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Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin

Dual-wavelengths studies with Mn^{2+}

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Previous studies of the early kinetics of rises in cytosolic free [Ca^{2+}] in fura-2-loaded human platelets suggested that: (1) Ca^{2+} entry slightly preceded internal discharge with thrombin and other agonists known to promote inositol lipid hydrolysis; (2) with ADP, Ca^{2+} entry occurred without measurable delay and clearly preceded internal Ca^{2+} discharge. In the present work, Mn^{2+} added to the external medium was used as a marker for Ca^{2+} entry. By using an excitation wavelength of 360 nm, a quench of fura-2 can be followed to report Mn^{2+} entry without 'contamination' of the signal by changes in [Ca^{2+}], because at this isosbestic wavelength Ca^{2+} does not alter fura-2 fluorescence. The present results show that, with thombin stimulation, readily discernible Mn^{2+} entry starts after discharge of internal Ca^{2+} and is maintained for many minutes. With ADP, Mn^{2+} entry starts without measurable delay (< 20 ms) and clearly precedes internal Ca^{2+} discharge. However, the enhanced Mn^{2+} permeability is only short-lived. These results, considered alongside previous data, point to the possible presence of at least three different receptor-mediated Ca^{2+}-entry mechanisms in human platelets, one of which may include regulation by the 'state of filling' of this dischargeable Ca^{2+} store.

INTRODUCTION

Stimulation of platelets by a number of different agonists results in a rise in $[Ca^{2+}]_i$ that appears to be due to both discharge of the internal stores and influx across the plasma membrane [1–4]. Stopped-flow fluorimetric studies of fura-2-loaded platelets have demonstrated that thrombin, vasopressin, platelet-activating factor and a prostaglandin endoperoxide (U44619) stimulate a rise in $[Ca^{2+}]_i$ with a delay of 180–250 ms, even at very high agonist concentrations [5,6]. The delay is 50–100 ms longer when Ca^{2+} influx is prevented either by chelation of external Ca^{2+} with EGTA or by blockade with extracellular Ni^{2+} [6]. These findings suggested that Ca^{2+} influx precedes the discharge of internal Ca^{2+} stores, but could not exclude the possibility that the absence of external Ca^{2+}, or its displacement by Ni^{2+}, interferes with ligand–receptor binding or subsequent signal transduction, and so artifactually delays store discharge. The difference in time courses of $[Ca^{2+}]_i$ rises evoked by ADP in the presence and absence of external Ca^{2+} was much more marked than with the other agonists; Ca^{2+} influx could occur without measurable delay (< 20 ms), whereas the internal release followed a time course similar to that seen with the other agonists. Again the question arises of possible artifactual consequences of chelation of external Ca^{2+} or addition of Ni^{2+}.

We have therefore investigated further the processes of Ca^{2+} mobilization in human platelets in nominally Ca^{2+}-free solutions without addition of EGTA and, more importantly, by using Mn^{2+} as an indicator of bivalent-cation influx. Both quin2 and fura-2 have high affinities for this ion, and their fluorescence is quenched by Mn^{2+} binding. When Mn^{2+} is added extracellularly, an agonist-stimulated quenching of the intracellular dye fluorescence can be observed and can be attributed to stimulated influx of Mn^{2+} [7–9]. We have previously shown that platelet agonists evoke Mn^{2+} influx in quin2- and fura-2-loaded platelets [8,9]. In previous platelet studies Mn^{2+} entry was monitored by recording fluorescence with 340 nm excitation, where Mn^{2+} decreased and Ca^{2+} increased the signal. However, with excitation at 360 nm fura-2 emission is independent of [Ca^{2+}], but fluorescence is still quenched by Mn^{2+}. The use of excitation at 360 nm thus allows the selective study of Mn^{2+} entry without interference in the signal caused by changes in $[Ca^{2+}]_i$ [10]. We report here studies of fura-2-loaded platelets, using excitation wavelengths of 340 nm and 360 nm measured simultaneously over 10 min time courses or separately over 5 s time courses.

EXPERIMENTAL

Platelets were prepared from freshly drawn human blood and loaded with fura-2 as previously described [6], and resuspended in a medium containing 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes and 10 mM-glucose, pH 7.4 at 37 °C. Hirudin (0.05 unit/ml) and apyrase (20 μg/ml) were added to prevent activation by residual traces of thrombin or ADP. This buffer is 'nominally Ca^{2+}-free', i.e. no $CaCl_2$ or chelator was added; 1 mM-$CaCl_2$, 1 mM-$MnCl_2$ and 4 mM-$NiCl_2$ were added as required. For measurement over several minutes, fluorescence was measured in a Spex dual-excitation-wavelength fluorimeter (Glen Creston Instruments, Stanmore, Middx., U.K.) The excitation wavelengths were 340 and 360 nm, with emission at 500 nm. Samples (0.7 ml) of cells were dispensed into cuvettes mounted

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Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DTPA, diethylenetriaminepenta-acetic acid.

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in a thermostatically controlled (37 °C) holder in the fluorimeter. The suspension was mixed with a plastic Pasteur pipette after each addition.

For measurement of sub-second kinetics, the fluorescence from fura-2-loaded platelets was recorded as previously described [5,6] by using a Hi-Tech Scientific SFA-II Rapid Kinetic Accessory (Hi-Tech Scientific, Salisbury, Wilts., U.K.) mounted in a Perkin–Elmer MPF-44A spectrophotometer, thermostatically controlled at 37 °C. Dye-loaded cells with or without added $CaCl_2$ were injected through one port and solution containing agonist, $MnCl_2$ and $NiCl_2$ (as required) through the other. Traces show the average of ten scans recorded at 15 s intervals. In each case, the record shown is typical of those from at least three independent cell preparations.

Fura-2 acetoxymethyl ester was from Molecular Probes, Junction City, OR, U.S.A.; ADP, apyrase, aspirin, DTPA, hirudin and bovine thrombin were from Sigma, Poole, Dorset, U.K. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Dual-excitation-wavelength fluorimetry

Fig. 1 shows simultaneous recording of fluorescence (500 nm emission) with excitation at 340 and 360 nm from fura-2-loaded platelets suspended in nominally Ca^{2+}-free medium. Addition of $MnCl_2$ initially caused a small step decrease in fluorescence at both excitation wavelengths. This step decrease is due to leaked extracellular fura-2, confirmed by use of the membrane-impermeant heavy-metal chelator DTPA, to remove extracellular Mn^{2+}, and has therefore been subtracted from each trace for clarity. The slow decline in fluorescence at both excitation wavelengths is due to a slow basal leak of Mn^{2+} into the cells and shown by testing for dye leakage with external DTPA. Addition of ADP (20 μM) or thrombin (1 unit/ml) caused a rapid increase in fluorescence at 340 nm, presumably owing to discharge of Ca^{2+} from intracellular stores, followed by a decline in fluorescence to well below the baseline. Both agonists also evoked a fall in fluorescence with excitation at 360 nm. This fall is due to the quenching of intracellular dye by a stimulated entry of Mn^{2+}, and not to the loss of dye from the cells again shown by application of DTPA. The extent of quenching was greater with thrombin than with ADP, the thombin-evoked response having an extended slower phase lasting several minutes until almost all the fura-2 was quenched. Ionomycin, which readily translocates Mn^{2+}, thus caused little further quenching after addition of thrombin, but a large additional quench was seen when ionomycin was added after ADP.

The most likely explanation for differences in the apparent ation of stimulated Mn^{2+} entry is that the entry process is activated only briefly with ADP, but persists in the continued presence of thrombin. This point was tested by adding Mn^{2+} after the application of the agonist, as shown in Figs. 1(*c*) and 1(*d*). Here, 4 min after ADP, addition of Mn^{2+} caused almost no quench, in contrast with the result in Fig. 1(*d*), where Mn^{2+} added 4 min after thrombin led to a rapid quenching of signal almost to autofluorescence levels. Similar results were obtained with time intervals of 1–6 min. The brief effects of ADP may reflect receptor desensitization. The ability of thrombin to maintain an enhanced entry for bivalent cations may result from continued, non-desensitized, signal transduction, or perhaps the introduction of a long-lived Ca^{2+}-entry mechanism into the membrane.

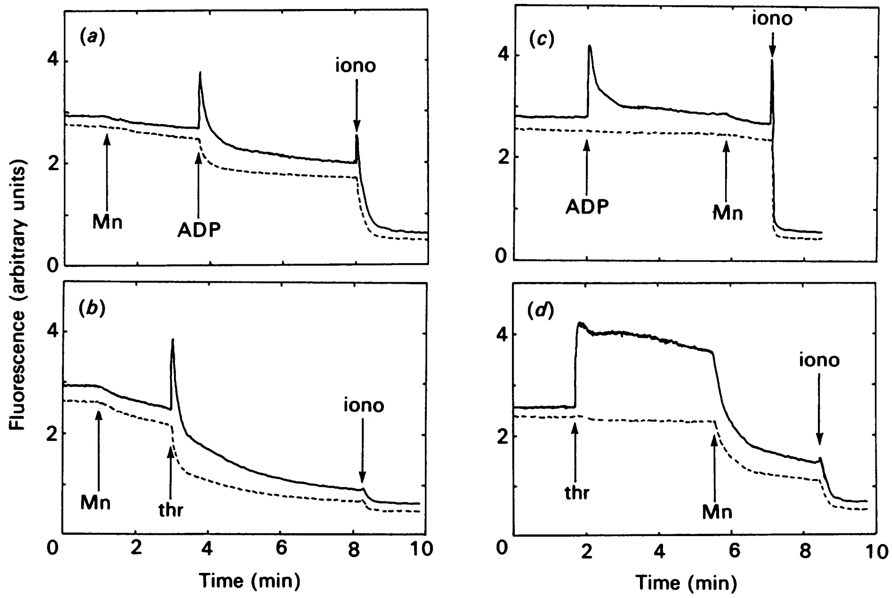

Fig. 1. Effect of ADP and thrombin on fluorescence of fura-2-loaded platelets in the presence of $MnCl_2$ (*a* and *b*) or of adding Mn in the presence of ADP or thrombin (*c* and *d*)

Fura-2 fluorescence (500 nm emission) was measured simultaneously with excitation at 340 nm (——) and 360 nm (----). Platelets were suspended in nominally Ca^{2+}-free medium, and the following additions were made as indicated: 1 mM-$MnCl_2$, 20 μM-ADP, 1 unit of thrombin/ml (thr), 2 μM-ionomycin (iono). At the end of the experiment, DTPA (2 mM) was added to chelate extracellular Mn^{2+}, and the step change in fluorescence was removed in order to correct for effects of Mn^{2+} on leaked extracellular fura-2.

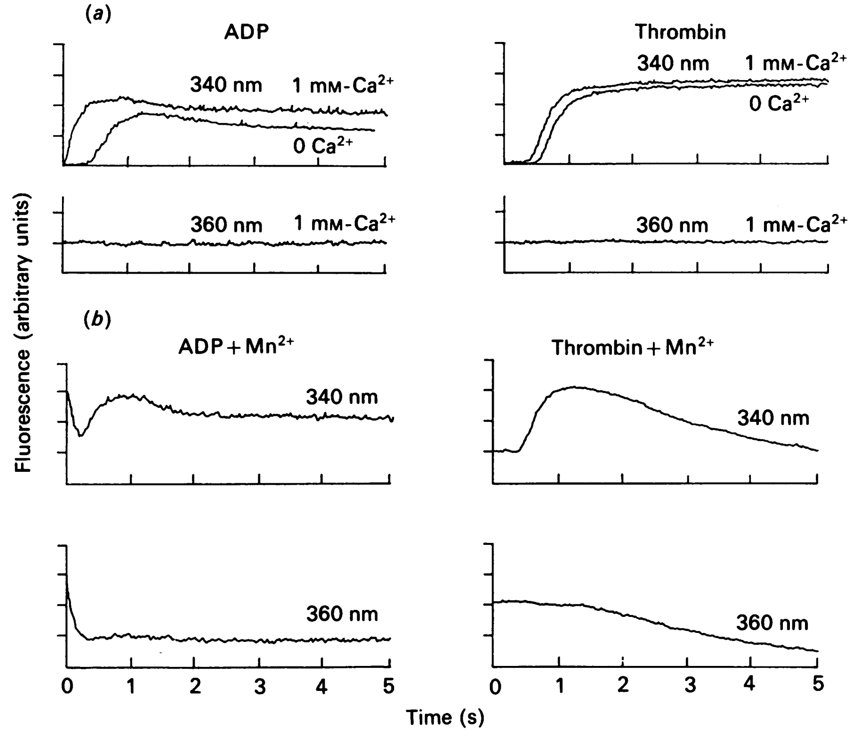

Fig. 2. Stopped-flow fluorimetry of fura-2-loaded platelets stimulated with 40 μM-ADP or thrombin (4 units/ml) in the presence and absence of external Ca^{2+} (*a*) or in the presence of 1 mM-$MnCl_2$ (*b*)

Platelets were suspended either in nominally Ca^{2+}-free medium or in medium containing 1 mM-Ca^{2+}, as indicated, and mixed with agonist at zero time. Fluorescence (500 nm emission) was measured with excitation at 340 nm or 360 nm in separate runs, with the same batch of cells. For clarity, the 360 nm trace in the absence of Ca^{2+} is omitted (it was at flat and featureless as that in the presence of Ca^{2+}). (*b*) Platelets stimulated with 40 μM-ADP or thrombin (4 units/ml) in the presence of 1 mM-Mn^{2+}. Platelets suspended in nominally Ca^{2+}-free medium were mixed with $MnCl_2$ and agonist at zero time. Fluorescence was measured with excitation at 340 nm or 360 nm in separate tests. Controls, with Mn^{2+} but no agonist, were subtracted digitally from the records.

This latter possibility would fit with the recent report of a Ca^{2+} channel in thrombin-stimulated platelet membranes that survives cell fractionation [11,12].

Stopped-flow fluorimetry

The initial Mn^{2+} entry or elevation in $[Ca^{2+}]_i$ stimulated by ADP or thrombin were too fast to be resolved in stirred cuvettes. Fig. 2(*a*) shows stopped-flow traces of fura-2 fluorescence from platelets in the presence or absence of 1 mM external Ca^{2+}. With excitation at 340 nm, 40 μM-ADP evoked an immediate rise in fluorescence, commencing within the mixing time of 20 ms in the presence of external Ca^{2+}; in the absence of external Ca^{2+} the onset of fluorescence rise was delayed by over 200 ms. This response is indistinguishable from that seen when contaminating external Ca^{2+} is chelated with 1 mM-EGTA, and can be attributed to discharge of internal Ca^{2+} stores. With excitation at 340 nm, thrombin (4 units/ml) evoked fluorescence rises that were delayed in both the presence and the absence of external Ca^{2+}. The delay was about 280 ms in the presence of external Ca^{2+} and about 360 ms in its absence. As expected, there were no changes in fluorescence with excitation at 360 nm.

Fig. 2(*b*) shows stopped-flow traces of fura-2 fluorescence from platelets stimulated in the presence of Mn^{2+} (final concn. 1 mM). (2 mM-$MnCl_2$ was added only to the agonist suspension to avoid quenching of the cellular fluorescence caused by passive Mn^{2+} leak into the cells before mixing. Traces have been corrected for passive leak of Mn^{2+} by subtraction of control traces from experiments in which cells were mixed with Mn^{2+} but no agonist. This passive leak over the short time course of these experiments was small, at only a few per cent of the total signal). In Fig. 2(*b*), ADP stimulated an immediate fall in fluorescence with excitation at 360 nm; with excitation at 340 nm an immediate fall in fluorescence was followed by a delayed rise. The initial fall in fluorescence at both excitation wavelengths indicates a rapid stimulated entry of Mn^{2+}. The later rise in fluorescence with excitation at 340 nm is presumably due to the delayed discharge of the internal Ca^{2+} stores. Fig. 2(*b*) shows that thrombin evoked a delayed rise in fluorescence with excitation at 340 nm, which is due to the discharge of intracellular Ca^{2+} stores. The onset time of this response is similar to that in the absence of extracellular Ca^{2+}, but the post-peak decline in fluorescence is more rapid, owing to stimulated Mn^{2+} entry and quenching of the intracellular fura-2 signal. With excitation at 360 nm, thrombin evoked a delayed quench in fluorescence. This quench, owing to Mn^{2+} entry, showed two phases: a slow initial phase, which was not easily resolved but appeared to commence at or a little after the start of the stimulated $[Ca^{2+}]_i$ rise, and then a more rapid phase which commenced after the peak of the

$[Ca^{2+}]_i$ rise, presumably when discharge of the internal Ca^{2+} stores was complete. Interestingly, the maximum rate of Mn^{2+}-induced quench was larger with ADP, although the final extent of the quench, and thus the amount of Mn^{2+} influx, was greater with thrombin.

In further tests, 4 mM-Ni^{2+} added at the same time as Mn^{2+} abolished the fluorescence quenching, with excitation at 360 nm, evoked by ADP or thrombin, showing that Ni^{2+} was able to block Mn^{2+} entry (results not shown).

Conclusions

These results confirm and extend our earlier observations that platelet agonists are able to stimulate Mn^{2+} entry [8,9] and support the concept of receptor-mediated Ca^{2+} entry in these cells. The study of Mn^{2+} influx with excitation at 360 nm, where the signal does not change in response to changes in $[Ca^{2+}]_i$, indicates that ADP stimulates only a transient Mn^{2+} influx, perhaps owing to rapid desensitization. In contrast, thrombin evoked a sustained Mn^{2+} influx which could almost completely quench the intracellular fura-2 fluorescence, and Mn^{2+} added 1–6 min after thrombin produced a substantial quench, showing that the bivalent-cation entry system remained 'open'.

Analysis of the kinetics of early Mn^{2+} entry shows convincingly that ADP evokes an immediate decrease (within 20 ms) in fluoresence intensity with excitation at 340 nm owing to Mn^{2+} entry, followed by a later rise owing to discharge of internal Ca^{2+} stores. This result has important implications: first, it indicates that the removal of extracellular Ca^{2+} and its replacement with another bivalent cation does not disrupt ADP–receptor binding or subsequent signal transduction; second, it shows that ADP generates an increase in plasma-membrane bivalent-cation permeability which precedes the discharge of the internal Ca^{2+} stores. Ca^{2+} entry is thus not a consequence, direct or indirect, of internal Ca^{2+} discharge. The rapidity of ADP-evoked Mn^{2+} entry is consistent with our previous suggestion [5,6] that ADP-receptor occupancy is closely coupled to the opening of plasma-membrane bivalent-cation channels. It is worth emphasizing again the ability of ADP to evoke a substantial discharge of internal Ca^{2+}, despite its being a weak or even ineffective stimulus for generation of InsP_3 (for references and further comment see refs. [5,6]).

The inability of thrombin to evoke an immediate entry of Mn^{2+} was expected from our previous observations of delayed $[Ca^{2+}]_i$ rises evoked by this agonist [5,6]. The slow initial rate of thrombin-stimulated Mn^{2+} entry made it difficult to detemine the exact time of onset of this response, but it appeared to be at the same time as, or perhaps slightly after, the onset of the increase in $[Ca^{2+}]_i$ evoked by thrombin in the presence of external Ca^{2+}. From the difference in the $[Ca^{2+}]_i$ response in the presence and absence of external Ca^{2+} (Fig. 2 and ref. [6]), we concluded that there is an early component to Ca^{2+} entry during stimulation with thrombin. We would have therefore expected a corresponding early, if small, Mn^{2+} entry. The lack of any marked quench at this point could have at least three explanations: (i) the entry is too small to pick out from the background; (ii) a form of Ca^{2+} entry mechanism is activated that does not readily pass Mn^{2+}, as seen in rat parotid cells [9]; (iii) there is no early Ca^{2+} entry, and the effect of Ca^{2+} removal or addition of Ni^{2+} in adding to the delay in thrombin-evoked fluorescence rises is indeed an artifact. However, it is clear that Mn^{2+} entry stimulated by thrombin accelerated at a time when $[Ca^{2+}]_i$ had reached a peak. Similar kinetics of thrombin-stimulated Mn^{2+} influx were seen in endothelial cells, where the response is slow enough to measure in cuvette experiments [10]. This increased rate of Mn^{2+} influx occurs at a time when the intracellular Ca^{2+} stores are likely to be depleted, and suggests that the state of the stores influence bivalent-cation entry across the plasma membrane. Models for Ca^{2+} entry dependent on the state of the intracellular Ca^{2+} store have been proposed previously (e.g. [13–15]). Perhaps a similar mechanism provides most of the Ca^{2+} entry in thrombin-stimulated platelets. The early Mn^{2+} influx stimulated by ADP and any initial small influx stimulated by thombin, seen before discharge of the internal Ca^{2+} stores, must be due to a different mechanism, most likely a direct Ca^{2+} channel into the cytoplasm.

These results add to the evidence for diversity of mechanisms of receptor-mediated Ca^{2+} entry and suggest the possible presence of three different mechanisms in human platelets: (i) a close coupling of ADP receptors to Ca^{2+} entry direct into the cytosol, for which Mn^{2+} is an effective substitute; (ii) an early phase of thrombin-stimulated entry, possibly activated by diffusible second messengers, that passes Mn^{2+} only poorly; (iii) a later phase of thrombin-evoked entry that is promoted by emptying of the dischargeable intracellular Ca^{2+} pool, and may be via pathways from the external medium into the pool and thence to the cytosol.

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