# Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets

Juan A. ROSADO and Stewart O. SAGE[1]

Department of Physiology, University of Cambridge, Downing St., Cambridge CB2 3EG, U.K.

Physical coupling between inositol 1,4,5-trisphosphate ($IP_3$) receptors and transient receptor potential (Trp) channels has been demonstrated in both transfected and normal cells as a candidate mechanism for the activation of store-mediated $Ca^{2+}$ entry (SMCE). We have investigated the properties of the coupling between the type II $IP_3$ receptor and naturally expressed human Trp1 (hTrp1) in human platelets. Treatment with xestospongin C, an inhibitor of $IP_3$ receptor function, abolished SMCE and coupling between the $IP_3$ receptor and hTrp1. The coupling was activated by depletion of the intracellular $Ca^{2+}$ stores, and was reversed by refilling of the stores. We have also examined the role of actin filaments in the activation and maintenance of the coupling. Stabilization of the cortical actin network with jasplakinolide prevented the coupling, indicating that, as with secretion, the actin filaments at the cell periphery act as a negative clamp which prevents constitutive coupling. In addition, the actin cytoskeleton plays a positive role, since disruption of the actin network inhibited the coupling when the $Ca^{2+}$ stores were depleted. These results provide strong evidence for the activation of SMCE by a secretion-like coupling mechanism involving a reversible association between $IP_3$ receptors and hTrp1 in normal human cells.

Key words: actin cytoskeleton, $Ca^{2+}$ influx, cytochalasin D, Trp channels.

## INTRODUCTION

Store-mediated $Ca^{2+}$ entry (SMCE), a mechanism regulated by the filling state of the intracellular $Ca^{2+}$ stores, is a major pathway for $Ca^{2+}$ influx in non-excitable cells. Several models have been proposed to account for the communication between the internal $Ca^{2+}$ stores and the plasma membrane. 'Conformational coupling' hypotheses propose physical coupling between elements in the endoplasmic reticulum and the plasma membrane [1], whereas 'diffusible messenger' hypotheses propose the release of a small molecule from the $Ca^{2+}$ stores that opens $Ca^{2+}$ channels in the plasma membrane, or the involvement of other diffusible elements (for a review, see [2]). Recently, a modified physical coupling model has been proposed to operate in several cell types. This secretion-like coupling model is based on the trafficking and coupling of portions of the endoplasmic reticulum with the plasma membrane. In this model, the actin cytoskeleton plays a key role [3–5].

Since the discovery of mammalian homologues of the *Drosophila* transient receptor potential (Trp) channels, these proteins have been proposed as candidates for the conduction of SMCE [6–8]. Evidence for Trp proteins mediating SMCE is provided by antisense studies, which show that interference with the expression of *trp* sequences affects the activation of SMCE [6]. In addition, the functional expression of Trp proteins enhances SMCE in several mammalian cells, including COS cells [6] and human salivary gland cells [8].

Several studies have reported coupling between the inositol 1,4,5-trisphosphate ($IP_3$) receptor ($IP_3R$) type III and Trp1, Trp3 and Trp6 in transfected cells [9–11], an interaction that has been shown to functionally regulate SMCE in patch–clamp studies in HEK-293 cells [12]. Recently we demonstrated a physical coupling between the $IP_3R$ type II and naturally expressed Trp1 in human platelets, which is activated *de novo* by depletion of the intracellular $Ca^{2+}$ stores [13]. In the present study we sought to expand our understanding of the characteristics of this coupling and its putative role in the activation and maintenance of SMCE.

## MATERIALS AND METHODS

### Materials

Fura 2 acetoxymethyl ester was from Texas Fluorescence (Austin, TX, U.S.A.). Apyrase (grade V), aspirin, thrombin, BSA, FITC-labelled phalloidin, Nonidet P40 and thapsigargin (TG) were from Sigma (Poole, Dorset, U.K.). Ionomycin, cytochalasin D (Cyt D) and xestospongin C (Xest C) were from Calbiochem (Nottingham, U.K.). Jasplakinolide (JP) was from Molecular Probes (Leiden, The Netherlands). Anti-hTrp1 polyclonal antibody (where hTrp1 is human Trp1) was from Alomone Laboratories (Jerusalem, Israel). Anti-($IP_3R$ type II) polyclonal antibody (C-20) and horseradish peroxidase-conjugated donkey anti-(goat IgG) antibody were from Santa Cruz (Santa Cruz, CA, U.S.A.). All other reagents were of analytical grade.

Abbreviations used: SMCE, store-mediated calcium entry; Trp, transient receptor potential; hTrp, human Trp; $IP_3$, inositol 1,4,5-trisphosphate; $IP_3R$, inositol 1,4,5-trisphosphate receptor; TG, thapsigargin; Cyt D, cytochalasin D; Xest C, xestospongin C; JP, jasplakinolide; HBS, Hepes-buffered saline; $[Ca^{2+}]_i$, intracellular free calcium concentration; TBST, Tris-buffered saline/0.1% Tween 20.

[1] To whom correspondence should be addressed (e-mail sos10@cam.ac.uk).

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### Platelet preparation

Fura 2-loaded platelets were prepared as described previously [14]. Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing 85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 ***g***, and aspirin (100 $\mu$M) and apyrase (40 $\mu$g/ml) were added. Platelet-rich plasma was incubated at 37 °C with 2 $\mu$M fura 2 acetoxymethyl ester for 45 min. Cells were then collected by centrifugation at 350 ***g*** for 20 min and resuspended in Hepes-buffered saline (HBS), pH 7.45, containing 145 mM NaCl, 10 mM Hepes, 10 mM D-glucose, 5 mM KCl and 1 mM $MgSO_4$, and supplemented with 0.1 % (w/v) BSA and 40 $\mu$g/ml apyrase.

The depletion and refilling of the intracellular $Ca^{2+}$ stores was performed as described by Jenner et al. [15]. Briefly, fura 2-loaded platelets were incubated with TG (1 $\mu$M) and ionomycin (50 nM) in the presence of 100 $\mu$M EGTA for 10 min at 37 °C. Cells were then washed twice with HBS (composition as above) to remove TG and ionomycin, and resuspended in nominally $Ca^{2+}$-free HBS or HBS containing 1 mM $CaCl_2$, and incubated for a further 20 min. The platelets were finally suspended in a $Ca^{2+}$-free HBS. The control consisted of platelets treated with vehicle instead of TG + ionomycin, but otherwise treated the same as the $Ca^{2+}$-depleted platelets.

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

Fluorescence was recorded from 1.5 ml aliquots of a magnetically stirred platelet suspension ($10^8$ cells/ml) at 37 °C using a Cairn Research Spectrophotometer (Cairn Research Ltd, Sittingbourne, Kent, U.K.) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura 2 340 nm/380 nm fluorescence ratio and calibrated using the method of Grynkiewicz et al. [16].

### Confocal microscopy

Samples of platelet suspension (200 $\mu$l) were transferred to 200 $\mu$l 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 $\mu$M) in PBS supplemented with 0.5 % (w/v) BSA. Cells were collected by centrifugation in an MSE Micro-Centaur Centrifuge (MSE Scientific Instruments) for 60 s at 3000 ***g*** and resuspended in PBS. Platelets were visualized using a Leica TCS4D confocal microscope.

### Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described previously [13]. Briefly, 500 $\mu$l aliquots of platelet suspension ($2 \times 10^9$ cell/ml) were lysed with an equal volume of lysis buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2 % SDS, 2 % sodium deoxycholate, 2 % Triton X-100, 2 mM $Na_3VO_4$, 2 mM PMSF, 100 $\mu$g/ml leupeptin and 10 mM benzamidine. Aliquots of platelet lysates (1 ml) were immunoprecipitated by incubation with 2 $\mu$g of anti-hTrp1 polyclonal antibody and 25 $\mu$l of Protein A–agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by SDS/8 %-PAGE, and separated proteins were transferred electrophoretically on to nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10 % (w/v) BSA in Tris-buffered saline/0.1 % Tween 20 (TBST) to block residual protein binding sites. Immunodetection of $IP_3R$ type II was achieved using the anti-$IP_3RII$ polyclonal antibody diluted 1:500 in TBST for 3 h. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated donkey anti-(goat IgG) antibody diluted 1:10000 in TBST, and then exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to preflashed photographic film. The density of bands on the film was measured using a scanning densitometer (Leica, Milton Keynes, U.K.).

## RESULTS

### Xest C blocks SMCE and coupling between $IP_3RII$ and hTrp1

Platelets have been shown to express three isoforms of the $IP_3R$: I, II and III. Isoform I is localized in the intracellular membranes, while isoform III has been found in the plasma membrane. In contrast, isoform II exhibits a dual distribution [17,18]. In an earlier study [13] we reported coupling between endogenously

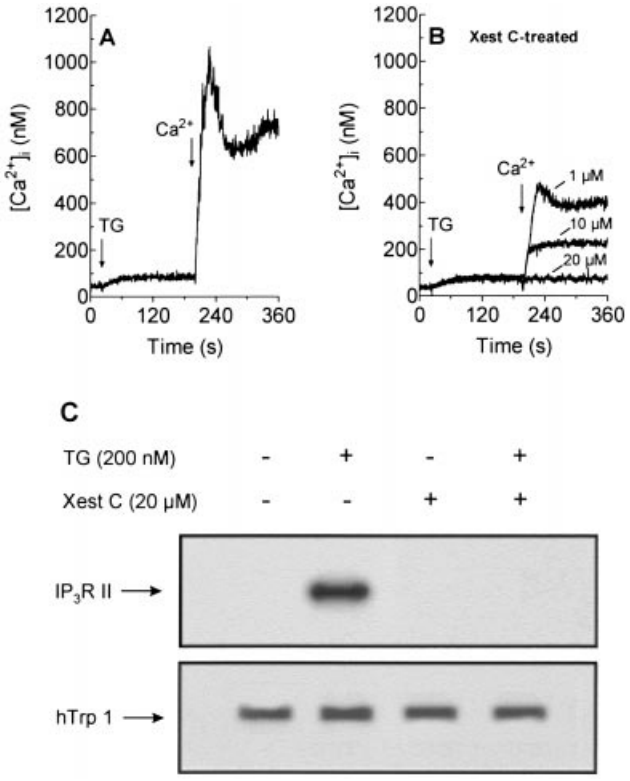


**Figure 1 Effects of Xest C on SMCE and coupling between $IP_3R$ type II and hTrp1**

(**A**, **B**) Fura 2-loaded human platelets were suspended in a $Ca^{2+}$-free medium and incubated for 30 min at 37 °C in the presence (**B**) or absence (**A**) of Xest C (1–20 $\mu$M). At the time of the experiment, 100 $\mu$M EGTA was added. Cells were then stimulated with TG (200 nM), followed by the addition of $CaCl_2$ (final concentration 300 $\mu$M) 3 min later to initiate $Ca^{2+}$ entry. (**C**) Platelets were incubated for 30 min in the presence or absence of 20 $\mu$M Xest C, as indicated. Cells were then stimulated or not with TG (200 nM). Samples were taken 5 s before and 3 min after the addition of TG, and lysed. Whole-cell lysates were immunoprecipitated with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-$IP_3RII$ polyclonal antibody (upper panel) or anti-hTrp1 antibody (lower panel), as described in the Materials and methods section. These results are representative of three to four independent experiments.

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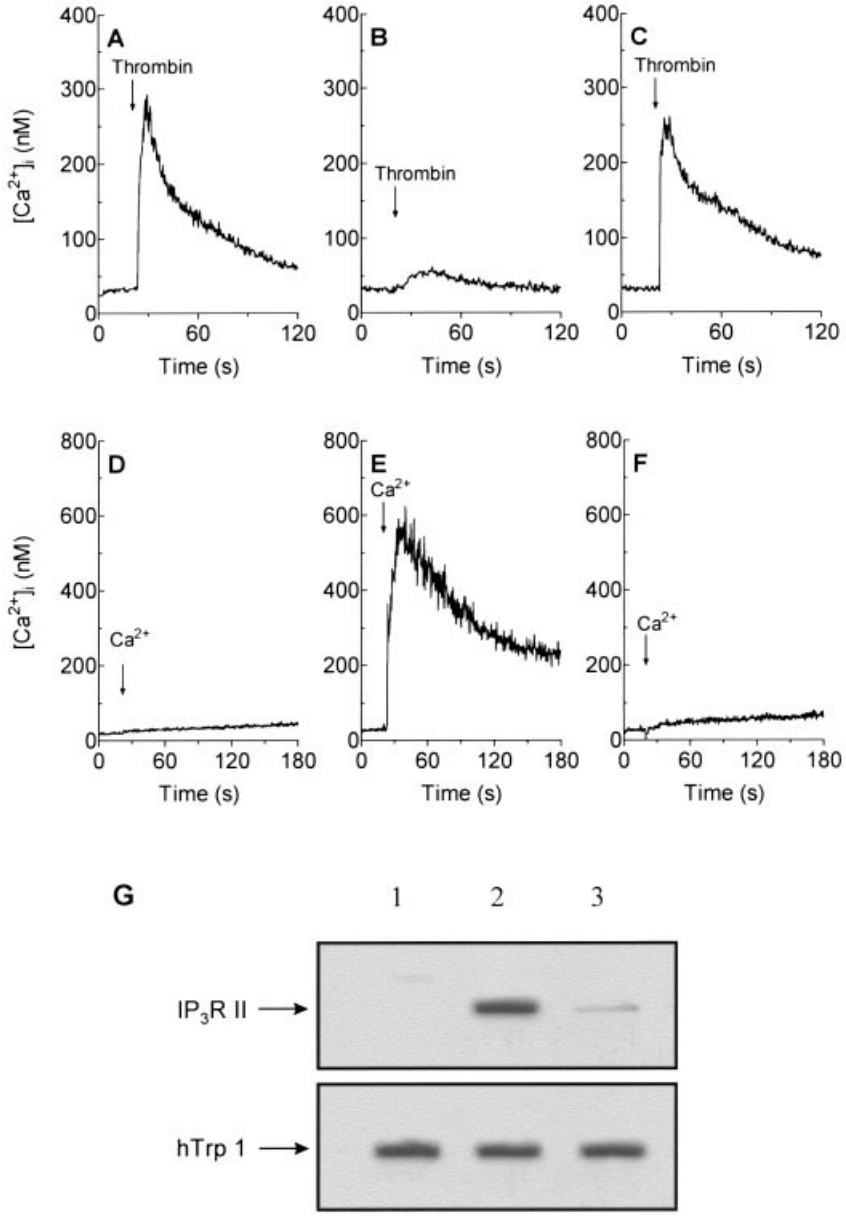


**Figure 2 Refilling of intracellular $Ca^{2+}$ stores reverses the coupling between $IP_3R$ type II and hTrp1**

(**A**)–(**F**) Fura 2-loaded human platelets were treated for 10 min with TG (1 $\mu$M) and ionomycin (50 nM) in a $Ca^{2+}$-free medium (100 $\mu$M EGTA was added), and incubated subsequently either with nominally $Ca^{2+}$-free HBS (**B** and **E**) or with HBS after the addition of 1 mM $CaCl_2$ (**C** and **F**). Traces in (**A**) and (**D**) show results from control platelets that had not been treated with TG and ionomycin, but were otherwise treated in the same way as the $Ca^{2+}$-depleted platelets. Cells were then suspended in a $Ca^{2+}$-free HBS and treated with thrombin (1 unit/ml) to release $Ca^{2+}$ from intracellular stores (**A**–**C**), or $Ca^{2+}$ (final concentration 300 $\mu$M) was added to initiate $Ca^{2+}$ entry (**D**–**F**). Elevations in $[Ca^{2+}]_i$ were monitored by using the 340/380 nm ratio, and traces were calibrated in terms of $[Ca^{2+}]_i$. (**G**) Human platelets were treated in a $Ca^{2+}$-free medium (100 $\mu$M EGTA added) for 10 min with 1 $\mu$M TG and 50 nM ionomycin (lanes 2 and 3) or vehicle (lane 1), and incubated subsequently either with nominally $Ca^{2+}$-free HBS (lane 2) or with HBS after the addition of 1 mM $CaCl_2$ (lanes 1 and 3). Whole-cell lysates were immunoprecipitated with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-$IP_3$RII polyclonal antibody (upper panel) or anti-hTrp1 antibody (lower panel), as described in the Materials and methods section. These results are representative of four separate experiments.

expressed hTrp1 and $IP_3R$ type II, but not types I or III, which was induced by depletion of the $Ca^{2+}$ stores. The conformational coupling model proposes that the $IP_3R$ is responsible for transmitting information from the endoplasmic reticulum to the plasma membrane [1]. According to this hypothesis, inhibition of the $IP_3R$ should result in the absence of coupling, and consequently of SMCE. To address this issue, we used Xest C, an inhibitor of $IP_3R$ function [19]. As reported previously [13], treatment of human platelets for 30 min with 20 $\mu$M Xest C abolished the thrombin-evoked release of $Ca^{2+}$ from the intracellular stores, confirming inhibition of $IP_3R$ function. In agreement with previous studies [13,20], incubation of human platelets for 30 min with Xest C decreased SMCE in a concentration-dependent manner (Figure 1B). Xest C significantly decreased SMCE by $52 \pm 7\%$, $76 \pm 8\%$ and $98 \pm 4\%$ at 1, 10 and 20 $\mu$M respectively, without having any effect on the TG-induced release of $Ca^{2+}$ from the stores ($P < 0.01$; $n = 3$–4) (Figure 1B).

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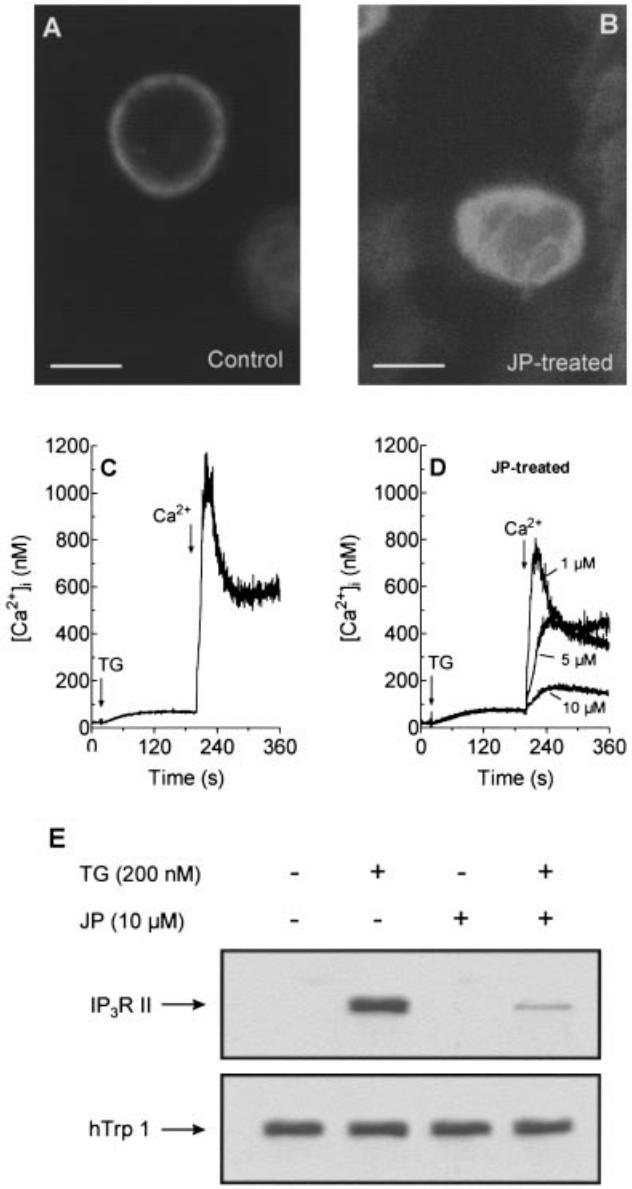


**Figure 3 Effects of JP on SMCE and on coupling between $IP_3R$ type II and hTrp1**

Human platelets were treated for 30 min at 37 °C in the absence (**A**, **C** and **E**) or presence of 10 $\mu$M JP (**B** and **E**) or various concentrations (1–10 $\mu$M) of JP (**D**), as indicated. (**A**, **B**) Confocal images of F-actin labelled with FITC-conjugated phalloidin in control (**A**) and JP-treated (**B**) human platelets. The bars represent 1 $\mu$m. (**C**, **D**) Fura 2-loaded platelets suspended in a $Ca^{2+}$-free HBS (100 $\mu$M EGTA was added) were then stimulated with TG (200 nM), and 3 min later $CaCl_2$ (final concentration 300 $\mu$M) was added to initiate $Ca^{2+}$ entry. (**E**) Platelets were suspended in a $Ca^{2+}$-free HBS (100 $\mu$M EGTA was added) and stimulated or not with TG (200 nM). Samples were taken 5 s before and 3 min after the addition of TG, and lysed. Whole-cell lysates were immunoprecipitated with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-$IP_3$RII polyclonal antibody (upper panel) or anti-hTrp1 antibody (lower panel), as described in the Materials and methods section. These results are representative of four independent experiments.

After immunoprecipitation with the anti-hTrp1 antibody, Western blotting revealed the presence of $IP_3$RII in samples from TG-treated, $Ca^{2+}$-store-depleted cells, but not in samples from resting cells (Figure 1C, lanes 1 and 2). As predicted, treatment, for 30 min with 20 $\mu$M Xest C completely blocked this coupling. These findings confirm that functional $IP_3$RII is required for its coupling with the hTrp1 protein, as well as for the activation of SMCE, in human platelets.

### Reversible coupling between $IP_3$RII and hTrp1

In order to investigate if the coupling between $IP_3$RII and hTrp1 is regulated directly by the filling state of the intracellular $Ca^{2+}$ stores, and thus reversed by refilling of the stores, we performed a series of experiments in which the $Ca^{2+}$ stores were depleted and then refilled. In a $Ca^{2+}$-free medium, the physiological agonist thrombin (1 unit/ml) evoked a rise in $[Ca^{2+}]_i$ in control platelets (which had only been treated with vehicles, but otherwise were subjected to all preparative procedures; Figure 2A), due to release of $Ca^{2+}$ from the stores. However, in cells where the $Ca^{2+}$ stores had been depleted by treatment for 10 min with TG (1 $\mu$M) and ionomycin (50 nM) and then incubated in a $Ca^{2+}$-free medium, the thrombin-evoked response was clearly reduced, confirming substantial depletion of the intracellular $Ca^{2+}$ stores (Figure 2B). Consistent with this, SMCE was substantially activated in these cells, as shown by the rise in $[Ca^{2+}]_i$ following addition of $CaCl_2$ (300 $\mu$M) to the platelet suspension (Figure 2E, cf. Figure 2D). When cells pretreated with TG and ionomycin were then incubated in medium containing 1 mM $CaCl_2$, the stores were significantly refilled, as shown by the greater release of $Ca^{2+}$ from the stores after treatment with thrombin (Figure 2C, cf. Figure 2B). The thrombin-induced $[Ca^{2+}]_i$ elevation in a $Ca^{2+}$-free medium was $99 \pm 13$ % of that observed in (completely untreated) control cells. As shown in Figure 2(F), in cells in which the $Ca^{2+}$ stores had been depleted and refilled, SMCE was reduced by 92 % compared with that in store-depleted cells (Figure 2E). As demonstrated previously [13], we found that $IP_3$RII and hTrp1 couple in response to depletion of the intracellular $Ca^{2+}$ stores (Figure 2G). Refilling of the $Ca^{2+}$ stores clearly reversed the extent of coupling (Figure 2G). These results strongly suggest that the coupling $IP_3$RII of hTrp1 is a dynamic process that is established by depletion of the $Ca^{2+}$ stores and reversed by their refilling.

### Existence of an actin clamp to prevent coupling between $IP_3$RII and hTrp1 and the activation of SMCE in resting platelets

SMCE has been proposed to be mediated by a secretion-like coupling model in several non-excitable cells, including platelets [3,5,20]. This mechanism is suggested to involve a physical and reversible interaction between the endoplasmic reticulum and the plasma membrane, which is based on trafficking, likely to involve a GTP-dependent step [14,21,22], of the endoplasmic reticulum towards the plasma membrane. In the secretion-like coupling model, the cortical actin filaments are proposed to play a negative regulatory role in $Ca^{2+}$ entry. To address how the cortical actin cytoskeleton regulates the coupling between the $IP_3$RII and hTrp1 we used JP, which induces the polymerization and re-organization of the actin filaments at the cell periphery. The structural changes induced by JP are shown in Figures 3(A) and 3(B). Consistent with this, we have reported that treatment of human platelets for 30 min with 10 $\mu$M JP enhanced platelet actin polymerization by 200 % [5]. Since it has been demonstrated that almost half of the actin in resting platelets is unpolymerized [23], our observations indicate that JP induces full actin polymerization, in agreement with studies by Patterson et al. [3]. After treatment of platelets for 30 min with 10 $\mu$M JP, the actin filaments became condensed into a tight layer under the plasma membrane (Figure 3B), which was much more dense than that

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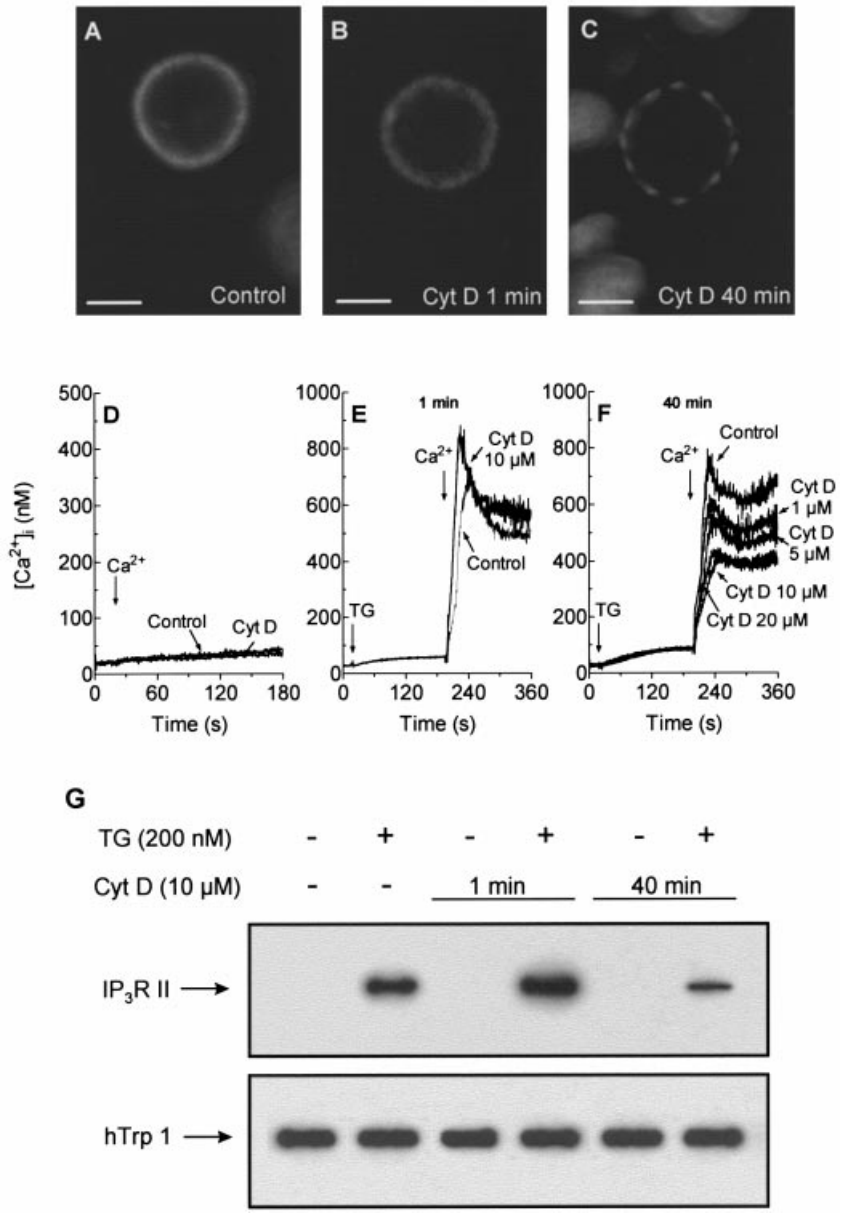


**Figure 4 Effects of Cyt D on SMCE and on coupling between $IP_3R$ type II and hTrp1**

(**A**–**C**) Confocal images of F-actin labelled with FITC-conjugated phalloidin in control platelets (**A**) or platelets treated with 10 $\mu$M Cyt D for 1 min (**B**) or 40 min (**C**). The bars represent 1 $\mu$m. (**D**–**F**) Fura 2-loaded human platelets were suspended in a $Ca^{2+}$-free medium and incubated for 1 min in the absence (**D**) or presence (**E**) of 10 $\mu$M Cyt D, or for 40 min with various concentrations (1–20 $\mu$M) of Cyt D (**F**), as indicated. At the time of the experiment, 100 $\mu$M EGTA was added. Cells either then had $CaCl_2$ added (final concentration 300 $\mu$M; **D**), or were stimulated with TG (200 nM) followed by the addition of $CaCl_2$ (final concentration 300 $\mu$M) 3 min later (**E** and **F**). (**G**) Platelets were incubated for 1 or 40 min in the presence or absence of 10 $\mu$M Cyt D, as indicated. Cells were then stimulated or not with TG (200 nM). Samples were taken 5 s before and 3 min after the addition of TG, and lysed. Whole-cell lysates were immunoprecipitated with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-$IP_3$RII polyclonal antibody (upper panel) or anti-hTrp1 antibody (lower panel), as described in the Materials and methods section. These results are representative of four independent experiments.

observed in control cells (Figure 3A). As shown in Figure 3, treatment of platelets for 30 min with JP reduced SMCE in a concentration-dependent manner, by $39 \pm 5\%$, $52 \pm 6\%$ and $90 \pm 4\%$ at concentrations of 1, 5 and 10 $\mu$M respectively (Figure 3D). JP (10 $\mu$M) substantially inhibited TG-induced coupling between $IP_3$RII and hTrp1. These findings suggest that the cortical actin network acts as a physical barrier to prevent coupling between the $IP_3$RII and hTrp1, as well as blocking the activation of SMCE, supporting a correlation between these processes.

### Biphasic effects of Cyt D on SMCE and on coupling between $IP_3$RII and hTrp1

Studies in secretory cells have demonstrated that the actin cytoskeleton has both inhibitory and facilitatory roles in regu-

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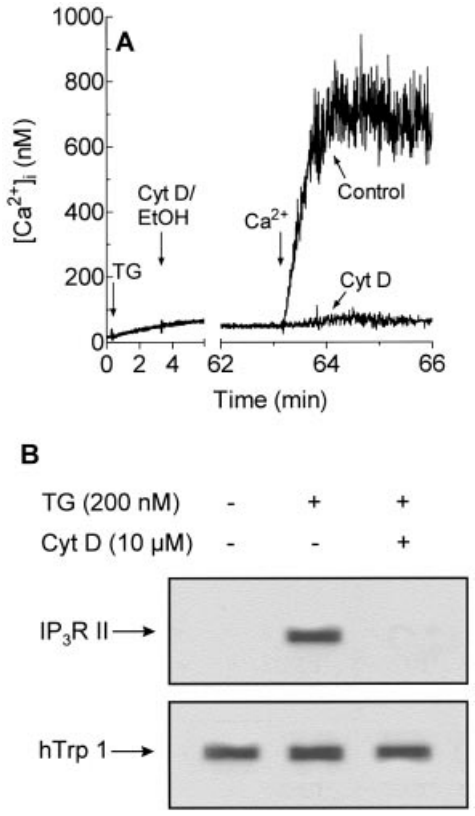


**Figure 5 Effects of Cyt D on the maintenance of SMCE and on the coupling between $IP_3R$ type II and hTrp1**

(**A**) Fura 2-loaded platelets suspended in a $Ca^{2+}$-free HBS were stimulated with TG (200 nM), and 3 min later 10 $\mu$M Cyt D or the vehicle (ethanol; EtOH) was added as indicated. $CaCl_2$ (final concentration 300 $\mu$M) was added to the medium 1 h after Cyt D or the vehicle, to initiate $Ca^{2+}$ entry. (**B**) Platelets were suspended in a $Ca^{2+}$-free HBS (100 $\mu$M EGTA was added). Cells were then treated with or without TG (200 nM), and 3 min later 10 $\mu$M Cyt D or the vehicle was added, as indicated, followed by incubation for 1 h and cell lysis. Whole-cell lysates were immunoprecipitated with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-$IP_3$RII polyclonal antibody (upper panel) or anti-hTrp1 antibody (lower panel), as described in the Materials and methods section. These results are representative of four independent experiments.

lated exocytosis [24,25]. To investigate further the role of the actin cytoskeleton in the coupling between $IP_3$RII and hTrp1, we examined the effects of Cyt D. We have reported recently that treatment of human platelets with 10 $\mu$M Cyt D inhibited actin polymerization in a time-dependent manner, showing a detectable effect within 1 min of treatment and complete inhibition after 40 min [14].

Treatment of platelets with 10 $\mu$M Cyt D for 1 min reduced the staining of the cortical actin filament network and evoked slight morphological changes in the distribution of the peripheral actin filaments (Figure 4B) compared with controls (Figure 4A). Further treatment with Cyt D (40 min) induced aggregation of the actin filaments into dense foci (Figure 4C), a phenomenon explained by the dissociation of the actin filaments from plasma membrane-binding proteins [26]. Although a brief (1 min) treatment with Cyt D potentiated both coupling between $IP_3$RII and hTrp1 and $Ca^{2+}$ entry when the $Ca^{2+}$ stores were depleted using TG (Figures 4E and 4G), no coupling or $Ca^{2+}$ entry were observed in unstimulated Cyt D-treated platelets (Figures 4D and 4G). Treatment with Cyt D for 40 min reduced SMCE in a concentration-dependent manner, with $21.9 \pm 5.7\%$, $31.2 \pm 6.7\%$, $50.3 \pm 4.0\%$ and $49.8 \pm 6.4\%$ inhibition at concentrations of 1, 5, 10 and 20 $\mu$M respectively ($P < 0.05$; $n = 4$) (Figure 4F). Consistent with the above, incubation of human platelets for 40 min with 10 $\mu$M Cyt D significantly reduced the coupling between the $IP_3$RII and hTrp1 ($P < 0.05$; $n = 4$) (Figure 4G). The inhibitory effect of long-term treatment with Cyt D was not due to non-specific cell damage, since TG (Figure 4F) and physiological agonists (results not shown) were still able to release $Ca^{2+}$ from the intracellular stores.

### Role of the actin cytoskeleton in the maintenance of the coupling between $IP_3$RII and hTrp1 and of SMCE

We have reported previously that the actin cytoskeleton is essential for the maintenance of SMCE, since treatment with Cyt D (10 $\mu$M) after the $Ca^{2+}$ stores had been depleted completely blocked $Ca^{2+}$ entry, an effect prevented by the inhibitor of actin depolymerization JP [5]. We have now studied the role of the actin cytoskeleton in the maintenance of the coupling between $IP_3$RII and hTrp1. Platelets were treated with TG (200 nM) in a $Ca^{2+}$-free medium, and 3 min later 10 $\mu$M Cyt D or the vehicle was added. At the time when Cyt D was added, coupling and $Ca^{2+}$ entry were already stimulated (see Figures 2 and 4). The addition of $Ca^{2+}$ after 1 h indicated considerable SMCE in control cells, whereas this process was completely inhibited in cells treated with Cyt D (Figure 5A). The TG-stimulated coupling between $IP_3$RII and hTrp1 was maintained for at least 1 h in control cells, but was completely reversed when the F-actin network was disrupted by the addition of Cyt D (Figure 5B). This is consistent with the hypothesis that support provided by the actin cytoskeleton is required to maintain SMCE [5], a role which may involve maintaining the close apposition of the endoplasmic reticulum and the plasma membrane, and hence coupling between proteins therein.

## DISCUSSION

In non-excitable cells, SMCE is a major pathway for $Ca^{2+}$ influx; however, the molecular basis of this mechanism is not completely understood, and two main questions still remain: to elucidate the mechanism of activation of this event and to identify the channel involved in $Ca^{2+}$ entry. Since the discovery of SMCE, different hypotheses (including direct 'conformational' and indirect coupling) have been proposed to account for its activation. Recently, a modification of the conformational coupling model has been proposed. This secretion-like coupling model, based on the trafficking and coupling of portions of the endoplasmic reticulum with the plasma membrane, has been suggested to operate in several cell types [3,5].

Several studies have proposed mammalian homologues of Trp proteins as candidates for the conduction of SMCE in several cell types [6–8,27]. In agreement with the conformational coupling hypothesis, physical interactions between Trp channels and $IP_3$Rs have been reported in several transfected cell lines [9,10,12,28,29]. Human platelets have been shown to contain mRNA for Trp1 and its splice variant Trp1A (D. Molin, E. den Dekker, G. Breikers, R. van Oerle, J. W. Akkerman and J. M. W. Heemskerk, personal communication), and we have confirmed the expression of hTrp1 in these cells [13]. In platelets, coupling between $IP_3$RII and Trp1 proteins occurs after depletion of the intracellular $Ca^{2+}$ stores, and not under resting conditions [13]. In the present study we provide new evidence indicating that the filling state of the intracellular $Ca^{2+}$ store regulates the coupling between these two proteins in human platelets. We have found that refilling of the internal stores reverses the coupling between

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$IP_3RII$ and Trp1 that is induced by store depletion in platelets. Therefore our results differ from those reported by others in cultured cell lines transfected with Trps [12,28], which show a constitutive coupling between Trp proteins and the $IP_3RIII$.

The conformational coupling model proposes that the $IP_3R$ transmits information to the $Ca^{2+}$ channel in the plasma membrane through a protein–protein interaction [1]. In agreement with this hypothesis, inhibition of $IP_3R$ function by Xest C abolished both SMCE and the coupling between $IP_3RII$ and Trp1 channels in human platelets, indicating that a functional $IP_3R$ is essential for the coupling, and hence the activation of SMCE.

We have also investigated the role of the actin cytoskeleton in facilitating or preventing the coupling between $IP_3RII$ and Trp1 proteins, and its influence on SMCE. Our findings indicate that the cortical actin filament network acts as a physical barrier to prevent constitutive coupling between the Trp1 and $IP_3RII$, as well as blocking the activation of SMCE, indicating a correlation between these processes. A similar effect was observed in secretory cells, where stabilization of the apical actin filaments using phalloidin prevented interaction of secretory vesicles with the plasma membrane [24].

Actin filaments also have a positive role in the secretory mechanism, since full inhibition of actin polymerization inhibited all phases of exocytosis, suggesting that this process cannot occur without a minimal actin structure [24,25]. Studies of the role of actin polymerization in the coupling between Trp1 and $IP_3RII$, and in the activation and maintenance of SMCE, are compatible with the idea that, as with secretion, a minimal remodelling of the actin filament network is required for the early steps in the activation of SMCE, which might include trafficking of portions of the endoplasmic reticulum towards the plasma membrane. However, we found that, although long treatments with Cyt D completely inhibited TG-induced actin polymerization [5], significant (if much reduced) SMCE and coupling between $IP_3RII$ and hTrp1 were still found. This may indicate that some portions of the endoplasmic reticulum are close enough to the plasma membrane to enable coupling when the stores are depleted, once areas of the cortical actin barrier have been eroded, and that this occurs even in the absence of driven, actin polymerization-mediated trafficking. Interestingly, no coupling between hTrp1 and $IP_3RII$ and no SMCE were observed after actin depolymerization in non-depleted platelets, indicating that these mechanisms are triggered by store depletion in these cells. These results demonstrate further a remarkable similarity between SMCE and secretion, since actin depolymerization does not elicit exocytosis, but potentiates agonist-stimulated responses [25]. In addition, these observations suggest that other signalling pathways may be involved in the activation of the coupling between Trp1 and $IP_3RII$, and also of SMCE. For example, we have reported previously that TG-induced Ras activation can mediate SMCE in an actin cytoskeleton-independent manner [14].

Consistent with our previous studies showing that support provided by the actin cytoskeleton is essential to maintain SMCE [5], we now report that a functional actin filament network is required for the maintenance of the coupling between hTrp1 and $IP_3RII$. We suggest that a dynamic cytoskeletal structure may be required to support the close interaction between the endoplasmic reticulum and the plasma membrane, which is essential for maintenance of the coupling between proteins therein.

The parallels between exocytosis and SMCE presented in this paper provide new evidence supporting the secretion-like coupling model as the hypothesis that best describes the activation of SMCE in human platelets. The remarkable correlation between the properties of the coupling between $IP_3RII$ and hTrp1 on the one hand, and the activation and maintenance of SMCE on the other, strongly suggests that coupling between $IP_3RII$ and hTrp1 underlies the activation and maintenance of SMCE in these cells.

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