RESEARCH COMMUNICATION

The effect of calcium-store depletion and refilling with various bivalent cations on tyrosine phosphorylation and Mn^{2+} entry in fura-2-loaded human platelets

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To investigate the possible involvement of tyrosine phosphorylation in the process of store-regulated Ca^{2+} entry, ionomycin (in the presence of EGTA) was used to deplete the intracellular Ca^{2+} stores of fura-2-loaded human platelets, and the effect of refilling with Ca^{2+}, Ba^{2+} or Sr^{2+} evaluated. Depletion of the intracellular Ca^{2+} stores resulted in an increase in protein tyrosine phosphorylation. This increase is reversed when the stores were refilled in Ca^{2+} or Sr^{2+}, but not Ba^{2+}. Refilling of the stores with Ca^{2+} or Sr^{2+}, but not Ba^{2+}, suppressed Mn^{2+} entry. These findings support the hypothesis that tyrosine phosphorylation plays a role in mediating store-regulated Ca^{2+} entry in human platelets and provides evidence for tyrosine phosphatase activity regulated by the Ca^{2+} content of the intracellular stores.

INTRODUCTION

Store-regulated (or 'capacitative') calcium entry has been demonstrated in many cell types [1], including platelets [2–4]. However, the mechanism of such Ca^{2+} entry is uncertain, and several models have been proposed. One suggestion is that protein-tyrosine phosphorylation plays a key role [5–8]). It has been proposed that the cytosolic and intracellular store Ca^{2+} concentrations antagonistically control platelet tyrosine phosphorylation and hence Ca^{2+} entry [5]. In this model elevated cytosolic Ca^{2+} is proposed to activate a tyrosine kinase, leading to enhanced tyrosine phosphorylation and so promotion of Ca^{2+} entry, perhaps by a direct effect on a plasma-membrane Ca^{2+} channel. Ca^{2+} entry is proposed to be reduced by the refilling of the internal Ca^{2+} stores, which would reduce tyrosine phosphorylation by activating a tyrosine phosphatase. In support of this hypothesis, inhibitors of tyrosine kinases have been shown in platelets to reduce agonist- and store-depletion-evoked Ca^{2+} entry without affecting the release of Ca^{2+} from intracellular stores [6,7]. Similar results have been reported in human fibroblasts [9]. To assess further the involvement of tyrosine phosphorylation in the process of store-regulated Ca^{2+} entry, we have now investigated the effects of Ca^{2+}-store depletion and refilling on protein tyrosine phosphorylation and bivalent-cation entry in fura-2-loaded human platelets. The internal Ca^{2+} stores were depleted using ionomycin in the presence of EGTA and the subsequent effects of refilling the stores with Ca^{2+}, Sr^{2+} or Ba^{2+} on protein tyrosine phosphorylation and Mn^{2+} entry were determined.

Some of these findings have been presented in a preliminary form [10].

EXPERIMENTAL

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Eugene, OR, U.S.A.). Apyrase (grade V), aspirin, bovine thrombin and prostaglandin E_1 were from Sigma (Poole, Dorset, U.K.). Ionomycin was from Calbiochem (Nottingham, U.K.). All other reagents were of analytical grade.

Methods

Fura-2-loaded platelets were prepared as described elsewhere [2]. Briefly, platelet-rich plasma was incubated with 2 μM fura-2/AM for 45 min at 37 °C. The cells were collected by centrifugation and resuspended in nominally Ca^{2+}-free Hepes-buffered saline (145 mM NaCl/5 mM KCl/10 mM sodium Hepes/1 mM $MgSO_4$/10 mM D-glucose/20 μg/ml apyrase, pH 7.4 at 37 °C). Fluorescence was recorded using a Cairn Research spectrophotometer (Cairn Research Ltd., Sittingbourne, Kent, U.K.) with excitation wavelengths of 340, 360 and 380 nm and emission at 500 nm. The platelet suspension was kept at 37 °C with constant stirring throughout. The release of bivalent cations from the intracellular stores was monitored using the fura-2 340/380 nm fluorescence ratio. Mn^{2+} quench of fluorescence was determined with excitation at 360 nm, the iso-emissive wavelength [2].

To deplete the intracellular Ca^{2+} stores, fura-2-loaded platelets were incubated in polypropylene tubes with 0.5 mM ionomycin in the presence of 1 mM EGTA (and no added Ca^{2+}) for 20 min at 37 °C. After incubation the platelets were collected by centrifugation in a MSE Micro-Centaur Centrifuge (MSE Scientific Instruments, Crawley, Sussex, U.K.) for 90 s at 3000 *g* and washed twice to remove ionomycin, in modified Hepes-buffered saline (nominally Ca^{2+} free), with the addition of 500 nM prostaglandin E_1, 0.1% BSA and 12 μl/ml acid-citrate/dextrose anticoagulant (2 g/100 ml D-glucose, 2.5 g/100 ml sodium citrate and 1.5 g/100 ml citric acid). The platelets were resuspended in normal Hepes-buffered saline and either 1 mM $CaCl_2$, $BaCl_2$, $SrCl_2$ or an equal volume of saline added, and then incubated for 10 min at 37 °C. The platelets were washed again with modified Hepes-buffered saline to remove external bivalent cations, and finally resuspended in nominally Ca^{2+}-free Hepes-buffered saline.

Abbreviation used: fura-2/AM, fura-2 acetoxymethyl ester.

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The control consisted of platelets treated with dimethyl sulphoxide as vehicle instead of ionomycin, but otherwise treated the same as Ca^{2+}-depleted cells.

Tyrosine phosphorylation was assessed by gel electrophoresis and Western blotting using a specific anti-phosphotyrosine antibody as described previously [6–8]. Densitometric measurements were made using a Quantimet 500 densitometer (Leica, Milton Keynes, U.K.). The integrated absorbance of the entire lane was estimated.

RESULTS AND DISCUSSION

Figure 1 shows five superimposed recordings of fura-2 fluorescence ratio from stirred platelet suspensions in the absence of extracellular Ca^{2+} (1mM EGTA added). In control platelets (which had been treated with dimethyl sulphoxide rather than ionomycin) (trace a), thrombin evoked a substantial rise in fluorescence ratio indicating a rise in intracellular $[Ca^{2+}]$. However, the thrombin-evoked response in ionomycin-treated platelets (trace b) was much reduced, confirming that partial depletion of the intracellular Ca^{2+} stores had been achieved. It was not possible to achieve greater depletion of the stores, as higher concentrations of ionomycin or stirring of the cells resulted in aggregation of the platelets. Traces c–e show the effect of thrombin stimulation of platelets pretreated with ionomycin and subsequently incubated after the removal of the ionophore with either 1 mM Ca^{2+}, Ba^{2+} or Sr^{2+} respectively. Thrombin evoked a rise in fura-2 ratio after incubation with each of these ions which was greater than that in the depleted cells (trace b), although the increase observed after incubation with Ba^{2+} was less than that observed with Ca^{2+} or Sr^{2+}. The thrombin-evoked response of Ca^{2+} refilled cells (trace c) was somewhat sluggish compared with that of untreated control cells (trace a). We cannot be certain as to the cause of this, but believe it reflects some deterioration of the thrombin reponse due to partial (and reversible) activation during ionomycin treatment. These findings confirm earlier reports that Ca^{2+}, Ba^{2+} and Sr^{2+} are taken up into the intracellular stores in platelets and are released into the cytoplasm upon thrombin stimulation [11].

Although it is difficult to compare the extent of refilling of the stores with these different bivalent cations, since they have different effects on fura-2 fluorescence and the affinity of the indicator for the ions differs ($Ca^{2+} > Ba^{2+} > Sr^{2+}$) [12], in these experiments Ba^{2+} appeared to enter the stores less well than Ca^{2+} or Sr^{2+}. This contrasts with the results of other workers, who reported refilling to a similar degree with all these ions in human platelets [11]. In mouse lacrimal acinar cells, Ca^{2+} and Sr^{2+}, but not Ba^{2+}, have been shown to refill the intracellular stores [13], suggesting differences in the entry pathways or the selectivity of the store Ca^{2+}-ATPases in two cell types.

To evaluate the effect of refilling the intracellular Ca^{2+} stores with the various bivalent cations on plasma-membrane bivalent-cation permeability, Mn^{2+} was used as a surrogate for Ca^{2+}. Figure 2 shows Mn^{2+} quench of fura-2 fluorescence at the iso-emissive wavelength. The magnitude of quenching observed after the addition of 500 mM $MnCl_2$ was much greater in Ca^{2+}-depleted platelets (trace b) than in undepleted controls (trace a). This confirms that the depletion of intracellular Ca^{2+} stores facilitates bivalent-ion influx. When the stores were refilled with Ca^{2+} (trace c) or Sr^{2+} (trace e), Mn^{2+} addition resulted in only a small degree of fluorescence quenching (less with Ca^{2+} than Sr^{2+}) similar to that observed in undepleted platelets (trace a). When Ba^{2+} was used to refill the Ca^{2+} stores (trace d), there was a large quench of fura-2 fluorescence on the addition of Mn^{2+}, similar to that observed with depleted cells (trace b). Hence, refilling the stores with Ba^{2+} did not suppress Mn^{2+} influx, whilst refilling with Ca^{2+} or Sr^{2+} did. Similar results have been reported previously [11]. These authors interpreted their findings as indicating that Sr^{2+}, but not Ba^{2+}, could replace Ca^{2+} at some key intrastore binding site, the occupation of which leads to the

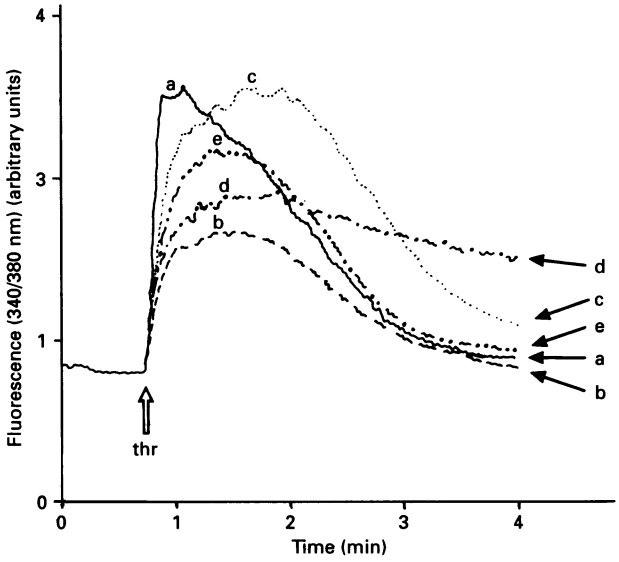

Figure 1 Refilling of platelet intracellular Ca^{2+} stores with various bivalent cations

Fura-2-loaded platelets were treated with ionomycin and subsequently incubated either with nominally Ca^{2+}-free Hepes-buffered saline (b) or with the addition of 1mM $CaCl_2$ (c), $BaCl_2$ (d) or $SrCl_2$ (e). Trace a shows fluorescence from control platelets which had not been treated with ionomycin, but were otherwise the same as Ca^{2+}-depleted platelets (trace b). Thrombin ('thr'; 0.5 unit/ml) was added as indicated in the presence of 1 mM EGTA. Traces show fura-2 340/380 nm fluorescence ratio. Traces are representative of five experiments.

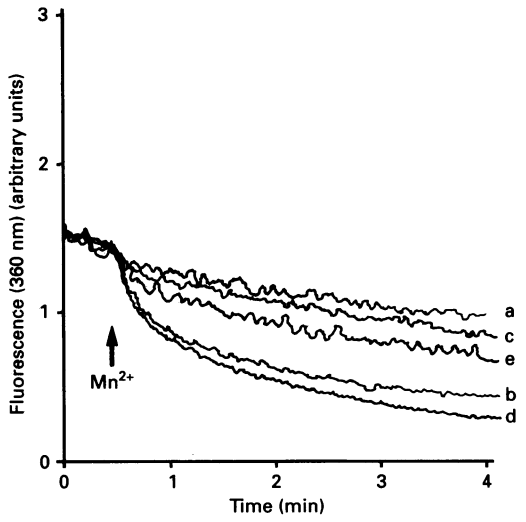

Figure 2 Effect of refilling with various bivalent cations on Mn^{2+} influx

Fura-2-loaded platelets were depleted and refilled as described in Figure 1. Mn^{2+} (500 μM) was added to the platelet suspension as indicated. Fura-2-fluorescence was measured with an excitation wavelength of 360nm, the iso-emissive wavelength. Traces are representative of five experiments.

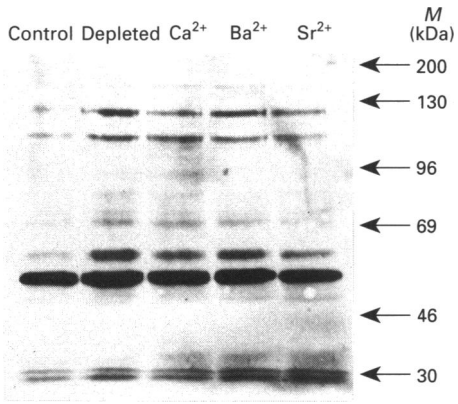

Figure 3 Effect of refilling with various bivalent cations on protein-tyrosine phosphorylation

Fura-2-loaded platelets were depleted and refilled as described in Figure 1 and samples removed for analysis after the final wash. Molecular masses (*M*) were determined using molecular-mass markers run in the same gel.

closure of the Ca^{2+} entry pathway [11]. Our results are consistent with this hypothesis, but might be alternatively or additionally explained by the apparent smaller extent of store refilling with Ba^{2+} compared with Ca^{2+} or Sr^{2+}.

Figure 3 shows the results of the protein phosphotyrosine analysis. Ca^{2+} store depletion using ionomycin (depleted) increased the protein phosphotyrosine content of most bands compared with undepleted platelets (control). The bands showing the most enhanced phosphorylation were at approx. 57 and 130 kDa. Refilling of the intracellular stores with Ca^{2+} or Sr^{2+} reduced protein phosphotyrosine levels back towards those seen in undepleted platelets. Refilling of the stores with Ba^{2+} did not significantly decrease protein tyrosine phosphorylation, which remained similar to that in depleted platelets. These effects are quantified in Table 1, which shows mean integrated absorbances of entire lanes from the protein phosphotyrosine analysis of four experiments. The results represented the percentage of that for the ionomycin-depleted control. Refilling of the stores with Ca^{2+} or Sr^{2+} reduced tyrosine phosphorylation to levels which were not significantly different (Student's paired *t* test; $0.5 > P > 0.1$) from those in undepleted (control) cells. These results are compatible with the idea that store refilling with these ions activates a Ca^{2+}-dependent tyrosine phosphatase which could be responsible for inactivating the Ca^{2+}-influx mechanism, as demonstrated using Mn^{2+} (Figure 2). In contrast, in cells refilled with Ba^{2+} the level of tyrosine phosphorylation remained significantly different ($0.05 > P > 0.02$) to undepleted platelets. This suggests that Ba^{2+} refilling of the internal store is unable to activate the tyrosine phosphatase, which may explain why refilling with this ion does not result in termination of bivalent influx (Figure 2).

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Table 1 Densitometric measurements of the phosphotyrosine analysis

Four experiments were performed as described in Figure 3 and the mean integrated absorbance of entire lanes was determined. The results are percentages (means ± S.E.M.) of those for the depleted control.

Condition	Absorbance (% of that for the depleted control)
Control	68.4 ± 7.6
Depleted	100 ± 0
Ca^{2+}-refilled	79.1 ± 6.4
Ba^{2+}-refilled	97.3 ± 4.4
Sr^{2+}-refilled	80.4 ± 3.7

In summary, these results indicate that depletion of the intracellular Ca^{2+} store in platelets results in an increase in protein tyrosine phosphorylation that is reversed when the stores are refilled with Ca^{2+} or Sr^{2+}, but not Ba^{2+}. The ability of refilling with a particular bivalent cation to reduce tyrosine phosphorylation correlated with its ability to reduce Mn^{2+} entry. These data thus support the hypothesis that tyrosine phosphorylation plays a role in mediating store-regulated Ca^{2+} entry in human platelets and provides evidence for a tyrosine phosphatase regulated by the Ca^{2+} content of the store. The presence of a store-regulated tyrosine phosphatase is also supported by reports that cooling Ca^{2+}-depleted platelets to inhibit Ca^{2+}-ATPase activity retards tyrosine dephosphorylation [5], and that Ca^{2+}-store depletion using thapsigargin promotes tyrosine phosphorylation even when a rise in intracellular [Ca^{2+}] is prevented by enhanced cytosolic Ca^{2+} buffering, so unmasking basal tyrosine kinase activity [8].

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