Farnesylcysteine analogues inhibit store-regulated Ca^{2+} entry in human platelets: evidence for involvement of small GTP-binding proteins and actin cytoskeleton

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We have investigated the mechanism of Ca^{2+} entry into fura-2-loaded human platelets by preventing the prenylation of proteins such as small GTP-binding proteins. The farnesylcysteine analogues farnesylthioacetic acid (FTA) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC), which are inhibitors of the methylation of prenylated and geranylgeranylated proteins respectively, significantly decreased thrombin-evoked increases in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the presence, but not in the absence, of external Ca^{2+} , suggesting a relatively selective inhibition of Ca^{2+} entry over internal release. Both these compounds and *N*-acetyl-*S*-farnesyl-L-cysteine, which had similar effects to those of FTA, also decreased Ca^{2+} entry evoked by the depletion of intracellular Ca^{2+} stores with thapsigargin. The inactive control *N*-acetyl-*S*-geranyl-L-cysteine was without effect. Patulin, an inhibitor of prenylation that is inert with respect to

methyltransferases, also decreased store-regulated Ca^{2+} entry. Cytochalasin D, an inhibitor of actin polymerization, significantly decreased store-regulated Ca^{2+} entry in a time-dependent manner. Both cytochalasin D and the farnesylcysteine analogues FTA and AGGC inhibited actin polymerization; however, when evoking the same extent of decrease in actin filament formation, FTA and AGGC showed greater inhibitory effects on Ca^{2+} entry, indicating a cytoskeleton-independent component in the regulation of Ca^{2+} entry by small GTP-binding-protein. These findings suggest that prenylated proteins such as small GTP-binding proteins are involved in store-regulated Ca^{2+} entry through actin cytoskeleton-dependent and cytoskeleton-independent mechanisms in human platelets.

Key words: Ca^{2+} influx, G-protein, haemostasis.

INTRODUCTION

The stimulation of platelets and other non-excitable cells by various agonists results in an increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) which consists of two components: the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} across the plasma membrane [1]. It has been established that in many non-excitable cells Ca^{2+} influx is controlled by the filling state of the intracellular Ca^{2+} stores [2]; however, the mechanism by which the filling state of the intracellular stores is communicated to the plasma membrane is still unknown. Current hypotheses fall into two main categories: indirect coupling and direct coupling [3]. Indirect coupling assumes the existence of an intermediate signal-transduction pathway involving the generation of a small messenger molecule that, directly or through different biochemical events, opens channels or inserts channels [4] into the plasma membrane, thus facilitating Ca^{2+} entry. In contrast, direct coupling models suggest a physical connection between the plasma membrane and that of the intracellular stores, perhaps as an interaction between an InsP_4 receptor in the plasma membrane and the InsP_3 receptor in the storage organelles [5].

Many different messenger molecules have been proposed to be involved in store-regulated Ca^{2+} entry (SRCE): cGMP [6], cytochrome P450 metabolites [7], a tyrosine kinase-dependent step [8,9], release of a Ca^{2+} influx factor from depleted stores [10] or a Ca^{2+} /calmodulin kinase-dependent step [11]. Fasolato et al. [12] have presented evidence for the involvement of small GTP-binding proteins in SRCE in RBL cells. In these cells, dialysis with non-hydrolysable analogues of GTP before the depletion of

stores prevented the activation of I_{CRAC} (calcium-release activated current) when the Ca^{2+} stores were depleted by using ionomycin. Similar results were obtained by Bird and Putney [13] in mouse lacrimal acinar cells and by Somasundaram et al. [4] in megakaryocytes, suggesting the involvement of small G-proteins such as the Rab-like proteins in the influx of Ca^{2+} . More recently, the small G-protein Rac has been suggested as a new candidate molecule involved in signalling upstream of growth-factor-induced Ca^{2+} influx in HeLa cells [14] or in thapsigargin (TG)-induced and somatostatin-induced Ca^{2+} entry in HEK cells stably expressing somatostatin receptor subtype 2 [15].

The Ras GTP-binding proteins have a pivotal role in several signal transduction pathways and differentiation processes [16–18]. Several reports have indicated that Ras superfamily proteins and several proteins that interact with Ras or are downstream of Ras in other cells are present in platelets [19–21]. In these cells, Ras is rapidly activated on the stimulation of the thrombin receptor, the prostaglandin H_2 /thromboxane A_2 receptor or protein kinase C, suggesting that Ras-mediated pathways might contribute to signal-response coupling in platelets [20].

Ras GTP-binding proteins, as well as nuclear lamins and the γ subunits of the heterotrimeric G-proteins, are prenylated and methylated post-translationally [22,23]; this modification involves the attachment of a farnesyl or geranylgeranyl group to the protein through a thioether linkage to a cysteine residue located four residues from the C-terminus, followed by the removal of the three C-terminal amino acids and reversible methylation of the newly exposed α -carboxy group of the farnesyl- or geranylgeranyl-cysteine residue [23,24]. The nature of the attached substituent depends on the specific sequence in the

Abbreviations used: AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S*-geranyl-L-cysteine; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; I_{CRAC} , calcium-release activated current; FC, *S*-all-*trans*-farnesyl-L-cysteine; FTA, farnesylthioacetic acid; SRCE, store-regulated Ca^{2+} entry; TG, thapsigargin.

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C-terminus of the protein [23]. Analysis of the candidate proteins has revealed that Ras, Rac, Rap1a and Rap2a are selectively farnesylated, whereas Rap1b, Rap2b, G25K, Rab and Rho are geranylgeranylated [23,25,26]. Prenylation and methylation are known to lead to the association of these proteins with membranes and to be important for their interaction with cell membrane components, for example interaction of the stimulatory and inhibitory GDP/GTP exchange proteins [27] and their effector proteins [22], possibly via prenylated protein–protein or protein–lipid interactions [15]. With Ras GTP-binding proteins, the prevention of this modification results in their inactivation [28,29].

The Ras superfamily proteins regulate the organization of the actin cytoskeleton in platelets and in many other cell types. Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, and also activates Rho [16,17,30], which in turn controls the assembly of actin stress fibres and focal adhesion complexes [31]. Cdc42 leads to the extension of filopodia [16,17,30] and Rap1 can regulate events that take place within seconds after the activation of platelets. Fischer et al. [19] have suggested a model in which Rap1b, integrated in the plasma membrane after prenylation and methylation, might form a site of attachment for the cytoskeleton, perhaps providing direction for the contractile mechanism or mediating the incorporation of membrane proteins into the cytoskeleton.

To investigate the possible role of small GTP-binding proteins of the Ras superfamily in SRCE in human platelets, we have used different *S*-all-*trans*-farnesyl-L-cysteine (FC) analogues such as farnesylthioacetic acid (FTA), a specific inhibitor of methylation of farnesylated proteins, or *N*-acetyl-*S*-farnesyl-L-cysteine (AFC), a substrate and also an inhibitor of the farnesyl methyltransferase [15,32], and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC), which specifically inhibits the methylation of geranylgeranylated proteins [15,33]. We also investigated whether the regulation of actin filament formation by Ras proteins could be important in mediating SRCE.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Texas Fluorescence (Austin, TX, U.S.A.). Apyrase (grade V), aspirin, BSA, colchicine, paraformaldehyde, Nonidet P40, FITC-labelled phalloidin, valinomycin, lanthanum chloride and TG were from Sigma (Poole, Dorset, U.K.). Cytochalasin D and C3 exoenzyme were from Calbiochem (Nottingham, Notts., U.K.). FTA, AFC, AGGC, *N*-acetyl-*S*-geranyl-L-cysteine (AGC) and the mycotoxin patulin were from Alexis Corporation (Nottingham, Notts., U.K.). Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Pan-Ras (Ab-3) monoclonal antibody was from Oncogene Science (Cambridge, MA, U.S.A.). Horseradish-peroxidase-conjugated sheep 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 (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 [9]. In brief, blood was drawn by venepuncture from healthy drug-free volunteers and mixed with one-sixth vol. 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*; aspirin (100 μ M) and apyrase (40 μ g/ml) were added. Platelet-rich plasma was incubated at 37 °C with 2 μ M fura-2/AM for 45 min. For co-loading with dimethyl BAPTA, 10 μ M dimethyl BAPTA/AM was added for the final 30 min of the fura-2 loading period. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in Hepes-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 Hepes, 10 D-glucose, 5 KCl, 1 MgSO₄ and supplemented with 0.1% BSA and 40 μ g/ml apyrase.

Measurement of [Ca²⁺]_i

Fluorescence was recorded from 1.5 ml aliquots of magnetically stirred platelet suspension (10⁸ cells/ml) at 37 °C with a Cairn Research Spectrophotometer (Cairn Research Ltd, Sittingbourne, Kent, U.K.) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. Changes in [Ca²⁺]_i were monitored with the ratio of fura-2 fluorescence at 340 and 380 nm and calibrated by the method of Grynkiewicz et al. [34]. Mn²⁺ influx was monitored as a quenching of fura-2 fluorescence at the isoemissive wavelength of 360 nm, presented on an arbitrary linear scale [35].

Determination of Ca²⁺ influx

Thrombin-evoked Ca²⁺ influx was measured as the integral of the rise in [Ca²⁺]_i above basal for 2½ min after the addition of thrombin in the presence of external Ca²⁺, corrected by subtraction of the integral over the same period for stimulation in the absence of external Ca²⁺ (with 100 μ M EGTA). When SRCE was stimulated after store depletion with TG, Ca²⁺ influx was estimated by using the integral of the rise in [Ca²⁺]_i for 2½ min after the addition of CaCl₂.

Protein tyrosine phosphorylation

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting [9]. Stimulation of platelets was terminated by mixing with an equal volume of 2× Laemmli's buffer [36] containing 10% (v/v) dithiothreitol, followed by heating for 5 min at 95 °C. One-dimensional SDS/PAGE was performed with 10% (w/v) polyacrylamide minigels and separated proteins were transferred electrophoretically, for 2 h at 0.8 mA/cm², in a semi-dry blotter (Hoefer Scientific, Newcastle-under-Lyme, Staffs., U.K.) to nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) to block residual protein-binding sites. Immunodetection of tyrosine phosphorylation was achieved with the anti-phosphotyrosine antibody 4G10 diluted 1:2500 in TBST for 1 h. The primary antibody was removed and blots were washed six times with TBST for 5 min each. To detect the primary antibody, blots were incubated with horseradish-peroxidase-conjugated sheep 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 with a Quantimet 500 densitometer (Leica, Milton Keynes, Bucks., U.K.) and the integrated attenuation of the entire lane was estimated.

Subcellular fractionation

Human platelet fractionation was performed with a procedure described previously [37]. In brief, platelets were pelleted in a microcentrifuge and the pellets were quickly resuspended in

0.5 ml of ice-cold 10 mM Tris/HCl buffer, pH 7.2, containing 158 mM NaCl, 1 mM EGTA, 50 $\mu\text{g}/\text{ml}$ leupeptin, 5 mM benzamidine, 1 mM PMSF and 1 mM Na_3VO_4 . The suspensions were sonicated and intact platelets were removed by centrifugation at 1500 g . The platelet lysate was centrifuged at 100 000 g at 4 °C for 60 min to obtain the membrane and cytosolic fractions. Membranes were washed with PBS containing 1 mM Na_3VO_4 at 4 °C and resuspended in 10 mM Tris/HCl buffer, pH 7.2, containing 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 50 $\mu\text{g}/\text{ml}$ leupeptin, 5 mM benzamidine, 1 mM PMSF and 1 mM Na_3VO_4 . Lysates were centrifuged at 16 000 g for 5 min to remove insoluble substances. Lysates of the subcellular fractions or total cell lysates (50 μg per well) were analysed by Western blotting with pan-Ras (Ab-3) monoclonal antibody diluted 1:500 in TBST. To detect the primary antibody, blots were incubated with horseradish-peroxidase-conjugated anti-mouse IgG antibody diluted 1:10 000 in TBST and exposed to enhanced chemiluminescence reagents for 1 min, as described above. Blots were then exposed to preflashed photographic film.

Measurement of F-actin content

The F-actin contents of resting and activated platelets were determined in accordance with the modifications [33] of a procedure described previously [38]. In brief, washed platelets (2×10^8 cells/ml) were activated in Hepes-buffered saline. 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% BSA. After incubation, the platelets were collected by centrifugation in an MSE Micro-Centaur Centrifuge (MSE Scientific Instruments, Crawley, Sussex, U.K.) for 90 s at 3000 g and resuspended in PBS. Staining of 2×10^7 cells/ml was measured with a Perkin-Elmer Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.). Samples were excited at 496 nm and emission was measured at 516 nm.

Statistical analysis

Analysis of statistical significance was performed with Student's unpaired t test. For multiple comparison, a one-way analysis of variance combined with the Dunnett or Tukey tests was used.

RESULTS

Inhibition of thrombin-evoked $[\text{Ca}^{2+}]_i$ elevation by FTA

Figure 1(A) shows that the addition of FTA (40 μM) to fura-2-loaded human platelets in stirred cuvettes at 37 °C resulted in a substantial inhibition of the elevation in $[\text{Ca}^{2+}]_i$ evoked by thrombin (0.1 unit/ml) in medium containing 1 mM Ca^{2+} . The initial peak $[\text{Ca}^{2+}]_i$ elevation above basal after treatment with agonist was decreased significantly from 468 ± 19 to 258 ± 9 nM (mean $\pm$ S.E.M.; $n = 10$; $P < 0.001$).

In the absence of external Ca^{2+} (100 μM EGTA added) FTA was without discernible effect on the thrombin-evoked rise in $[\text{Ca}^{2+}]_i$, indicating no effect on the release of Ca^{2+} from the internal stores (Figure 1B). The initial peak elevation in $[\text{Ca}^{2+}]_i$ above basal after treatment with agonist was 205 ± 11 nM in control and 199 ± 10 nM in FTA-treated cells ($n = 9$). The lack of effect of FTA on thrombin-evoked release of Ca^{2+} from intracellular stores suggested that FTA was selectively inhibiting Ca^{2+} entry over internal release. If we consider the entry of Ca^{2+}

Figure 1 Effect of FTA on thrombin-evoked elevations in $[\text{Ca}^{2+}]_i$ in the presence and the absence of external Ca^{2+}

Human platelets were loaded with fura-2 and resuspended in a Ca^{2+} -free medium as described in the Materials and methods section. Elevations in $[\text{Ca}^{2+}]_i$ were monitored by using the 340-to-380 nm ratio and traces were calibrated in terms of $[\text{Ca}^{2+}]_i$. At the time of the experiment either 1 mM CaCl_2 (A) or 100 μM EGTA (B) was added. Cells were stimulated with thrombin (0.1 unit/ml) at the time indicated, in the presence of 40 μM FTA (traces b) or vehicle (DMSO, traces a). The traces shown are representative of nine or ten separate experiments.

stimulated by thrombin (see the Materials and methods section), FTA significantly inhibited thrombin-induced Ca^{2+} influx by $56 \pm 2\%$ ($P < 0.001$). FTA alone induced a small increase in $[\text{Ca}^{2+}]_i$ both in the presence and in the absence of extracellular Ca^{2+} (Figures 1A and 1B); the mechanism of this previously reported FTA-evoked elevation in $[\text{Ca}^{2+}]_i$ is unclear [15].

Inhibition of TG-induced store-regulated $[\text{Ca}^{2+}]_i$ entry by FTA

In a Ca^{2+} -free medium, TG, a specific inhibitor of the Ca^{2+} -ATPase of internal stores (sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase [39]), evoked a prolonged elevation of $[\text{Ca}^{2+}]_i$ in platelets owing to the release of Ca^{2+} from intracellular pools. The subsequent addition of Ca^{2+} (300 μM) to the external medium induced a sustained increase in $[\text{Ca}^{2+}]_i$, indicative of SRCE (Figure 2A). The addition of FTA subsequent to TG decreased Ca^{2+} entry in a concentration-dependent manner (Figures 2A and 2B). FTA significantly decreased SRCE by $24 \pm 3\%$, $41 \pm 2\%$ and $73 \pm 3\%$ ($n = 9$) at 10, 20 and 40 μM respectively (Figure 2B; $P < 0.05$).

Figure 2 Concentration dependence of the inhibition by FTA of TG-evoked SRCE in human platelets

(A) Fura-2-loaded human platelets were stimulated with TG (200 nM) in a Ca²⁺-free medium (100 μM EGTA); 3 min later CaCl₂ (final concentration 300 μM) was added to the medium to initiate Ca²⁺ entry. [Ca²⁺]_i was monitored as described in the legend to Figure 1. The addition of increasing concentrations of FTA (10 μM, trace b; 20 μM, trace c; 40 μM, trace d) or vehicle (DMSO, trace a) was made 45 s before the addition of Ca²⁺, as shown by the arrow marked FTA. Traces are representative of nine independent experiments. (B) Histograms indicating the percentage of Ca²⁺ entry in the presence of different concentrations of FTA relative to their control (vehicle was added). Ca²⁺ entry was determined as described in the Materials and methods section. Values are means ± S.E.M.; significance values indicate differences compared with TG-treated cells in the absence of FTA. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

FTA inhibits the TG-stimulated entry of extracellular Mn²⁺

Mn²⁺ was used to evaluate the effect of FTA on TG-evoked bivalent-cation influx. This cation can be used as a surrogate for Ca²⁺ entry, given its quenching effect on fura-2 [35]. Fura-2 was excited at the isoemissive wavelength, 360 nm, to permit the monitoring of quenching of fluorescence by Mn²⁺. The addition of Mn²⁺ (300 μM) to platelets with TG-depleted intracellular Ca²⁺ stores resulted in a sustained quenching of fluorescence (Figure 3, trace e) compared with that in non-depleted cells (Figure 3, trace a). When FTA was added after TG, there was an attenuation in the rate of Mn²⁺ entry. The initial rate of Mn²⁺-evoked fluorescence quenching in platelets treated with TG and then with FTA was significantly decreased to 77 ± 4%, 59 ± 4% and 29 ± 5% (*n* = 6–8) of the rate observed in cells treated with TG alone, at 10, 20 and 40 μM respectively (Figure 3; *P* < 0.05). These results indicate that FTA inhibits store-depletion-induced Mn²⁺ entry in a concentration-dependent manner.

Figure 3 Effect of FTA on store-depletion-evoked entry of extracellular Mn²⁺

Human platelets were loaded with fura-2 and resuspended in a Ca²⁺-free medium as described in the Materials and methods section. Fura-2 fluorescence was measured at an excitation wavelength of 360 nm, the isoemissive wavelength. Platelets were stimulated with TG (200 nM) 3 min before the addition of MnCl₂ (final concentration 300 μM). Mn²⁺ was added to untreated control cells (trace a) or TG-depleted cells (traces b–e). FTA at 10 μM (trace d), 20 μM (trace c) and 40 μM (trace b), or vehicle (DMSO, trace e), was added 45 s before Mn²⁺. Traces are representative of six to eight separate experiments.

Effect of different FC analogues on SRCE

To investigate further the role of protein prenylation or methylation in SRCE in human platelets, we tested the effect of other FC analogues. Because FTA is a potent inhibitor of farnesylcysteine methyltransferase but is not a substrate, it was of interest to investigate the effect of AFC, another FC analogue that is a substrate, as well as a specific inhibitor, of farnesylcysteine methyltransferase [40,41]. The results shown in Figure 4(A) indicate that AFC significantly inhibited TG-induced SRCE by 55 ± 2% at 40 μM (*n* = 11; *P* < 0.01). As with FTA, AFC on its own induced a small increase in [Ca²⁺]_i. To investigate the role of geranylgeranylated proteins in SRCE we tested the effect of AGGC, an FC analogue that specifically inhibits the methyl esterification of geranylgeranylated proteins such as Rab or Rho. As shown in Figure 4(B), AGGC significantly attenuated TG-evoked Ca²⁺ entry into platelets by 47 ± 2% in comparison with non-treated cells at 30 μM (*n* = 11; *P* < 0.01). In contrast, AGC (which is a negative control for FC analogues in having a similar structure to the compounds studied but lacking their biological activity [33]) had no effect on TG-evoked SRCE. As shown in Figure 4(C), even at 200 μM (*n* = 10), AGC was without effect. This result suggests that FC analogues do not have any non-specific effects as Ca²⁺ chelators or as Ca²⁺ entry blockers. This finding is also supported by the different patterns of inhibition of TG-evoked SRCE when FTA or lanthanum are added after entry has been initiated by the addition of CaCl₂ (Figure 5). Lanthanum (100 μM), an inhibitor of non-selective cation channels [3], blocked Ca²⁺ entry immediately on addition; however, FTA (40 μM) showed a very different pattern, with a delay of approx. 30 s before the onset of inhibition.

FTA, AFC and AGGC are specific inhibitors of farnesyl or geranylgeranyl methyltransferases. To evaluate whether the effect on Ca²⁺ entry observed was due to the inhibition of small G-proteins and not to an inhibition of methyltransferase activity, we tested the effect of the mycotoxin patulin, a metabolite isolated from *Byssoschlamys nivea* that blocks protein prenylation without having any inhibitory effect on methyltransferase activity

Figure 4 Effect of several FC analogues and patulin on TG-evoked SRCE in human platelets

Fura-2-loaded human platelets were stimulated with TG (200 nM) in a Ca^{2+} -free medium (100 μM EGTA); 3 min later, CaCl_2 (final concentration 300 μM) was added to the medium. $[\text{Ca}^{2+}]_i$ was monitored as described in the legend to Figure 1. FC analogues or patulin were added 45 s before Ca^{2+} , as shown by the arrows. (A) Cells were treated with 40 μM AFC (trace b) or vehicle (DMSO, trace a). (B) Platelets were treated with 30 μM AGGC (trace b) or vehicle (DMSO, trace a). (C) Cells were treated with 200 μM AGC (trace b) or vehicle (DMSO, trace a). (D) Platelets were treated with 100 μM patulin (trace b) or vehicle (ethanol, trace a). Traces shown are representative of 9–11 separate experiments.

Figure 5 Comparative effects of FTA and lanthanum on TG-evoked SRCE in human platelets

Fura-2-loaded human platelets were stimulated with TG (200 nM) in a Ca^{2+} -free medium (100 μM EGTA); 3 min later, CaCl_2 (final concentration 300 μM) was added to the medium. $[\text{Ca}^{2+}]_i$ was monitored as described in the legend to Figure 1. The addition of FTA (40 μM , trace b), lanthanum chloride (100 μM , trace c) or vehicle (trace a) was made 2 min after the addition of Ca^{2+} , as shown by the arrow. Traces are representative of six independent experiments.

[42]. Figure 4(D) shows that the addition of patulin (100 μM) to fura-2-loaded human platelets significantly inhibited TG-evoked Ca^{2+} entry by $35 \pm 1\%$ ($n=9$; $P < 0.001$). This concentration of

patulin was previously found to inhibit the prenylation of proteins in FM3A cells [42].

It has been demonstrated that depolarization of the membrane potential decreases the driving force for agonist- and store-depletion-evoked Ca^{2+} entry. To check whether the inhibitory effects of the FC analogues could be attributable to changes in membrane potential, we studied the effects of FTA and AGGC on Ca^{2+} influx in the presence of the K^+ ionophore valinomycin, which stabilizes the platelet membrane potential close to the K^+ equilibrium potential [43]. FTA and AGGC inhibited Ca^{2+} influx to the same extent in the presence and in the absence of valinomycin (3 μM ; results not shown). This finding indicates that the effects of the FC analogues are not due to a decrease in the membrane potential. This is in agreement with previous studies [15].

Importance of geranylgeranylated proteins in thrombin-evoked $[\text{Ca}^{2+}]_i$ mobilization

Figure 6(A) shows that the addition of AGGC (30 μM) to fura-2-loaded human platelets significantly decreased the elevation in $[\text{Ca}^{2+}]_i$ evoked by thrombin (0.1 unit/ml) in medium containing 1 mM Ca^{2+} from 468 ± 19 nM to 290 ± 13 nM ($n=7-10$; $P < 0.001$), at the initial peak. AGGC significantly decreased thrombin-stimulated Ca^{2+} entry by $38 \pm 3\%$ ($P < 0.001$).

The possibility that the inhibition of methyl esterification of geranylgeranylated proteins might interfere with the production of InsP_3 , through the inhibition of heterotrimeric G-proteins, was tested for by examining the release of the intracellular Ca^{2+} stores. The addition of AGGC (30 μM) before thrombin had no effect on the release of Ca^{2+} from the internal stores, as indicated by experi-

Figure 6 Effect of AGGC on thrombin-evoked $[Ca^{2+}]_i$ elevations in the presence and the absence of external Ca^{2+}

Human platelets were loaded with fura-2 and resuspended in a Ca^{2+} -free medium as described in the Materials and methods section. Elevations in $[Ca^{2+}]_i$ were determined as described in the legend to Figure 1. At the time of the experiment either 1 mM $CaCl_2$ (A) or 100 μ M EGTA (B) was added. Cells were stimulated with 0.1 unit/ml thrombin at the time indicated in the absence (DMSO added; trace a) or presence of 30 μ M AGGC (trace b). The traces shown are representative of seven to ten separate experiments.

ments performed in the presence of 100 μ M EGTA (Figure 6B). The initial peak elevation of $[Ca^{2+}]_i$ was 209 ± 11 nM in the presence of AGGC and 205 ± 11 nM in controls ($n = 7-10$).

Effect of FTA on tyrosine phosphorylation induced by depletion of the intracellular Ca^{2+} stores

We have previously reported [9] that tyrosine kinase activity is required for SRCE in human platelets. To test whether FC analogues affect tyrosine kinase activity we have examined the effect of FTA on store-depletion-evoked tyrosine phosphorylation in these cells. Platelets heavily loaded with the Ca^{2+} chelator dimethyl BAPTA were used for this study so as to eliminate Ca^{2+} -evoked but not store-depletion-evoked tyrosine phosphorylation [44]. Ca^{2+} stores were depleted with TG (250 nM) and ionomycin (50 nM); FTA (40 μ M) was then added. We found that FTA (40 μ M) inhibited Ca^{2+} entry to the same extent in cells treated with TG and ionomycin as in cells treated with TG alone (results not shown). In each experiment we checked that the rise 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 Table 1, FTA did not modify

Table 1 Effects of FTA on the phosphotyrosine content of store-depleted BAPTA-loaded platelets

Dimethyl BAPTA-loaded platelets were treated with 250 nM TG and 50 nM ionomycin to deplete the intracellular Ca^{2+} stores. The addition of 40 μ M FTA or vehicle (DMSO, control) was made 45 s before the addition of Ca^{2+} (135 s after the addition of TG and ionomycin). 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/PAGE [10% (w/v) gel] and subsequent Western blotting with a specific anti-phosphotyrosine antibody; the presence of phosphotyrosine residues was quantified by densitometry in Western blots as described. The results are the integrated attenuances for entire lanes under each condition. Results are expressed as fold increases (means $\pm$ S.E.M. for six separate experiments) over the integrated attenuance of platelet proteins before Ca^{2+} store depletion.

Time (s)	Control	40 μ M FTA
-10	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
45	1.09 $\pm$ 0.02	1.12 $\pm$ 0.05
170	1.38 $\pm$ 0.03	1.37 $\pm$ 0.04
300	1.50 $\pm$ 0.14	1.50 $\pm$ 0.13

Figure 7 Effect of FTA on the membrane association of Ras proteins

Human platelets were stimulated with TG (200 nM) in a Ca^{2+} -free medium; 3 min later, $CaCl_2$ (final concentration 300 μ M) was added. The addition of 40 μ M FTA or the vehicle (control) was made 45 s before the addition of Ca^{2+} as described in the legend to Figure 2. Samples were taken 5 s before the addition of TG (Basal) and 30 s after the addition of $CaCl_2$ (Control and FTA). The subcellular distribution of Ras proteins between the cytosolic (C) and membrane (M) fractions was analysed by Western blotting with a pan-Ras antibody. The amount of Ras present in cells treated with 40 μ M FTA or vehicle (DMSO) was estimated by Western blotting of total cell lysates. The arrow represents the position of the putative Ras proteins. These results are representative of at least three others.

phosphotyrosine levels at the highest concentration used in this study, indicating that FTA (40 μ M) does not have a general inhibitory effect on tyrosine kinase activity stimulated by the depletion of intracellular Ca^{2+} stores.

Effect of FTA on the subcellular localization of Ras proteins

It has been reported that the inhibition of Ras methylation by farnesylthiosalicylic acid impairs the membrane association of Ras proteins, which is an important process in Ras activation [45]. We therefore investigated whether the inhibition of SRCE in human platelets by FTA might be associated with alterations in Ras subcellular localization. As shown in Figure 7, in resting platelets, pan-Ras (Ab-3) immunoreactive proteins could be detected in both the cytosolic and membrane fractions. In agreement with previous studies [45], after stimulation with TG, Ras was found to be localized predominantly in the membrane fraction of vehicle-treated platelets. In contrast, most of the Ras proteins in the FTA-treated platelets were localized in the cytosolic fraction (Figure 7; $n = 4$). In total cell lysates the levels of Ras proteins in FTA-treated platelets were not different from those in control cells (Figure 7).

Effect of C3 exoenzyme on SRCE

To investigate further the role of small GTP-binding proteins in SRCE in human platelets, we examined the effect of C3 exo-

Figure 8 Effect of C3 exoenzyme on TG-evoked SRCE in human platelets

Fura-2-loaded human platelets were incubated in the absence (trace a) or presence (trace b) of 100 $\mu\text{g}/\text{ml}$ C3 exoenzyme for 2 h at 37 °C and then stimulated with TG (200 nM) in a Ca^{2+} -free medium (100 μM EGTA); 3 min later, CaCl_2 (final concentration 300 μM) was added to the medium. $[\text{Ca}^{2+}]_i$ was monitored as described in the legend to Figure 1. Traces are representative of eight independent experiments.

enzyme. *Clostridium botulinum* C3 exoenzyme is a 25 kDa enzyme that inactivates Rho A by ADP-ribosylation [46]. As shown in Figure 8, treatment of human platelets for 2 h at 37 °C with 100 $\mu\text{g}/\text{ml}$ C3 exoenzyme significantly decreased Ca^{2+} entry by $70 \pm 4\%$ ($n=8$; $P < 0.001$) without having any effect on the TG-evoked release of Ca^{2+} from the intracellular stores.

Role of actin cytoskeleton in SRCE

Members of the Ras superfamily of small GTP-binding proteins are believed to regulate the organization of the actin cytoskeleton. To test whether the actin cytoskeleton has a role in Ca^{2+} influx, cytochalasin D was used to disrupt the cytoskeletal network by blocking the formation of actin microfilaments [47,48]. Human platelets were pretreated with 10 μM cytochalasin D for different durations at 37 °C; actin filament content was determined with FITC-phalloidin. As shown in Table 2, cytochalasin D was without significant effect on the actin filament content of unstimulated platelets when treated for up to 40 min. However, cytochalasin D prevented TG-stimulated actin filament formation in platelets. This effect was dependent on the time of preincubation: it showed a significant decrease after 20 min of preincubation with cytochalasin D ($n=6$; $P < 0.01$) and a complete inhibition after 40 min of preincubation.

Figure 9 Inhibition of SRCE in human platelets by cytochalasin D

Fura-2-loaded platelets were incubated at 37 °C with 10 μM cytochalasin D for various durations (1, 10, 20 and 40 min) or with vehicle (ethanol; black bars), for the same duration, in medium that was nominally Ca^{2+} -free. At the time of the experiment 100 μM EGTA was added. Cells were then stimulated with TG (200 nM) before the addition of CaCl_2 (final concentration 300 μM), exactly as described in the legend to Figure 2(A) (trace a). Histograms indicate the percentages of Ca^{2+} entry, measured as described in the Materials and methods section, relative to control (vehicle added) for each of the indicated incubation durations. The values are mean $\pm$ S.E.M. for five separate determinations; significance values indicate differences compared with their respective controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Next we tested whether the inhibition of actin filament formation affects the TG-evoked release of Ca^{2+} from intracellular stores or SRCE. Platelets previously preincubated with 10 μM cytochalasin D for various durations showed an identical release of Ca^{2+} from the intracellular pools after stimulation with TG in comparison with untreated cells (results not shown). This indicates that the accumulation of Ca^{2+} in the internal stores was unaffected by preincubation with cytochalasin D. The subsequent addition of Ca^{2+} demonstrated that treatment with cytochalasin D attenuated SRCE in a time-dependent manner in comparison with vehicle-treated controls (Figure 9). A short preincubation with cytochalasin D caused a small increase in Ca^{2+} influx; however, Ca^{2+} entry was significantly decreased after 20 or 40 min of preincubation with cytochalasin D by $29 \pm 1\%$ and $49 \pm 3\%$ of control respectively ($n=5$; $P < 0.01$). The effect of cytoskeletal disruption on SRCE seemed to be dependent on actin microfilaments. Pretreatment of platelets at 37 °C for 40 min with colchicine, an agent that disrupts microtubules and inhibits tubulin polymerization [49], at 1 or 20 μM had no effect on SRCE. The integral of the rise in $[\text{Ca}^{2+}]_i$ above basal for $2\frac{1}{2}$ min after the addition of Ca^{2+} , taking data every 1 s, was $77510 \pm 8793 \text{ nM}\cdot\text{s}$ in control and 77494 ± 8230 and

Table 2 Effect of duration of preincubation with cytochalasin D on the actin filament content of unstimulated or TG-stimulated platelets

Platelets were incubated with 10 μM cytochalasin D or vehicle (ethanol) at 37 °C for different durations. Platelets were then treated with 200 nM TG before the addition of 300 μM CaCl_2 by following the protocol described in the legend to Figure 2(A) (trace a). Samples were removed 5 s before the addition of TG and 30 s after the addition of CaCl_2 ; actin filament content was determined as described in the Materials and methods section. Results given are actin filament contents as percentages of basal and are expressed as means $\pm$ S.E.M. for six separate determinations. ** $P < 0.01$ compared with the actin filament content in TG-activated platelets in the absence of cytochalasin D.

Stimulatory agent	Duration of preincubation (min)...	Filamentous actin (% of basal)				
		0	1	10	20	40
None		100.0 $\pm$ 0.0	100.4 $\pm$ 3.0	100.5 $\pm$ 2.0	101.0 $\pm$ 1.7	100.1 $\pm$ 3.2
TG		136.9 $\pm$ 1.3	128.3 $\pm$ 3.8	126.6 $\pm$ 1.8	111.9 $\pm$ 2.5**	98.9 $\pm$ 4.1**

Figure 10 Comparison of the effects of FTA, AGGC and cytochalasin D on TG-induced F-actin formation in platelets

Platelets were treated with cytochalasin D (10 μ M) as described in the legends to Figure 9 and Table 2, or with FTA (40 μ M) or AGGC (30 μ M) as mentioned in the legends to Figures 2(A) and 4(B), or with vehicle (control). Samples were removed 5 s before the addition of TG and 30 s after the addition of CaCl_2 ; actin filament content was determined as described in the Materials and methods section. Values given are TG-evoked actin filament formation as percentages of control; results are expressed as means $\pm$ S.E.M. for six separate determinations. *** P < 0.001 compared with platelets preincubated for 20 min with cytochalasin D; † P < 0.001 compared with control cells.

Table 3 Effect of FTA or AGGC on the actin filament content of unstimulated or thrombin-stimulated platelets

Platelets were treated with 40 μ M FTA or 30 μ M AGGC or vehicle (DMSO) 45 s before the addition of 0.1 unit/ml thrombin at 37 °C by following the protocol described in the legends to Figures 1(A) and 6(A). Samples were removed 30 s after the addition of FTA, AGGC or DMSO, and 30 s after the addition of thrombin; actin filament content was determined as described in the Materials and methods section. Results are actin filament contents as percentages of basal and are expressed as mean $\pm$ S.E.M. for eight separate determinations. ** P < 0.01 compared with the actin filament content in thrombin-activated platelets in the absence of FTA or AGGC.

Stimulatory agent	Filamentous actin (% of basal)		
	DMSO	FTA	AGGC
None	100.0 $\pm$ 0.0	97.9 $\pm$ 1.3	94.5 $\pm$ 2.4
Thrombin	164.7 $\pm$ 3.3	115.5 $\pm$ 2.4**	114.8 $\pm$ 3.3**

78248 $\pm$ 7821 nM·s in cells pretreated with 1 and 20 μ M colchicine respectively.

Figure 10 compares the effects on TG-induced actin filament formation of preincubation for different durations with 10 μ M cytochalasin D with treatment with 40 μ M FTA or 30 μ M AGGC by following the protocols shown in Figures 2(A) and 4(B). FTA and AGGC significantly decreased TG-stimulated actin filament formation by 70 $\pm$ 2% and 69 $\pm$ 2% of control respectively (n = 6; P < 0.001). With 20 min of preincubation, cytochalasin D decreased TG-evoked actin polymerization by 70 $\pm$ 3% of control. Actin polymerization stimulated by TG was similar after treatment with FTA, AGGC or 20 min preincubation with cytochalasin D and significantly different from that after preincubation with cytochalasin D for 1, 10 and 40 min (P < 0.001).

Actin filament polymerization induced by thrombin is shown in Table 3. Stimulation of human platelets with the physiological

agonist thrombin markedly increased actin filament content. Treatment with 40 μ M FTA by following the protocol shown in Figure 1(A) significantly decreased thrombin-induced actin filament formation (n = 8; P < 0.01). Similar results were obtained when platelets were treated with 30 μ M AGGC (n = 8; P < 0.01).

DISCUSSION

A large number of post-translational modifications of proteins have been described that affect an equivalently extensive range of protein structures and functions. Prenylated proteins are a recently discovered class of post-translationally modified proteins. Prenylation has been shown to affect protein–lipid or protein–protein interactions; a possible role for protein prenylation in the control of cellular function has been suggested [23].

The results presented here demonstrate that FC analogues inhibit SRCE induced by agonist action or the depletion of intracellular Ca^{2+} stores by TG in human platelets by preventing the prenylation/methylation of proteins. Although in platelets two different groups of proteins with known signal transduction properties have been shown to undergo prenylation and methylation (small GTP-binding proteins and γ subunits of heterotrimeric G-proteins), we suggest that, as in other cells [12–15], the effects observed on SRCE were due entirely to the inactivation of small GTP-binding proteins by FC analogues. Previous studies have suggested that prenylation might be important for the interaction between $\beta\gamma$ complexes and the α subunit but that it is not required for $\beta\gamma$ assembly [50,51]. However, it has recently been demonstrated that the prenylation of γ subunits of heterotrimeric G-proteins has only a negligible effect on signal transduction processes, which can be correlated with a decrease in hydrophobicity of the $\beta\gamma$ subunits [52]. This conclusion is strongly supported by the fact that prenylation has no effect on the ability of $\beta\gamma$ complex to interact with α subunits or on their ability to adhere to membranes or activate phospholipases or phosphoinositide 3-kinase [52]. In agreement with this, our results show that the treatment of platelets with FC analogues had no effect on thrombin-evoked Ca^{2+} release from intracellular stores, suggesting that FC analogues do not interfere with the production of InsP_3 by modulating the activity of heterotrimeric G-proteins and subsequently phospholipase C.

FC analogues have been shown to prevent the post-translational processing and membrane translocation of small GTP-binding proteins, and thus to inhibit their activation [28,29,53]; they have been widely used to study the involvement of small GTP-binding proteins in several signal transduction pathways, for example the involvement of Ras in the responses to chemotactic agonists [53] and β_2 -integrin-induced signalling and cytoskeletal rearrangements in neutrophils [33], or the mediation by Rho of the induction of expression of the gene for cyclin E [54]. Small GTP-binding proteins, such as Rab-like proteins in mouse lacrimal acinar cells [13] or Rac in HeLa cells [14] and HEK-293 cells [15], have been suggested to be involved in store-depletion-induced Ca^{2+} entry.

The finding that FTA (an inhibitor of methylation of farnesylated proteins) selectively inhibited agonist-induced Ca^{2+} entry without having any effect on the release of Ca^{2+} from internal stores was confirmed with the use of TG, a cell-permeable compound that releases Ca^{2+} from the intracellular stores by inhibiting the Ca^{2+} -ATPase without a change in the level of inositol phosphates [55]. Our results showing that the inhibitory role of FTA in TG-induced Ca^{2+} entry was dependent on concentration are in agreement with the observation of the ability of FTA to inhibit Ca^{2+} entry in HEK cells [15]. The inhibitory effect of FTA was confirmed by using AFC, an FC

analogue that is an inhibitor, as well as a substrate, of farnesyl methyltransferase. These results have both similarities to and differences from previous studies. In HEK cells, AFC significantly inhibited the Ca^{2+} entry stimulated by TG at $50\ \mu\text{M}$ [15]. In contrast, our results are different from those of Huzoor-Akbar et al. [56], who concluded that AFC inhibits agonist-evoked Ca^{2+} mobilization without having any effect on ionomycin-induced Ca^{2+} entry. A possible explanation could be the different concentrations of the inhibitor used (as shown in Figures 2 and 3, the effects of FC analogues are clearly dependent on concentration). In addition we show that treatment with FTA inhibited depletion-evoked Mn^{2+} entry, confirming that FTA inhibits store-regulated bivalent-cation entry.

Experiments with AGGC show that, as well as farnesylated proteins, geranylgeranylated small GTP-binding proteins are involved in SRCE when stimulated by an agonist or, in agreement with previous studies [15], by the depletion of intracellular Ca^{2+} stores with TG. In addition, we show here that treatment with AGGC has no effect on the thrombin-evoked release of Ca^{2+} from intracellular stores in human platelets. In contrast, other authors have reported that geranylgeranylated proteins are not involved in β_2 -integrin-induced Ca^{2+} entry in neutrophils, although they are involved in actin filament reorganization in these cells [33].

The inhibition of Ca^{2+} entry by FC analogues is not due to non-specific effects as chelators of Ca^{2+} or as Ca^{2+} entry blockers, as demonstrated by the lack of effect of AGC, an inactive control of FC analogues [33], and the latency in onset of inhibition of TG-induced SRCE evoked by FTA in comparison with instantaneous inhibition evoked by lanthanum added after Ca^{2+} . In addition, the effect of FC analogues is not likely to be explained by inhibition of tyrosine kinase activity as indicated by the lack of effect of FTA, at the highest concentration used in this study, on tyrosine phosphorylation stimulated by the depletion of intracellular Ca^{2+} stores. Because the FC analogues used are inhibitors of specific farnesyl or geranylgeranyl methyltransferases, we examined whether the effect on Ca^{2+} entry was due to the inhibition of small GTP-binding proteins rather than an inhibition of methyltransferase activity itself. The results obtained with the mycotoxin patulin, an inhibitor of protein prenylation that is inert with respect to methyltransferases [42], demonstrated that, in agreement with previous studies [15], the effect of FC analogues is not likely to be explained by the inhibition of methyltransferase activity.

Prenylation and mainly methylation are essential for the association of Ras proteins with membranes [45]. We therefore investigated the effect of FTA on the association of Ras with membranes. Our results, in agreement with others [45,57], show that FTA impaired the membrane association of Ras stimulated by TG. Because methylation is the only reversible step of prenylation/methylation [24], FTA could exert its effect by inhibiting this process, thereby inhibiting the association of Ras proteins with membranes, which has been reported to be an important process for Ras activation [45]. Our results agree with previous studies reporting that methylation is a key step for the association of prenylated proteins with membranes through a hydrophobic mechanism [24]. Analysis of the farnesylated intermediate of Ras for its ability to associate with membranes showed that less than 20% of that intermediate associates with the membrane; in contrast, after methylation the association was approx. 80% [57].

Consistent with results obtained using FC analogues were those obtained with *C. botulinum* C3 exoenzyme, which inactivates Rho A by ADP-ribosylation, that supported the involvement of small GTP-binding proteins in SRCE in human platelets. Our results provide the first evidence for the involvement of small

GTP-binding proteins in SRCE in platelets, a mechanism proposed for several other cell types. We note that in RBL cells, a more recent report has suggested that GTP-binding proteins are not necessary for the activation of I_{CRAC} [58]. The discrepancies between these studies are unclear.

It remains to be elucidated how small GTP-binding proteins might act to mediate SRCE. Many cell functions, including maintenance of morphology, aggregation, motility, smooth-muscle contraction and membrane ruffling, are regulated by small GTP-binding proteins of the Ras superfamily through the dynamic reorganization of the actin cytoskeleton [16,17,30,31]. To determine whether the platelet cytoskeleton has a role in SRCE in platelets, we used an inhibitor of actin polymerization, cytochalasin D, a fungal metabolite that blocks the formation of actin microfilaments by preventing monomer addition at the growing end of the polymer [59]. Cells treated with cytochalasin D retained their ability to respond to Ca^{2+} -mobilizing agents such as TG, which indicates that this treatment did not affect the ability of platelets to store Ca^{2+} in intracellular compartments. Our results demonstrate clearly that cytochalasin D decreases Ca^{2+} entry after store depletion in human platelets. This is in agreement with recent reports describing the effect of actin cytoskeleton disruption in vascular endothelial cells [60]. However, Pedrosa-Ribeiro et al. [61] have reported essentially the opposite results in NIH 3T3 cells. The reason for these discrepant results is not clear. It is possible that in NIH 3T3 cells SRCE does not depend on a precise structural relationship between intracellular stores and plasma membrane Ca^{2+} channels. The role of the cytoskeleton in store-operated Ca^{2+} entry was shown to be selective for actin filaments because colchicine, an agent that disrupts microtubules, had no effect on Ca^{2+} influx.

The effect of cytochalasin D was confirmed by measurement of actin filament content in the cells, showing that cytochalasin D inhibited TG-evoked actin filament polymerization without having any effect on unstimulated platelets. These observations are in agreement with previous results [47] and confirm that if 'treadmilling' (the net polymerization at one end of the filament and depolymerization at the other) occurs in resting platelets it must occur very slowly. Inhibition of actin polymerization by cytochalasin D was observed to be dependent on the duration of incubation of the cells with the agent, in agreement with previous studies [48,60]. A similar effect was observed for the inhibition in Ca^{2+} entry, which is in agreement with Holda and Blatter [60]. However, complete inhibition of actin polymerization by cytochalasin D after 40 min of preincubation decreased Ca^{2+} entry by only 50%. These results suggest that SRCE is only partly dependent on actin filament polymerization in human platelets.

Because the activation of small GTP-binding proteins is required for actin polymerization [16,17,30,31,38], we investigated the effects of the FC analogues FTA and AGGC on actin filament formation in platelets. Our results show that both FTA and AGGC clearly inhibited actin polymerization stimulated by TG. In addition, FTA and AGGC also decreased thrombin-evoked actin filament formation. To our knowledge, this is the first demonstration of the inhibitory effect of FC analogues on actin polymerization. However, the inhibitory effect of FTA and AGGC on Ca^{2+} entry cannot be completely explained by the inhibition of actin polymerization because treatment with cytochalasin D that caused the same extent of inhibition of actin filament formation (20 min preincubation) had a much smaller effect than FTA or AGGC on Ca^{2+} entry. These results suggest the involvement of both farnesylated and geranylgeranylated small GTP-binding proteins in SRCE in human platelets that is mediated by mechanisms that are both dependent on and independent of actin cytoskeleton.

Our observations shed new light on the mechanisms involved in SRCE in human platelets. The results presented are consistent with the notion that the signal for Ca^{2+} entry after the depletion of intracellular stores involves a GTP-dependent step. Although speculative, different models could be proposed to explain how small GTP-binding proteins might be involved in SRCE by actin cytoskeleton-dependent or -independent mechanisms. (1) GTP-binding proteins might act as messengers to gate the Ca^{2+} channel directly or through the activation of an unidentified diffusible messenger, as suggested previously [12,13]. (2) On the basis of the role of small GTP-binding proteins in vesicular trafficking [62] and the observation that a number of channels and carrier proteins undergo regulation by vesicular trafficking [63], the activation of small GTP-binding proteins could mediate the transport of vesicles that carry Ca^{2+} channels to be inserted in the plasma membrane. Such a model has been proposed for rat megakaryocytes [4]. In contrast, the physical trafficking of endoplasmic reticulum towards the plasma membrane is another mechanism to be considered [3]. Recently, Rho D has been proposed as a candidate for a molecule that co-ordinates vesicle transport with the rearrangement of the actin cytoskeleton [64]. (3) The control of actin reorganization by Ras proteins [16,17,30,31] could participate in the maintenance of a cytoskeletal structure supporting a direct coupling between the intracellular stores and the plasma membrane. InsP_3 receptors located in the plasma membrane have been shown to be shifted by treatment with cytochalasin B, suggesting that the InsP_3 -sensitive organelle might be linked to the plasma membrane through the actin microfilaments [65]. Therefore the small GTP-binding proteins could have a key regulatory role in a model based on a combination of indirect and direct coupling between the stores and plasma membrane in SRCE in human platelets.

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REFERENCES

- Rink, T. J. and Sage, S. O. (1990) *Annu. Rev. Physiol.* **52**, 431–439
- Putney, Jr., J. W. (1990) *Cell Calcium* **11**, 611–624
- Parekh, A. B. and Penner, R. (1997) *Physiol. Rev.* **77**, 901–930
- Somasundaram, B., Norman, J. C. and Mahaut-Smith, M. P. (1995) *Biochem. J.* **309**, 725–729
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Pandol, S. J. and Schoeffield-Payne, M. S. (1990) *J. Biol. Chem.* **265**, 12846–12853
- Álvarez, J., Montero, M. and García-Sancho, J. (1991) *Biochem. J.* **274**, 193–197
- Vostal, J. G., Jackson, W. L. and Shulman, N. R. (1991) *J. Biol. Chem.* **266**, 16911–16916
- Sargeant, P., Farndale, R. W. and Sage, S. O. (1993) *J. Biol. Chem.* **268**, 18151–18156
- Randriampita, C. and Tsien, R. Y. (1993) *Nature (London)* **364**, 809–814
- Gailly, P., Hermans, E. and Gillis, J. M. (1996) *J. Physiol. (London)* **491**, 635–646
- Fasolato, C., Hoth, M. and Penner, R. (1993) *J. Biol. Chem.* **268**, 20737–20740
- Bird, G. S. and Putney, Jr., J. W. (1993) *J. Biol. Chem.* **268**, 21486–21488
- Peppelenbosch, M. P., Tertoolen, L. G. J., de Vries-Smits, A. M. M., Qiu, R.-G., M'Rabet, L., Symons, M. H., de Laat, S. W. and Bos, J. L. (1996) *J. Biol. Chem.* **271**, 7883–7886
- Xu, X., Gilbert, B. A., Rando, R. R., Chen, L. and Tashjian, Jr., A. H. (1996) *Mol. Pharmacol.* **50**, 1495–1501
- Ridley, A. J. and Hall, A. (1994) *EMBO J.* **13**, 2600–2610
- Nobes, C. D. and Hall, A. (1995) *Cell* **81**, 53–62
- Rosado, J. A., Salido, G. M., Jensen, R. T. and García, L. J. (1998) *Biochim. Biophys. Acta* **1404**, 412–426
- Fischer, T. H., Gatliff, M. N., McCormick, F., Duffy, C. M. and White, II, G. C. (1994) *J. Biol. Chem.* **269**, 17257–17261
- Shock, D. D., He, K., Wencel-Drake, J. D. and Parise, L. V. (1997) *Biochem. J.* **321**, 525–530
- Wolthuis, R. M., Franke, B., van Triest, M., Bauer, B., Cool, R. H., Camonis, J. H., Akkerman, J. W. and Bos, J. L. (1998) *Moll. Cell. Biol.* **18**, 2486–2491
- Ando, S., Kaibuchi, K., Sasaki, T., Hiraoka, K., Nishiyama, T., Mizuno, T., Asada, M., Nunoi, H., Matsuda, I., Matsuura, Y. et al. (1992) *J. Biol. Chem.* **267**, 25709–25713
- Sinensky, M. and Lutz, R. J. (1992) *Bioessays* **14**, 25–31
- Zhang, F. L. and Casey, P. J. (1996) *Annu. Rev. Biochem.* **65**, 241–269
- Farrell, F. X., Yamamoto, K. and Lapetina, E. G. (1993) *Biochem. J.* **289**, 349–355
- Graham, T. E., Pfeiffer, J. R., Lee, R. J., Kusewitt, D. F., Martinez, A. M., Foutz, T., Wilson, B. S. and Oliver, J. M. (1998) *J. Immunol.* **161**, 6733–6744
- Hori, Y., Kikuchi, A., Isomura, M., Katayama, M., Miura, Y., Fujioka, H., Kaibuchi, K. and Takai, Y. (1991) *Oncogene* **6**, 515–522
- Hancock, J. F., Magee, A. I., Childs, J. E. and Marshall, C. J. (1989) *Cell* **57**, 1167–1177
- Shafer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S.-H. and Rine, J. (1989) *Science* **245**, 379–385
- Olson, M. F., Ashworth, A. and Hall, A. (1995) *Science* **269**, 1270–1272
- Symons, M. (1996) *Trends Biochem. Sci.* **21**, 178–181
- Tan, E. W., Pérez-Sala, D., Cañada, F. J. and Rando, R. R. (1991) *J. Biol. Chem.* **266**, 10719–10722
- Molony, L., Ng-Sikorski, J., Hellberg, C. and Andersson, T. (1996) *Biochem. Biophys. Res. Commun.* **223**, 612–617
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Sage, S. O., Merritt, J. E., Hallam, T. J. and Rink, T. J. (1989) *Biochem. J.* **258**, 923–926
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C. and Fisher, G. J. (1992) *J. Biol. Chem.* **267**, 15585–15590
- Hartwig, J. H., Kung, S., Kovacsos, T., Janmey, P. A., Cantley, L. C., Stossel, T. P. and Toker, A. (1996) *J. Biol. Chem.* **271**, 32986–32993
- Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B. and Sugimura, T. (1989) *Agents Actions* **27**, 17–23
- Ma, Y.-T., Shi, Y.-Q., Lim, Y. H., McGrail, S. H., Ware, J. A. and Rando, R. R. (1994) *Biochemistry* **33**, 5414–5420
- Pérez-Sala, D., Gilbert, B. A., Rando, R. R. and Cañada, F. J. (1998) *FEBS Lett.* **426**, 3319–3324
- Miura, S., Hasimu, K. and Endo, A. (1993) *FEBS Lett.* **318**, 88–90
- Mahaut-Smith, M. P., Rink, T. J., Collins, S. C. and Sage, S. O. (1990) *J. Physiol. (London)* **428**, 723–735
- Sargeant, P., Farndale, R. W. and Sage, S. O. (1994) *Exp. Physiol.* **79**, 269–272
- Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y. and Kloog, Y. (1995) *J. Biol. Chem.* **270**, 22263–22270
- Morii, N., Teru-uchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narumiya, S. (1992) *J. Biol. Chem.* **267**, 20921–20926
- Fox, J. E. B. and Phillips, D. R. (1981) *Nature (London)* **292**, 650–652
- Tsakiridis, T., Vranic, M. and Klip, A. (1994) *J. Biol. Chem.* **269**, 29934–29942
- Salmon, E. D., McKeel, M. and Hays, T. (1984) *J. Cell Biol.* **99**, 1066–1075
- Higgins, J. B. and Casey, P. J. (1994) *J. Biol. Chem.* **269**, 9067–9073
- Matsuda, T., Takao, T., Shimonishi, Y., Murata, M., Asano, T., Yoshizawa, T. and Fukada, Y. (1994) *J. Biol. Chem.* **269**, 30358–30363
- Parish, C. A., Smarcka, A. V. and Rando, R. R. (1996) *Biochemistry* **35**, 7499–7505
- Phillips, M. R., Pillinger, M. H., Staud, R., Volker, C., Rosenfeld, M. G., Weissmann, G. and Stock, J. B. (1993) *Science* **259**, 977–980
- Tanaka, T., Tatsuno, I., Noguchi, Y., Uchida, D., Oeda, T., Narumiya, S., Yasuda, T., Higashi, H., Kitagawa, M., Nakayama, K. et al. (1998) *J. Biol. Chem.* **273**, 26772–26778
- Mason, M. J., Garcia-Rodriguez, C. and Grinstein, S. (1991) *J. Biol. Chem.* **266**, 20856–20862
- Huzoor-Akbar, Wang, W., Kornhauser, R., Volker, C. and Stock, J. B. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 868–872
- Hancock, J. F., Cadwallader, K. and Marshall, C. J. (1991) *EMBO J.* **10**, 641–646
- Fierro, L. and Parekh, A. B. (1999) *Pflügers Arch.* **437**, 547–552
- Wang, W. H., Cassola, A. and Giebisch, G. (1994) *Am. J. Physiol.* **267**, F592–F598
- Holda, J. R. and Blatter, L. A. (1997) *FEBS Lett.* **403**, 191–196
- Pedrosa-Ribeiro, C. M., Reece, J. and Putney, Jr., J. W. (1997) *J. Biol. Chem.* **272**, 26555–26561
- Rothman, J. E. and Orci, L. (1992) *Nature (London)* **355**, 409–415
- Bradbury, N. A. and Bridges, R. J. (1994) *Am. J. Physiol.* **267**, C1–C24
- Murphy, C., Saffrich, R., Grummt, M., Gournier, H., Rybin, V., Rubino, M., Auvinen, P., Lütcke, A., Parton, R. G. and Zerial, M. (1996) *Nature (London)* **384**, 427–432
- Rossier, M. F., Bird, G. St. J. and Putney, Jr., J. W. (1991) *Biochem. J.* **274**, 643–650