Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion

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Cytoplasmic free $[Ca^{2+}]$, $[Ca^{2+}]_i$, was measured in human platelets using the intracellularly-trapped, fluorescent indicator quin2. Basal $[Ca^{2+}]_i$ with the Ca^{2+}-ionophore ionomycin revealed apparent thresholds for shape-change, 5-HT release and aggregation of approx. 0.5 μM, 0.8 μM and 2 μM. Thrombin raised $[Ca^{2+}]_i$ to 3 μM fast enough for the Ca^{2+} to have triggered the cell activation. However, thrombin released more 5-HT than ionomycin could, and in Ca^{2+}-free medium thrombin evoked shape-change and secretion even when $[Ca^{2+}]_i$ remained near basal levels throughout, suggesting the existence of alternative triggers for shape-change and secretory exocytosis.

Platelet *Calcium* *Fluorescent indicator* *Shape-change* *Secretion* *Aggregation*

1. INTRODUCTION

A rise in cytoplasmic free $[Ca^{2+}]$, $[Ca^{2+}]_i$, has been regarded as the final common pathway for the cytoskeletal rearrangement of platelet shape change, the secretion of granule contents and aggregation [1–4], though the evidence is largely indirect. Ca-ionophores can produce shape-change, secretion and aggregation (e.g., [4,5]) and micromolar $[Ca^{2+}]$ can cause secretion from platelets made permeable to Ca buffers by high voltage discharge [6,7], but actual measurements of $[Ca^{2+}]_i$ have not been previously achieved in such small cells. Here, we report measurements of $[Ca^{2+}]_i$ in human platelets, made with a new fluorescent indicator, quin2 [8,9] that can be trapped in the cytoplasm of populations of intact cells of any size. Responses to the Ca-ionophore ionomycin [5,10], were compared with those to the natural agonist thrombin in order to:

(i) Reveal approximate $[Ca^{2+}]$ thresholds for shape-change, secretion and aggregation;

(ii) Examine the $[Ca^{2+}]_i$ changes evoked by thrombin;

(iii) Assess how far thrombin acts via elevation in $[Ca^{2+}]_i$.

The most striking finding was that conditions could be found where thrombin activated the cells at or near basal $[Ca^{2+}]_i$, and even without any rise in $[Ca^{2+}]_i$.

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2. EXPERIMENTAL

Platelets were separated from freshly drawn, citrated plasma on a Sepharose 2B column equilibrated with: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 10 mM Hepes, 5 mM glucose, pH 7.55 at room temperature (pH 7.4 at 37°C). The gel-filtered platelets were incubated for 25 min at 37°C with 5 μM quin2 acetyoxymethyl ester, which permeates the cell membrane and is hydrolysed to regenerate and trap the hydrophilic quin2. The quin2 content was ~1 mmol/cell. The suspension was again gel-filtered to remove any extraneous dye, and the external $[Ca^{2+}]$ restored to 1 mM. Fluorescence was recorded at 37°C in a Perkin Elmer 44A fluorimeter. Quin2 shows a ~6-fold fluorescence enhancement on binding Ca^{2+}, the binding being 1:1, and the apparent K_d 115 nM (with 130 mM K^+, 20 mM Na^+, 1 mM free Mg^{2+}, pH 7.05) [9]. The $[Ca^{2+}]_i$ calibration procedure is detailed in [8,9]. The basic idea is to

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release the trapped quin2 into the medium at the end of the experiment and record the signal at known $[Ca^{2+}]$. The available evidence showed that as in lymphocytes [9] quin2 is very largely free in, and confined to, the cytoplasm and hence reports cytoplasmic $[Ca^{2+}]$. Digitonin (50 μM), which disrupts plasma membrane while leaving mitochondria largely intact, released all the dye indicating that there is no accumulation in these organelles. Nor did dye seem to get into secretory granules since thrombin-evoked discharge of granule contents did not release the intracellular quin2.

Shape-change and aggregation were assessed by a standard turbidimetric technique [11] at 37°C, stirring at 1100 rev./min. The fluorimeter cuvette was stirred only intermittently so the platelets did not aggregate and correlation between $[Ca^{2+}]_i$ measurement and aggregation responses is offered with caution. However, shape-change occurs with or without continuous stirring and correlations here should be valid. Release of 5-HT was measured in the fluorimeter cuvette simultaneously with quin2 fluorescence. Cells were loaded with 1.7 μM ^{14}C-labelled 5-HT (Amersham) along with the quin2. Imipramine (2 μM) was added just prior to stimulation to prevent re-uptake of secreted 5-HT. Triplicate 100μl aliquots were taken into ice-cold microtubes and immediately centrifuged at 14 000 × *g* for 40 s, and supernatant ^{14}C measured by scintillation counting.

3. RESULTS AND DISCUSSION

Fig.1 shows aggregometer and fluorescence responses to thrombin and ionomycin with 1 mM external $[Ca^{2+}]$, $[Ca^{2+}]_o$. A fully activating concentration of thrombin evoked a rise in $[Ca^{2+}]_i$ from the basal level near 100 nM to ~3 μM, fast enough to have triggered the shape-change and aggregation. Fig.1B shows that 50 nM ionomycin produced a very similar rise in $[Ca^{2+}]_i$ and also gave shape-change and substantial aggregation. Since the only significant action of ionomycin should be to translocate Ca^{2+}, it seems that the elevation of $[Ca^{2+}]_i$ to ~3 μM could largely account for the thrombin-evoked aggregometer responses. Also shown are responses to lower concentrations of ionomycin. 20 nM ionomycin raised $[Ca^{2+}]_i$ to ~800 nM and produced shape-change but no aggregation and 10 nM ionomycin raised $[Ca^{2+}]_i$ to ~500 nM and neither aggregation nor shape-change. A series of experiments of this type in platelets from several subjects indicated threshold for shape-change around 400–600 nM $[Ca^{2+}]_i$. For ionomycin to promote aggregation, a $[Ca^{2+}]_i$ of ~2 μM seemed to be required. This range of $[Ca^{2+}]_i$ is similar to the $[Ca^{2+}]$ levels required to stimulate myosin light-chain phosphorylation in digitonin-treated platelets [12].

The rises in $[Ca^{2+}]_i$ could be due to Ca^{2+} influx or internal release, and to assess this the agonists were applied with very low external $[Ca^{2+}]$ (~50 nM) to minimise or eliminate influx. Fig.2a shows that thrombin and ionomycin (50 nM) now gave rapid elevation of $[Ca^{2+}]_i$ to 200–300 nM demonstrating an internally releasable pool, but showing that at normal $[Ca^{2+}]_o$ (1 mM) most of the much larger response was due to triggered influx. Both agonists seemed to release the same Ca^{2+} pool since application of one after the other showed virtual occlusion of the fluorescence response (e.g., fig.2b and 3e). A Ca^{2+}-ionophore is expected to transport Ca^{2+} out of Ca-sequestering organelles such as dense tubular system. It has been suggested that thrombin might act via thromboxane A_2 [3] and we find that inhibiting thromboxane production with indomethicin or aspirin partially blocks this response to thrombin. (As we have discussed in [9] the presence of quin2 will reduce $[Ca^{2+}]_i$ transients produced by internal release. The $[Ca^{2+}]_i$ reached inside unloaded cells in low $[Ca^{2+}]$ solution, would be higher than the 200–300 nM seen here, and from the aggregometer responses we guess the peak $[Ca^{2+}]_i$ is ~500 nM.)

The striking feature of experiments like those shown in fig.2a is that although ionomycin expectedly fails to activate the cells with $[Ca^{2+}]_i$ rising to only 200–300 nM, thrombin gives shape-change and partial aggregation. The possibility that ionomycin exerts inhibitory effects seems unlikely since subsequent application of thrombin still produces the cellular responses. Fig.2b shows that thrombin could evoke the aggregometer responses of shape-change even while $[Ca^{2+}]_i$ remained below the normal basal level. These cells had been kept in Ca-free solution to deplete cellular Ca^{2+} and EGTA was added to chelate contaminating Ca^{2+}. Ionomycin raised $[Ca^{2+}]_i$ from 60–90 nM, showing there was still internally releasable Ca^{2+},

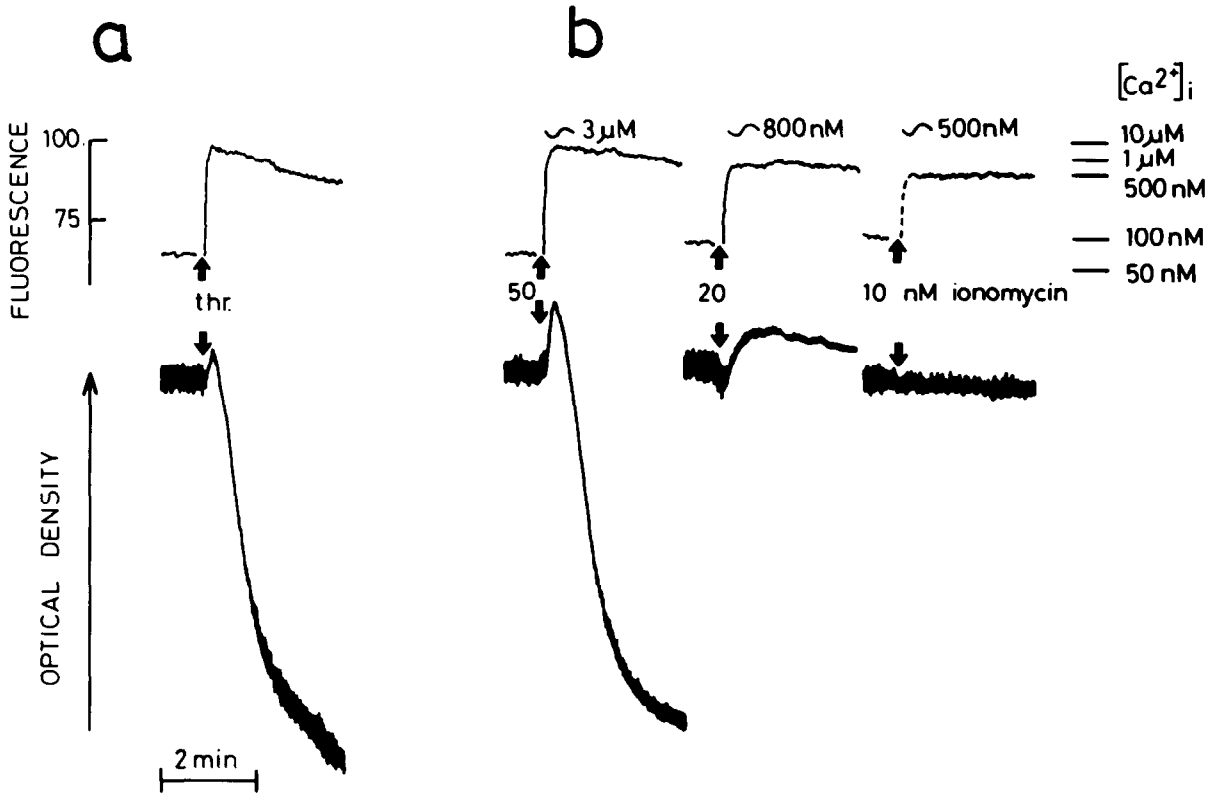

Fig.1. Quin2 fluorescence (upper trace) and aggregometer (lower trace) responses with 1 mM external Ca^{2+}, (a) to 0.4 units/ml human thrombin (Sigma) and (b) to different levels of ionomycin (Squibb) added from 10 μM DMSO stock, as indicated. The appropriate $[Ca^{2+}]_i$ calibration scale is shown at the right of the fluorescence records. Excitation was at 339 nm and emission at 500 nm. Gaps in the fluorescence record show periods for addition or stirring.

but evoked no aggregometer response. Addition of thrombin now gave no significant change in $[Ca^{2+}]_i$ but did stimulate shape-change. These findings suggest that thrombin can activate shape-change by Ca-independent pathways, or possibly by increasing the Ca-sensitivity of rate-limiting steps.

Fig.3 shows a similar experimental analysis of 5-HT secretion and $[Ca^{2+}]_i$. Panels a–c show corresponding $[Ca^{2+}]_i$ rises and 5-HT release in response to ionomycin. A peak $[Ca^{2+}]_i$ of 700 nM gave no release, 1.5 μM gave 40% and ~3 μM 68% release. These results are representative of 25 trials in cells from 3 subjects, which suggest an apparent $[Ca^{2+}]_i$ threshold of 0.7–1 μM for ionophore stimulated secretion, with substantial release at 3 μM. These data would fit reasonably well on the $[Ca^{2+}]$/secretion relation seen in platelets made permeable to Ca-buffers by high-voltage discharge [6,7]. In 1 mM Ca solution, our usual dose of thrombin, 0.4 units/ml, produced faster and more substantial 5-HT release, 90% by 15 s, than did 50 nM ionomycin, although the $[Ca^{2+}]_i$ rises were similar. Thrombin could also stimulate release at $[Ca^{2+}]$ levels that were ineffective when produced by ionomycin. Fig.3e shows the response at 1 mM $[Ca^{2+}]_o$ to a bath of thrombin of reduced potency. $[Ca^{2+}]_i$ rose to only 420 nM, but there was still 90% release. Even at very low $[Ca^{2+}]_o$, as in fig.3d, with $[Ca^{2+}]_i$ reaching only 250 nM there was still 70% release. In 8 such tests the stimulated $[Ca^{2+}]_i$ was 200 ± 22 (SE) nM with 65 ± 6 (SE) % release. By contrast, as shown in panel f, ionomycin produces no secretion from cells in low $[Ca^{2+}]_o$. But

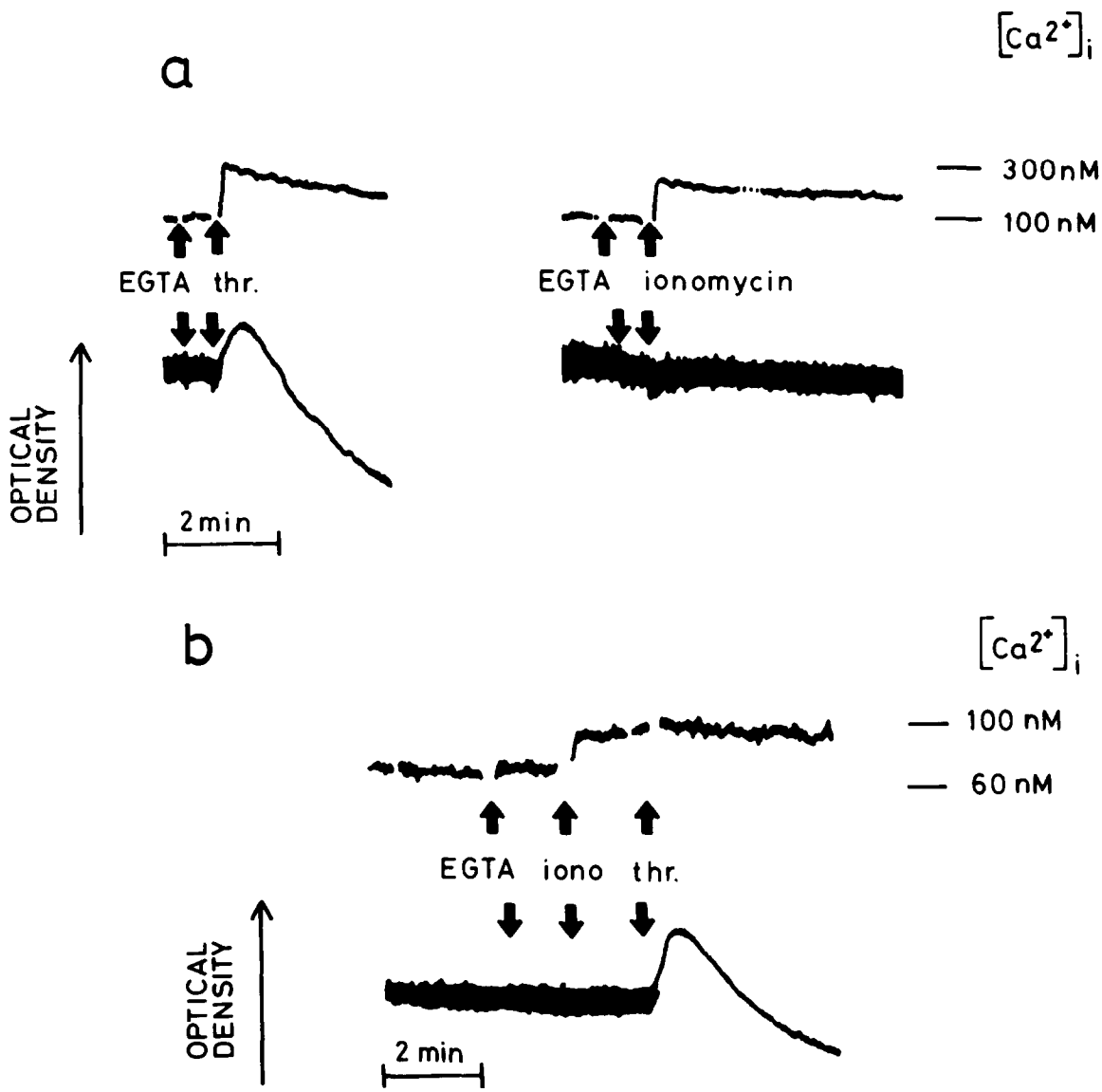

Fig.2. Fluorescence (upper traces) and aggregometer responses to 0.4 units/ml thrombin and 50 nM ionomycin in very low external $[Ca^{2+}]$: (a) 2 mM EGTA was added (with 2 mM Tris base to prevent acidification) to reduce $[Ca^{2+}]_o$ to 50 nM; (b) platelets were kept in nominally Ca-free solution throughout. 1 mM EGTA was added as indicated to chelate contaminating Ca^{2+}.

subsequent addition of thrombin evoked 70% release without further changing the $[Ca^{2+}]_i$. These findings show that $[Ca^{2+}]_i$ in the micromolar range is an effective stimulus for 5-HT secretion in intact platelets, but it seems that, as with shape-change, thrombin may be additionally able to trigger secretory exocytosis by as yet unidentified Ca-independent pathways.

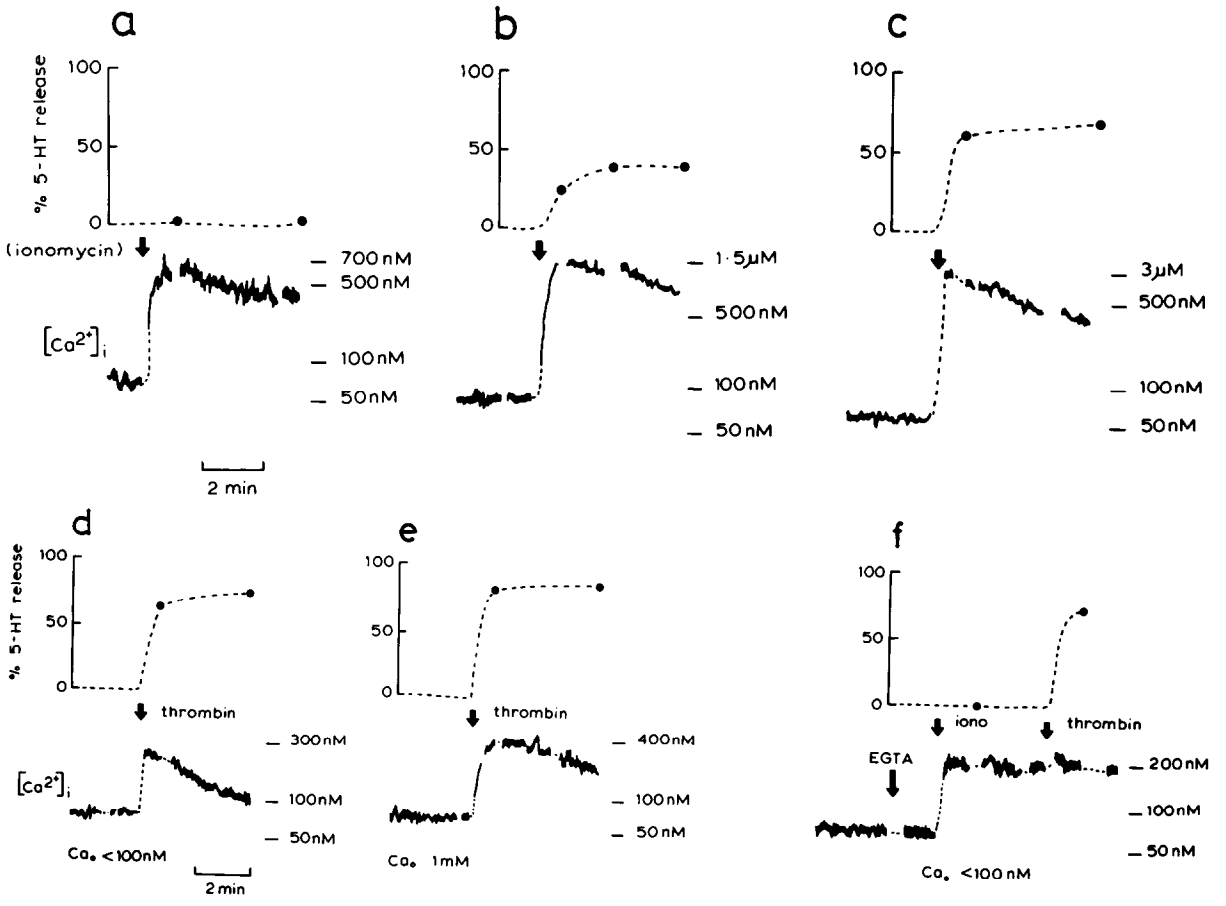

Fig.3. $[Ca^{2+}]_i$ and 5-HT secretion expressed as % release of 5-[^{14}C]HT in the cells just prior to stimulation (a) 35 nM ionomycin added; (b,c) 50 nM ionomycin with 1 mM $[Ca^{2+}]_o$ (The different $[Ca^{2+}]_i$ transients and secretions in (b,c) probably result from the difficulty in precisely matching application of hydrophobic reagents, and the apparent steepness of the ionomycin dose–response relation in this region); (d,e) responses to thrombin in very low, and normal $[Ca^{2+}]_o$; (f) shows responses first to 50 nM ionomycin and then 0.4 units/ml thrombin with $[Ca^{2+}]_o$ lowered to 50 nM. The dotted lines are drawn for guidance only.

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