Vol. 139, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 29, 1986 Pages 308-314

THROMBIN AND IONOMYCIN CAN RAISE PLATELET CYTOSOLIC Ca^{2+} TO MICROMOLAR LEVELS BY DISCHARGE OF INTERNAL Ca^{2+} STORES: STUDIES USING FURA-2

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Received July 14, 1986

SUMMARY: In the presence of 1 mM EGTA, the addition of the calcium ionophore ionomycin to human platelets loaded with 30 μM fura-2 could elevate $[Ca^{2+}]_i$ from less than 100 nM to a maximum of greater than 3 μM, presumably by discharge of Ca^{2+} from internal stores. Under the same conditions thrombin could maximally increase $[Ca^{2+}]_i$ to a peak of greater than 1 μM which then declined to near resting levels within 3-4 minutes; by contrast in platelets loaded with 1 mM quin2 thrombin could raise $[Ca^{2+}]_i$ to only about 200 nM. In the presence of 1 mM Ca^{2+} the peak response to thrombin in fura-2-loaded platelets was higher (1.4 μM) than that observed in the presence of EGTA (1.1 μM) and the elevation in $[Ca^{2+}]$ was prolonged, presumably by Ca^{2+} influx. These results with fura-2-loaded platelets indicate that mobilisation of internal Ca^{2+} can contribute a substantial proportion of the early peak $[Ca^{2+}]_i$ evoked by thrombin directly confirming the deductions from previous work with different loadings of quin2. Under natural conditions the major role of Ca^{2+} influx may be to prolong the $[Ca^{2+}]_i$ rise rather than to make it larger.

In many cell types stimulus-response coupling involves an elevation in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, as a consequence of Ca^{2+} influx and/or mobilisation of Ca^{2+} from intracellular stores. Ca^{2+} influx may be gated directly by the opening of receptor operated or voltage sensitive, Ca^{2+} channels in the plasma membrane (1). Intracellular Ca^{2+} can be mobilised by specific intracellular second messengers such as inositol(1,4,5) trisphosphate (2).

In human platelets loaded with quin2, agonists such as thrombin, platelet activating factor and vasopressin elicit an elevation in $[Ca^{2+}]_i$ which is reduced several fold by the removal of external Ca^{2+}; typically from around 0.5-1 μM to 0.1-0.2 μM (3,4,5). Such a reduction in the $[Ca^{2+}]_i$ signal by external Ca^{2+} removal is however largely dependent upon the amount of cytosolic quin2 available to

0006-291X/86 $1.50

buffer out Ca^{2+} discharged from internal stores. Recently new fluorescent Ca^{2+} probes have been synthesised by Tsien and colleagues (6), including the compound fura-2, to be used at intracellular loading concentrations as low as 20 - 30 µmol/litre of cell water (7,8) [c.f. 0.5 - 1 mmol/litre of cell water needed for good signals with quin2] which adds much less Ca^{2+} buffering capcity to the cytosol. We report here an analysis of $[Ca^{2+}]_i$ in fura-2 loaded platelets stimulated by ionomycin and thrombin, with particular reference to the contribution of Ca^{2+} mobilisation from internal stores.

Methods and Materials

Blood obtained from healthy volunteers, was mixed with one- sixth volume acid citrate dextrose and platelet rich plasma (PRP) obtained as previously described (4). PRP was then incubated at 37°C in the presence of either 20 µM quin2-AM for 30 min, or 3 µM fura-2-AM for 45 min. The cells were then spun down (350 g, 20 min, 15-20°C) in the presence of 20 µg apyrase/ml, the supernatant plasma discarded and the cells resuspended in physiological saline (145 mM NaCl, 5 mM-KCl 1 mM-$MgSO_4$, 10 mM-Hepes and 10 mM-glucose, pH 7.4, 15-20°C) containing 10 µM indomethacin, at 1.5 - 2 x 10^8 cells/ml. EGTA (1 mM) or $CaCl_2$ (1 mM) was added to individual aliquots of cells at least 5 min prior to agonist additions.

Fluorescence (339 nm ex., 500 nm em.) and optical density measurements were carried out at 37°C using 0.8 ml aliquots of cells. When optical density measurements were recorded the cells were continuously stirred, otherwise stirring was stopped immediately after the agonist addition to the cell suspension. $[Ca^{2+}]_i$ was calculated as described for quin2 (9) and for fura-2 (10) using Ca^{2+}-dye dissociation constants of 115 nM and 224 nM respectively. Fluoresence from external dye, assessed by the addition of excess EGTA to separate samples, was subtracted from the total fluoresence signal. Quin2 loading was 1-1.5 mmoles/litre of cell water; fura-2 loading (L) ws 50-70 µmoles/litre of cell water; fura-2 loading (L) was 50-70 µmoles/litre of cell water [L = (Fmax/3 x cell autofluorescence) x 30 µM; and Rink T.J. and Tsien R.Y., unpublished observations].

The traces shown are typical of replicate (2-4) determinations within the same batch of cells; similar results were observed in at least 3 different experiments using platelets from different donors.

Ionomycin from Calbiochem, England; quin2-acetoxymethylester from Lancaster Synthesis, Morecambe, England; fura-2-acetoxymethyl ester from Molecular Probes Inc., Junction City, Oregon; and digitonin from B.D.H. Ltd., England, were disssolved in dimethyl sulphoxide. Indomethacin (Sigma) was dissolved at a concentration of 10 mM in methanol. Human thrombin was from Calbiochem; apyrase was from Sigma and EGTA was from Fluka.

Results and Discussion

Figure 1 shows typical fluoresence records from fura-2-loaded platelets suspended in the presence of 1 mM EGTA. From the

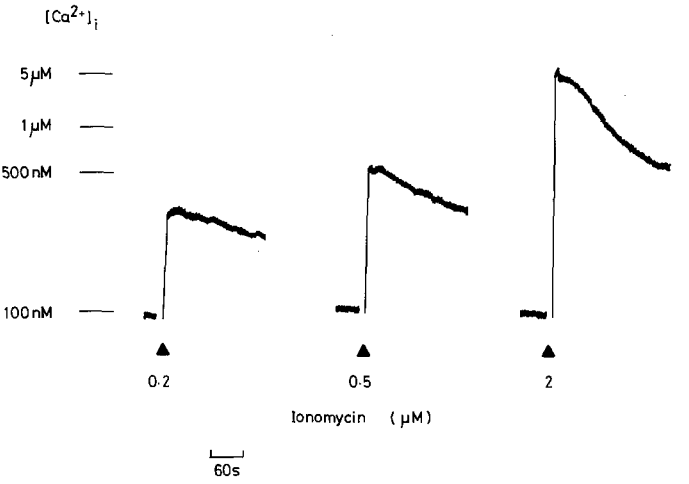

Figure 1. Mobilisation of internal Ca^{2+} stores by ionomycin. Ionomycin, at the concentrations indicted, was added to fura-2-loaded platelets, suspended in the presence of 1 mM EGTA.

calibration of the fluoresence records the mean resting $[Ca^{2+}]_i$ was calculated to be 95 ± 2 nM (S.E., n = 9). Ionomycin translocates Ca^{2+} across membranes independent of receptor-mediated events and causes a rapid, concentration-dependent elevation in $[Ca^{2+}]_i$. 200 nM ionomycin raised $[Ca^{2+}]_i$ to 310 nM which then fell slowly to around 230 nM 3-4 minutes after agonist addition; 500 nM ionomycin raised the $[Ca^{2+}]_i$ to 500 nM which then decreased to around 300 nM. At an optimal concentration of ionomycin (2 μM) the $[Ca^{2+}]_i$ increased to 3.36 μM and then decreased over the next 3-4 minutes to level out at around 500 nM.

These results show that under conditions where the extracellular $[Ca^{2+}]$ is substantially lower ($< 10^{-8}$M), than that in the cytosol, ionomycin can mobilise sufficient Ca^{2+} to elevate $[Ca^{2+}]_i$ to micromolar levels.

Figure 2 shows representative fluorescence and optical density records from fura-2-loaded platelets activated by thrombin (0.01-0.3 U/ml) in the presence of 1 mM EGTA. The mean resting $[Ca^{2+}]_i$ in these platelets was calculated to be 81 ± 3 nM (S.E., n = 13). A low concentration of thrombin (0.01 U/ml) caused no measurable change in $[Ca^{2+}]_i$; 0.03, 0.1 and 0.3 U/ml evoked peak rises in $[Ca^{2+}]_i$ to 110, 200 and 710 nM respectively. In response to 0.1 and 0.3 U/ml thrombin there is a rapid fall in the signal that approaches resting levels within 2-3 minutes. This return to near resting levels in response to thrombin is shown better in figure 4. The simultaneously recorded optical density traces show that the changes in $[Ca^{2+}]_i$ in response to

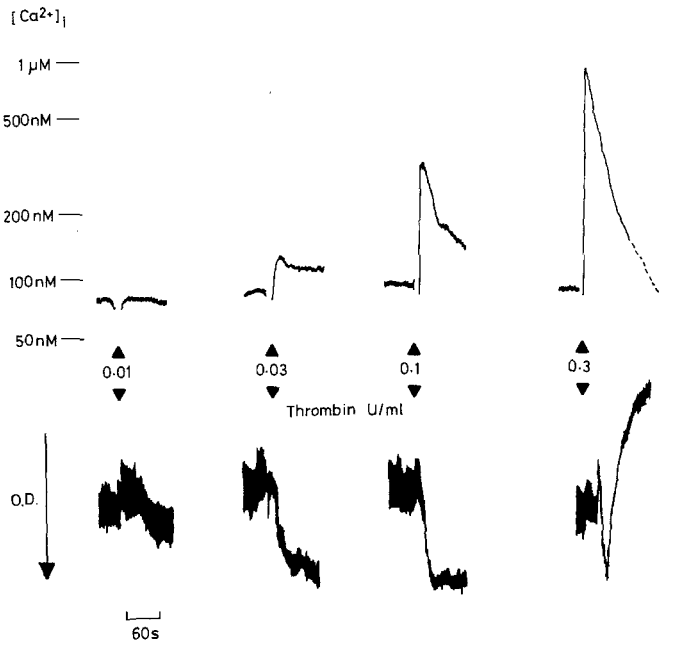

Figure 2. Fluoresence and optical density traces from thrombin stimulated, fura-2-loaded platelets. Thrombin, at the concentrations indicated, was added to platelets suspended in the presence of 1 mM EGTA.

thrombin (plus EGTA) are accompanied by shape change and, in the case of 0.3 U/ml thrombin, aggregation.

Figure 3 shows typical fluoresence and optical density records from quin2-loaded platelets activated by thrombin (0.01-0.3 U/ml) in the presence of 1 mM EGTA. The mean resting $[Ca^{2+}]_i$ in these cells was 63 ± 2 nM (S.E., n = 12). As in the fura-2-loaded platlets (fig. 2) 0.01 U/ml thrombin caused no increase in $[Ca^{2+}]_i$; 0.03, 0.1 and 0.3 U/ml thrombin raised $[Ca^{2+}]_i$ to 84, 102 and 152 nM respectively. The blunted responses to thrombin in quin2-loaded platelets are due to the high intracellular buffering exerted by the millimolar loading concentrations of this dye. The lower resting $[Ca^{2+}]_i$ value measured by quin2 may reflect a genuinely lower level or an error in the calibrations for the intracellular signals from quin2 or fura-2. The available data would suggest (see 6) that fura-2 is more likely to give an off-set absolute $[Ca^{2+}]_i$ value than quin2 would. However in any case the error in the absolute $[Ca^{2+}]_i$ calibration is small in comparison to stimulus evoked changes in $[Ca^{2+}]_i$.

The optical density records in figure 3 indicate that these quin2-loaded cells undergo a concentration-dependent shape change in

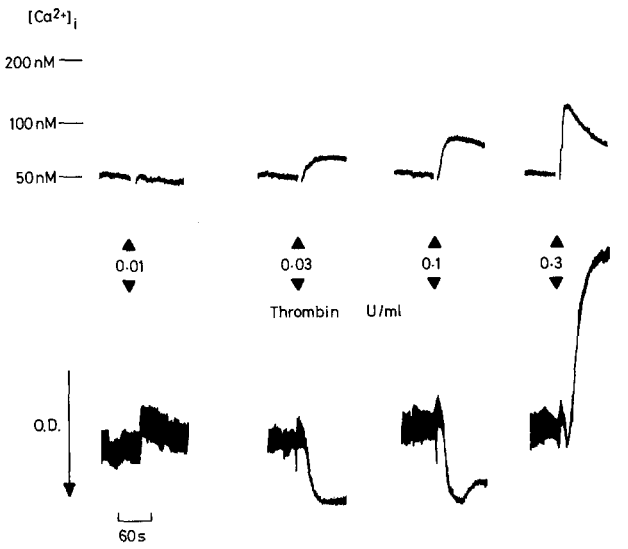

Figure 3. Fluoresence and optical density traces from thrombin stimulated, quin2-loaded platelets. Thrombin, at the concentration indicated, was added to platelets suspended in the presence of 1 mM EGTA.

response to thrombin and that 0.3 U/ml thrombin also caused a small aggregatory response. Thus the concentration dependence of the responses are similar in fura-2 and quin2-loaded cells; the main effect of quin2 therefore is to buffer the $[Ca^{2+}]_i$ change caused by the triggered release of a finite store of Ca^{2+}. These observations with fura-2 and quin2 also support the notion that available $[Ca^{2+}]_i$ is not the only determinant of platelet shape change.

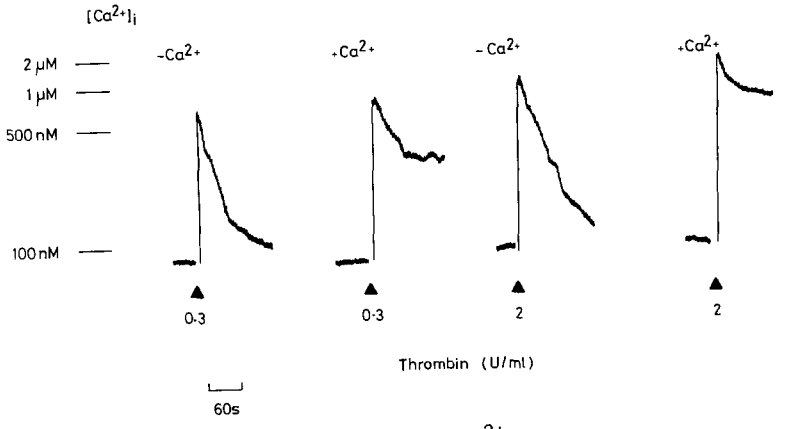

Figure 4. Mobilisation versus influx of Ca^{2+} in thrombin stimulated, fura-2-loaded platelets. Thrombin (0.3 or 2 U/ml) was added to fura-2-loaded platelets in the presence of either 1 mM EGTA ($-Ca^{2+}$) or 1 mM Ca^{2+} ($+Ca^{2+}$).

In figure 4 typical fluoresence records are shown from fura-2-loaded platelets comparing the responses to two concentrations of thrombin in the presence and absence of external Ca^{2+}. As in figure 2, thrombin (0.3 U/ml) in the presence of EGTA raised the $[Ca^{2+}]_i$ from a mean resting value of 91 ± 3 nM (S.E., n = 11) to a peak value of 620 nM which then rapidly declined to near resting levels. In the presence of 1 mM Ca^{2+}, 0.3 U/ml thrombin raised the $[Ca^{2+}]_i$ to a peak of 750 nM which was followed by a plateau phase at around 500 nM. 2 U/ml thrombin plus EGTA raised the $[Ca^{2+}]_i$ to a peak of around 1.1 μM which again rapidly declined to near resting levels whereas in the presence of 1 mM external Ca^{2+}, 2 U/ml thrombin raised the $[Ca^{2+}]_i$ to around 1.4 μM which then dropped to an elevated steady level of 750-800 nM.

These results indicate that thrombin is capable of mobilising enough Ca^{2+} from intracellular stores to provide an initial spike in $[Ca^{2+}]_i$ up to micromolar levels. Even so the peak levels measured here may still be slightly reduced compared with normal cells lacking the small extra buffering of the fura-2. However in the presence of physiological levels of external Ca^{2+}, the peak $[Ca^{2+}]_i$ response to thrombin is greater indicating some contribution from Ca^{2+} influx. In addition Ca^{2+} influx appears to be important in maintaining an elevated $[Ca^{2+}]_i$ following thrombin addition. A similar relationship has been observed in vascular endothelial cells and smooth muscle cells loaded with fura-2 (11,12) and has been proposed for Ca^{2+} mediated responses in many cells (13).

Most studies with quin2 in platelets and in other cells such as PC12 cells (14), neutrophils (15) and smooth muscle cells (16) state the observed $[Ca^{2+}]_i$ values obtained following stimulation and these will underestimate the normal contribution of mobilisation versus influx. Since different cell types may vary in their relative utilisation of intracellular or extracellular Ca^{2+} it is useful to use low loadings of fura-2 to get a more realistic assessment of the contribution from either source.

In the absence of external Ca^{2+} the rapid return of the $[Ca^{2+}]_i$ signal to near resting levels in response to thrombin (and compared to ionomycin) implies more effective sequestration and/or extrusion of the discharged Ca^{2+}. A possible mechanism for such negative feedback control of Ca^{2+} mobilisation by thrombin could involve activtion of a protein kinase C pathway as has been previously described (17,18).

Fura-2 is a highly sensitive probe for measuring $[Ca^{2+}]_i$ which has certain clear advantages over quin2, even when used simply in a

single channel fluorimeter, for instance, as demonstrated in this paper, when analysing the contributions of Ca^{2+} influx and mobilisation of internal Ca^{2+}.

Acknowledgements

This work was supported by a grant to Dr R.F. Irvine from the M.R.C. We thank Professor R.D. Keynes for making available facilities in the Physiological Laboratory, Cambridge, and Dr R.F. Irvine and Mr S.O. Sage for their helpful comments.

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