Biochem. J. (1992) 283, 379–383 (Printed in Great Britain)

Spiking in cytosolic calcium concentration in single fibrinogen-bound fura-2-loaded human platelets

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Fura-2-loaded human platelets were immobilized on a fibrinogen-coated surface and the cytosolic free calcium concentration ($[Ca^{2+}]_i$) was measured in single platelets by low-light-level video-ratio image-processing of the optical probe signal. Some fibrinogen-bound platelets showed repetitive spiking in $[Ca^{2+}]_i$ with a mean frequency of about 2/min, which increased to 5/min in the presence of ADP. Other cells showed no activity until the addition of agonist. When immobilized in the presence of prostaglandin I_2 and the fibrinogen antagonist Arg-Gly-Asp-Ser, the platelets adhered less firmly to fibrinogen, and in many $[Ca^{2+}]_i$ remained low and constant. Subsequent activation of such platelets with ADP evoked oscillations in $[Ca^{2+}]_i$ with a peak frequency of about 5/min and which persisted for at least 5 min. These results indicate that human platelets, like many other non-excitable cells, have an elaborate system of calcium signalling involving spiking.

INTRODUCTION

The elevation of cytosolic free calcium concentration ($[Ca^{2+}]_i$) plays a pivotal messenger role in platelet activation [1–3]. On stimulation with physiological agonists like ADP and thrombin, platelets in suspension show a rapid increase in $[Ca^{2+}]_i$ to about 10 fold the resting level. This increase results from mobilization of Ca^{2+} from internal stores as well as receptor-mediated influx of Ca^{2+} from the surrounding medium (e.g. [2]).

Stimulation of many other non-excitable cells, including macrophages, hepatocytes, endothelial and pancreatic cells, results in elevated $[Ca^{2+}]_i$. When the responses of individual cells of these types were analysed, the elevations in $[Ca^{2+}]_i$ were resolved as repetitive spikes or oscillations. Such oscillatory fluctuations in $[Ca^{2+}]_i$ are usually found after the occupation of receptors which act via the phosphoinositidase C signalling pathway [4]. Knowing the importance of phosphoinositidase C for signal transduction in platelets, this raises a question as to the organization of the calcium response in individual cells of this type. To date, $[Ca^{2+}]_i$ has been examined in single platelets in only a few recent studies. Flow-cytometric analysis has revealed heterogeneity in calcium responses evoked by ADP and thrombin in cell populations [3,5]. Preliminary microscopic-imaging studies of fura-2-loaded glass-bound human platelets suggested once-only elevations in $[Ca^{2+}]_i$ evoked by ADP and thrombin in most cells, and no clear oscillations were seen [6,7]. However, a recent imaging study using rabbit platelets has demonstrated oscillations in $[Ca^{2+}]_i$ evoked by 5-hydroxytryptamine (5-HT) [8].

Earlier attempts to study $[Ca^{2+}]_i$ in single human platelets were complicated by activation before agonist addition when the cells contacted glass. Here we report studies in which fura-2-loaded platelets were first allowed to bind to a natural substrate, fibrinogen, and then stimulated with ADP or thrombin. Most platelet agonists evoke the activation of fibrinogen receptors [1,9], which are complexes of glycoproteins IIb and IIIa. Increased activation of fibrinogen-binding sites on the platelet surface is one of the earliest responses upon activation, and the interaction of platelets with fibrinogen is thought to be the initial step in aggregate formation and thereby the generation of the haemostatic plug. The attachment of platelets to fibrinogen thus provides a physiologically appropriate means of immobilization for study by digital video calcium imaging.

EXPERIMENTAL

Platelet preparation

Human platelet-rich plasma was prepared as previously described [10], and the cells were loaded with fura-2 by incubation with fura-2 acetoxymethyl ester (3 μM) in the presence of aspirin (100 μM) and apyrase (20 μg/ml) for 1 h at 37 °C. After centrifugation, the cells were resuspended in a buffer containing 145 mM-NaCl, 10 mM-glucose, 10 mM-sodium Hepes, 5 mM-KCl, 1 mM-$MgCl_2$ and 1% (w/v) BSA (pH 7.4 at 37 °C). To prevent activation during immobilization, the following inhibitors were added: apyrase (20 μg/ml) to remove traces of ADP, prostaglandin I_2 (5 μM) to increase cyclic AMP levels, and the fibrinogen/fibronectin antagonist Arg-Gly-Asp-Ser (RGDS) (10 μM). In the initial experiments, prostaglandin I_2 and RGDS were absent. The platelet density was about 5×10^8/ml.

Platelet immobilization and activation

A glass coverslip (22 mm diameter; Horwell, London, U.K.) was cleaned with ethanol/2 M-HCl (3:1, v/v) and then coated with fibrinogen (10 mg/ml in saline) for 1 h at room temperature. The coverslip was washed with buffer (composition as above) containing apyrase (20 μg/ml) and 10 μM-RGDS and mounted in an open perfusion chamber (thermostatically controlled at 37 °C) which was placed on an inverted microscope (Nikon Diaphot). Fura-2-loaded platelets (1 ml of suspension) were allowed to settle on the coating for 10 min, after which unattached cells were removed by rapid washing with 5 ml of buffer. The immobilized platelets were then perfused at a rate of 0.5 ml/min with buffer containing either EGTA (1 mM) or $CaCl_2$ (1 mM). After 2 min of recording, activation was started by the addition of 1 ml of agonist-containing buffer, after which perfusion was continued with the same agonist-containing medium.

Abbreviations used: RGDS, Arg-Gly-Asp-Ser; 5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, cytosolic free calcium concentration.

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Video-imaging

Fura-2 fluorescence was measured with a Magical video-imaging system (Joyce–Loebl, Gateshead, Tyne and Wear, U.K.), interfaced to a low-light-level CCD (charged coupled device) detector (Photonic Sciences, Robertsbridge, Sussex, U.K.), operating at a video frame rate of one image every 40 ms. A computer-controlled filter wheel alternated filters of 340 and 380 nm (10 and 13 nm half-bandwidths, respectively) into the light path, and light was collected through a 440 nm dichroic long-pass filter and finally through a 510 nm band-pass filter (40 nm half-bandwidth). Nikon × 40 and × 100 quartz oil-immersion objectives were used, and the light path contained quartz throughout the u.v.-passing sections. Images were hardware-averaged in real time to reduce noise, and the average was digitized to 256 grey levels with a fast 8-bit analogue-to-digital converter. Images were then passed into a 32 Mb image memory and could be reconstructed for analysis of temporal changes. 256 × 256 pixel images were employed, and individual wavelength images for fura-2 were divided (340/380 nm) to provide a high-resolution ratio image updated every 0.8 s. By entering a calibration look-up table (using the formula of Grynkiewicz *et al.* [11]), a calibrated calcium image was obtained at each time point. These could be analysed by defining a geometric region around individual cells or a field of cells to produce a graph of ionized calcium as a function of time (for further details, see [12–14]).

We employed calibration parameters obtained on a number of other cell types for minimum and maximum fluorescence at low and high Ca^{2+} values evoked by exposing fura-2-loaded cells to either 10 mM-EGTA or 1 mM-Ca^{2+} in the presence of 2 μM-ionomycin. Despite the fact that we used quartz neutral-density filters to reduce excitation light and thus reduce bleaching, we could not reliably calibrate signals from dye loaded into platelets, presumably because of the low dye content of these small cells. However, the parameters used for calibration are nearly identical for most animal cell types studied with this equipment. Furthermore, they gave estimates of resting and peak $[Ca^{2+}]_i$ in most experiments similar to those determined in population studies using a spectrofluorimeter.

Materials

Fibrinogen (type IV), thrombin (both from bovine plasma), prostaglandin I_2, ADP and BSA were obtained from Sigma (Poole, Dorset, U.K.). RGDS was from Bachem (Bubendorf, Switzerland). Other chemicals were of reagent grade.

RESULTS

Single fura-2-loaded fibrinogen-bound platelets often showed repetitive spikes of elevated $[Ca^{2+}]_i$ (Fig. 1*a*), not unlike the fluctuations observed in other non-excitable cells [4,8,15–17]. There was noticeable heterogeneity in $[Ca^{2+}]_i$ transients between individual platelets (Fig. 1). The mean frequency of peaks was 1.7 ± 0.3/min (S.E.M., $n = 17$). This calcium response suggested that many of the immobilized platelets were already in an activated state, which was confirmed by their spread shape on the fibrinogen. Nevertheless, these platelets responded to ADP, as 40 μM-ADP increased the frequency of $[Ca^{2+}]_i$ spikes to 5.1 ± 0.8/min (S.E.M., $n = 8$ platelets). The peak amplitude of the spikes was not noticeably changed by ADP (Fig. 1*a*). Some cells appeared unactivated before the addition of ADP, which evoked spikes in $[Ca^{2+}]_i$ (Fig. 1*b*). As far as could be analysed at the levels of resolution possible in these experiments, elevations in $[Ca^{2+}]_i$ occurred homogeneously throughout the whole cell (results not shown).

We tried to change the immobilization conditions so that the platelets bound to fibrinogen with minimal or no activation. This was achieved by the addition during platelet settlement of prostaglandin I_2, which inhibits platelet responses and fibrinogen-receptor activation by increasing platelet cyclic AMP, and the tetrapeptide RGDS, a fibrinogen-receptor antagonist [18,19]. The RGDS concentration was found to be critical: 20 μM prevented adherence of almost all of the platelets to the fibrinogen coating, whereas 10 μM gave a sufficient number of loosely bound cells. When perfused with buffer containing 1 mM-$CaCl_2$ and 10 μM-RGDS (but no prostaglandin I_2 or apyrase), in most platelets $[Ca^{2+}]_i$ remained low and constant for several minutes (Fig. 2*a*), although occasional spikes were seen in 15 out of 45 cells at a frequency of 1.6 ± 0.2/min (S.E.M., $n = 15$). In buffer containing 1 mM-EGTA and 10 μM-RGDS, again $[Ca^{2+}]_i$ spikes were mostly absent (Fig. 2*b*), and the number of adhered platelets tended to be lower. This probably reflected the known reduction in platelet binding to fibrinogen in the presence of Ca^{2+} chelators [1].

In both $CaCl_2$- and EGTA-containing media, ADP induced spikes in $[Ca^{2+}]_i$ in the presence of RGDS (Fig. 2). There was no substantial difference between responses in $CaCl_2$- and EGTA-containing media: spikes achieved similar peak concentrations and were generated for several minutes under both conditions. The spike frequency evoked by 40 μM-ADP was 3.7 ± 0.5/min (S.E.M., $n = 9$) in the absence of extracellular Ca^{2+} and 5.1 ± 0.9/min (S.E.M., $n = 10$) in its presence. This difference was not significant (Student t test, $0.5 > P > 0.1$). Under both con-

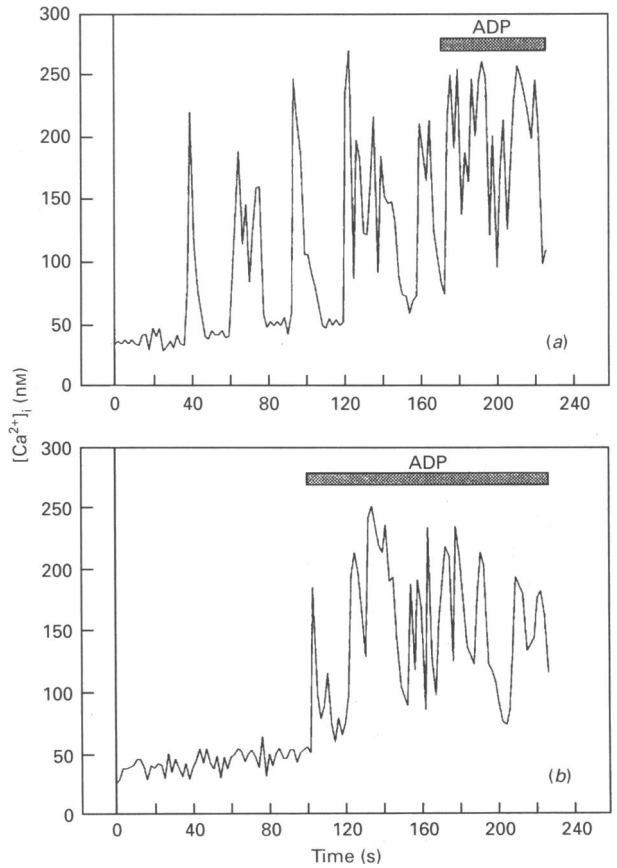

Fig 1. Oscillations in $[Ca^{2+}]_i$ in fibrinogen-bound platelets

Aspirin-treated fura-2-loaded platelets were allowed to bind to a fibrinogen-coated coverslip in the presence of apyrase (20 μg/ml). The immobilized platelets were perfused with buffer containing 1 mM-$CaCl_2$ (without apyrase); 40 μM-ADP was added as indicated. Representative data are shown for two platelets.

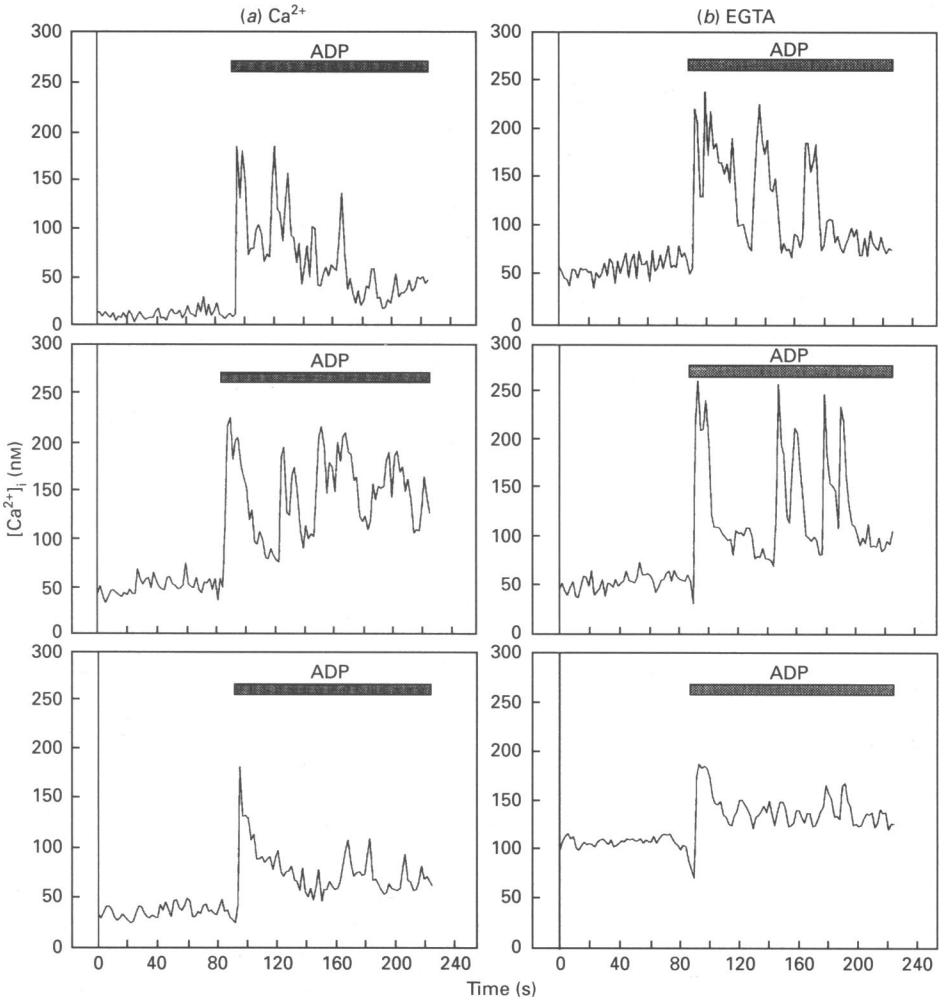

Fig 2. Oscillatory response in platelets bound to fibrinogen in the presence of inhibitors and activated by ADP

Aspirin-treated platelets were allowed to bind to a fibrinogen-coated coverslip in the presence of apyrase (20 μg/ml). 5 μM-prostaglandin I_2 and 10 μM-RGDS. Immobilized platelets were perfused with buffer (containing RDGS, but without apyrase or prostaglandin I_2) in the presence of either 1 mM-$CaCl_2$ ('Ca^{2+}'; *a*) or 1 mM-EGTA ('EGTA'; *b*). ADP (40 μM) was added as indicated. Representative data are shown as obtained in two platelets under each condition (upper four traces). Also shown is the signal for total fields of 14 (*a*) or 9 (*b*) platelets (lower two traces).

ditions, when the fluorescence signal from a whole field of cells was analysed, ADP was found to evoke a simple $[Ca^{2+}]_i$ transient, similar to that seen in a population of cells (Fig. 2, lower traces). This shows that the response of the cell population is an average of individual oscillatory responses. To mimic physiological conditions subsequent experiments were carried out in the presence of 1 mM extracellular $CaCl_2$.

The frequency of spiking was little influenced by the concentration of ADP. Spike frequencies were 4.6 ± 0.8 (S.E.M., $n = 9$) with 0.4 μM-ADP, 4.7 ± 0.7 (S.E.M., $n = 9$) with 4 μM-ADP and 5.1 ± 0.9 (S.E.M., $n = 10$) with 40 μM-ADP.

Thrombin-evoked responses of single platelets in the presence of RGDS differed from those evoked by ADP. Thrombin (0.1 unit/ml) evoked a relatively long-lasting elevation in $[Ca^{2+}]_i$ and, again, some heterogeneity between individual platelets was seen (Fig. 3). In some cells the plateau level of $[Ca^{2+}]_i$ was constant for several minutes (Fig. 3*a*), whereas in others $[Ca^{2+}]_i$ decreased back towards basal levels over tens of seconds (Fig. 3*b*). At a lower concentration (0.01 unit/ml), some evidence of thrombin-evoked oscillations was obtained, although this activity was not as clear-cut as that evoked by ADP (results not shown). Peak levels of $[Ca^{2+}]_i$ evoked by thrombin were similar to those evoked by ADP. Cells stimulated by ADP subsequently responded to thrombin (results not shown).

DISCUSSION

We report here a study of calcium signalling in single human platelets using digital fluorescence imaging. To fulfil the requirement of keeping the cells stationary during experimentation, we have investigated the responses of platelets immobilized by attachment to their physiological substrate, fibrinogen.

When platelets were allowed to attach to fibrinogen-coated coverslips in the absence of inhibitors, many cells appeared activated and showed spikes in $[Ca^{2+}]_i$. Fibrinogen itself is unlikely to activate platelets [1]. Thus the $[Ca^{2+}]_i$ transients observed in the absence of agonists were probably a consequence of contact activation with uncoated glass. After immobilization in the presence of prostaglandin I_2 and RGDS, activated spiking platelets were seen far less frequently. Examination of such preparations by scanning electron microscopy revealed discoid unactivated platelets as well as cells that had spread and developed small pseudopodia (J. W. M. Heemskerk, unpublished work). Although it cannot be ruled out that a selected population

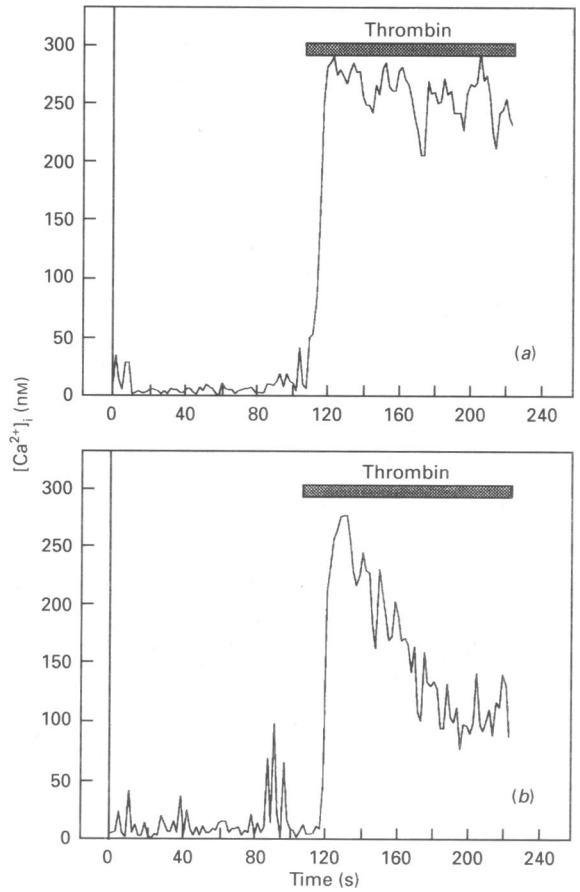

Fig 3. Elevations in $[Ca^{2+}]_i$ in platelets bound to fibrinogen and activated with thrombin

Aspirin-treated fura-2-loaded platelets were immobilized and perfused in the presence of 1 mM-$CaCl_2$ as described for Fig. 2. At the time indicated, thrombin (0.1 unit/ml) was added. Representative responses in two cells are shown.

of activated platelets adhered to the fibrinogen, the absence of spiking in the majority of cells, as well as the appearance of discoid platelets and the consistent agonist-evoked $[Ca^{2+}]_i$ responses, strongly suggests that many of the immobilized cells were unactivated before the addition of agonists.

In human platelets, ADP was able to evoke spiking in $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, indicating that the basis of the mechanism is release of Ca^{2+} from internal stores. The platelet oscillations continued for many minutes in the absence of external Ca^{2+}, whereas those in human umbilical-vein endothelial cells cease a few spikes after Ca^{2+} removal [20] and those in pancreatic cells may cease as soon as extracellular Ca^{2+} is removed [21]. This suggests that human platelets have substantial intracellular stores [22] and that much of the Ca^{2+} released by weak agonists is resequestered rather than lost across the plasma membrane.

Our results with human platelets contrast with the finding that 5-HT evoked oscillations in $[Ca^{2+}]_i$ in rabbit platelets in the presence, but not in the absence, of extracellular Ca^{2+} [8]. This may reflect the difference in the species or in the agonist. Rabbit, like rat platelets [22], may have much smaller mobilizable Ca^{2+} stores than human platelets.

Oscillations in $[Ca^{2+}]_i$ have been demonstrated in many types of non-excitable cell (e.g. [4]). The basis of oscillation is believed to be the periodic release of Ca^{2+} from intracellular stores, which is initiated by the agonist-evoked formation of Ins(1,4,5)P_3. The mechanisms which underlie the oscillations can be explained by several models, differing mainly in whether the concentration of Ins(1,4,5)P_3 is assumed to oscillate [23] or not [4]. Our results give no direct information as to the mechanism of oscillation in platelets and are compatible with both types of model. The observation that platelet $[Ca^{2+}]_i$ oscillates after activation with both ADP and low doses of thrombin (0.01 unit/ml) may be an indication that the spiking is generated by low levels of Ins(1,4,5)P_3, since low phosphoinositidase C activity has been reported in platelets activated with low concentrations of thrombin [24] as well as ADP [25]. However, some workers report no detectible Ins(1,4,5)P_3 production in ADP-stimulated human platelets [26], raising the possibility that oscillations in $[Ca^{2+}]_i$ may be generated in the absence of sustained Ins(1,4,5)P_3 production. Thrombin at higher concentrations is a potent activator of phosphoinositidase C in human platelets (e.g. [26,27]). The sustained rise in $[Ca^{2+}]_i$ evoked by thrombin at a concentration of 0.1 unit/ml may reflect this. Similarly, in other cell types, low doses of agonist may evoke oscillations in $[Ca^{2+}]_i$, whereas a high dose evokes a sustained plateau in elevated $[Ca^{2+}]_i$ (e.g. [20]).

In summary, we have demonstrated for the first time oscillations in $[Ca^{2+}]_i$ in fura-2-loaded human platelets bound to their natural substrate fibrinogen. The phenomenon of agonist-evoked oscillations in $[Ca^{2+}]_i$ observed in many non-excitable cell types also appears to play a role in signal transduction in these important cell fragments.

This work was supported in part by a grant from the Netherlands Organisation for Scientific Research (NWO) to J. W. M. H. We thank Applied Imaging International for financial assistance. S. O. S. held a Royal Society 1983 University Research Fellowship.

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Received 7 August 1991/22 October 1991; accepted 29 October 1991