Wortmannin inhibits store-mediated calcium entry and protein tyrosine phosphorylation in human platelets

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Abstract The effects of the WT on store-mediated Ca^{2+} entry and protein tyrosine phosphorylation were investigated in fura-2-loaded human platelets. Wortmannin (2 μM) attenuated the rise in $[Ca^{2+}]_i$ caused by Ca^{2+} entry while having no effect on the mobilisation of Ca^{2+} from internal stores. It also reduced store-depletion-evoked protein tyrosine phosphorylation. These findings demonstrate that WT is an inhibitor of tyrosine phosphorylation and store-mediated calcium entry and provide further evidence for the involvement of a tyrosine phosphorylation step in the link between Ca^{2+} store depletion and Ca^{2+} influx in human platelets.

Key words: Thapsigargin; Tyrosine phosphorylation; Store-mediated calcium influx; Wortmannin; Platelet

1. Introduction

In many cell types including human platelets, depletion of intracellular Ca^{2+} stores evokes influx of Ca^{2+} across the plasma membrane [1–3]. However, the mechanism of this store-mediated (or 'capacitative') Ca^{2+} entry [4] remains uncertain. One proposal is that protein tyrosine phosphorylation plays a role. Vostal and coworkers suggested that cytosolic and intracellular store Ca^{2+} antagonistically control tyrosine phosphorylation and hence Ca^{2+} entry in human platelets [5]. In this model, store-depletion is proposed to activate a tyrosine kinase resulting in enhanced tyrosine phosphorylation and hence promotion of Ca^{2+} influx. Internal store refilling is proposed to activate a tyrosine phosphatase which would reduce tyrosine phosphorylation and terminate Ca^{2+} influx [5]. This hypothesis is supported by the finding that various known tyrosine kinase inhibitors reduce Ca^{2+} entry evoked by agonists and by store-depletion in platelets whilst having little or no effect on the release of Ca^{2+} from intracellular stores [6, 7]. Similar results have been reported with other cell types [8–10]. We have shown that depletion of the intracellular Ca^{2+} store in platelets results in an increase in protein tyrosine phosphorylation which is reversed when the stores are refilled with divalent cations [11]. The ability of refilling with a particular divalent cation to reduce tyrosine phosphorylation correlates with its ability to reduce Mn^{2+} entry [11]. These observations support the existence of a tyrosine phosphatase activity associated with full intracellular Ca^{2+} stores.

The fungal metabolite wortmannin (WT) has been shown to inhibit several agonist evoked platelet responses including serotonin secretion and aggregation [12]. In a number of cell types, including platelets, WT has been reported to suppress Ca^{2+} entry evoked by both agonists and store-depletion [13,14]. Hence, we have investigated the effects of WT on store-depletion evoked Ca^{2+} influx and protein tyrosine phosphorylation in human platelets.

2. Materials and methods

2.1. Materials

Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Apyrase (grade V), aspirin, thapsigargin and WT were from Sigma (Poole, Dorset, UK). Ionomycin was from Calbiochem (Nottingham, UK). All other reagents were of analytical grade.

2.2. Methods

Human platelets were prepared as described elsewhere [1]. Briefly, platelet-rich plasma was incubated at 37°C with 2 μM Fura-2/AM for 45 min. Cells were collected by centrifugation and resuspended in Hepes-buffered saline (145 mM NaCl, 5 mM KCl, 10 mM NaHepes, 1 mM $MgSO_4$, 10 mM D-glucose, 20 μg/ml apyrase, pH 7.4 at 37°C). Fluorescence was recorded from aliquots of stirred platelet suspensions at 37°C using a Cairn Research Spectrophotometer (Cairn Research Ltd., Sittingbourne, Kent, UK) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were monitored using the 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz et al. [15].

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting using a specific anti-phosphotyrosine antibody essentially as described previously [6] but blocking with 10% (w/v) bovine serum albumin instead of milk protein. Densitometric measurements were made with the use of a Quantimet 500 densitometer (Leica, Milton Keynes, UK).

3. Results and discussion

Fig. 1 shows the effect of WT on the change in platelet $[Ca^{2+}]_i$ following the sequential additions of thapsigargin and Ca^{2+} to platelets initially in the absence of external Ca^{2+} (presence of 0.5 mM EGTA). Preincubation with WT had no effect on the thapsigargin-evoked $[Ca^{2+}]_i$ rise. After addition of thapsigargin the mean peak $[Ca^{2+}]_i$ elevations above basal were 45 ± 5 nM (mean ± S.E.M.) in controls, 52 ± 7 nM with 100 nM WT (Student's paired t test, difference of means, $0.5 > P > 0.1$, $n = 7$) and 39 ± 6 nM with 2 μM WT ($0.1 > P > 0.05$, $n = 8$). However, preincubation with 2 μM WT significantly reduced the rise in $[Ca^{2+}]_i$ occurring after the addition of 2 mM Ca^{2+} (Fig. 1b). The peak of the rise in $[Ca^{2+}]_i$ on Ca^{2+} addition was 549 ± 96 nM in the presence of WT compared with 1079 ± 135 nM in controls ($0.01 > P > 0.001$, $n = 8$). At a lower concentration of WT (100 nM), there was no effect on Ca^{2+} entry after addition of external Ca^{2+} (Fig. 1c). After the addition of Ca^{2+} the peak $[Ca^{2+}]_i$ rise was 1110 ± 281 nM in the presence of WT compared with 1286 ± 216 nM in controls ($0.5 > P > 0.1$, $n = 7$).

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Abbreviations: Fura-2/AM, fura-2 acetoxymethyl ester; WT, wortmannin; $[Ca^{2+}]_i$, intracellular calcium

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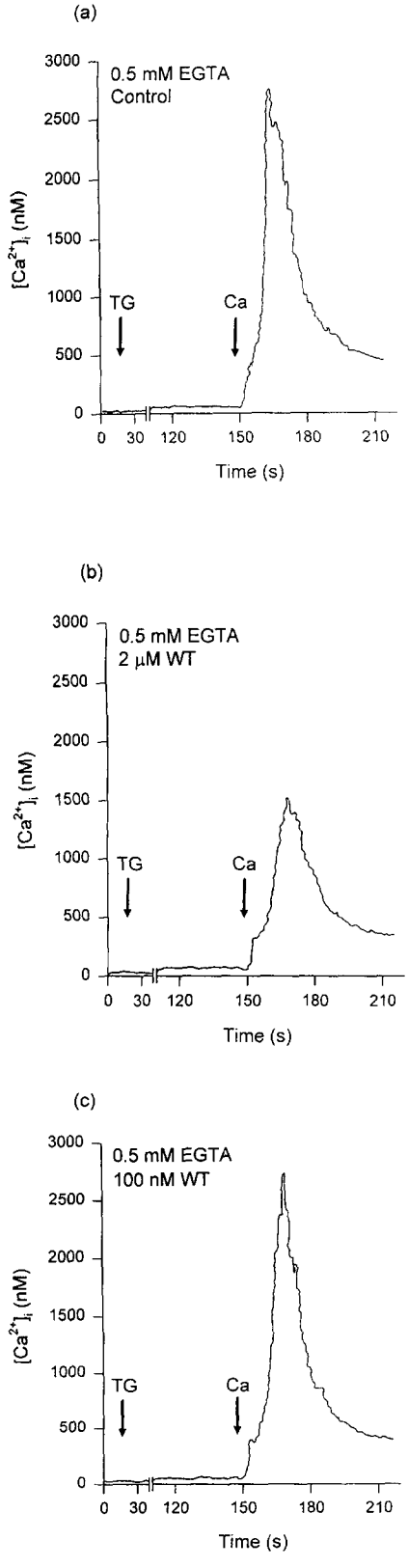

Fig. 1. The effect of WT on the $[Ca^{2+}]_i$ elevation evoked by thapsigargin. Fluorescence recordings from fura-2-loaded human platelets incubated, for 10 min at 37°C, with either 2 μM WT (b), 100 nM WT (c), or the vehicle, DMSO (a), in the presence of 0.5 mM EGTA. 250 nM thapsigargin (TG) was added as indicated, followed by the subsequent addition of 2 mM Ca^{2+} to assess Ca^{2+} entry.

These data demonstrate that WT at high concentrations attenuates store-depletion evoked calcium entry while having no affect on the thapsigargin-evoked mobilisation of Ca^{2+} from internal stores.

Next we examined the effect of WT on store-depletion evoked tyrosine phosphorylation in platelets. Samples were taken during the time course of the fluorescence experiments and protein tyrosine phosphorylation determined. Typical results are shown in Fig. 2. A 10 min incubation with 2 μM WT decreased protein tyrosine phosphorylation evoked by store depletion with inhibition most evident at specific bands of approximately 57, 69 and 130 kDa. Wortmannin, at this concentration, also suppresses thrombin-evoked protein tyrosine phosphorylation (data not shown). Incubation with the lower concentration of WT (100 nM) did not affect the phosphotyrosine levels. These effects are quantified in Fig. 3, which shows mean integrated absorbencies of the entire lane from the protein phosphotyrosine analysis of three experiments. Results are expressed as a percentage of the integrated optical density of platelets before stimulation. Hence, at high concentrations, WT almost completely suppressed the rise in tyrosine phosphorylation evoked by depletion of the intracellular calcium stores. Therefore at micromolar concentrations, WT is a potent tyrosine kinase inhibitor and greatly reduces store depletion-evoked protein tyrosine phosphorylation. Wortmannin has also been reported to inhibit tyrosine phosphorylation in human neutrophils [16].

Similar effects of high concentrations of WT on thapsigargin-evoked Ca^{2+} entry in human platelets have been reported previously [14]. This inhibition was suggested to result from a specific inhibition of myosin-light chain kinase which was therefore proposed to play a role in Ca^{2+} entry [14]. Our results show that WT leads to inhibition of protein tyrosine phosphorylation within the cell an effect which reduces store-mediated Ca^{2+} influx [5–11].

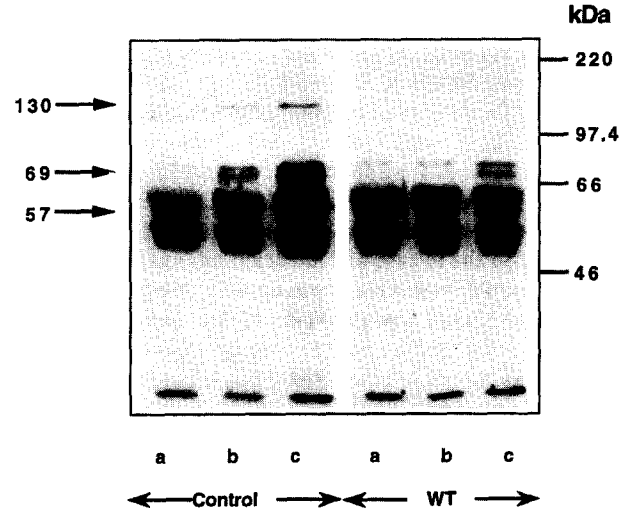

Fig. 2. The effect of WT on store-depletion-evoked protein tyrosine phosphorylation. 200 μM aliquots were taken from a stirred fura-2-loaded platelet suspension at 20 s prior to (a), and 10 s (b) and 105 s (c) after, the addition of 250 nM thapsigargin with 50 nM ionomycin. Platelet suspensions were pre-incubated for 10 min at 37°C with either 2 μM WT (right hand lanes) or control, DMSO (left hand lanes). Proteins were analysed by 8% SDS-PAGE and subsequent Western blotting with a specific antiphosphotyrosine antibody.

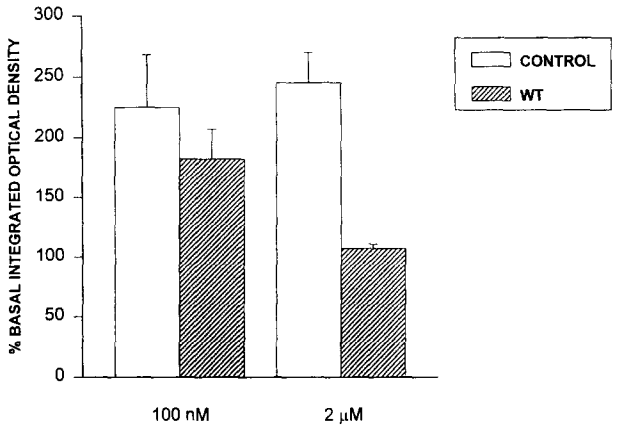

Fig. 3. Densitometric measurements of the phosphotyrosine analysis. Three experiments were performed as described for Fig. 2 on platelets from three different donors and the presence of phosphotyrosine residues quantified by densitometry. The data represent the integrated optical density (mean + SEM) for the entire lane 105 s after the thapsigargin and ionomycin addition.

In human neutrophils, WT attenuates *N*-formyl-Met-Leu-Phe (fMLP) receptor-mediated phospholipase-D activation, without directly affecting the phospholipase activity [17]. This was later found to correlate with an inhibition of phosphoinositide 3-kinase (PI 3-kinase) upstream of the phospholipase [18]. At concentrations of 100 nM or less WT has been shown to specifically and directly block PI 3-kinase [19]. At this concentration WT had no affect on store-mediated calcium influx or protein tyrosine phosphorylation. Consequently, the observed effects at high concentrations is unlikely to be due to a specific inhibition of PI 3-kinase. In a recent report in Swiss 3T3 cells, WT was demonstrated to be a potent inhibitor of phospholipase A_2, as well as PI 3-kinase, at nM concentrations [20]. However, inhibition of phospholipase A_2 cannot explain observations reported here as aspirinated platelets were used.

4. Conclusions

In summary, we have demonstrated that WT at micromolar concentrations effectively suppresses store-depletion evoked calcium influx while having no affect on the thapsigargin-evoked mobilisation of Ca^{2+} from internal stores. At the same concentrations WT also reduced store-depletion-evoked protein tyrosine phosphorylation. Concentrations in the range at which wortmannin inhibits PI-3 kinase were without these effects. Hence, at micromolar concentrations, WT acts as a non-specific tyrosine kinase inhibitor. These findings are compatible with a role for the involvement of a tyrosine phosphorylation step in the link between depletion of Ca^{2+} stores and Ca^{2+} entry in human platelets.

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