Tyrosine kinases activate store-mediated Ca^{2+} entry in human platelets through the reorganization of the actin cytoskeleton

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We have recently reported that store-mediated Ca^{2+} entry in platelets is likely to be mediated by a reversible trafficking and coupling of the endoplasmic reticulum with the plasma membrane, a model termed 'secretion-like coupling'. In this model the actin cytoskeleton plays a key regulatory role. Since tyrosine kinases have been shown to be important for Ca^{2+} entry in platelets and other cells, we have now investigated the possible involvement of tyrosine kinases in the secretion-like-coupling model. Treatment of platelets with thrombin or thapsigargin induced actin polymerization by a calcium-independent pathway. Methyl 2,5-dihydroxycinnamate, a tyrosine kinase inhibitor, prevented thrombin- or thapsigargin-induced actin polymerization. The effects of tyrosine kinases in store-mediated Ca^{2+} entry were found to be entirely dependent on the actin cytoskeleton. PP1, an inhibitor of the Src family of proteins, partially inhibited store-mediated Ca^{2+} entry. In addition, depletion of intracellular Ca^{2+} stores stimulated cytoskeletal association of the cytoplasmic tyrosine kinase $pp60^{src}$, a process that was sensitive to treatment with cytochalasin D and PP1, but not to inhibition of Ras proteins using prenylcysteine analogues. Finally, combined inhibition of both Ras proteins and tyrosine kinases resulted in complete inhibition of Ca^{2+} entry, suggesting that these two families of proteins have independent effects in the activation of store-mediated Ca^{2+} entry in human platelets.

Key word: Src.

INTRODUCTION

In non-excitable cells, the main mechanism for Ca^{2+} influx is store-mediated Ca^{2+} entry (SMCE), where the filling state of the intracellular Ca^{2+} stores regulates the entry of Ca^{2+} across the plasma membrane (PM) [1]. However, how depletion of the Ca^{2+} stores activates Ca^{2+} entry is not well understood. Hypotheses have considered both indirect and direct coupling mechanisms. Indirect coupling assumes the existence of a diffusible messenger generated by the Ca^{2+}-storage organelles, such as a calcium influx factor [2], cytochrome P450 metabolites [3], cGMP [4] or small GTP-binding proteins [5]. Alternatively, direct coupling (conformational coupling) proposes a physical interaction between the endoplasmic reticulum (ER) and the PM [6]. Recently, a secretion-like-coupling model has been proposed in smooth-muscle cell lines and human platelets [7,8] that is based on a reversible coupling of the ER with the PM which involves trafficking of the ER towards the PM.

In the secretion-like-coupling model, the actin cytoskeleton plays an important regulatory role, as it does in secretion. A key role resides in the cortical actin filaments that can prevent the coupling between the ER and the PM [7,8]. The actin cytoskeleton has been shown to be important for SMCE in different cells types [9,10], including platelets [6,8]. Consistent with this, small GTP-binding proteins, which play a central role in several transduction pathways through the organization of the actin cytoskeleton, have been shown to be important for SMCE in many cell types [5,11,12]. We have recently reported that the effect of small GTP-binding proteins is partially mediated through the reorganization of the actin cytoskeleton in human platelets [5].

Platelet activation is accompanied by a dramatic increase in tyrosine phosphorylation of many cellular proteins [13]. Several protein-tyrosine kinases, such as those of the Src family, FAK and Syk, are involved in these events [13]. The cytoplasmic tyrosine kinase $pp60^{src}$ is particularly abundant in platelets [14]. A role for protein-tyrosine phosphorylation in the regulation of SMCE has been proposed on the basis of the correlation between an increase in phosphotyrosine levels and the filling state of the intracellular Ca^{2+} stores, as well as the effects of different tyrosine kinase inhibitors, such as methyl 2,5-dihydroxycinnamate (M-2,5-DHC) and genistein, on agonist- and thapsigargin (TG)-evoked Ca^{2+} entry [15–18]. In the present study we sought to expand our understanding of the involvement of tyrosine kinases in SMCE in platelets. We report here that inhibition of tyrosine phosphorylation blocks actin polymerization, which might play a key role in the regulation of SMCE by tyrosine kinases. Our findings also indicate that Ras proteins and tyrosine kinases play a complementary role in the activation of SMCE. We have also investigated the translocation to the actin cytoskeleton of the protein-tyrosine kinase, $pp60^{src}$, after depletion of the internal Ca^{2+} stores in human platelets.

MATERIALS AND METHODS

Materials

Fura 2 acetoxymethyl ester (fura 2/AM) was from Texas Fluorescence (Austin, TX, U.S.A.). Apyrase (grade VII), aspirin, BSA, paraformaldehyde, Nonidet P40, M-2,5-DHC, fluorescein isothiocyanate-labelled phalloidin, thrombin and TG were from Sigma (Poole, Dorset, U.K.). Cytochalasin D and ionomycin were from Calbiochem (Nottingham, U.K.). PP1, farnesylthioacetic acid (FTA) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC) were from Alexis Corporation (Nottingham, U.K.). Anti-phosphotyrosine monoclonal antibody (4G10) and anti-$pp60^{src}$ monoclonal antibody GD11 were from Upstate Bio-

Abbreviations used: SMCE, store-mediated Ca^{2+} entry; $[Ca^{2+}]_i$, intracellular free calcium concentration; TG, thapsigargin; HBS, Hepes-buffered saline; PM, plasma membrane; ER, endoplasmic reticulum; M-2,5-DHC, methyl 2,5-dihydroxymethylcinnamate; FTA, farnesylthioacetic acid; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; FC, (*S*)-all-*trans*-farnesyl-L-cysteine; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid; AM, acetoxymethyl ester.

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technology (Lake Placid, NY, U.S.A.). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham (Little Chalfont, Bucks., U.K.). Dimethyl bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid/AM (dimethyl BAPTA/AM) was from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade.

Platelet preparation

Fura 2-loaded platelets were prepared as described previously [5]. Briefly, blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 ***g*** and aspirin (100 μM) and apyrase (40 μg/ml) were added. Platelet-rich plasma was incubated at 37 °C with 2 μM fura 2/AM for 45 min. For loading with dimethyl BAPTA, cells were incubated for 30 min at 37 °C with 10 μM dimethyl BAPTA/AM. Cells were then collected by centrifugation at 350 ***g*** for 20 min and resuspended in Hepes-buffered saline (HBS) containing (in mM): 145 NaCl, 10 Hepes, 10 D-glucose, 5 KCl, 1 $MgSO_4$, (pH 7.45) and supplemented with 0.1 % (w/v) BSA and 40 μg/ml apyrase.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Fluorescence was recorded from 1.5 ml aliquots of magnetically stirred platelet suspension (10^8 cells/ml) at 37 °C using a Cairn Research (Faversham, Kent, U.K.) spectrophotometer, with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura 2 340/380 fluorescence ratio. It was not possible to calibrate traces when M-2,5-DHC was present because of its effect on fura 2 fluorescence, which results in a small decrease in the 340/380 fluorescence ratio. For this reason, in controls M-2,5-DHC was added immediately prior to the addition of Ca^{2+} in order to produce the same fluorescence effect such that traces could be compared. Previous experiments have confirmed that M-2,5-DHC is without effect on agonist-evoked $[Ca^{2+}]_i$ increases for up to 5 min of incubation [16].

Determination of Ca^{2+} entry

Ca^{2+} influx in platelets which had been store depleted using TG was estimated using the integral of the increase in the 340/380 fluorescence ratio for 2.5 min after addition of $CaCl_2$ [5]. When platelets were preincubated with inhibitors, Ca^{2+} entry was corrected by subtraction of the 340/380 fluorescence ratio due to leakage of the indicator. Thrombin-evoked Ca^{2+} entry was measured as the integral of the increase in the 340/380 fluorescence ratio above basal for 90 s after the addition of thrombin in the presence of external Ca^{2+}, corrected by subtraction of the integral over the same period of stimulation in the absence of external Ca^{2+} (with 100 μM EGTA).

Measurement of F-actin content

The F-actin content of resting and activated platelets was determined with modifications [19] according to a previously published procedure [20]. Briefly, washed platelets (2×10^8 cells/ml) were activated in HBS. Samples of platelet suspension (200 μl) were transferred to 200 μl of ice-cold 3 % (w/v) formaldehyde in PBS for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025 % (v/v) Nonidet P40 detergent dissolved in PBS. Platelets were then incubated for 30 min with FITC-labelled phalloidin (1 μM) in PBS supplemented with 0.5 % (w/v) BSA. After incubation the platelets were collected by centrifugation in an MSE Micro-Centaur Centrifuge (MSE Scientific Instruments, Crawley, Sussex, U.K.) for 60 s at 3000 ***g*** and resuspended in PBS. Staining of 2×10^7 cells/ml was measured using a Perkin–Elmer (Norwalk, CT, U.S.A.) fluorescence spectrophotometer. Samples were excited at 496 nm and emission was at 516 nm.

Protein tyrosine phosphorylation

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting [16]. Platelet stimulation was terminated by mixing them with an equal volume of 2 × Laemmli's buffer [21] with 10 % dithiothreitol, followed by heating for 5 min at 95 °C. One-dimensional SDS/PAGE was performed with 10 % (w/v) polyacrylamide mini-gels and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm², in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs., U.K.) on to nitrocellulose for subsequent probing. Blots were incubated overnight with 10 % (w/v) BSA in Tris-buffered saline (20 mM Tris base/137 mM NaCl, pH 7.6 at 37 °C) with 0.1 % Tween 20 (TBST) to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved using the anti-phosphotyrosine antibody 4G10 diluted 1:2500 in TBST for 1 h. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody diluted 1:10000 in TBST, washed six times in TBST, and exposed to enhanced-chemiluminescence reagents for 1 min. Blots were then exposed to preflashed photographic film. Densitometric measurements were made using a Quantimet 500 densitometer (Leica, Milton Keynes, U.K.) and the integrated absorbance of the entire lane was estimated.

Analysis of cytoskeleton-associated pp60^{src}

Human platelet fractionation was carried out as described previously [22]. Briefly, activated and control platelets (2×10^9 cells/ml) were immediately lysed with an equal volume of 2 × Triton buffer [2 % Triton X-100/2 mM EGTA/100 mM Tris/HCl (pH 7.2)/100 μg/ml leupeptin/2 mM PMSF/10 mM benzamidine/2 mM Na_3VO_4] at 4 °C for 30 min. Platelet lysate was centrifuged at 16000 ***g*** for 5 min. The supernatant was removed, and the pellet (cytoskeleton-rich fraction) was solubilized into the original volume in Laemmli's buffer [21], boiled for 5 min and subjected to Western blotting as described previously using the anti-pp60^{src} monoclonal antibody GD11.

Statistical analysis

Analysis of statistical significance was performed using Student's *t*-test. For multiple comparisons, one-way analysis of variance combined with the Dunnett test was used.

RESULTS

Effect of M-2,5-DHC on thrombin- or TG-induced actin polymerization in human platelets

We have previously shown that the physiological agonist thrombin and TG, an inhibitor of the endomembrane Ca^{2+}-ATPase ['SERCA' (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase)], induce actin polymerization in human platelets [5]. Treatment

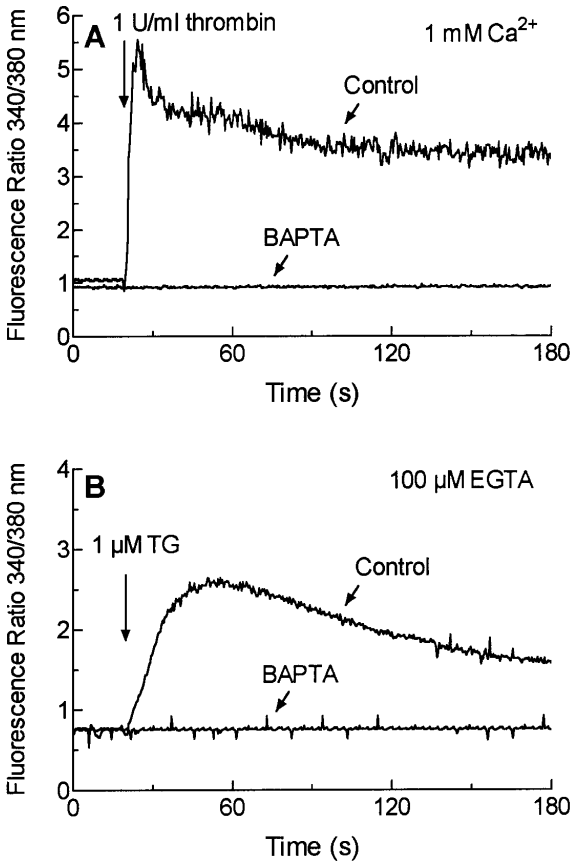

Figure 1 Effects of dimethyl BAPTA on thrombin- or TG-induced calcium mobilization in platelets

Human platelets were incubated at 37 °C for 30 min in the presence or absence of 10 μM dimethyl BAPTA. At the time of experiment, 1mM Ca^{2+} or 100 μM EGTA was added as indicated. Cells were then stimulated with 1 unit/ml thrombin (**A**) or 1 μM TG (**B**). Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio as described in the Materials and methods section. Traces shown are representative of five independent experiments.

of platelets with 1 unit/ml thrombin in medium containing 1 mM Ca^{2+} increased F-actin content by $61 \pm 2.5\%$. Depletion of the internal Ca^{2+} stores using 1 μM TG in a Ca^{2+}-free medium also raised F-actin content by $38.3 \pm 4.4\%$. In order to investigate whether this event is entirely mediated by the elevation of $[Ca^{2+}]_i$, we loaded platelets with dimethyl BAPTA, an intracellular Ca^{2+} chelator, by incubating the cells for 30 min at 37 °C with 10 μM dimethyl BAPTA/AM. As shown in Figure 1, dimethyl BAPTA loading prevented both thrombin- and TG-evoked $[Ca^{2+}]_i$ elevations, but only decreased thrombin- or TG-induced actin polymerization by 21.0 ± 4.9 and $27.1 \pm 11.1\%$ respectively (Table 1; $P < 0.05$; $n = 4$–5).

Treatment of human platelets with various concentrations of the tyrosine kinase inhibitor M-2,5-DHC inhibited thrombin- or TG-induced actin polymerization in a concentration-dependent manner, with IC_{50} values of 0.30 ± 0.14 and 0.15 ± 0.20 μg/ml respectively, and complete inhibition at 1 μg/ml (Table 1; $P < 0.05$; $n = 4$–5). A similar effect was observed in dimethyl BAPTA-loaded platelets. Treatment of dimethyl BAPTA-loaded platelets with M-2,5-DHC also resulted in a concentration-dependent inhibition of thrombin- and TG-induced actin polymerization, with IC_{50} values of 0.28 ± 0.13 and 0.10 ± 0.02 μg/ml respectively, and complete inhibition at a concentration of 1 μg/ml (Table 1; $P < 0.05$; $n = 4$–5).

We previously reported that M-2,5-DHC inhibits thrombin-evoked tyrosine phosphorylation [23]. Store-depletion-induced protein-tyrosine phosphorylation was assessed by gel electrophoresis and Western blotting with a specific antiphosphotyrosine antibody. Platelets heavily loaded with the Ca^{2+} chelator dimethyl BAPTA were used for this study so as to eliminate Ca^{2+}-dependent, but not store-depletion-dependent, tyrosine phosphorylation [17]. Dimethyl BAPTA-loaded platelets were incubated for 30 min at 37 °C in the presence of M-2,5-DHC or the vehicle and Ca^{2+} stores were depleted using TG (250 nM) in the presence of a low concentration of ionomycin (50 nM; required for extensive depletion of the intracellular Ca^{2+} stores in platelets where two Ca^{2+} stores with high and low Ca^{2+} leakage rates have been described [24,25]). In each experiment we checked that the increase in $[Ca^{2+}]_i$ evoked by TG and ionomycin was abolished by BAPTA loading (results not shown). Samples for protein phosphorylation analysis were taken from the spectrophotometer cuvette 10 s before, and 45, 170 and 300 s after, the addition of TG and ionomycin. As shown in Figure 2, pre-incubation for 30 min with M-2,5-DHC (1 μg/ml) significantly reduced protein-tyrosine phosphorylation relative to its control in store-depleted cells at all time points checked ($P < 0.05$; $n = 4$).

Table 1 Effect of preincubation with M-2,5-DHC on the actin-filament content of unstimulated or either thrombin- or TG-stimulated platelets

Dimethyl BAPTA-loaded platelets or control platelets (DMSO was added) were incubated for 30 min at 37 °C in the absence or presence of various concentrations of M-2,5-DHC as indicated. Platelets were then treated with either 1 unit/ml thrombin in a medium containing 1 mM Ca^{2+} or 1 μM TG in a Ca^{2+}-free medium (300 μM EGTA was added). Samples were removed 5 s before and 3 min after adding thrombin or TG and actin filament content was determined as described in the Materials and methods section. Values given are thrombin- or TG-induced actin-filament formation as a percentage of control (thrombin- or TG-stimulated non-treated platelets) and results are expressed as means $\pm$ S.E.M. of four or five separate determinations. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the F-actin content in the absence of M-2,5-DHC.

			Filamentous actin (% of control)			
Treatment	Stimulatory agent	[M-2,5-DHC] (μg/ml) ...	0	0.1	0.3	1
DMSO	None		100.0 ± 0.0	99.4 ± 2.6	98.7 ± 0.7	101.8 ± 1.6
	Thrombin		161.1 ± 2.5	$143.6 \pm 3.2^*$	$117.8 \pm 1.5^{**}$	$101.7 \pm 2.1^{***}$
	None		100.0 ± 0.0	95.9 ± 8.3	105.5 ± 4.8	98.8 ± 2.6
	TG		150.0 ± 3.7	$116.1 \pm 6.0^*$	$110.6 \pm 6.8^{**}$	$104.0 \pm 7.7^{**}$
BAPTA	None		100.0 ± 0.0	100.0 ± 1.5	101.2 ± 1.7	99.4 ± 1.5
	Thrombin		148.2 ± 3.2	$134.3 \pm 2.9^*$	$115.6 \pm 1.2^{**}$	$100.5 \pm 1.6^{***}$
	None		100.0 ± 0.0	96.6 ± 4.0	95.2 ± 4.1	103.2 ± 3.2
	TG		133.2 ± 5.9	$110.2 \pm 7.3^*$	$104.9 \pm 5.4^*$	$102.3 \pm 4.6^{**}$

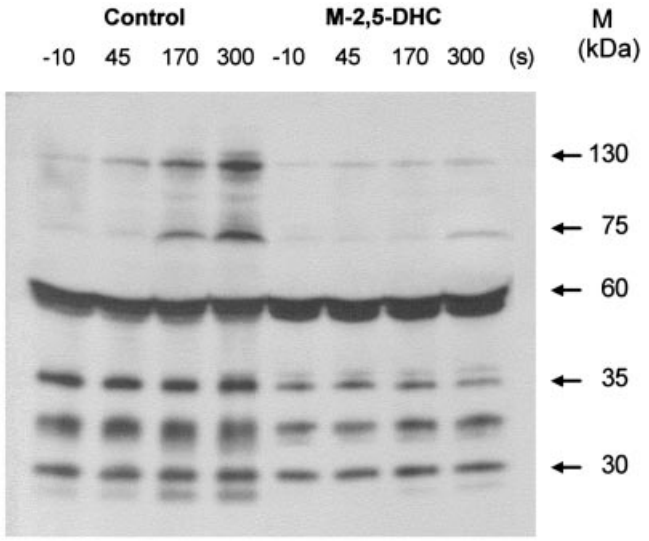

Figure 2 Effect of M-2,5-DHC on store-depletion-evoked protein-tyrosine phosphorylation in dimethyl BAPTA-loaded platelets

Dimethyl BAPTA-loaded platelets were preincubated for 30 min at 37 °C with either 1 μg/ml M-2,5-DHC or DMSO ('Control') and then were treated with 250 nM TG and 50 nM ionomycin to deplete the intracellular Ca^{2+} stores. Samples were taken from the platelet suspension at 10 s before, and 45, 170 and 300 s after, the addition of TG and ionomycin. Platelet proteins were analysed by SDS/10% -PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody as described in the Materials and methods section. Molecular masses ('M') indicated on the right were determined using molecular-mass markers run in the same gel.

Role of the actin cytoskeleton in the M-2,5-DHC-induced decrease in TG-evoked SCME in human platelets

In the absence of extracellular Ca^{2+}, addition of TG (200 nM) to fura 2-loaded platelets in stirred cuvettes at 37 °C induced an elevation in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores. Subsequent addition of $CaCl_2$ (300 μM) to the external medium resulted in a sustained increase in $[Ca^{2+}]_i$, indicative of SMCE (Figure 3).

Pretreatment of human platelets for 30 min at 37 °C with M-2,5-DHC decreased SMCE in a concentration-dependent manner (Figure 3A). M-2,5-DHC significantly decreased SMCE by $12 \pm 3\%$, $26 \pm 5\%$, $52 \pm 3\%$ and $53 \pm 6\%$ at 0.1, 0.3, 1 and 3 μg/ml respectively (Figure 3A; $P < 0.05$; $n = 4$). Since both 1 and 3 μg/ml M-2,5-DHC inhibit SMCE to the same extent, and 1 μg/ml abolished actin polymerization induced by both thrombin and TG, we used this concentration of M-2,5-DHC throughout subsequent parts of the study.

We have previously reported that treatment of human platelets for 40 min with cytochalasin D, an inhibitor of actin polymerization, prevents TG-induced actin polymerization in a concentration-dependent manner, reaching a complete inhibition at the concentration 10 μM [5]. Treatment of platelets for 40 min with 10 μM cytochalasin D decreased TG-induced SMCE by $52.0 \pm 3.2\%$ (Figure 3B; $P < 0.01$; $n = 6$). Simultaneous treatment with both 1 μg/ml M-2,5-DHC and 10 μM cytochalasin D resulted in a level of inhibition similar to that obtained with either of the agents separately, $47.5 \pm 3.7\%$ (Figure 3B; $P < 0.01$; $n = 6$). These findings suggest that the effect of tyrosine kinases might be entirely dependent on the actin cytoskeleton.

Effect of M-2,5-DHC on the maintenance of SMCE

Previously we reported that the integrity of the actin cytoskeleton is essential for the maintenance of SMCE in human platelets [8].

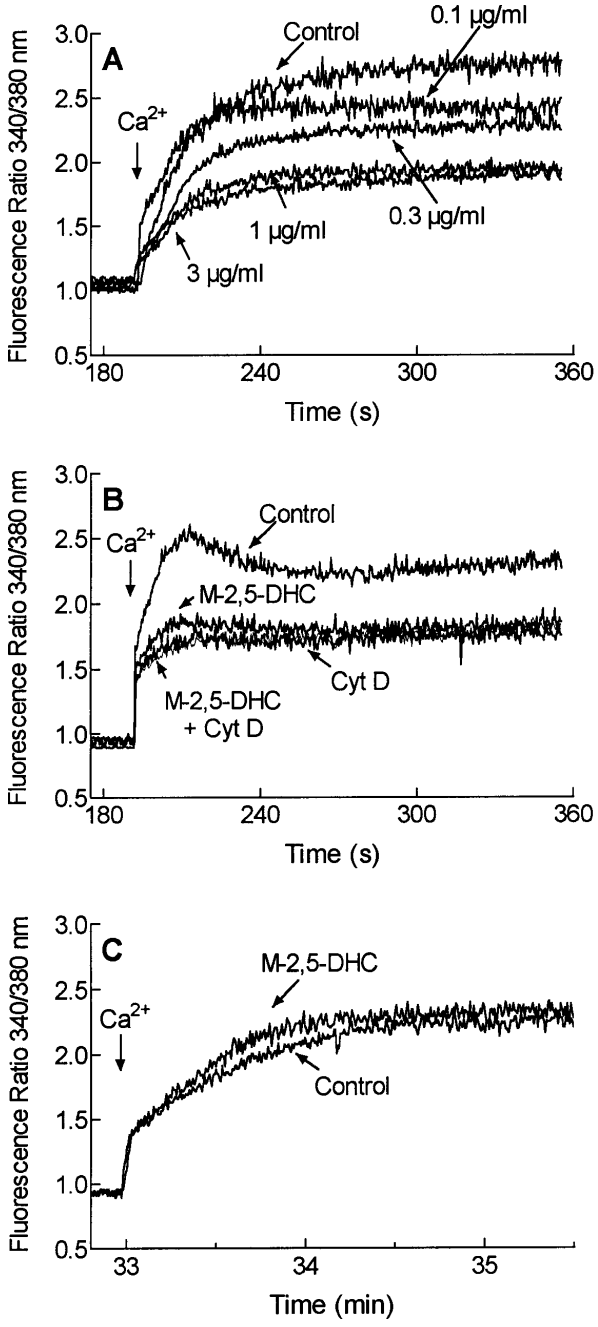

Figure 3 Role of tyrosine kinases and the actin cytoskeleton in SMCE

Fura 2-loaded human platelets were incubated at 37 °C in the presence of either increasing concentrations of M-2,5-DHC (0.1–3 μg/ml) for 30 min (**A**) or in the presence of 1 μg/ml M-2,5-DHC for 30 min (M-2,5-DHC), 10 μM cytochalasin D for 40 min (cytochalasin D), both (M-2,5-DHC + cytochalasin D) (**B**) or the vehicles (Control). At the time of the experiment 100 μM EGTA was added. Cells were then stimulated with TG (200 nM) and, 3 min later, $CaCl_2$ (final concn. 300 μM) was added to the medium to initiate Ca^{2+} entry. (**C**) Human platelets were suspended in a Ca^{2+}-free medium (100 μM EGTA was added) as described in the Materials and methods section. Cells were then stimulated with TG (200 nM) and, 3 min later, 1 μg/ml M-2,5-DHC or the vehicle ('Control') was added. $CaCl_2$ (final concn. 300 μM) was added to the medium 30 min later. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio as described in the Materials and methods section. Because of the effect of M-2,5-DHC in the 340/380 fluorescence ratio, in control or cytochalasin D-treated cells, M-2,5-DHC was added immediately before the addition of Ca^{2+} to produce the same fluorescence effect such that traces could be compared. Traces are representative of four to eight independent experiments. Times shown on the *x*-axis are those elapsed from the start of the incubation with inhibitor or vehicle.

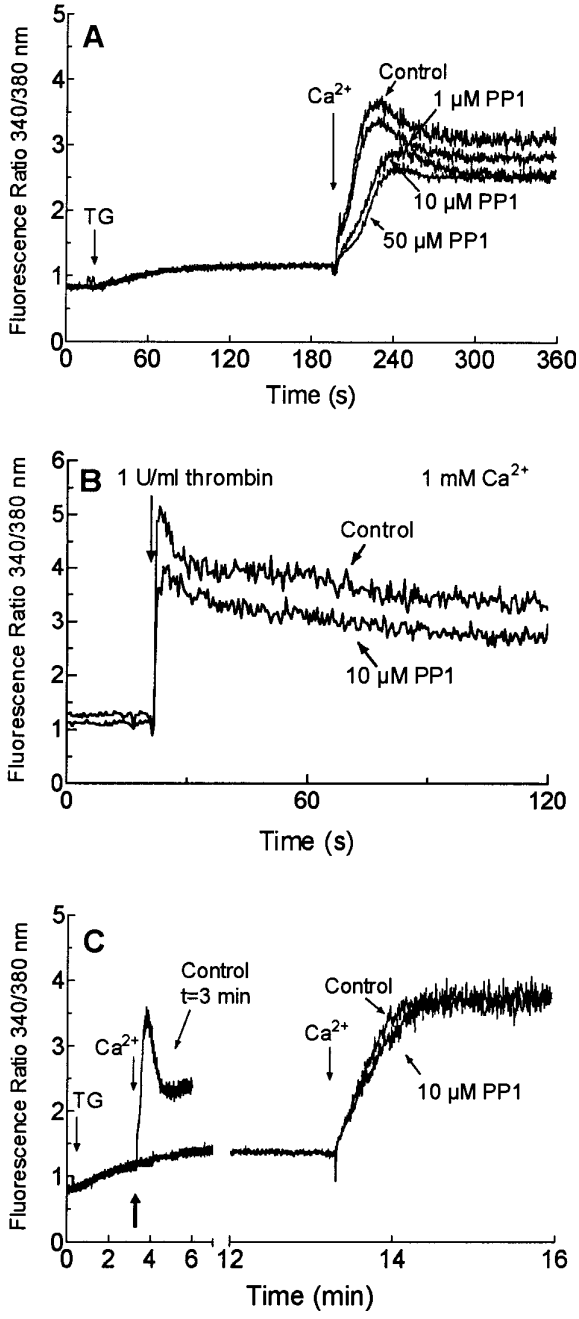

Figure 4 Effect of PP1 on thrombin- or TG-evoked Ca^{2+} entry

(**A**) Fura 2-loaded human platelets were incubated at 37 °C for 10 min in the presence of increasing concentrations of PP1 (1–50 μM) or the vehicle ('Control'). At the time of the experiment, 100 μM EGTA was added. Cells were then stimulated with TG (200 nM) and, 3 min later, $CaCl_2$ (final concn. 300 μM) was added to the medium to initiate Ca^{2+} entry. Traces shown are representative of 11 independent experiments. (**B**) Fura 2-loaded human platelets were incubated for 10 min at 37 °C in the presence of 10 μM PP1 or the vehicle ('Control'). At the time of the experiment, 1 mM Ca^{2+} was added. Cells were then stimulated with thrombin (1 unit/ml) at the time indicated. Traces shown are representative of four separate experiments. (**C**) Fura 2-loaded human platelets were suspended in a Ca^{2+}-free medium (100 μM EGTA added) as described in the Materials and methods section. Cells were then stimulated with TG (200 nM) and, 3 min later, 10 μM PP1 or the vehicle ('Control') were added, as indicated by the heavy arrow. $CaCl_2$ (final concn. 300 μM) was added to the medium 3 min after TG to check that Ca^{2+} entry was activated at the time of inhibitor addition ('Control', $t = 3$ min) or 10 min after PP1 or the vehicle to initiate Ca^{2+} entry ('Control'). Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio as described in the Materials and methods section. Traces are representative of six independent experiments.

To investigate the role of tyrosine kinases in the maintenance of SMCE, we examined the effect of M-2,5-DHC on Ca^{2+} entry in platelets after SMCE had been previously stimulated using TG.

Figure 3(C) shows the effect of adding M-2,5-DHC to store-depleted human platelets. M-2,5-DHC (1 μg/ml) or the vehicle (DMSO) was added 3 min after TG and cells were then incubated for a further 30 min before the addition of Ca^{2+} to the medium (final concn. 300 μM) to initiate Ca^{2+} entry. At the time when M-2,5-DHC was added, Ca^{2+} entry was already stimulated (results not shown, but see Figure 4B). Addition of M-2,5-DHC after activation of SMCE did not significantly modify the Ca^{2+} entry (Figure 3C; $P = 0.31$; $n = 5$). Under these conditions M-2,5-DHC did not reverse the actin-filament formation stimulated by TG ($n = 6$; results not shown). These findings are compatible with a role for tyrosine kinases in the activation of SMCE, but not in its maintenance. In addition, these observations indicate that M-2,5-DHC does not act as a Ca^{2+} channel blocker, Ca^{2+} chelator or a non-specific inhibitor of actin polymerization.

Effect of the src inhibitor PP1 on Ca^{2+} entry and actin polymerization

PP1, a potent and selective inhibitor of the Src family of protein tyrosine kinases [26], was used to investigate the involvement of these proteins in SMCE. As shown in Figure 4(A), treatment of human platelets for 10 min at 37 °C with PP1 resulted in a concentration-dependent inhibition of TG-stimulated Ca^{2+} entry. Treatment with PP1 significantly decreased SMCE by $16 \pm 4\%$, $32 \pm 4\%$ and $35 \pm 7\%$ at 1, 10 and 50 μM respectively (Figure 4A; $P < 0.05$; $n = 4$). PP1 did not modify the resting cytosolic Ca^{2+} level, nor did it have any effect on TG-induced Ca^{2+} release from the intracellular stores, indicating that accumulation of Ca^{2+} in the internal stores was unaffected by treatment with PP1 (Figure 4A). Since the effect of 10 and 50 μM PP1 on SMCE was not significantly different, we used 10 μM PP1 throughout the remainder of the study.

Treatment of platelets with 10 μM PP1 resulted in a significant inhibition of thrombin-evoked Ca^{2+} elevation (Figure 4B). PP1 reduced Ca^{2+} entry evoked by 1 unit/ml thrombin by $30 \pm 5\%$ ($P < 0.05$; $n = 4$) without having any effect on release of Ca^{2+} from the intracellular stores (results not shown).

As shown for M-2,5-DHC, treatment of human platelets for 10 min with 10 μM PP1, after SMCE had been activated by the addition of TG, did not alter the entry of extracellular Ca^{2+} (Figure 4C; $n = 6$). These observations suggest that Src family tyrosine kinases might be involved in the activation, but not in the maintenance, of SMCE, and, furthermore, indicate that PP1 is not acting as a Ca^{2+} chelator or Ca^{2+} channel blocker.

Exposure of platelets to 10 μM PP1 for 10 min did not modify the F-actin content in resting platelets or in either thrombin- or TG-stimulated platelets ($n = 6$; results not shown). Similar results were obtained when PP1 was added to the platelet suspension after treatment with TG (after activation of SMCE). PP1 did not reverse TG-stimulated actin polymerization in these cells ($n = 6$; results not shown).

Translocation of $pp60^{src}$ to the cytoskeletal fraction in store-depleted platelets

Recent studies have shown that tyrosine kinases of the Src family associate with the platelet cytoskeleton upon stimulation with thrombin, a process that is important for phosphorylation of their substrates [22,27,28]. To investigate the possibility that the tyrosine kinase $pp60^{src}$ is specifically associated with the platelet cytoskeleton after depletion of the internal Ca^{2+} stores, Western

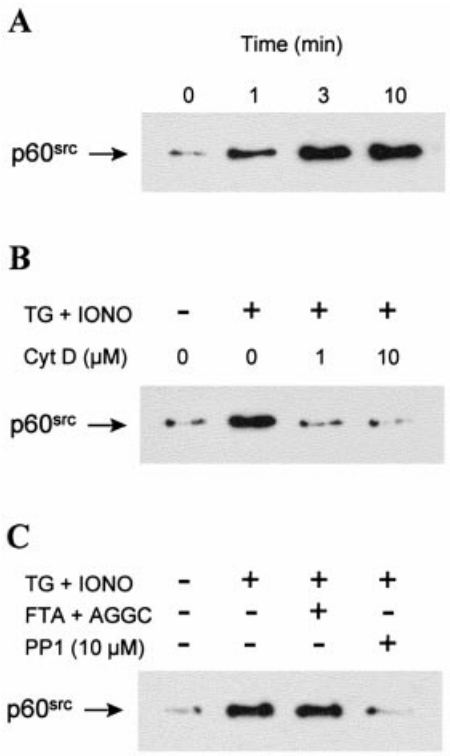

Figure 5 Cytoskeletal association of pp60^{src} after depletion of the intracellular Ca^{2+} stores

Dimethyl BAPTA-loaded human platelets were incubated at 37 °C with TG (250 nM) + ionomycin (50 nM) and then lysed with a Triton X-100 buffer and centrifuged to separate the detergent-insoluble fraction. These samples were subjected to SDS/PAGE and analysed by Western blotting with the specific anti-pp60^{src} antibody GD11 as described in the Materials and methods section. Bands were revealed using chemiluminiscence, and were quantified by scanning densitometry. (**A**) Time course of pp60^{src} incorporation into the cytoskeletal fraction in platelets treated with TG and ionomycin. (**B**) Platelets were pretreated for 40 min in the absence or presence of various concentrations of cytochalasin D as indicated. Cells were then stimulated for a further 3 min with no addition or with TG and ionomycin. (**C**) Platelets were incubated in the absence or presence of either FTA (40 μM) plus AGGC (30 μM) or PP1 (10 μM) for 10 min at 37 °C before stimulation for a further 3 min with TG and ionomycin. The panels show results from a representative experiment of at least three others. The arrow represents the position of pp60^{src}.

immunoblot analysis was performed on the cytoskeletal fraction of resting and Ca^{2+}-store-depleted platelets which had been treated with TG (250 nM) and ionomycin (50 nM). Platelets heavily loaded with dimethyl BAPTA were used for this study so as to eliminate Ca^{2+}-dependent, but not store-depletion-dependent, association of pp60^{src} with the cytoskeleton. The cytoskeletal fractions were probed for the presence of the protein tyrosine kinase pp60^{src}. Only a small amount of this protein was detected, associated with the cytoskeleton of resting platelets (Figure 5A). When platelets were treated with TG and ionomycin, the amount of pp60^{src} associated with the cytoskeleton increased in a time-dependent manner (Figure 5A). An increase in pp60^{src} association with the cytoskeleton was detectable after treatment with TG and ionomycin for 1 min (Figure 5A). There was a 1.8 ± 0.3-fold increase in association after 1 min and this reached a maximum of 3.67 ± 0.34-fold within 3 min (Figure 5A; $n = 4$). This maximum level was maintained for at least 10 min.

To test whether the association of pp60^{src} with the cytoskeletal fraction depends on the actin-filament polymerization that occurs in response to Ca^{2+} store depletion in platelets, cells were treated with cytochalasin D prior to addition of TG and ionomycin. The incorporation of pp60^{src} into the cytoskeleton in store-depleted cells was inhibited when platelets were treated for 40 min with cytochalasin D in a concentration-dependent manner. Treatment of platelets with 1 μM cytochalasin D decreased the association of pp60^{src} with the cytoskeleton by $78 \pm 3\%$, reaching a complete inhibition at 10 μM cytochalasin D (Figure 5B; $n = 4$). These results are in agreement with those obtained in previous studies [28]. Treatment of platelets with 10 μM cytochalasin D for 40 min has previously been reported to abolish actin polymerization stimulated by TG in these cells [5]. The ability of cytochalasin D to abolish cytoskeletal association of pp60^{src} after depletion of the intracellular Ca^{2+} stores provides evidence that this kinase incorporates into the reorganizing platelet actin cytoskeleton rather than binding non-specifically with the detergent-insoluble fraction. To investigate whether activation of pp60^{src} is required for its association with the cytoskeleton, we examined the effect of PP1. PP1 inhibited pp60^{src} incorporation into the cytoskeletal fraction by $97 \pm 5\%$ (Figure 5C; $n = 4$), indicating that activation of pp60^{src} is required prior to its translocation to the cytoskeleton.

We have previously reported that (*S*)-all-*trans*-farnesyl-L-cysteine (FC) analogues partially inhibit actin polymerization [5]. Hence we investigated the effect of FTA and AGGC on pp60^{src} association with the cytoskeleton. As shown in Figure 5(C), treatment of platelets with FTA (40 μM) combined with AGGC (30 μM) did not significantly modify the incorporation of pp60^{src} into the cytoskeletal fraction following depletion of the intracellular Ca^{2+} stores using TG and ionomycin.

Complementary effects of tyrosine kinases and Ras proteins on SMCE in platelets

We have recently demonstrated that FC analogues, such as FTA and AGGC, which prevent methylation of farnesylated or geranylgeranylated Ras respectively, partially inhibit SMCE in human platelets in a concentration-dependent manner, suggesting the involvement of Ras proteins in SMCE in these cells [5]. We found this effect to be partially mediated by actin filament polymerization [5]. Since the effect of tyrosine kinases seems to be dependent on the actin cytoskeleton, we have investigated the combined effects of these two families of proteins in the activation of SMCE.

Human platelets were incubated at 37 °C with the tyrosine kinase inhibitor M-2,5-DHC (1 μg/ml) for 30 min, the FC analogues FTA (40 μM) and AGGC (30 μM) for 10 min or all of these agents in combination. The platelets were then stimulated with TG (200 nM) in a Ca^{2+}-free medium and, 3 min later, Ca^{2+} (300 μM) was added to initiate Ca^{2+} entry. As shown in Table 2, treatment of platelets with FTA plus AGGC inhibited SMCE by 72%. Interestingly, inhibition of both tyrosine kinase and Ras protein effects abolished TG-induced SMCE (Table 2; $n = 5$). These findings suggest that Ras proteins and tyrosine kinases play a complementary role in the activation of SMCE.

Since the inhibition of tyrosine kinase activity using M-2,5-DHC resulted in full inhibition of actin polymerization, whereas, as previously reported, some actin polymerization remains after treatment with FC analogues [5], we investigated whether the remaining Ca^{2+} entry after treatment with FC analogues can be fully prevented by complete inhibition of actin polymerization. Consistent with the above, treatment of platelets at 37 °C with a combination of the cytoskeletal disrupter cytochalasin D and the FC analogues, FTA and AGGC, completely abolished TG-induced SMCE (Table 2; $n = 5$).

Table 2 Combined inhibitory effects of M-2,5-DHC or cytochalasin D and FC analogues on TG-stimulated store-mediated Ca^{2+} entry

Fura 2-loaded human platelets were incubated at 37 °C with 1 μg/ml M-2,5-DHC for 30 min, 40 μM FTA plus 30 μM AGGC for 10 min, 10 μM cytochalasin D for 40 min, a combination of FTA plus AGGC with either M-2,5-DHC or cytochalasin D or the vehicles. At the time of the experiment 100 μM EGTA was added. Cells were then stimulated with TG (200 nM) and, 3 min later, $CaCl_2$ (final concn. 300 μM) was added to the medium to initiate Ca^{2+} entry. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio as described in the Materials and methods section. Data indicate the percentage inhibitions of Ca^{2+} entry relative to respective controls (vehicle was added). Ca^{2+} entry was estimated as described in the Materials and methods section. Values are means ± S.E.M. of five separate determinations.

Inhibitory agent	Ca^{2+} entry (% of inhibition)
M-2,5-DHC	52.4 ± 3.4
FTA + AGGC	72.8 ± 2.1
Cytochalasin D	52.0 ± 3.2
FTA + AGGC + M-2,5-DHC	99.8 ± 8.6
FTA + AGGC + cytochalasin D	96.2 ± 1.5

Table 3 Combined inhibitory effects of PP1, cytochalasin D and FC analogues on TG-stimulated SMCE

Fura 2-loaded human platelets were incubated at 37 °C with 10 μM PP1 for 10 min, 10 μM cytochalasin D for 40 min, 40 μM FTA for 10 min, 30 μM AGGC for 10 min or a combination of several inhibitors as indicated. At the time of the experiment 100 μM EGTA was added. Cells were then stimulated with TG (200 nM) and, 3 min later, $CaCl_2$ (final concn. 300 μM) was added to the medium to initiate Ca^{2+} entry. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio. and traces were calibrated in terms of $[Ca^{2+}]_i$ as described in the Materials and methods section. Data indicate the percentage inhibitions of Ca^{2+} entry relative to respective controls (vehicle was added). Ca^{2+} entry was estimated as described in the Materials and methods section. Values are means ± S.E.M. of eight to twelve separate determinations.

Inhibitory agent	Ca^{2+} entry (% of inhibition)
PP1	32.0 ± 4.3
Cytochalasin D	52.0 ± 3.2
PP1 + cytochalasin D	49.7 ± 2.6
FTA	54.9 ± 3.1
FTA + PP1	70.4 ± 1.7
AGGC	59.4 ± 3.3
AGGC + PP1	69.5 ± 1.1
FTA + AGGC	72.8 ± 2.1
FTA + AGGC + PP1	97.7 ± 0.8

Combined effects of PP1 and FC analogues on TG-induced SMCE

Since tyrosine kinase activity and actin polymerization are required for the Ca^{2+} entry remaining after treatment with FC analogues, we further investigated whether Src tyrosine kinases, which require a functional actin cytoskeleton for recruitment and phosphorylation of their substrates, are involved in the residual response. To examine the role of the actin cytoskeleton in Src mediation of SMCE, we studied the effect of cytochalasin D alone and in combination with PP1. As shown in Table 3, the combination of cytochalasin D and PP1 decreased TG-induced SMCE by 50 %, similar to results that were obtained with cytochalasin D alone. The absence of an additive effect indicates that Src-mediated SMCE requires remodelling of the actin cytoskeleton.

Treatment of human platelets at 37 °C with 10 μM PP1 combined with either 40 μM FTA, 30 μM AGGC, or both, for 10 min resulted in an additive inhibitory effect (Table 3; n = 8–12). In addition, treatment of platelets with PP1 and both FC analogues resulted in complete inhibition of TG-stimulated SMCE (Table 3; n = 12).

DISCUSSION

Human platelets possess high levels of protein tyrosine kinases, particularly proteins of the Src family. Platelets show a substantial increase in protein tyrosine phosphorylation in response to their activation, suggesting an important role for this process in platelet physiology. Over the last few years, several studies have presented evidence that tyrosine kinases are important for SMCE in a number of cell types [18,29–31], including platelets [15–17,23]. Recently, a new model has been proposed for SMCE, based on a reversible trafficking and coupling of the ER with the PM, where the actin cytoskeleton plays a key regulatory role [7,8]. In the present study we provide evidence for a relationship between tyrosine kinases and the secretion-like coupling model through the activation of actin polymerization in human platelets.

We have previously reported that treatment of platelets with thrombin or TG induced actin polymerization, an event that is essential for the activation and maintenance of SMCE, as demonstrated by using cytochalasin D or latrunculin A, two inhibitors of actin polymerization [5,8]. A role for the actin cytoskeleton in SMCE has also been reported in endothelial cells [9] and type I astrocytes [10]. Significantly, we now report that the effect of thrombin or TG on actin polymerization is not dependent on elevations in $[Ca^{2+}]_i$, as demonstrated by loading the cells with the intracellular Ca^{2+} chelator dimethyl BAPTA. TG evoked an increase in actin polymerization, even though there was no detectable increase in $[Ca^{2+}]_i$ above basal levels. We have recently reported that, in platelets, depletion of the intracellular Ca^{2+} stores induces translocation of Ras proteins from the cytosol to the PM, a process that is required for their activation [5]. This event has been shown to be independent of changes in $[Ca^{2+}]_i$ [5]. Ras proteins have been shown to mediate many cell functions through the reorganization of the actin cytoskeleton [32]. Therefore we propose that activation of Ras proteins after depletion of the intracellular Ca^{2+} stores might be involved in the reorganization of the actin cytoskeleton which is required for the activation and maintenance of SMCE in human platelets. It remains to be elucidated how Ras proteins regulate actin polymerization, but the classical second messengers PtdIns(4,5)P_2, diacylglycerol, Ca^{2+} and cAMP, generally assumed to be involved in actin polymerization, are not required in the Ras-mediated actin polymerization; however, there is evidence that tyrosine kinases might be involved in these events [33]. These findings suggest that Ca^{2+}-store depletion stimulates a dynamic mechanism to activate Ca^{2+} entry involving calcium-independent actin reorganization and polymerization. This observation makes a model for the activation of SMCE involving the actin cytoskeleton compatible with electrophysiological data, which show that the calcium release-activated current, I_{CRAC}, can be activated when the intracellular Ca^{2+} stores are depleted in the absence of an increase in $[Ca^{2+}]_i$ [34].

The effect of thrombin and TG on actin polymerization was inhibited by treatment of platelets with the tyrosine kinase inhibitor M-2,5-DHC. The role of tyrosine kinases in actin polymerization is controversial. In lymphocytes, changes in membrane lipid composition induced by phospholipase $C_{\gamma\text{-}1}$, known to be activated by protein tyrosine phosphorylation, might initiate actin polymerization and membrane-site redistribution [35]. More recently, Chintala et al. [36] reported that vanadate and phenylarsine oxide, inhibitors of tyrosine phosphatases, induced both elevation of the levels of protein tyrosine phosphorylation and inhibition of actin polymerization in glioma cells. The results presented here clearly demonstrate that, in platelets, store depletion-induced actin polymerization involves a tyrosine phosphorylation-dependent step.

Tyrosine phosphorylation has been shown to be required for SMCE in several different cell types, including platelets, on the basis of inhibitory effects of tyrosine kinase inhibitors on Ca^{2+} entry and on correlations between depletion of Ca^{2+} stores and the increase in phosphotyrosine levels [15–18,23]. The findings that inhibition of tyrosine phosphorylation or actin polymerization reduced SMCE to the same extent, and that no further inhibition was observed when both pathways were blocked, suggest that the involvement of tyrosine kinases in SMCE is entirely mediated by the actin cytoskeleton, rather than through phosphorylation of a Ca^{2+} entry channel as previously suggested [15]. The support provided by the actin cytoskeleton has been shown to be required for the activation of cytoskeleton-related tyrosine kinases, such as focal adhesion-associated tyrosine kinases and pp60^{src} [22,37,38]. In addition, a large number of tyrosine-phosphorylated proteins have been found in the actin-rich cytoskeletal fraction in human platelets, suggesting that association with the actin cytoskeleton is essential for the activity of tyrosine kinases in these cells [22]. Therefore, tyrosine kinase-mediated actin polymerization may be important in providing a physical support for both the coupling mechanism itself and the activity of other tyrosine kinases that may be involved in mediating SMCE in these cells.

Since actin polymerization has been previously shown to be required for both activation and maintenance of SMCE in human platelets [8], we further investigated the role of tyrosine kinases in the maintenance of Ca^{2+} entry. Inhibition of tyrosine phosphorylation after initiation of SMCE did not reverse TG-induced Ca^{2+} entry or actin polymerization. This indicates that M-2,5-DHC itself is not a Ca^{2+} channel blocker or an inhibitor of actin polymerization. Consistent with the lack of effect of M-2,5-DHC on the F-actin content, our results provide evidence that tyrosine phosphorylation is not required for the maintenance of SMCE in human platelets. This suggests that tyrosine kinases are important for the activation of SMCE, but that their activity is not required for the maintenance of the process (either in the continued provision of the actin filament support for the coupling process or the coupling mechanism itself).

Activation of human platelets with thrombin or other agonists results in a transient activation of pp60^{src} in the detergent-soluble fraction followed by redistribution to the cytoskeleton [13]. Several members of the Src family have been described in platelets: pp60^{src} is the most abundant and other members (pp55^{fgr}, pp56^{lck}, pp59^{fyn}, pp62^{yes}, pp56^{lyn} and pp59/56^{hck}) are present at a significantly lower level than pp60^{src} [13,39]. To investigate whether pp60^{src} and other Src proteins are important for the activation of SMCE in human platelets, we examined the effect of PP1, an inhibitor of the Src family of tyrosine kinases that potently inhibits pp60^{src} [26]. Experiments using PP1 indicate that Src proteins might be involved in the activation, but not in the maintenance, of SMCE. These results are consistent with the above-reported role of tyrosine kinases in the activation of SMCE, but not in its maintenance.

Recently a role for pp60^{src} in SMCE has been reported in 3T3-like embryonic fibroblasts [30]. pp60^{src} translocates to the cytoskeleton upon stimulation in a number of cell types, including platelets, a process that requires the integrity of the actin-filament network [22,27]. In addition, thrombin rapidly activates pp60^{src} in human platelets before it translocates to the cytoskeletal fraction, where most of its potential substrates are located [22]. Our results provide evidence that pp60^{src} associates with the cytoskeletal fraction after depletion of the intracellular Ca^{2+} stores by a mechanism that is independent of elevations in $[Ca^{2+}]_i$. To our knowledge this is the first time that pp60^{src} has been shown to associate with the cytoskeleton in direct response to the depletion of the internal Ca^{2+} stores. The cytoskeletal incorporation of pp60^{src} depends on the actin-filament polymerization that occurs in response to depletion of the intracellular Ca^{2+} stores, as demonstrated by the inhibitory effect of cytochalasin D, an agent that inhibits actin polymerization without modifying the F-actin content of resting platelets [5]. The ability of cytochalasin D to inhibit cytoskeletal incorporation of pp60^{src} indicates that the presence of this protein in the cytoskeletal fraction is due to its association with the reorganizing cytoskeleton rather than it being trapped in the detergent-insoluble fraction. Our results are in agreement with a recent report that described the ability of cytochalasin D to inhibit the cytoskeletal association of pp60^{src} upon stimulation with thrombin in human platelets [28]. In addition, the association of pp60^{src} with the actin cytoskeleton requires prior activation of this protein. This conclusion is supported by the ability of PP1 to inhibit the cytoskeletal association of pp60^{src} in human platelets.

It remains to be elucidated how depletion of the intracellular Ca^{2+} stores induces activation of proteins of the Src family. Members of the Src family of kinases contain an SH2 domain that serves to bind substrates after they are phosphorylated at tyrosine residues [40] and, when Src is inactive, is bound to a phosphorylated tyrosine residue in the C-terminus of Src [41]. The actin reorganization might be important in bringing Src proteins into proximity to cytoskeletal-associated substrates. Thus phosphotyrosine-containing proteins that bind to SH2 domains compete with, and prevent, autoinhibition evoked by intramolecular binding of SH2 domains to phosphorylated tyrosine residues. Activated Src proteins might recruit signalling molecules such as phosphatidylinositol 3′-kinase [42], which we have previously reported to be involved in SMCE in human platelets [43], to specific submembraneous locations at the early stages of platelet activation. Consistent with this, we found that the involvement of Src proteins in SMCE is not mediated by actin polymerization, which instead appears to be required for the cytoskeletal association of pp60^{src}. The biochemical mechanism for store-depletion-induced Src activation deserves further investigation.

We have recently reported that using FC analogues to inhibit the membrane-association of the Ras family of small GTP-binding proteins, a process that is essential for their activation [44], partially inhibited SMCE in human platelets [5]. In addition we have reported that the role of these proteins is partially mediated by actin polymerization, although we found that concentrations of FC analogues that prevented Ras membrane association did not completely inhibit actin polymerization induced by thrombin or depletion of the internal Ca^{2+} stores using TG [5]. On the basis of our finding that inactivation of Ras proteins, combined with full inhibition of actin polymerization (either using cytochalasin D or by inhibition of tyrosine phosphorylation), abolishes SMCE, we suggest that Ras proteins and tyrosine kinases might have independent effects in the activation of SMCE through the reorganization of the actin cytoskeleton.

We have found that Src proteins are not required for actin polymerization; however, this process is a downstream step required for the association of pp60^{src} with the cytoskeletal fraction, a process essential for pp60^{src} to exert its function. Consistent with this, our results indicate that the role of Src proteins in SMCE is entirely dependent on actin polymerization. This conclusion is supported by the inhibitory effect of cytochalasin D on the cytoskeletal association of pp60^{src} and the lack of any further inhibition of Ca^{2+} entry by PP1 when added together with cytochalasin D. Our results suggest that Src and Ras proteins might have independent effects in the activation of

SMCE, indicating that some Src activity remains after inhibition of Ras proteins, which is consistent with the remaining actin polymerization when FC analogues are used. In agreement with the above, treatment of platelets with FC analogues did not alter cytoskeletal-association of $pp60^{src}$ in these cells. Therefore we suggest that actin-filament polymerization, which involves the combined activity of Ras proteins and unidentified tyrosine kinases, and the redistribution of signalling molecules such as the tyrosine kinase $pp60^{src}$ or phosphoinositide kinases to the re-organizing actin cytoskeleton, may represent a crucial step in regulating SMCE in human platelets.

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