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The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets

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Platelet activation is associated with the phosphorylation of a number of platelet proteins at tyrosine residues. The significance of this is unknown. Here we have investigated the effects of two tyrosine kinase inhibitors, methyl 2,5-dihydroxycinnamate and genistein, on thrombin-evoked protein tyrosine phosphorylation and Ca^{2+} signal generation in fura-2-loaded human platelets. Both compounds inhibited thrombin-evoked tyrosine phosphorylation and reduced the elevation of $[Ca^{2+}]_i$ in the presence, but not the absence, of external Ca^{2+}. This suggested a selective inhibition of thrombin-evoked Ca^{2+} entry but not release from internal stores. Both compounds also reduced thrombin-evoked Mn^{2+} entry. In contrast, selective blockade of protein kinase C with Ro 31/8220–002 potentiated the thrombin-evoked Ca^{2+} signal. These data are compatible with a role for protein tyrosine phosphorylation contributing to thrombin-evoked Ca^{2+} entry in human platelets.

Thrombin; Tyrosine phosphorylation; Ca^{2+}; Tyrosine kinase inhibitor; Fura-2; Platelet

1. INTRODUCTION

Agonist-stimulation of platelet plasma membrane receptors results in the Phospholipase C-mediated metabolism of inositol phospholipids and the production of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) [1–3]. $InsP_3$ is responsible for the mobilisation of Ca^{2+} from the internal stores [4]. The mechanism which accounts for the sustained phase of Ca^{2+} entry following agonist-stimulation remains unclear.

During platelet activation many proteins are phosphorylated at tyrosine residues (e.g. [5–9]), although the functional significance of this is uncertain. Increased tyrosine phosphorylation may be an integral component of platelet signal transduction [10]. Platelets possess several protein-tyrosine kinases such as the cellular *src* and *fyn* gene products [11–13]. The cytoplasmic tyrosine kinase $pp60^{c\text{-}src}$ is particularly abundant in platelets [7,13].

Evidence is now emerging that many elements of the signal transduction pathway interact at specific recognition sites, termed SH2-domains [14,15] . These regions are found on many different substrates, and permit the association of substrate with receptors that possess intrinsic tyrosine kinase activity. For example, Phospholipase C-γ has been found to associate with, and be phosphorylated by, several tyrosine kinase receptor systems [16–19]. It is therefore possible that the SH2-domains of the cytoplasmic tyrosine kinases present in platelets, such as *src* and *fyn*, are also involved in the control of PLC-γ activity [20,21].

It has recently been suggested that the cytosolic and intracellular store calcium concentration may antagonistically control platelet tyrosine phosphorylation [22]. In this model elevated cytosolic calcium is proposed to activate a tyrosine kinase leading to phosphorylation of certain platelet proteins and the promotion of calcium entry. A calcium replete internal store is proposed to activate a tyrosine phosphatase such that store refilling would reduce tyrosine phosphorylation and, therefore, Ca^{2+} entry.

In many cell types, including platelets, it has been demonstrated that the depletion of the intracellular Ca^{2+} stores promotes divalent cation entry [23–26], although the nature of the coupling from intracellular Ca^{2+} stores to plasma membrane remains unclear. It has been proposed that cytochrome *P*-450, or one of its metabolites, might couple store depletion to Ca^{2+} entry in human platelets [27] and other cells [28]. This model was proposed on the basis of inhibition of Mn^{2+} and Ca^{2+} entry by inhibitors of cytochrome *P*-450. However, our previous work suggests against a role for the cytochrome in mediating agonist-evoked divalent cation entry [29,30]. A model in which stored and cytosolic

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Abbreviations $[Ca^{2+}]_i$, cytosolic calcium concentration; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SH2, *src* homology region.

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Ca^{2+} exert an antagonistsic control over Ca^{2+} entry is functionally indistinguishable from store-regulated entry and might provide an alternative explanation for this phenomenon in platelets.

To assess the possible role of tyrosine phosphorylation in agonist-evoked Ca^{2+} signal generation we have investigated the effects of the inhibitors of tyrosine kinases, genistein [31,32] and methyl 2,5-dihydroxycinnamate [33,34], using fura-2-loaded human platelets. These inhibitors were compared with Ro 31/8220–002 and Ro 31/7549–001, selective inhibitors of protein kinase C [35]. To confirm the tyrosine kinase inhibitors were effective in platelets, the actions of methyl 2,5-dihydroxycinnamate and genistein on protein tyrosine phosphorylation were assessed by gel electrophoresis and Western blotting with a specific anti-phosphotyrosine antibody.

2. EXPERIMENTAL

Fura-2-loaded human platelets were prepared as previously described [29]. Changes in intracellular free calcium concentration, $[Ca^{2+}]_i$, were monitored using the 340/380 nm fluorescence ratio. In experiments involving genistein or diadzein, changes in $[Ca^{2+}]_i$ were judged from the 340/380 nm ratio since both compounds interfered with fura-2 fluorescence. Genistein was without effect on agonist-evoked Ca^{2+} signals for upto 2 min of incubation, so controls were performed with inhibitor applied immediately prior to addition of agonist. Methyl 2,5-dihydroxycinnamate had negligible effects on fluorescence such that the 340/380 nm ratio could be calibrated in terms of $[Ca^{2+}]_i$ as previously described [36].

Tyrosine phosphorylation was detected by gel electrophoresis and Western blotting [9]. Platelet stimulation was terminated by the addition of an equal volume of Laemmli's buffer [37] with 10% 2-mercaptoethanol followed by heating for 5 mins at 95°C. One-dimensional SDS electrophoresis was performed with 8% polyacrylamide minigels and separated proteins were electrophoretically transferred, for 15 h at 1.5 A, onto Immobilon (Millipore) membranes for subsequent probing. The blots were incubated for 1 h with 5% (w/v) milk protein in Tris-buffered saline with 0.02% Tween (TBST) to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved using a specific anti-phosphotyrosine monoclonal murine antibody (05–321, UBI, USA) diluted 1:2000 in TBST, for 1 h. The primary antibody was removed, and the blots washed six times in TBST. To detect the primary antibody the blots were incubated with HRP-conjugated ovine anti-mouse antibody (NA931, Amersham) diluted 1:6000 in TBST, washed six times in TBST and exposed to ECL reagents for 1 min. Blots were exposed to pre-flashed photographic film for 1 5 min.

2.1. *Materials*

Methyl 2,5-dihydroxycinnamate was a kind gift of Dr. A. Hudson, Wellcome Research Laboratories, Beckenham, UK. Ro 31/8220–002 and Ro 31/7549–001 were kindly provided by Dr. T.J. Hallam, Roche Research Centre, Welwyn Garden City, UK. Apyrase, aspirin, EGTA and Bovine thrombin were from Sigma (Poole, Dorset, UK). All other reagents were of analytical grade.

3. RESULTS AND DISCUSSION

3.1. *Effects of methyl 2,5-dihydroxycinnamate*

Fig. 1a shows that preincubation for 30 min with the tyrosine kinase inhibitor methyl 2,5-dihydroxycinnamate (1 μg/ml), a stable analogue of erbstatin, reduced the Ca^{2+} elevation evoked by thrombin (1 U/ml) in medium containing 1 mM Ca^{2+} from 981 ± 135 nM to 306 ± 59 nM (S.E.M., $n = 10$), at the initial peak. This difference was statistically significant (Student's t-test, difference of the means, $0.001 > P$).

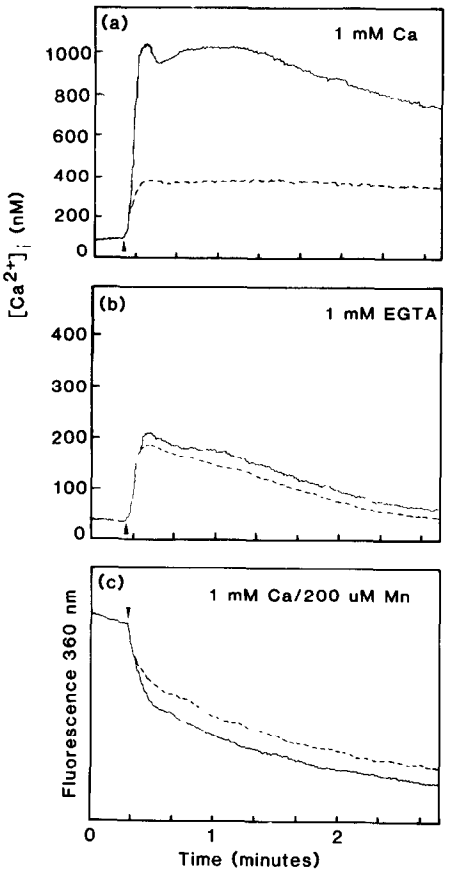

Fig. 1. Effect of methyl 2,5-dihydroxycinnamate on $[Ca^{2+}]_i$ rises and Mn^{2+} entry evoked by thrombin in human platelets. Fura-2-loaded platelets were incubated with 1 μg/ml methyl 2,5-dihydroxycinnamate (dashed lines) or the vehicle, DMSO (solid lines) as control, for 30 mins at 37°C in stirred cuvettes. At the time of experiments either 1 mM Ca^{2+} (a), 1 mM EGTA (b) or 1 mM Ca^{2+} and 200 μM Mn^{2+} (c) were added to cells. Thrombin (1 U/ml), was added as shown. The 340/380 nm ratio was calibrated in terms of $[Ca^{2+}]_i$ (top two panels). In the bottom panel the trace shows quench of fura-2 fluorescence with excitation at 360 nm, confirming divalent cation entry.

The possibility that the inhibition of tyrosine phosphorylation by methyl 2,5-dihydroxycinnamate may interfere with the production of inositol 1,4,5-trisphosphate ($InsP_3$), through inhibition of PLC-γ, was tested for by examination of the release of the intracellular Ca^{2+} stores. A preincubation with methyl 2,5-dihydroxycinnamate (1 μg/ml; 30 min) had little effect upon the release of Ca^{2+} from the internal stores, as indicated by experiments performed in the presence of 1 mM EGTA (Fig. 1b). The initial, peak elevation of $[Ca^{2+}]_i$ was 153 ± 19 nM after preincubation with methyl 2,5-dihydroxycinnamate (100 μM; 30 min) and 193 ± 14

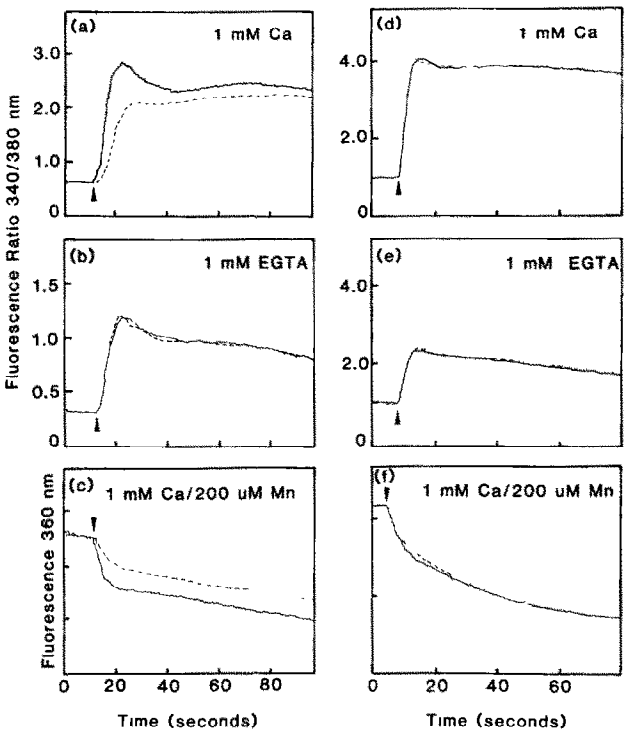

Fig. 2. Effects of genistein or diadzein on thrombin-evoked $[Ca^{2+}]_i$ rises and Mn^{2+} entry in human platelets. Fura-2-loaded platelets were incubated for 30 min, in stirred cuvettes at 37°C, with either 100 μM genistein, left hand panels, or 100 μM diadzein, right hand panels (dashed lines) or the vehicle, DMSO, as control (solid lines). At the time of experiments either 1 mM Ca^{2+} (a) and (d), 1 mM EGTA (b) and (e) or 1 mM Ca^{2+} and 200 μM Mn^{2+} (c) and (f) were added. All other details are as for Fig. 1, except that changes in $[Ca^{2+}]_i$ were judged from the 340/380 nm ratio.

nM in controls (S.E.M., $n = 10$). This difference was not significant (Student's t-test, difference of the means, $0.5 > P > 0.1$).

The lack of a significant effect of methyl 2,5-dihydroxycinnamate on the release of Ca^{2+} from the intracellular stores suggests that the effect of the inhibitor on PLC-γ is small. Therefore, in platelets, at least, tyrosine kinase-mediated regulation of PLC-γ is unlikely to be an important contribution to the initial discharge of the internal Ca^{2+} stores. These data suggest that methyl 2,5-dihydroxycinnamate is relatively selective at inhibiting thrombin-evoked Ca^{2+} entry.

The inhibition of divalent cation influx was confirmed by reduction of thrombin-evoked Mn^{2+} quench of fura-2 fluorescence in medium containing 1 mM Ca^{2+} and 200 μM Mn^{2+} (Fig. 1c).

3.2. *Effects of genistein and diadzein*

Fig. 2 shows results obtained with another tyrosine kinase inhibitor, genistein, and its inactive analogue, diadzein. Preincubation with genistein (100 μM; 30 min) reduced the thrombin-evoked rise in $[Ca^{2+}]_i$, as indicated by the 340/380 nm fluorescence ratio, in medium containing 1 mM Ca^{2+} by about 35% (Fig. 2a), at the peak of response, as compared with controls. Responses in medium containing 1 mM EGTA were unaffected by a similar preincubation with genistein (Fig. 2b), indicating that the inhibitor was without effect on the release of Ca^{2+} from the intracellular stores. The inhibition of agonist-evoked divalent cation entry by genistein was further demonstrated by the finding that genistein reduced the thrombin-evoked Mn^{2+} quench of fura-2 fluorescence in medium containing 200 μM Mn^{2+} (Fig. 2c). Preincubation with diadzein (100 μM; 30 min) was without effect on thrombin-evoked Ca^{2+} entry, release of Ca^{2+} from internal stores and Mn^{2+} entry (Fig. 2d, e and f, respectively).

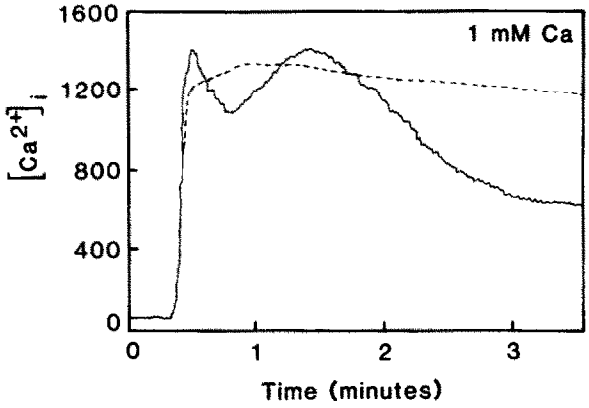

Fig. 3. Effect of the selective protein kinase C inhibitor, Ro 31/8220–002, on the rise in $[Ca^{2+}]_i$ evoked by thrombin in human platelets. Fura-2-loaded platelets were incubated with 3 μM Ro 31/8220–002 (dashed line) or the vehicle (solid line) for 20 min in stirred cuvettes at 37°C before addition of 1 U/ml thrombin. The 340/380 nm fluorescence ratio was calibrated in terms of $[Ca^{2+}]_i$.

3.3. *Effects of the specific protein kinase C inhibitor Ro 31/8220–002*

Although these results suggest that inhibition of tyrosine kinase activity selectively reduces thrombin-evoked Ca^{2+} entry, the selectivity of presently available inhibitors is uncertain, and effects on other kinases cannot be excluded. However, the results presented here are unlikely to be due to inhibition of protein kinases A, C or G, all of which have inhibitory effects on Ca^{2+} signal generation in platelets [38]. If these kinases were being inhibited, Ca^{2+} signals might be expected, if anything, to be potentiated, rather than reduced. This point is addressed in Fig. 3. Preincubation with the selective protein kinase C inhibitor Ro 31/8220–002 (3 μM; 20 min) slightly, but not significantly, decreased the peak $[Ca^{2+}]_i$ rise evoked by thrombin, 1283 ± 97 nM compared with 1484 ± 157 nM in controls (S.E.M., $n = 3$, Student's t-test, difference of the means $0.5 > P > 0.1$), and significantly slowed the subsequent decline of the maintained elevated $[Ca^{2+}]_i$ phase, 1112 ± 98 nM compared with 715 ± 103 nM (S.E.M., $n = 3$, $0.05 > P$), at

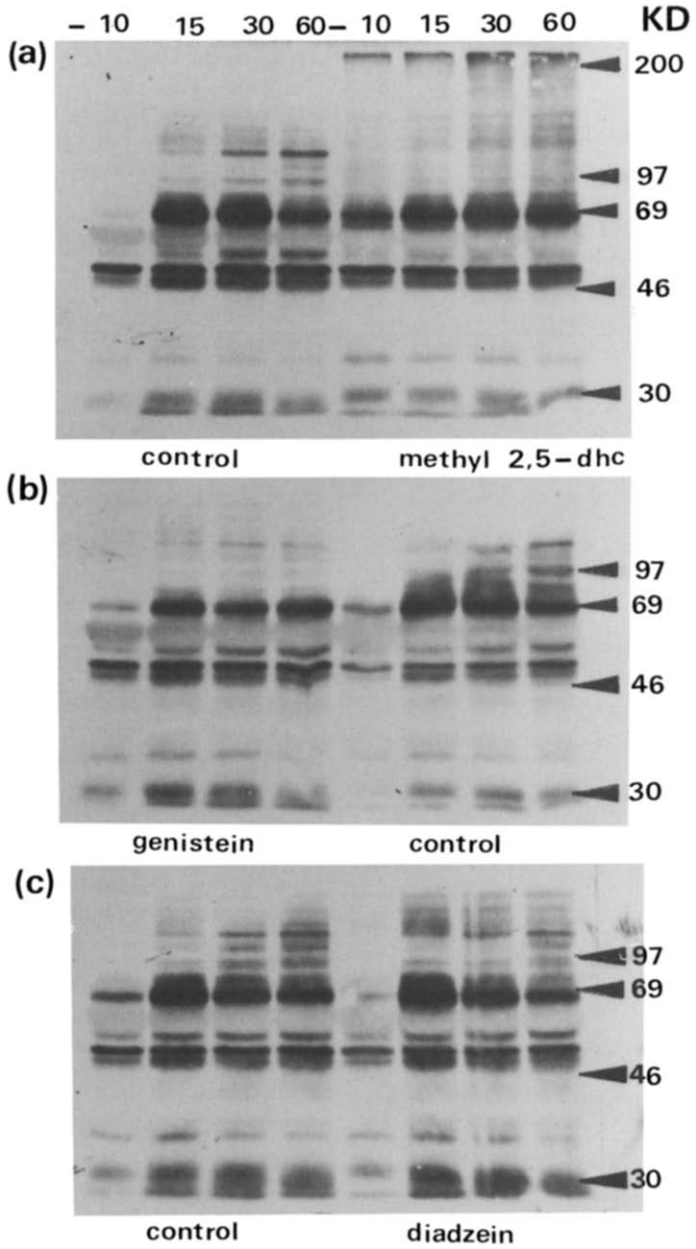

Fig. 4. Effects of genistein, diadzein and methyl 2,5-dihydroxycinnamate on changes of tyrosine phosphorylation in thrombin stimulated human platelets. 100 μl aliquots were taken from a cuvette of stirred, fura-2-loaded platelet suspension at 10 s before agonist (−10), and 15, 30 and 60 s after addition of thrombin (1 U/ml). Platelet suspension had been preincubated for 30 min at 37°C in stirred cuvettes with either methyl 2,5-dihydroxycinnamate (methyl 2,5-dhc) (1 μg/ml) (a) (four right-hand lanes), genistein (100 μM) (b) (four left-hand lanes) or diadzein (100 μM) (c) (four right-hand lanes), or the vehicle, DMSO, (all other lanes) as control. Proteins were analysed by 8% SDS-PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody as described in section 2.

3 min post stimulation. Similar results were obtained with another selective PKC inhibitor, Ro 31/7549–001.

3.4. *Examination of protein tyrosine phosphorylation*

The inhibition of agonist-evoked Ca^{2+} and Mn^{2+} entry by the tyrosine kinase inhibitors suggests that tyrosine phosphorylation may be a component of platelet Ca^{2+} signal generation. However, effects other than on tyrosine phosphorylation cannot be ruled out. To assess the effectiveness of the tyrosine kinase inhibitors used here against agonist-evoked tyrosine phosphorylation in fura-2-loaded human platelets, samples were taken during the timecourses of fluorescence experiments and protein tyrosine phosphorylation determined. Typical gels are shown in Fig. 4. A 30 min incubation with methyl 2,5-dihydroxycinnamate (1 μg/ml) or genistein (100 μM), (Fig. 4a and b), reduced thrombin-stimulated protein tyrosine phosphorylation, with inhibition most evident at certain bands (eg. genistein most strongly inhibited tyrosine phosphorylation

of proteins 97–200KD; methyl 2,5-dihydroxycinnamate inhibited two specific bands of 97KD and approximately 140KD, Fig. 4a and b). Diadzein (100 μM), the inactive analogue, was without significant effect on thrombin-evoked tyrosine phosphorylation (Fig. 4c).

3.5. *Conclusions*

In conclusion, we have demonstrated that two tyrosine kinase inhibitors, methyl 2,5-dihydroxycinnamate and genistein, effectively reduced thrombin-evoked protein tyrosine phosphorylation in human platelets. Both compounds inhibited thrombin-evoked Ca^{2+} and Mn^{2+} influx whilst having little effect on thrombin-evoked release of Ca^{2+} from the intracellular stores. These data are compatible with a role for protein tyrosine phosphorylation in the generation of agonist-evoked Ca^{2+} entry in human platelets.

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