A role for cofilin in the activation of store-operated calcium entry by *de novo* conformational coupling in human platelets

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Abstract

Store-operated Ca\textsuperscript{2+} entry (SOCE) is a major mechanism for Ca\textsuperscript{2+} influx in platelets and other cells. De novo conformational coupling between elements in the plasma membrane and Ca\textsuperscript{2+} stores, where the actin cytoskeleton plays an important regulatory role, has been proposed as the most likely mechanism to activate SOCE in platelets. Here we have examined for the first time changes in platelet F-actin levels on a sub-second timescale. Using stopped-flow fluorimetry and a quenched-flow approach we provide evidence for the involvement of cofilin in actin filament reorganization and SOCE in platelets. Thrombin (0.1 unit/ml) evoked an initial decrease in F-actin that commenced within 0.1 s and reached a minimum 0.9 s after stimulation, prior the activation of SOCE. F-actin then increased, exceeding basal levels again approximately 2.5 s after stimulation. Thrombin also induced cofilin dephosphorylation and activation, which paralleled the changes observed in F-actin, and rapid Btk activation. Inhibition of cofilin dephosphorylation by LFM-A13 resulted in the loss of net actin depolymerization and an increased delay in SOCE initiation. These results suggest that cofilin is important for the rapid actin remodeling necessary for the activation of SOCE in platelets through de novo conformational coupling.

Key words: Cofilin, actin cytoskeleton, Ca\textsuperscript{2+} entry, LFM-A13, platelets.
Introduction

In non-excitable cells, such as platelets, store-operated calcium entry (SOCE), regulated by the filling state of the intracellular Ca\(^{2+}\) stores, is a major mechanism for calcium influx.\(^1\)-\(^5\) Different models that have been presented to account for the activation of SOCE in distinct cell types can be grouped into two main categories that propose either indirect or direct coupling between the Ca\(^{2+}\) stores and the plasma membrane (PM). Indirect coupling models assume the generation of a small diffusible molecule that operates as a Ca\(^{2+}\) influx factor (CIF) so gating Ca\(^{2+}\) channels in the PM.\(^6\)-\(^8\) On the other hand, direct or constitutive conformational coupling models propose a physical interaction between Ca\(^{2+}\) channels in the PM and IP\(_3\) receptors in the membrane of the intracellular Ca\(^{2+}\) stores.\(^5\),\(^9\) It has also been suggested that SOCE might be activated by the insertion of preformed channels into the PM by vesicle fusion in a “secretion-like” coupling model.\(^10\),\(^11\)

A modification of the constitutive conformational coupling model proposes a dynamic and reversible conformational coupling based on the transport of portions of the endoplasmic reticulum (ER) containing IP\(_3\) receptors to the PM to facilitate de novo protein coupling.\(^9\),\(^12\)-\(^14\) De novo conformational coupling requires a mechanical support provided by the actin cytoskeleton.\(^14\) In addition, the cortical actin cytoskeleton plays a regulatory role by acting as a negative clamp that prevents constitutive coupling and activation of SOCE.\(^4\),\(^5\),\(^14\),\(^15\) The de novo conformational coupling model requires that the cortical actin barrier be rapidly disrupted following cell stimulation to allow the coupling to occur.\(^9\),\(^12\),\(^16\) Earlier determinations of agonist-evoked changes in platelet F-actin have been made on a timescale of seconds or minutes;\(^17\)-\(^19\) however, we are not aware of determinations of F-actin content on the same timescale as agonist-evoked Ca\(^{2+}\) entry, which can commence within a second of stimulation.\(^16\),\(^20\)
Proteins involved in actin filament remodeling include a large number of actin binding proteins (ABPs), which control actin reorganization by direct contact with F-actin or with G-actin monomers. Some ABPs, like tropomodulin or Cap Z, bind to the barbed ends of F-actin, preventing the addition of more G-actin subunit. Twinfilin, verprolin/WIP, DNase I and thymosin β4, inhibit actin polymerization by sequestering G-actin units. The role of other ABPs on actin remodeling, such as profilin and Srv2, is more subtle. Profilin-bound actin monomers cannot add to the pointed end but add to the barbed end, and Srv2 appears to be a shuttle transferring actin monomers from coflin to profilin, and allowing nucleotide exchange on the monomers. Other ABPs, such as gelsolin, actively reduce the length of actin filaments by promoting the cleavage of the filaments. Cofilin accelerates depolymerization, though the mechanism of this effect is not completely understood.

ABPs can be regulated by several intracellular mechanisms. A major pathway involves the activation of phosphatidylinositol 3-kinase (PI3K) by a LIM kinase-Rho-protein kinase C (PKC)-Ca²⁺/calmodulin dependent pathway. Recent studies have presented an alternative pathway for actin reorganization based on the equilibrium between Ser/Thr kinases and phosphatases. Especially relevant is the regulation of cofilin by the Ser/Thr phosphatase, slingshot, which dephosphorylates the residue Ser³ allowing cofilin to bind to the actin cytoskeleton and reduce the length of the actin filament by removing G-actin monomers from the barbed end.

Here we have investigated the temporal relationship between Ca²⁺ entry and actin filament reorganization on a sub-second time scale. The results suggest that actin depolymerization precedes release of Ca²⁺ from intracellular stores and the activation of Ca²⁺ entry, the former indicating that it is independent of rises in cytosolic free Ca²⁺ concentration ([Ca²⁺]c). Rapid actin depolymerization requires cofilin dephosphorylation and is compatible with membrane
trafficking underlying the *de novo* conformational coupling of the type II inositol 1,4,5-trisphosphate receptor (IP$_3$RII) to the store-operated Ca$^{2+}$ entry channel hTRPC1 with subsequent activation of SOCE.$^{12,16}$
Materials and methods

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, bovine serum albumin, dithiothreitol, and fluorescein isothiocyanate-conjugated (FITC) phalloidin and thrombin, were from Sigma (Poole, Dorset, United Kingdom). LFM-A13 was from Calbiochem (Nottingham, United Kingdom). Anti phospho-Btk (Y-223) antibody and anti-Btk antibody were from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-phosphoSer\textsuperscript{3}-cofilin antibody, anti-cofilin antibody (C-20), horseradish peroxidase-conjugated donkey anti-goat IgG antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were from Santa Cruz (Santa Cruz, CA, U.S.A.). All other reagents were of analytical grade.

Platelet Preparation

Fura-2-loaded platelets were prepared as described previously.\textsuperscript{14} Briefly, blood was obtained from drug-free healthy volunteers. Approval was obtained from the University of Cambridge institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki. Blood was 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 700g and aspirin (100 μM) and apyrase (40 μg/ml) added. Platelet-rich plasma was incubated at 37 °C with 2 μM fura-2/AM for 45 min. Cells were then collected by centrifugation at 350g for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO\textsubscript{4}, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 μg/ml apyrase.
**Stopped-flow kinetic measurements**

The kinetics of fluorescence change from fura-2-loaded platelets was investigated by stopped-flow fluorimetry at 37 °C using a Hi-Tech Scientific SF-61SX2 Single-Mixing Stopped-Flow System (Hi-Tech Ltd., Salisbury, Wilts., U.K.) with an excitation wavelength of 340 or 360 nm and emission at 500 nm. Dye-loaded cells (100 µl; 4 x 10^8 cells/mL) and an agonist solution (100 µl) were introduced into the sample flow circuit via separate reservoirs at the top of the sample-handling unit. Mn²⁺ influx was monitored as a quenching of fura-2 fluorescence at the isoemissive wavelength of 360 nm, presented on an arbitrary linear scale. Results were corrected for the effects of quenching extracellular fura-2 and basal leak of Mn²⁺ into the cells by subtraction of agonist-free control runs. To reduce leakage of Mn²⁺ into the cells before the experiment, 50 µM MnCl₂ was added to the cell suspension and 350 µM MnCl₂ was added to the agonist solution, giving a final concentration of 200 µM extracellular Mn²⁺ after mixing.

**F-actin kinetic measurements**

The kinetics of F-actin reorganization were determined in samples stimulated for various times before fixing using a rapid quench flow system (Hi-Tech Ltd., Salisbury, Wilts., U.K.). Briefly, the cell suspension (75 µl) and an agonist solution (75 µl) were introduced into the sample flow circuit via separate reservoirs at the top of the sample-handling unit and then mixed after the times indicated with 75 µl 3 % (w/v) formaldehyde in phosphate-buffered saline (PBS) before storing on ice for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025 % (v/v) Nonidet P-40 detergent dissolved in PBS. Platelets were then incubated for 30 min with fluorescein isothiocyanate-labeled phalloidin (1 µM) in PBS supplemented with 0.5 % (w/v) bovine serum albumin. After incubation the platelets were collected by centrifugation for 60 s at 3000 g and resuspended in PBS. Staining of cells was measured using a Perkin-Elmer fluorescence spectrofluorimeter (Perkin-Elmer, Norwalk, CT). Samples were excited at 496 nm and emission was at 516 nm.
Western blotting

Platelets were stimulated for various times at 37 °C using a Hi-Tech Scientific RQF-63 Rapid Quench-Flow System\textsuperscript{16} as above for F-actin determinations except that the stimulation was terminated by mixing with 75 µl of 2X Laemmli’s buffer\textsuperscript{41} with 10 % dithiothreitol followed by heating for 5 min at 95 °C. One-dimensional SDS-electrophoresis was performed on 12.5 % sodium dodecyl sulphate-polyacrylamide gels and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm\textsuperscript{2}, in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs., United Kingdom) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10 % (w/v) BSA in tris-buffered saline with 0.1 % Tween 20 (TBST) to block residual protein binding sites. Blocked membranes were then incubated with the anti phospho-Btk (Y-223) antibody or the anti-Btk antibody diluted 1:1000 in TBST or with the anti-phosphoSer\textsuperscript{3}-cofilin antibody diluted 1:250 in TBST or with anti-cofilin antibody diluted 1:100 in TBST for 1 h. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody or horseradish peroxidase-conjugated donkey anti-goat IgG antibody, diluted 1:2000 and 1:2500 in TBST, respectively; washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry.

Statistical Analysis

Analysis of statistical significance was performed using Student’s unpaired \textit{t}-test. For multiple comparisons, one-way analysis of variance combined with the Dunnet’s tests was used.
Results

Latencies of Ca$^{2+}$ release from intracellular stores and of Mn$^{2+}$ entry stimulated by the physiological agonist thrombin

Human platelets were stimulated with 0.1 U/mL thrombin, a concentration which activates SOCE but not non-capacitative Ca$^{2+}$ entry,$^{16,42}$ and the latencies of thrombin-evoked release of Ca$^{2+}$ from intracellular stores and of thrombin-evoked Mn$^{2+}$ entry were determined by stopped-flow fluorimetry. Fura 2-loaded platelets were rapidly mixed with thrombin at a final concentration of 0.1 U/mL in the presence of 100 µM EGTA and 200 µM MnCl$_2$. Recording fura 2 fluorescence at an excitation wavelength of 340 nm indicated that the delay in onset of thrombin-induced Ca$^{2+}$ release from the intracellular stores was 1.64 ± 0.09 s (mean ± SE; Figure 1; n=20). At the isoemissive wavelength of 360 nm, Mn$^{2+}$ quench of fura 2 fluorescence was first detected with latencies of 2.14 ± 0.09 s (Figure 1; n=20). As shown previously,$^{16}$ mixing cells with agonist-free HBS containing 100 µM EGTA and 200 µM MnCl$_2$ did not modify fura 2 fluorescence at either excitation wavelength (not shown), confirming that the mixing procedure per se did not activate the cells.

Time course of thrombin-evoked actin filament reorganization

It has been reported that actin filament reorganization is necessary for the activation of SOCE in a number of cells, including smooth muscle cells,$^{43}$ corneal endothelial cells,$^{7}$ pancreatic acinar cells,$^{5}$ glioma C6 cells$^{44}$ and platelets.$^{14}$ To investigate the time course of actin filament remodelling, platelet samples were prepared by quenched flow for subsequent staining with FITC-phalloidin. The delay times between the rapid mixing of cells with 0.1 U/mL thrombin and the subsequent fixation with 3 % formaldehyde in PBS (see the Materials and methods section) were set at 100-200 ms intervals for the first 2 s and at 500 ms for subsequent timepoints, commencing 100 ms after the mixing of cells with thrombin. Thrombin (0.1 U/mL)
evoked an initial decrease in platelet F-actin that commenced within 100 ms and reached a minimum 0.9 s after stimulation at 70.7 ± 4.4 % of the resting level (Figure 1; \( P<0.05; n=21 \)). The F-actin content then increased, exceeding basal levels again approximately 2.5 s after stimulation. As reported above for Ca\(^{2+} \) mobilization, mixing cells with agonist free HBS did not evoke significant changes in F-actin content (Figure 1; dashed line).

**Time course of coflin phosphorylation evoked by thrombin**

The actin depolymerizing factor coflin is an ABP involved in actin reorganization that is negatively regulated by phosphorylation at Ser\(^3 \) and reactivated by dephosphorylation.\(^{34,36} \) In view of the initial net actin depolymerization induced by thrombin we have investigated the time course of changes in coflin phosphorylation after treatment with 0.1 U/mL thrombin. To do this, platelet samples were prepared by quenched flow for subsequent SDS/PAGE and Western-blot analysis. The delay times between the rapid mixing of cells with thrombin and the subsequent mixing with Laemmli’s buffer (see the Materials and methods section) were set as described above for determination of F-actin content. Thrombin (0.1 U/mL) evoked an initial dephosphorylation of coflin that commenced within 100 ms and reached a minimum 0.9 s after stimulation at 78.1 ± 7.7 % of the resting level, as detected by Western blotting with an anti-phosphoSer\(^3 \)-cofilin antibody (Figure 2, top panel and graph; \( P<0.05; n=15 \)). Cofilin phosphorylation at Ser\(^3 \) then increased, exceeding resting levels again approximately 1.5 s after stimulation. Western blotting with an anti-cofilin antibody revealed that a similar amount of the protein was loaded in all lanes (Figure 2, lower panel).

**Time course of actin filament reorganization and coflin phosphorylation evoked by thrombin in the presence of LFM-A13**

We have recently demonstrated the involvement of Bruton’s tyrosine kinase (Btk) in actin remodelling and SOCE in thapsigargin-treated human platelets.\(^{45} \) Hence we have investigated
whether the role of Btk in SOCE might be mediated by coflin and the regulation of actin reorganization. The activation of Btk was analysed by Western blotting using a rabbit monoclonal phosphospecific anti-Btk antibody that only detects Btk autophosphorylated at the tyrosine residue 223, which has been shown to be the full activated form of Btk. Treatment of human platelets with thrombin (0.1 U/mL) evoked a rapid activation of Btk that was detectable within 100 ms and reached a maximum 2.5 s after stimulation at 385 ± 27 % of the resting level (Figure 3, top panel; n=6). Western blotting with an anti-Btk antibody revealed that a similar amount of protein was loaded in all lanes (Figure 3, lower panel).

Pretreatment of platelets for 10 min with 10 μM LFM-A13, which abolishes Btk activation, inhibited the thrombin-evoked dephosphorylation of coflin detected in the first 1.5 s after stimulation with the agonist, and enhanced thrombin-evoked coflin phosphorylation as detected by Western blotting with an anti-phosphoSer3-cofilin antibody (Figure 4; n=6). Consistent with this, treatment of the cells for 10 min with 10 μM LFM-A13 abolished thrombin-evoked net actin depolymerization (Figure 5). After treatment with LFM-A13 the F-actin content 0.9 s after treatment with thrombin (0.1 U/mL) was 110 % of basal compared with 70% of basal in controls (Table 1; P<0.001).

Treatment of platelets with LFM-A13 did not significantly increase the latency of thrombin-evoked Ca2+ release. The thrombin-evoked increase in [Ca2+]c was first detected after a delay of 1.85 ± 0.14 s after a 10 min pretreatment with 10 μM LFM-A13 (n=6), compared with 1.64 ± 0.09 s in controls (Table 1; P=0.20). In contrast, the latency of thrombin-evoked Mn2+ entry was increased by the Btk inhibitor (Table 1). Mn2+ entry was first detected 2.60 ± 0.17 s (n=10) after agonist addition following a 10 min pretreatment with 10 μM LFM-A13, compared with 2.14 ± 0.09 s in controls (P<0.05). We have previously shown that pretreatment of human platelets for 10 min with 10 μM LFM-A13 decreased thrombin-evoked Ca2+ entry by about 30 % without having any effect on thrombin-evoked release of Ca2+ from the intracellular stores.
Discussion

We have previously proposed that SOCE in human platelets may be activated by a de novo conformational coupling model in which Ca\(^{2+}\) store depletion leads to trafficking of portions of the ER towards the PM to allow coupling between IP\(_3\)RII in the ER membrane and hTRPC1 in the PM\(^{12-16}\). In support of this model, we have shown that SOCE is reduced by inhibitors of polymerization such as cytochalasin D or latrunculin A, which impair reorganization of the cytosolic actin network that provides support to the transport of the ER towards the PM or by stabilization of the membrane cytoskeleton using jasplakinolide, which interfere with the depolymerization of the membrane cytoskeleton and which might thus prevent the approach of the ER and PM through the dense cortical F-actin layer present in platelets\(^{14}\). We have also shown in co-immunoprecitation experiments that Ca\(^{2+}\) store depletion following treatment with thapsigargin (TG) or the physiological agonist thrombin results in de novo coupling of IP\(_3\)RII and hTRPC1\(^{12,15,16}\). This coupling is inhibited by agents that interfere with remodeling of the actin cytoskeleton\(^{15}\) and is reversed if the Ca\(^{2+}\) stores are allowed to refill\(^{48}\). The coupling between IP\(_3\)RII and hTRPC1 is closely temporally correlated with the activation of Ca\(^{2+}\) release from the ER and the activation of Ca\(^{2+}\) entry in thrombin-stimulated platelets\(^{16}\), supporting the hypothesis that this coupling event may underlie the activation of SOCE.

Rapid coupling of IP\(_3\)RII in the ER membrane to hTRPC1 in the PM requires an equally rapid reorganization of the actin cytoskeleton to allow the approach of the ER and PM, which are normally separated by a dense cortical layer of F-actin\(^{14}\). Here we have monitored for the first time thrombin-stimulated changes in platelet F-actin content on a sub-second timescale. Using a quenched-flow approach to fix platelet samples at varying times after stimulation with 0.1 U/mL thrombin, we have shown that there is an initial decrease in F-actin content that commenced within 100 ms of stimulation. The F-actin content of the cells reached
a minimum around 70% of the resting level 0.9 s after stimulation and then began to increase again, exceeding the resting level about 2.5 s after stimulation. The early decrease in platelet F-actin content preceded the release of Ca\(^{2+}\) from intracellular stores, which was determined by stopped-flow fluorimetry in parallel experiments on the same platelet preparations to occur about 1.6 s after stimulation with thrombin. This early decrease and subsequent rise in platelet F-actin content may play an important role in the trafficking of portions of the ER towards the PM. The fact that the actin remodeling preceded Ca\(^{2+}\) store release might explain the close temporal correlation between Ca\(^{2+}\) release and the coupling of IP\(_3\)RII to hTRPC1 that we have previously reported\(^{16}\).

Since remodeling of the actin cytoskeleton occurred prior to the release of Ca\(^{2+}\) from intracellular stores, it cannot be dependent on either Ca\(^{2+}\) store depletion or a rise in [Ca\(^{2+}\)]\(_c\). To investigate the mechanism of early thrombin-evoked changes in platelet F-actin, we focused on the possible role of coflin, an actin binding protein which promotes actin depolymerization by removing G actin subunits from the barbed ends of the filaments.\(^{21,25-38}\) Cofilin has been shown to be activated by the Ser\(\rightarrow\)Thr phosphatase, slingshot, which dephosphorylates the residue Ser\(^{3}\).\(^{33-39}\) Cofilin dephosphorylation has been shown to be involved in transient association with the actin cytoskeleton,\(^{49}\) decreasing coflin affinity for actin after phosphorylation at Ser\(^{3}\).\(^{50}\) Western blotting using platelet samples prepared by quenched flow from the same cell preparations used for F-actin measurements revealed that the platelet content of coflin phosphorylated on Ser\(^{3}\) changed with a similar timecourse to the content of F-actin. Thrombin (0.1 U/mL) evoked an initial dephosphorylation of coflin that commenced within 100 ms, reached a minimum 0.9 s after stimulation at around 78 % of the resting level and then coflin phosphorylation at Ser\(^{3}\) increased again, exceeding resting levels about 1.5 s after stimulation. The close temporal correlation between the changes in platelet phosphocofilin and F-actin contents are compatible with coflin playing a role in the observed early actin depolymerisation.
A role for coflin in the early thrombin-evoked actin depolymerisation is supported by our observations with the Btk inhibitor, LFM-A13. Pretreatment of platelets for 10 min with 10 μM LFM-A13, which we have previously shown abolishes Btk activation, inhibited the early coflin dephosphorylation and the actin depolymerisation evoked by thrombin. Our results demonstrate that thrombin induced rapid Btk phosphorylation and activation, which is consistent with a role for Btk in the activation of coflin and actin filament reorganization stimulated by thrombin. We have previously shown that Btk inhibition reduced Ca\(^{2+}\) entry evoked following thrombin-evoked Ca\(^{2+}\) store depletion. Here we found that Btk inhibition increased the latency of thrombin-evoked Ca\(^{2+}\) entry as assessed by Mn\(^{2+}\) quench of fura-2 fluorescence. These alterations in the activation of Ca\(^{3+}\) entry following Btk inhibition might be explained by a failure of SOCE activation by de novo conformational coupling when actin depolymerization is inhibited. The residual Ca\(^{2+}\) entry observed following inhibition of actin depolymerization may be accounted for by the cytoskeleton-independent SOCE pathway activated via extracellular signal-related kinase 1/2 (ERK1/2) that we have previously described.

Another mechanism of regulating coflin activity is through its binding to phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)), which inhibits the actin-binding ability of coflin. This mechanism has been shown to be important for the spatial regulation of coflin activity. The PIP\(_2\)- and actin-binding sites have been localized in residues W104-M115 of the coflin primary sequence. In addition to the described mechanism of coflin activation through the activation of Btk, thrombin might also regulate coflin activity by the activation of phospholipase C, which might cooperate to remodel the actin cytoskeleton through PIP\(_2\) hydrolysis, leading to activation of coflin.

In summary, we have shown that platelet activation by thrombin is associated with rapid depolymerization of F-actin prior to the release of Ca\(^{2+}\) from intracellular stores and the
activation of Ca\textsuperscript{2+} entry. This early actin depolymerization and later polymerization may be important in allowing trafficking of portions of the ER towards the PM to allowing the \textit{de novo} coupling of IP\textsubscript{3}RII to hTRPC1, which we have suggested may underlie the activation of SOCE in human platelets\textsuperscript{12-16, 48}. The activation of cofilin by its dephosphorylation at Ser\textsuperscript{3} parallels the early thrombin-evoked actin depolymerization and appears to be essential for this to occur. In NIH3T3 cells, cofilin dephosphorylation has been shown to lie downstream of Ras activation and to involve two Ras effector pathways: Raf-MEK and PI3K\textsuperscript{56}. We have previously demonstrated that inhibition of Ras\textsuperscript{57}, MEK\textsuperscript{51} or PI3K\textsuperscript{58} results in inhibition of SOCE in human platelets. These earlier data are compatible with an important role for cofilin activity in the activation of SOCE by a reversible \textit{de novo} coupling model in human platelets.

Acknowledgements

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FIGURE LEGENDS

Fig. 1. Comparison of the latency and time course of thrombin-evoked Ca$^{2+}$ release, Mn$^{2+}$ entry and actin reorganization. Top panel, Fura 2-loaded human platelets were rapidly mixed with thrombin (at 0 s) at a final concentration of 0.1 U/mL in the presence of 100 μM of EGTA and 200 μM MnCl$_2$. Fura-2 fluorescence was recorded at excitation wavelengths of 340 nm (black trace, left axis) and 360 nm (grey trace, right axis) Traces are representative of twenty runs made on cell preparations from ten donors. Bottom panel, Human platelets were rapidly mixed with 0.1 U/mL thrombin (●) or with HBS (dashed line) and incubated at 37 ºC for various time periods (1-5 seconds) before mixing with formaldehyde (3% in PBS). Actin filament content was determined as described in the Materials and methods section. Results shown are presented as percentage of the F-actin content in resting cells and expressed as mean ± SE of twenty one runs made on cell preparations from twelve donors. Vertical dashed lines represent the starting times for Ca$^{2+}$ release and Mn$^{2+}$ entry. * P<0.05 compared with F-actin content in resting cells.

Fig. 2. Comparison of the latency and time course of thrombin-evoked cofilin phosphorylation and actin reorganization. Platelets were rapidly mixed with thrombin at final concentration of 0.1 U/mL or with agonist-free HBS solution (control at 0 s), and incubated at 37 ºC for various time periods (1-5 s) before mixing with lysis buffer (for cofilin phosphorylation) or with formaldehyde (3% in PBS; for F-actin measurement) using a quenched flow system. For cofilin phosphorylation, proteins were separated by SDS/PAGE followed by Western blotting with either anti-phosphoSer$^{3}$-cofilin antibody (top panel) or anti-cofilin antibody (bottom panel) as described in the Materials and methods section. Bands were revealed using chemiluminiscence, and were quantified using scanning densitometry. Positions of molecular-mass markers are shown on the right. Actin filament content was determined as described in the Materials and methods section. Graph represents the quantification of cofilin.
phosphorylation (●) and F-actin content (○). Values are mean ± SE of fifteen runs made on cell preparations from eleven donors expressed as the percentage of cofilin phosphorylation or F-actin content in resting cells. * P<0.05 compared with cofilin phosphorylation in resting cells.

**Fig. 3. Thrombin-evoked rapid Btk phosphorylation and activation in human platelets.**

Platelets were rapidly mixed with thrombin at final concentration of 0.1 U/mL or with agonist-free HBS solution (control at 0 s), and incubated at 37 ºC for various time periods (0.1-2.5 s) before mixing with lysis buffer using a quenched flow system. Proteins were separated by SDS/PAGE followed by Western blotting with either anti-phospho-Btk (Y-223) antibody (top panel) or anti-Btk antibody (bottom panel) as described in the Materials and methods section. Bands were revealed using chemiluminiscence, and were quantified using scanning densitometry. Positions of molecular-mass markers are shown on the right. Graph represents the quantification of Btk phosphorylation. Values are mean ± SE of six runs made on cell preparations from six donors expressed as the percentage of Btk phosphorylation in resting cells.

**Fig. 4. Effect of Btk inhibition on the latency and time course of thrombin-evoked cofilin phosphorylation.** Human platelets were preincubated for 10 min in the presence of 10 μM LFM-A13 (○) or the vehicle (■) and then rapidly mixed with thrombin (0.1 U/mL) or with agonist-free HBS solution (control at 0 s), and incubated at 37 ºC for various time periods before mixing with lysis buffer using a quenched flow system. Proteins were separated by SDS/PAGE followed by Western blotting with either anti-phosphoSer³-cofilin antibody (top panels) or anti-cofilin antibody (bottom panels) as described in the Material and methods section (C, control; T, LFM-A13-treated cells). Bands were revealed using chemiluminiscence, and were quantified using scanning densitometry. Positions of molecular-mass markers are shown on the right. Graph represents the quantification of cofilin phosphorylation. Values are mean ± SE of six runs made on cell preparations from six donors expressed as the percentage
of coflin phosphorylation in resting cells.

**Fig. 5. Effect of LFM-A13 on the latency and time course of thrombin-evoked actin reorganization.** Human platelets were preincubated for 10 min in the presence of 10 μM LFM-A13 (○) or the vehicle (■) and then rapidly mixed with 0.1 U/mL thrombin and incubated at 37 °C for various time periods before mixing with formaldehyde (3% in PBS). Actin filament content was determined as described in the Material and methods section. Results are presented as the percentage of the F-actin content of non-stimulated cells and expressed as mean ± SE of six runs made on cell preparations from six donors.
Table 1
Effect of Btk inhibition on thrombin-evoked actin reorganization and Ca\textsuperscript{2+} mobilization

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<tr>
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<th>Control</th>
<th>LFM-A13</th>
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<tr>
<td>F-actin content</td>
<td>70.7 ± 9.8</td>
<td>110.3 ± 4.36***</td>
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<tr>
<td>(% control)</td>
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<tr>
<td>Ca\textsuperscript{2+} release (Latency (s))</td>
<td>1.64 ± 0.09</td>
<td>1.85 ± 0.14</td>
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<tr>
<td>Ca\textsuperscript{2+} entry (Latency (s))</td>
<td>2.14 ± 0.09</td>
<td>2.60 ± 0.17*</td>
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Human platelets were preincubated for 10 min with 10 μM LFM-A13 and then were rapidly mixed with 0.1 U/mL thrombin. F-actin content was determined 0.9 s after thrombin stimulation as described in the Materials and methods section. Results shown are presented as percentage of the F-actin content in resting cells and expressed as mean ± SE of six independent experiments. *** *P* < 0.001. Fura-2 fluorescence was recorded at excitation wavelengths of 340 nm (for Ca\textsuperscript{2+} release) and 360 nm (for Ca\textsuperscript{2+} entry). Values are expressed as mean ± SE of at least six runs made on cell preparations from six donors seven to ten separate experiments. * *P* < 0.05.
Figure 1
Figure 2

[Image of a Western blot analysis showing P-Cofilin and Cofilin bands over time (0 to 5 seconds) and molecular weight markers (0 to 30 kDa). Below, a graph showing Cofilin phosphorylation and F-actin content over time (0 to 5 seconds).]
Figure 4

![Diagram showing time course of P-Cofilin and Cofilin phosphorylation](image-url)
Figure 5
A role for cofilin in the activation of store-operated calcium entry by de novo conformational coupling in human platelets

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