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Stimulation of Ca^{2+} efflux from fura-2-loaded platelets activated by thrombin or phorbol myristate acetate

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We investigated the restoration of $[Ca^{2+}]_i$ in fura-2-loaded human platelets following discharge of internal Ca^{2+} stores in the absence of external Ca^{2+}. After stimulation by thrombin $[Ca^{2+}]_i$ returned from a peak level of 0.6 μM to resting levels within 4 min. When ionomycin discharged the internal stores the recovery was slower with $[Ca^{2+}]_i$ still elevated at around 0.5 μM after 5 min. Thrombin added shortly after ionomycin could accelerate the recovery of $[Ca^{2+}]_i$ and restore resting levels within 5 min, an effect that was mimicked by phorbol-12-myristate-13-acetate (PMA). Since the continued presence of ionomycin precluded reuptake into the internal stores we conclude that thrombin and PMA stimulate Ca^{2+} efflux, perhaps via protein kinase C actions on a plasma membrane Ca^{2+} pump.

Platelet; Ca^{2+}; Fura-2; Ca^{2+} efflux; Protein kinase C

1. INTRODUCTION

In blood platelets agonist-induced rises in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) are often transient or have a phasic component [1–3], even in the continued presence of the agonist. In the absence of external Ca^{2+} the recovery phase of a $[Ca^{2+}]_i$ signal may reflect a desensitisation of Ca^{2+} mobilisation followed by extrusion of Ca^{2+} from the cell and reuptake into internal stores, or there could be persistent mobilisation with stimulated Ca^{2+} efflux and/or re-uptake, or some combination of these possibilities.

We present here experiments using fura-2-loaded human platelets suspended in the absence of external Ca^{2+} which allowed us to analyse the contribution of extrusion and reuptake of Ca^{2+} following thrombin stimulation, and the influence of thrombin or phorbol myristate acetate (PMA) on Ca^{2+} extrusion. The initial observation was that, in the absence of external Ca^{2+}, recovery of $[Ca^{2+}]_i$ following thrombin stimulation was faster and more complete than that following discharge of internal stores by the Ca^{2+} ionophore ionomycin. The continued presence of ionomycin is expected to prevent resequestration into internal storage pools by releasing Ca^{2+} as soon as it is taken up. In addition, ionomycin has a relatively low affinity for Ca^{2+}, EC_{50} of approx. 50 μM $[Ca^{2+}]_o$ for an elevation of $[Ca^{2+}]_i$ in quin2-loaded human platelets (Simpson, A.W. and Rink, T.J., unpublished). Thus ionomycin is ineffective in translocating Ca^{2+} from the cytosol where $[Ca^{2+}]_i$ is normally below micromolar. In the presence of ionomycin recovery of an elevated $[Ca^{2+}]_i$ is presumably therefore due almost entirely to Ca^{2+} extrusion.

Thrombin stimulation of platelets is known to activate protein kinase C (PKC) [4,5] as a consequence of receptor-stimulated generation of diacylglycerol. PMA is a pharmacological activator of PKC [6] and is reported to inhibit an elevation in $[Ca^{2+}]_i$ in various cell types [7,8] in-

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cluding platelets [9]. PMA is also reported to stimulate Ca^{2+} efflux from neutrophils [10]. Using ionomycin-stimulated platelets, the influence of thrombin and PMA on Ca^{2+} efflux could therefore be investigated to determine whether such a stimulation of extrusion is present in these cells.

2. EXPERIMENTAL

Platelet-rich plasma was prepared from freshly drawn blood as in [1]. Platelets were then loaded with 50–80 μmol fura-2/l cell water by incubating platelet-rich plasma for 45 min at 37°C in the presence of 3 μM fura-2 acetoxy methyl ester. 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 containing: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM dextrose (pH 7.4) at 37°C. The cell count was 1.5–2 × 10^8 platelets/ml. 10 μM indomethacin was present to eliminate any thromboxane formation and 1 mM EGTA was added to individual 0.8 ml aliquots of cells to maintain the external $[Ca^{2+}]$ below 10^{-8} M. Fura-2 fluorescence was measured at 37°C in a Perkin-Elmer MPF44A spectrophotometer with excitation and emission wavelengths of 339 and 500 nm, respectively; $[Ca^{2+}]_i$ was calculated as described [11] using a Ca^{2+}/dye dissociation constant for fura-2 of 224 nM.

The fluorescence records 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.

2.1. *Materials*

Fura-2 acetoxy methyl ester was obtained from Molecular Probes (Junction City, OR); ionomycin, platelet-activating factor (PAF) and human thrombin were from Calbiochem (Cambridge, England); PMA was from Sigma (England).

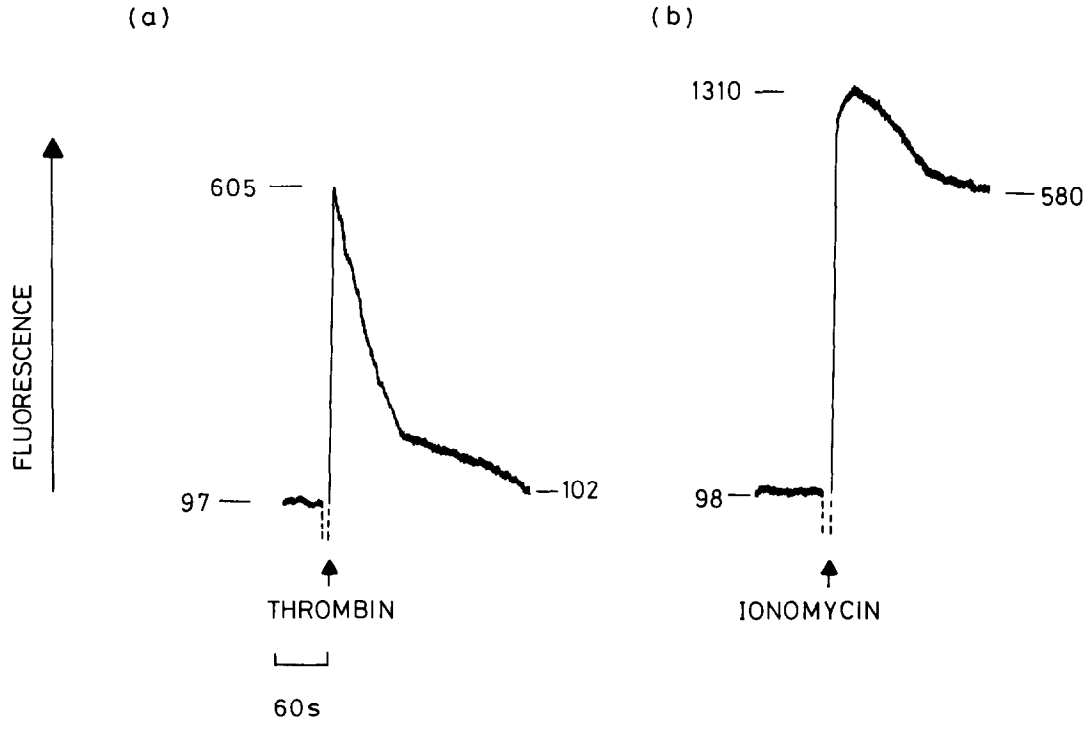

Fig.1. Discharge of internal Ca^{2+} stores by thrombin and ionomycin. Fura-2 fluorescence from human platelets suspended in the presence of 1 mM EGTA and activated by (a) 0.3 U/ml thrombin or (b) 1 μM ionomycin. $[Ca^{2+}]_i$ values in nM were calculated as described in section 2.

3. RESULTS AND DISCUSSION

In fig.1 fluorescence records are shown from fura-2-loaded platelets stimulated by 0.3 U/ml thrombin (a) and 1 μM ionomycin (b). Thrombin caused a rapid elevation in $[Ca^{2+}]_i$ from a resting level of just under 100 nM to a peak value of over 600 nM. Thereafter the $[Ca^{2+}]_i$ returned to resting levels within 4 min. Ionomycin similarly evoked a rapid elevation in $[Ca^{2+}]_i$ up to a maximum of 1.3 μM (fig.1b), but following an initial decline, the response to ionomycin decayed much more slowly, recovering only to 580 nM after 5 min. The elevations in $[Ca^{2+}]_i$ caused by both thrombin and ionomycin are a consequence of Ca^{2+} mobilisation from internal stores since, with 1 mM EGTA present, the external $[Ca^{2+}]$ was less than 10^{-8} M. The subsequent fall in the signal indicates removal of Ca^{2+} from the cytosol into internal stores or extrusion across the plasma membrane. For thrombin, acting through a receptor-operated mechanism, both active extrusion and sequestration by Ca^{2+}-pumping organelles are expected to contribute to the recovery of $[Ca^{2+}]_i$. However, with ionomycin the observed fall in the $[Ca^{2+}]_i$ signal can probably occur only by Ca^{2+} extrusion; the continued presence of ionomycin is expected to 'short-circuit' sequestering organelles. (For reasons outlined in section 1, ionomycin is not expected to translocate Ca^{2+} across the plasma membrane under the experimental conditions used.)

The recovery from an elevation in $[Ca^{2+}]_i$ in response to thrombin (fig.1a) could result from Ca^{2+} re-uptake into internal organelles, stimulated extrusion, or both. We next examined the effect of thrombin on $[Ca^{2+}]_i$ elevated by ionomycin as shown in fig.2, where re-uptake should have been precluded. Fig.2a shows the control response to ionomycin; a rapid elevation in $[Ca^{2+}]_i$ to 1.3 μM followed by a fall to 500 nM after 5 min. Addition of thrombin to platelets 60 s after ionomycin (fig.2b) caused no further elevation in $[Ca^{2+}]_i$, implying that the ionomycin had maximally discharged the thrombin-sensitive Ca^{2+} pool. However, thrombin did stimulate the recovery of the $[Ca^{2+}]_i$ so that it returned to the basal level within 4 min. To test our hypothesis that the recovery reflected Ca^{2+} extrusion and not refilling of the internal pool we looked at the response to another agonist, PAF, capable of discharging Ca^{2+} from internal stores [1]. Fig.2 shows that the addition of PAF after the application of ionomycin and thrombin did not raise $[Ca^{2+}]_i$, indicating that the internal pool was indeed depleted. By contrast, ad-

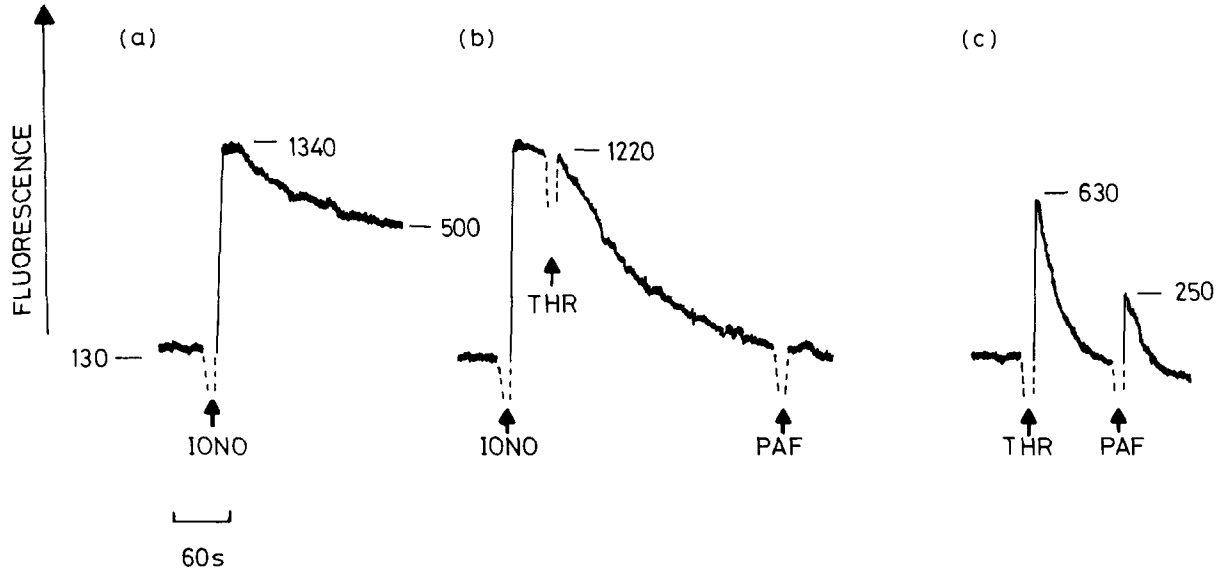

Fig.2. Stimulation of Ca^{2+} efflux by thrombin. Fura-2 fluorescence from human platelets suspended in the presence of 1 mM EGTA and activated by (a) 1 μM ionomycin (IONO), (b) ionomycin followed by 0.3 U/ml thrombin (THR), and (c) thrombin followed by 20 ng/ml platelet activating factor (PAF).

dition of PAF after thrombin alone (fig.2c) did raise $[Ca^{2+}]_i$, to 250 nM. Thus after thrombin alone the internal pool is at least partly refilled implying that some of the recovery of $[Ca^{2+}]_i$ under these conditions is due to repletion of the dischargeable pool. It is worth noting that the recovery of $[Ca^{2+}]_i$ following thrombin alone (figs 1a,2c) is faster than that seen when thrombin follows ionomycin (fig.2b) indicating the additional contribution of Ca^{2+} sequestration in the absence of ionomycin.

An obvious question is what mechanisms of activating Ca^{2+} extrusion could be available to the cell? Both Ca^{2+}-calmodulin- and PKC-stimulated Ca^{2+} pumping are known [10,12]. The former is unlikely as such a mechanism could not account for the difference between thrombin and ionomycin which both raise $[Ca^{2+}]_i$; also a recent report [13] has identified a Ca^{2+}-ATPase in platelets similar to that in erythrocyte membranes but insensitive to calmodulin. Since thrombin causes activation of PKC [4], whereas ionomycin, in aspirin-treated platelets, does not [14], we looked at the influence of PMA, a direct activator of PKC [6] that does not elevate $[Ca^{2+}]_i$ in fura-2-loaded platelets [11], on Ca^{2+} efflux. Typical results are shown in fig.3. Addition of PMA 60 s after ionomycin had an effect similar to that evoked by thrombin (fig.2b) in enhancing the decline in the $[Ca^{2+}]_i$ which dropped from near 1 μM to 170 nM within 4 min. These results concur with previous observations of a PKC-regulated Ca^{2+} efflux mechanism, for example in neutrophils [10] or lymphocytes [15]. This effect of PMA on platelet $[Ca^{2+}]_i$ can be considered additional to the inhibitory effect of PMA on mobilisation of Ca^{2+} reported in platelets by MacIntyre et al. [9]. Thus, protein kinase C appears to interact with platelet $[Ca^{2+}]_i$ in at least three ways: it is synergistic to promote platelet activation; it inactivates or desensitises the receptor/Ca^{2+} gating transduction mechanism; and it promotes the return of $[Ca^{2+}]_i$ to resting levels by stimulating removal of Ca^{2+} from the cell. The basic mechanisms by which PKC-stimulated Ca^{2+} efflux operates could involve enhanced Na^+-Ca^{2+} exchange or stimulation of an ATP-dependent Ca^{2+} pump in the platelet plasma membrane. The question of whether Ca^{2+} pumping from platelets occurs by an Na^+-Ca^{2+} exchanger or a Ca^{2+}-ATPase is contentious and not directly addressed by this paper. Our guess is that Ca^{2+}-ATPase is more important, a view supported by a recent report of such activity in platelets [13]. Studies combining the use of $^{45}Ca^{2+}$ and fluores-

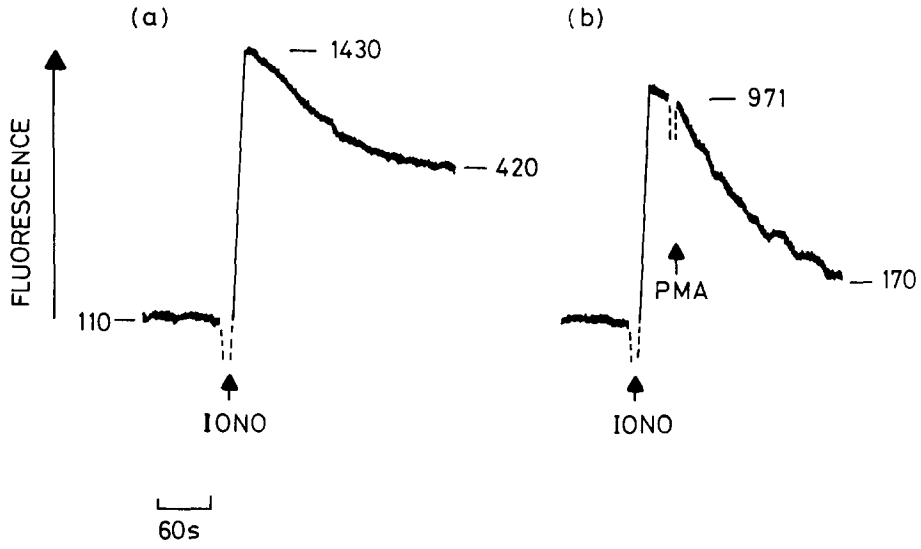

Fig.3. Stimulation of Ca^{2+} efflux by PMA. Fura-2 fluorescence from human platelets suspended in the presence of 1 mM EGTA and activated by (a) 1 μM ionomycin (IONO) or (b) ionomycin followed by 100 nM phorbol myristate acetate (PMA).

cent Ca^{2+}-indicator dyes in the presence and absence of external Na^+ are currently under way in our laboratories to analyse further these matters.

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REFERENCES

[1] Hallam, T.J., Sanchez, A. and Rink, T.J. (1984) Biochem. J. 218, 819–827.
[2] Hallam, T.J. and Rink, T.J. (1985) J. Physiol. 368, 131–146.
[3] Pollock, W.K. and Rink, T.J. (1986) Biochem. Biophys. Res. Commun., in press.
[4] Sano, K., Takai, Y., Yaminishi, J. and Nishizuka, Y. (1982) J. Biol. Chem. 258, 2010–2013.
[5] Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. and Nishizuka, Y. (1982) Cell Calcium 3, 323–335.
[6] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[7] Drummond, A.H. (1985) Nature 315, 752–755.
[8] Di Virgilio, F., Pozzan, T., Wollheim, C.B., Vicenti, L.M. and Meldolesi, J. (1985) J. Biol. Chem. 261, 32–35.
[9] MacIntyre, D.E., McNicol, A. and Drummond, A.H. (1985) FEBS Lett. 180, 160–164.
[10] Lagaste, H., Pozzan, T., Waldvogel, F.A. and Lew, P.D. (1984) J. Clin. Invest. 73, 878–883.
[11] Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) Biochem. J. 235, 869–877.
[12] Caroni, P. and Carafoli, E. (1980) J. Biol. Chem. 256, 3263–3270.
[13] Enyedi, A., Sarkadi, B., Foldes-Papp, Z., Monostory, S. and Gardos, G. (1986) J. Biol. Chem. 261, 9558–9563.
[14] Rittenhouse, S.E. and Horne, W.C. (1984) Biochem. Biophys. Res. Commun. 123, 393–397.
[15] Lichtman, A.H., Segel, G.B. and Lichtman, M.A. (1981) J. Biol. Chem. 256, 6148–6154.