_i^{2+} spikes (Fig. 2*A*; $n = 2$) or repetitive Ca_i^{2+} spiking (Fig. 2*B*; $n = 3$). These data demonstrate that mobilization of Ca_i^{2+} by ADP in single human platelets is a reversible and repeatable response.

Single platelet Ca_i^{2+} events activated by thrombin

The effect of thrombin was also investigated in platelets that displayed a low, constant calcium level for at least 1 min following immobilization. The upper left trace in Fig. 3 shows an example of the effect of thrombin application (1 u ml^{-1}) on the $[Ca^{2+}]_i$ of an isolated, non-activated human platelet. As can be seen more clearly in the lower left expanded record (*a*), Ca_i^{2+} increased in the form of a brief burst of spikes of variable amplitude, followed by a recovery to basal Ca^{2+} levels. In contrast to ADP, the $[Ca^{2+}]_i$ continued to spike following removal of thrombin. The spiking response was observed throughout the duration of the experiment, which in this platelet was a total of 67 min. The right upper trace in Fig. 3 shows the 340 nm/380 nm ratio after a break in the time axis of 37 min. The second expanded portion of the trace (*b*) illustrates the typical variability in the amplitude of the Ca_i^{2+} spiking events induced by thrombin, which complicated detailed analysis of the pattern of Ca_i^{2+} elevations in these single platelets. This type of repetitive Ca_i^{2+} spiking response was induced by thrombin in the majority of cells tested (15/17); the remaining two platelets displayed a single transient elevation of $[Ca^{2+}]_i$ upon application of thrombin (data not shown). The former group of platelets exhibited repetitive Ca_i^{2+} spiking following termination of the agonist application (duration 99 s to 5 min in different experiments) and continuous perfusion with agonist-free saline. These recordings lasted between 8 and 67 min total duration ($n = 15$), and therefore suggest that thrombin-evoked Ca_i^{2+} spiking in human platelets is not reversed by removal of exogenously applied ligand using bath perfusion. To further analyse the thrombin-induced Ca^{2+} spiking, the 340 nm/380 nm ratios above basal levels were integrated for two time periods: the duration of thrombin application and an

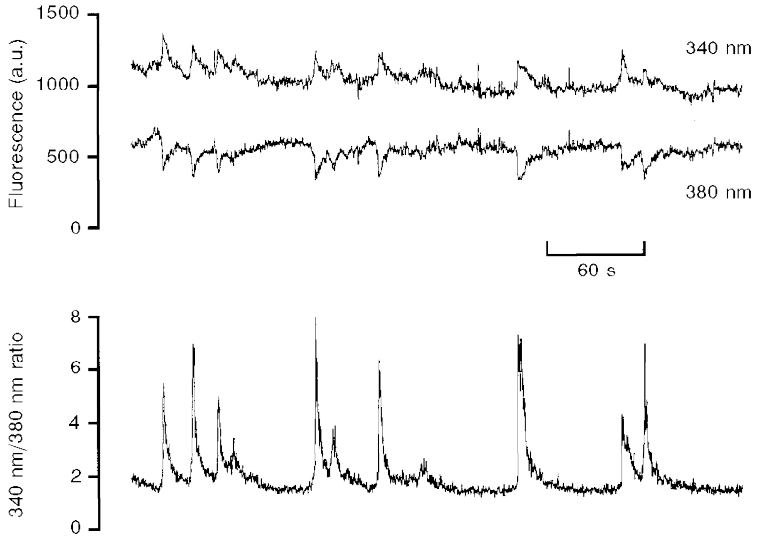

Figure 1. 'Contact' activation of Ca_i^{2+} spiking in a single human platelet

Fura-2 fluorescence signals from a single platelet immobilized at the tip of a glass pipette. The upper graph shows the background-corrected fluorescence signals at 340 and 380 nm excitation and the lower graph shows the 340/380 ratio. a.u., arbitrary units.

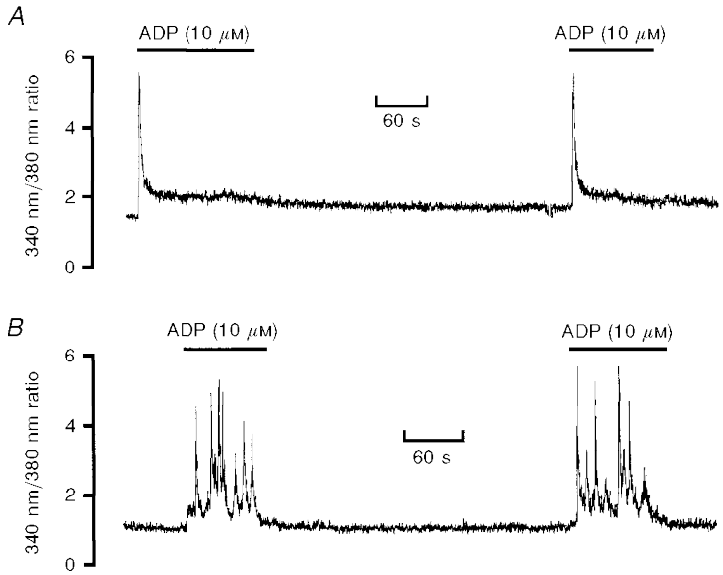

Figure 2. Reversible and repeatable ADP-dependent Ca_i^{2+} mobilization

Effect of ADP (10 μM) on $[Ca^{2+}]_i$ of a single isolated human platelet. The bars indicate timing of agonist application from a nearby puffer pipette. *A*, platelet in which the $[Ca^{2+}]_i$ increase occurred in the form of a single spike followed by a raised plateau level. *B*, platelet displaying repetitive $[Ca^{2+}]_i$ spikes in response to ADP.

equivalent time period at the end of the experiment. In experiments lasting at least 25 min, the integrated Ca^{2+} increase at the end of the experiment was $118 \pm 29\%$ ($n = 7$) of the integrated Ca^{2+} increase during thrombin exposure. Together, these data indicate that under the conditions used here, induction of Ca_i^{2+} spiking in human platelets by thrombin is essentially irreversible.

DISCUSSION

In the present study, we have used photometric recordings of fura-2 fluorescence to study changes in $[Ca^{2+}]_i$ in single human platelets immobilized at the tip of a glass pipette by gentle suction. Previous studies of single platelet Ca_i^{2+} responses have measured changes in fura-2 fluorescence from platelets immobilized onto either uncoated glass

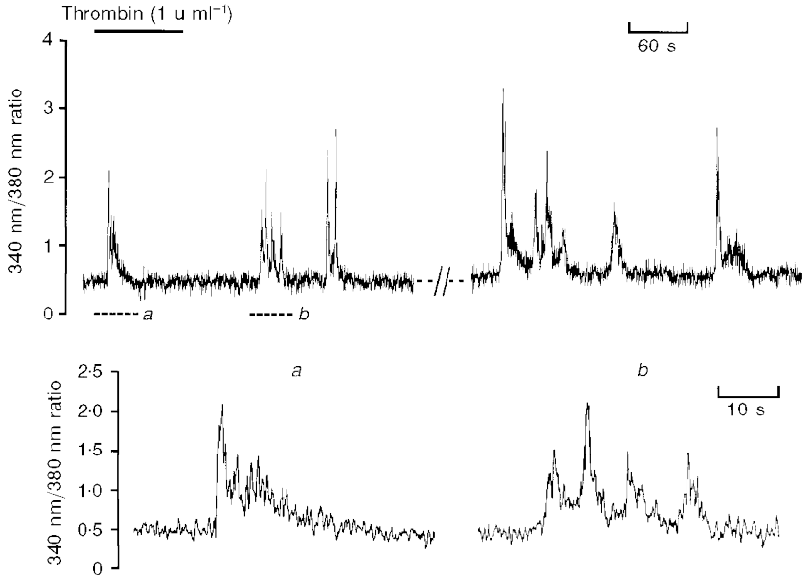

Figure 3. Continuous Ca_i^{2+} spiking evoked by thrombin in a single human platelet

Effect of thrombin (1 u ml^{-1}) application on $[Ca^{2+}]_i$ of a single fura-2-loaded human platelet. The upper graph shows two sections of the recording, the left trace during the initial application of thrombin, the right trace after a gap of 37 min. The lower graphs (*a* and *b*) show expanded portions of the upper left trace as indicated by the dashed lines.

coverslips (Hallam *et al.* 1986) or glass coverslips coated with various substances such as fibrinogen (Heemskerk *et al.* 1992, 1993, 1997), fibronectin (Ozaki *et al.* 1992), polyetheneimine (Nishio *et al.* 1991) or poly-L-lysine (Ariyoshi & Salzman, 1995). A significant problem with recordings from platelets adhered onto coated or uncoated glass coverslips is the presence of spontaneous Ca_i^{2+} spiking indicating activation either by the glass surface or the method of attachment, for example fibrinogen (Heemskerk *et al.* 1992). Unstimulated platelets can attach to immobilized fibrinogen via a process mediated primarily by glycoprotein IIbIIIa; however, the initial attachment is followed by a general spreading reaction and irreversible adhesion (Savage & Ruggeri, 1991). In the present study, 89% of platelets attached to the pipette tip showed no sign of contact activation of Ca^{2+} spiking, implying that our method of immobilization was able to overcome glass activation in the majority of platelets. Presumably this resulted from the relatively small surface area of glass in contact with the platelet when using pipette tips with diameters of less than 1 μm. Advantages of the methodology developed in this study are (i) long duration recordings of $[Ca^{2+}]_i$, in some experiments for more than an hour, which permit studies of the reversibility of Ca_i^{2+} responses in single platelets and (ii) isolation of individual platelets which allows responses to individual agonists to be monitored without complicating effects of paracrine signalling.

The Ca_i^{2+} spiking induced in a small proportion of platelets by immobilization at the tip of a glass pipette appeared to result from contact with the glass surface of the holding pipettes since the response was present in the absence of exogenously applied agonists and was not reversed by perfusion that resulted in the removal of other cells. Although platelets are well known to adhere to and become activated by glass surfaces (Hellem, 1960), the precise events that trigger glass-dependent activation are not understood. A large proportion of platelets (89%) isolated by gentle suction to a glass pipette lacked spontaneous Ca_i^{2+} events, and enabled an investigation of the effects of agonists in individual platelets. ADP (10 μM) induced Ca_i^{2+} mobilization in the form of either a single spike or multiple spikes of Ca_i^{2+} increase. Thrombin also evoked a single Ca_i^{2+} spike in two platelets; however, in the majority of cells (15/17), repetitive Ca_i^{2+} spiking was evoked by this protease. Both thrombin and ADP were used at maximally activating concentrations, suggesting that thrombin is more effective at inducing Ca_i^{2+} spiking than ADP. The different Ca_i^{2+} spiking patterns of individual platelets may represent true single platelet heterogeneity as suggested by other workers (Hallam *et al.* 1986; Tao *et al.* 1996) due, for example, to different receptor densities or variability in Ca^{2+} homeostatic mechanisms. Alternatively, variations in the level of fura-2 loading may account for the single cell heterogeneity due to different levels of cytosolic Ca^{2+} buffering.

The data presented here indicate an important difference in the single cell Ca^{2+} response to ADP compared with thrombin. ADP-induced Ca_i^{2+} responses were completely reversible and repeatable, whereas the Ca_i^{2+} spiking response to thrombin continued following termination of application and saline perfusion. Reports in the literature indicate that ADP stimulates at least three distinct receptors in platelets: (i) an ionotropic $P2X_1$ receptor (MacKenzie *et al.* 1996), (ii) a metabotropic $P2Y_1$ receptor that is coupled to phospholipase-Cβ and (iii) a second G-protein-coupled receptor coupled to inhibition of adenylate cyclase (Daniel *et al.* 1998). Activation of $P2Y_1$, rather than $P2X_1$, purinoceptors is likely to account for the $[Ca^{2+}]_i$ elevations observed in the present study, since removal of extracellular Ca^{2+} had little effect on the responses (J. F. Hussain & M. P. Mahaut-Smith, unpublished observations). The reversible nature of the platelet Ca_i^{2+} response to ADP is a property predicted from studies of P2Y receptors in other cells, including megakaryocytes (Uneyama *et al.* 1993). The thrombin receptor belongs to the family of protease-activated receptors (PAR) and human platelets possess PAR 1 (Vu *et al.* 1991), PAR 3 (Ishihara *et al.* 1997) and PAR 4 (Xu *et al.* 1998). Thrombin acts by cleaving the receptor to create a tethered ligand and therefore PAR receptors can only be activated once by the protease (Vu *et al.* 1991; Brass, 1997). In cell lines, thrombin receptors shut off soon after activation, at least partly as a result of receptor phosphorylation (Ishii *et al.* 1993, 1994). Certain cells, for example endothelial cells, have a large reserve of intracellular thrombin receptors that can rapidly replenish those activated on the surface membrane (Brass, 1997); however, platelets lack internally stored thrombin receptors (Molino *et al.* 1997). Approximately 40% of thrombin receptors remain on the platelet surface following activation (Molino *et al.* 1997), and may account for the persistent activation of Ca^{2+} spiking in our experiments provided that receptor phosphorylation is limited. A possibility to bear in mind is that platelet–platelet interactions may be required for phosphorylation and such interactions would be prevented under our experimental conditions. Alternatively, it is possible that the initial exogenous application of agonist does not activate the entire thrombin receptor population and that following saline perfusion thrombin remains bound to the platelet membrane and is capable of activating further receptors. These issues require further investigation using thrombin receptor agonists and antagonists which are currently being developed (Brass, 1997). However, regardless of the mechanism, platelets appear to possess a signalling pathway coupled to Ca^{2+} mobilization that can be persistently activated by thrombin.

In conclusion, we have isolated single fura-2-loaded human platelets at the tip of a glass pipette and measured spiking of $[Ca^{2+}]_i$ in response to contact- and agonist-induced activation using fluorescence photometry. Long-term Ca_i^{2+} spiking was observed following activation by glass contact and by thrombin, which could not be reversed by saline

perfusion. In contrast ADP-induced $[Ca^{2+}]_i$ responses were completely reversed following removal of agonist and were fully repeatable. The clear difference in the reversibility of $[Ca^{2+}]_i$ spiking to ADP and thrombin may reflect the different roles played by these agonists during haemostasis.

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