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Dual role of tubulin-cytoskeleton in store-operated calcium entry in human platelets

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Abstract

Two mechanisms for store-operated Ca^{2+} entry (SOCE) regulated by two independent Ca^{2+} stores, the dense tubular system (DTS) and the acidic stores, have been described in platelets. We have previously suggested that coupling between the type II IP_3 receptor (IP_3RII) and hTRPC1, involving reorganization of the actin microfilaments, play an important role in SOCE. However, the involvement of the tubulin microtubules, located beneath the plasma membrane, remains unclear. Here we show that the microtubule disrupting agent colchicine reduced Ca^{2+} entry stimulated by low concentrations (0.1 U/mL) of thrombin, which activates SOCE mostly by depleting acidic Ca^{2+}-store. Consistently, colchicine reduced SOCE activated by 2,5 di-(tertbutyl)-1,4-hydroquinone (TBHQ), which selectively depletes the acidic Ca^{2+} stores. In contrast, colchicine enhanced SOCE mediated by depletion of the DTS, induced by high concentrations of thapsigargin (TG), which depletes both the acidic Ca^{2+} stores and the DTS, the major releasable Ca^{2+} store in platelets. These findings were confirmed by using Sr^{2+} as a surrogate for Ca^{2+} entry. Colchicine attenuated the coupling between IP_3RII and hTRPC1 stimulated by thrombin while it enhanced that evoked by TG. Paclitaxel, which induces microtubular stabilization and polymerization, exerted the opposite effects on thrombin- and TG-evoked SOCE and coupling between IP_3RII and hTRPC1 compared with colchicine. Neither colchicine nor paclitaxel altered the ability of platelets to extrude Ca^{2+}. These findings suggest that tubulin microtubules play a dual role in SOCE, acting as a barrier that prevents constitutive SOCE regulated by DTS, but also supporting SOCE mediated by the acidic Ca^{2+} stores.

Keywords: Human Platelets; SOCE; Tubulin-microtubule; Colchicine; Paclitaxel

1. Introduction

Store-operated Ca^{2+} entry (SOCE) is the main mechanism for Ca^{2+} entry in non-excitable cells, including human platelets [1–5]. We have previously suggested that SOCE in platelets may be modulated by a reversible interaction between the IP_3 receptors (IP_3Rs) located in the membrane of the stores and canonical transient potential receptors channel (TRPCs) in the plasma membrane in which reorganization of the actin cytoskeleton plays a role [4,7,8].

In human platelets, the tubulin cytoskeleton is organized in a core-coil structure located in the subplasmalemmal region maintaining the discoid shape of the resting platelets [9–12]. Electron and confocal microscopy have revealed that stimulation of platelets with agonists such as thrombin results in a rapid disappearance of tubulin-microtubules from the cytosol by disorganization of the core-coil structure, allowing shape change and emission of pseudopodia [10,11]. Disruption of the microtubular network using colchicine or nocodazole has been shown to have major effects on intracellular signaling in different cell types, including platelets [13,14], that can be reversed by using paclitaxel, an agent that prevents microtubule disassembly, restoring the platelet spreading process necessary for platelet aggregation [14].

Microtubule-associated proteins have been shown to be regulated by Ca^{2+} or Ca^{2+}-sensitive kinases like STOP, which is regulated by phosphorylation through the activity of the calmodulin kinase type II [15]. Other proteins involved in intracellular Ca^{2+} signaling, such as PLCγ 1 [16] or the

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neuronal calcium sensor caldendrin [17] associate with microtubules and modulate its structure. Treatment of bovine brain cells with SK&F96365, an inhibitor of Ca^{2+}-channels, has a direct depolymerizing effect on microtubules, which has been suggested as a possible cause of its effect on Ca^{2+} homeostasis [18]. These results suggest the existence of a tight correlation between Ca^{2+} signaling and the tubulin cytoskeleton; however, little is known about the involvement of tubulin microtubule reorganization in the activation of SOCE.

Human platelets posses two separate agonist-releasable Ca^{2+} stores differentiated by the distinct sensitivities of the SERCA isoforms located on each store to thapsigargin (TG) and 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ). The major store, the dense tubular system (DTS) expresses SERCA2b, which is inhibited by low concentrations of TG and is insensitive to TBHQ [19]. The second store has recently been identified as an acidic store [20] and expresses SERCA3, which shows a lower sensitivity to TG but is sensitive to TBHQ [19–22]. Both stores are sensitive to the physiological agonist thrombin [23], which activates three different receptors in human platelets: the protease-activated receptors PAR-1 and PAR-4 and the glycoprotein GPIb-IX-V [24]. Thrombin shows a high affinity for GPIb-IX-V and higher affinity for PAR-1 than for PAR-4, and thus it is believed that activation of human platelets by low doses of thrombin is predominantly mediated by GPIb-IX-V and PAR-1 [24,25]. Depletion of both Ca^{2+} stores results in the activation of two different pathways for SOCE [26].

Here we have investigated the possible role of the tubulin microtubules in SOCE mediated by depletion of both intracellular Ca^{2+} stores, the DTS and the acidic compartments, either using physiological agonists, such as thrombin, or by pharmacological depletion of the stores using TG in human platelets.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Texas Fluorescence (Austin Texas, U.S.A.). Apyrase (grade VII), aspirin, bovine serum albumin

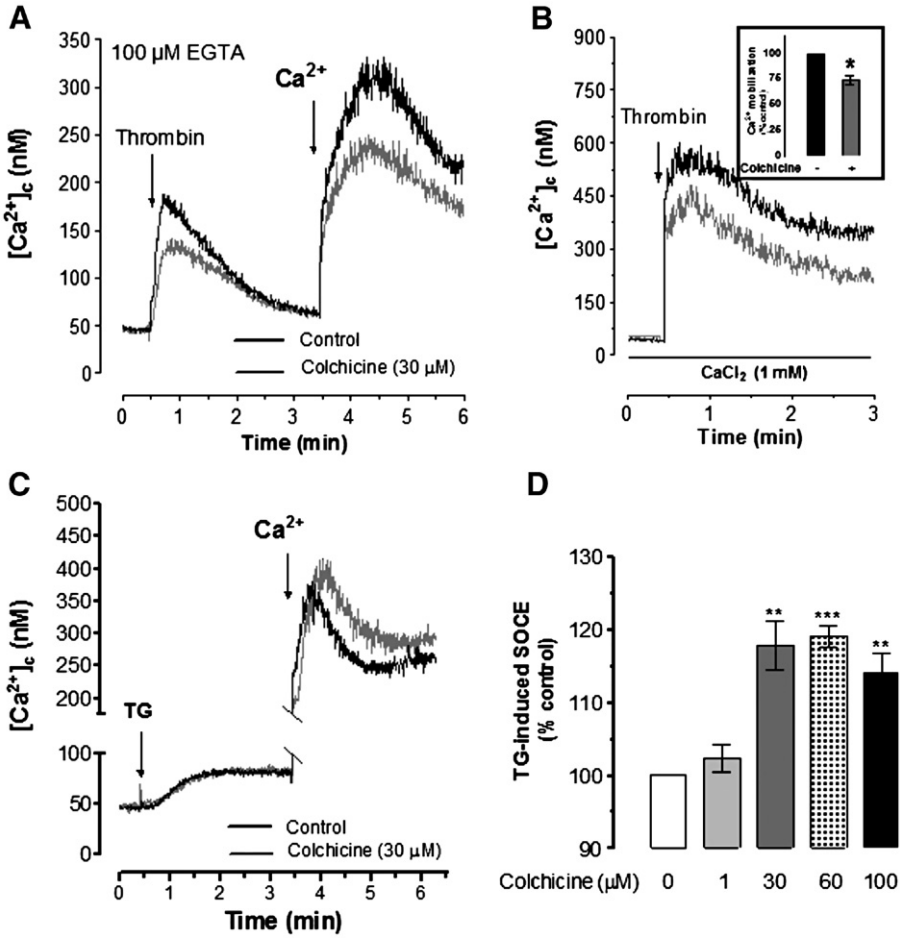

Fig. 1. Effect of colchicine on thrombin- and TG-induced Ca^{2+} entry in human platelets. A–C, Fura-2-loaded human platelets were preincubated for 30 min at 37 °C, with 30 μM colchicine or the vehicle (DMSO). Cells were then stimulated in a Ca^{2+} free medium (100 μM EGTA was added) with thrombin (0.1 U/mL; A) or TG (200 nM; C), followed by addition of 300 μM $CaCl_2$ (final concentration) to the medium three min later to initiate calcium entry, or were stimulated with thrombin in a medium containing 1 mM Ca^{2+} (B). Changes in cytosolic calcium concentration $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Inset Fig. 1B: histograms show Ca^{2+} mobilization induced by thrombin in the absence or presence of colchicine estimated as described in Experimental procedures. D, histograms show TG-induced SOCE in the presence of increasing concentrations of colchicine (1–100 μM) estimated as described under Experimental procedures (n=4–6). * $P<0.05$, ** $P<0.01$ *** $P<0.001$ compared to platelets not treated with colchicine.

(BSA), thrombin, thapsigargin (TG), 2,5 di-(tertbutyl)-1,4-hydroquinone (TBHQ), ionomycin (Iono), paclitaxel (PTx), colchicine, dithiothreitol (DTT), sodium dodecyl sulphate (SDS), ionic detergent tween 20, Na_3VO_4 and ECL reagents were from Sigma (Poole, Dorset, U.K.). Anti-IP_3R type II antibody and Horseradish peroxide-conjugated donkey anti-goat IgG were from Santa Cruz (Santa Cruz, CA, U.S.A.). Horseradish peroxide-conjugated donkey anti-rabbit IgG and protein A-agarose were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-hTRPC1 antibody was from Alomone Laboratories (Jerusalem, Israel). Wide range molecular weight markers were from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK). All other reagents were of analytical grade.

2.2. Platelet preparation

Fura-2-loaded human platelets were prepared as described previously [4] as approved by Local Ethical Committees. Briefly, blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 *g* and aspirin (100 μM) and apyrase (40 μg/mL) added. Platelet-rich plasma was incubated at 37 °C with 2 μM fura-2/AM for 45 min. Cells were then collected by centrifugation at 350 g for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.4 and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase.

2.3. Measurement of cytosolic free calcium concentration ($[Ca^{2+}]_c$)

Fluorescence was recorded from 2 mL aliquots of magnetically stirred cell suspensions (10^8 cells/mL) at 37 °C using a Fluorescence Cairn Research Spectrophotometer (Cairn Research, Faversham, UK) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. [6, 27]. Ca^{2+} entry was estimated using the integral of the rise in $[Ca^{2+}]_c$ for three min after its addition, taking a sample every second, and was expressed as the percentage of control [28]. When platelets were preincubated with various compounds, Ca^{2+} entry was corrected by subtraction of the change in 340/380 nm fluorescence (due to leakage of the indicator) that occurred when Ca^{2+} was added to vehicle-treated controls.

In a number of experiments, Sr^{2+} was used to monitor divalent cation entry. This was done to avoid complications arising from the stimulation of the platelet plasma membrane Ca^{2+}-ATPase (PMCA) by the drugs used, since Sr^{2+} is transported with lower affinity than Ca^{2+} by this Ca^{2+}-ATPase [28,29]. Sr^{2+} entry was measured in a Ca^{2+}-free HBS containing EGTA (100 μM) to minimize the effects of contaminating Ca^{2+}. Cytosolic Sr^{2+} was monitored using the fura-2 340/380 nm fluorescence ratio. Agonist evoked Sr^{2+} entry was calculated using the integral of the rise in the 340/380 nm fluorescence ratio for three min after addition of $SrCl_2$.

To compare the rate of decay of $[Ca^{2+}]_c$ to basal values, after treatment of platelets with TG + Iono we used the constant of the exponential decay described previously [4]. Traces were fitted to the equation $y = A(1 - e_1^{-KT})e_2^{-KT}$, where K_1 and K_2 are the constants of the exponential increase and decay respectively, T is time and A is the span.

2.4. Immunoprecipitation and Western blotting

250 μl aliquots of platelet suspension (4×10^8 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 phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, and 10 mM benzamidine. Aliquots (0.5 mL) were then immunoprecipitated by incubation 1.5 μg/mL of anti-IP_3R type II antibody and protein A-agarose overnight at 4 °C. Immunoprecipitates were resolved by 8% SDS-PAGE, and separated proteins were transferred onto nitrocellulose membranes. Immunodetection of IP_3R type II or hTRPC1 was achieved using the anti-IP_3RII antibody diluted 1:500 in TBST for 3 h or the anti-hTRPC1 antibody diluted 1: 200 in TBST for 2 h, respectively. To detect the primary antibody, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody or horseradish peroxidase-conjugated donkey anti-goat IgG antibody diluted 1:5000 in TBST supplemented with 10% BSA for 1 h. Membranes were then exposed to enhanced chemiluminescence (ECL) reagents for 5 min. Blots were then exposed to a photographic film. Membrane reprobing for protein loading control was performed by removing bound antibodies by incubation for 30 min at 50 °C with stripping buffer containing 100 mM 2-mercaptoethanol, 65.5 mM Tris, and 2% SDS, pH 6.7, followed by Western blotting with the appropriate antibodies as described above.

2.5. Statistical analysis

The results are expressed as the means ± S.E.M. Analysis of statistical significance was performed using Student's unpaired *t*-test. For multiple comparisons, one-way analysis of variance combined with the Dunnett tests was used. Only values with $P < 0.05$ were considered significant.

3. Results

3.1. Effect of colchicine on Ca^{2+} entry in platelets

Fura-2-loaded human platelets were used to assess the role of the tubulin microtubules in Ca^{2+} entry evoked by the physiological agonist thrombin or the pharmacological SERCA inhibitor TG, using colchicine, a tubulin microtubule disrupting agent [13]. As previously reported [30,31], in the absence of external Ca^{2+}, 0.1 U/mL thrombin evoked a transient increase in $[Ca^{2+}]_c$ due to depletion of the acidic Ca^{2+} stores and a concentration-dependent discharge of the dense tubular system (DTS; Fig. 1A). Subsequent addition of 300 μM Ca^{2+} to the platelet suspension induced a rapid increase in $[Ca^{2+}]_c$, indicative of Ca^{2+} entry. Ca^{2+} influx induced by thrombin at 0.1 U/mL must be attributed to store depletion since the ability

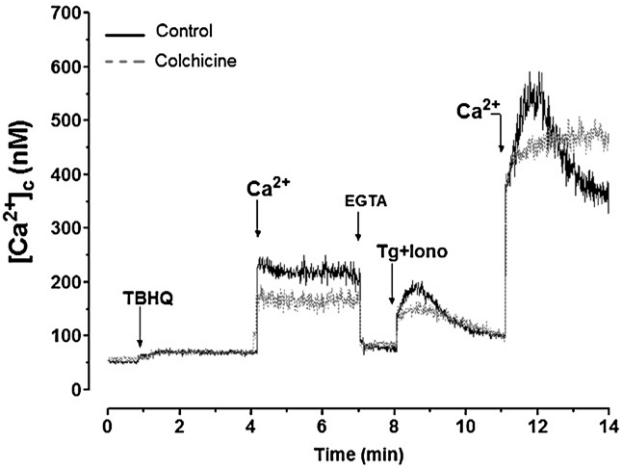

Fig. 2. Effect of colchicine on Ca^{2+} entry induced by depletion of TBHQ-sensitive (acidic stores) and insensitive (DTS) stores. Fura-2-loaded human platelets were treated for 30 min in the absence (Control) or presence of 30 μM colchicine and then suspended in a Ca^{2+}-free medium (100 μM EGTA was added). Cells were treated for 3 min with TBHQ (20 μM) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Extracellular Ca^{2+} was chelated again by addition of EGTA (final concentration 1.6 mM) and then platelets were stimulated with TG (10 nM) plus Iono (50 nM) followed by the addition of $CaCl_2$ (2 mM) to allow Ca^{2+} entry. Changes in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Traces are representative from six to eleven independent experiments.

of thrombin to induce non-capacitative Ca^{2+} entry has only been demonstrated at high concentrations (≥1 U/mL) [30]. As shown in Fig. 1A, platelet incubation with 30 μM of colchicine, for 30 min at 37 °C, reduced thrombin-evoked Ca^{2+} release by 12.0±5.9% (n=8). Pretreatment with colchicine significantly reduced thrombin-evoked Ca^{2+} entry by 13.5±2.5% (P<0.05, n=8). Similar results were observed when cells, preincubated for 30 min with 30 μM colchicine or the vehicle, were stimulated with 0.1 U/mL thrombin in a medium containing 1 mM Ca^{2+}. Under these conditions, the initial peak elevation in $[Ca^{2+}]_c$ above basal after treatment with thrombin was significantly reduced from 490±12 nM in control cells to 385±31 nM in colchicine-treated cells (Fig. 1B). The histogram represents the integral of the rise in $[Ca^{2+}]_c$ above basal after treatment with thrombin for 3 min, taking a sample every second and expressed as percentage of control. As shown in Fig. 1B, inset, colchicine reduced thrombin-evoked Ca^{2+} mobilization by 21.4±6.7% (P<0.05; n=4–6).

The DTS and acidic Ca^{2+} stores express SERCA2b and SERCA3, respectively, which show high and low sensitivity to TG [19,22,32]. Treatment of platelets with a high concentration of TG (200 nM), which induced depletion of both Ca^{2+} stores, evokes a sustained increase in $[Ca^{2+}]_c$. Subsequent addition of extracellular Ca^{2+} induced a rise of $[Ca^{2+}]_c$ that was found to be greater than that induced by 0.1 U/mL thrombin (Fig. 1C vs 1A). Cell preincubation with 30 μM colchicine significantly increased TG-induced SOCE by 17.7±3.5% (Fig. 1C; P<0.01; n=9), without having any effect on TG-induced Ca^{2+} release (Fig. 1C). The effect of colchicine on TG-induced SOCE was found to be concentration dependent, reaching a maximal effect at 60 μM colchicine, which was not statistically different from that observed at 30 μM (Fig. 1D; n=4–6); therefore, we used 30 μM colchicine throughout this study.

We have recently described two different pathways for SOCE in platelets activated by depletion of the DTS or the acidic Ca^{2+} stores [26]. Previous studies have reported that low (0.1 U/mL) concentrations of thrombin and high (200 nM) concentrations of TG show a different ability to discharge the Ca^{2+} stores [31]. While the ability of thrombin to discharge the acidic stores is greater than that to deplete the DTS, TG shows a

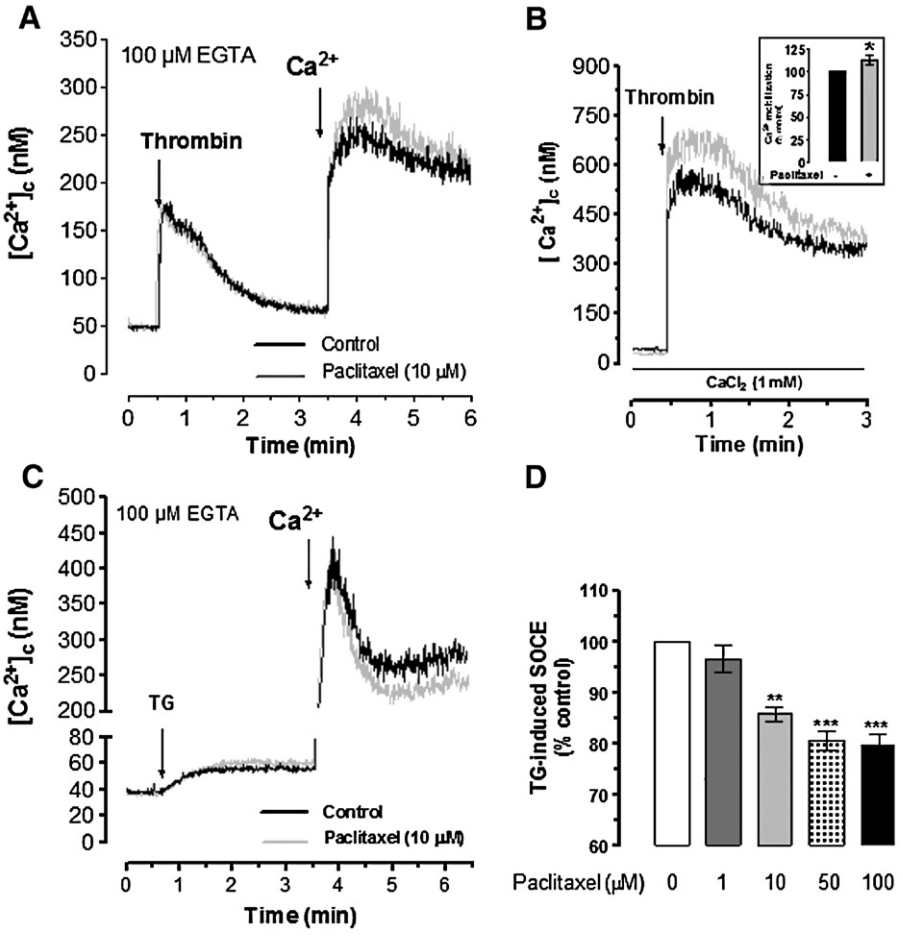

Fig. 3. Effect of paclitaxel on thrombin- and TG-induced Ca^{2+} entry in human platelets. A–C, Fura-2-loaded human platelets were preincubated for 30 min at 37 °C, with 10 μM paclitaxel or the vehicle (DMSO). Cells were then stimulated in a Ca^{2+} free medium (100 μM EGTA was added) with thrombin (0.1 U/mL; A) or TG (200 nM; C), followed by addition of 300 μM $CaCl_2$ (final concentration) to the medium three min later to initiate calcium entry, or were stimulated with thrombin in a medium containing 1 mM Ca^{2+} (B). Changes in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Inset Fig. 3*B*, histograms show Ca^{2+} mobilization induced by thrombin in the absence or presence of paclitaxel estimated as described in Experimental procedures. D, histograms show TG-induced SOCE in the presence of increasing concentrations of paclitaxel (1-100 μM) estimated as described under Experimental procedures (n=4-6). * P<0.05, ** P<0.01 *** P<0.001 compared to platelets not treated with paclitaxel.

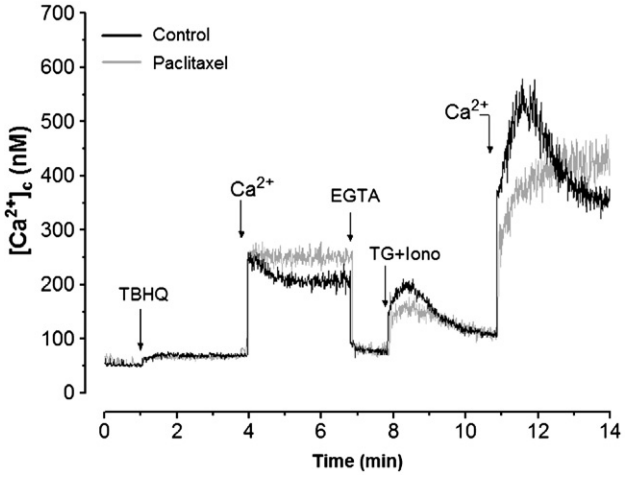

Fig. 4. Effect of paclitaxel on Ca^{2+} entry induced by depletion of TBHQ-sensitive (acidic stores) and insensitive (DTS) stores. Fura-2-loaded human platelets were treated for 30 min in the absence (Control) or presence of 10 μM paclitaxel and then suspended in a Ca^{2+}-free medium (100 μM EGTA was added). Cells were treated for 3 min with TBHQ (20 μM) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Extracellular Ca^{2+} was chelated again by addition of EGTA (final concentration 1.6 mM) and then platelets were stimulated with TG (10 nM) plus Iono (50 nM) followed by the addition of $CaCl_2$ (2 mM) to allow Ca^{2+} entry. Changes in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Traces are representative from six to eleven independent experiments.

similar ability to discharge both stores. The results presented here suggest that tubulin microtubules might play different roles in Ca^{2+} entry depending of the Ca^{2+} store depleted. To confirm this hypothesis we investigated the effect of colchicine on SOCE induced by sequential depletion of the Ca^{2+} stores individually as previously described [26]. Treatment of platelets in a Ca^{2+}-free medium (100 μM EGTA was added) with 20 μM TBHQ, which selectively and completely depletes the acidic stores [26,32], induced a sustained increase in $[Ca^{2+}]_c$ due to release from the acidic compartments; subsequent addition of $CaCl_2$ (300 μM) 3 min later induced a larger, prolonged increase in $[Ca^{2+}]_c$ indicative of Ca^{2+} entry (Fig. 2). The extracellular Ca^{2+} was chelated 3 min later by addition of EGTA (1.6 mM) and cells were then treated with TG (10 nM)+Iono (50 nM) for a further 3 min, which induced a transient increase in $[Ca^{2+}]_c$ due to Ca^{2+} release from the DTS. The subsequent addition of $CaCl_2$ (2 mM) resulted in a rise in $[Ca^{2+}]_c$ indicative of Ca^{2+} entry (Fig. 2). The difference between TG+Iono- and TBHQ-induced SOCE is indicative of Ca^{2+} influx mediated by depletion of the DTS [26]. Treatment of human platelets with colchicine significantly reduced TBHQ-induced SOCE by 13.3±3.5% but increased SOCE mediated by depletion of the DTS by 15.1±5.4% (Fig. 2; $n=6–11$, $P<0.05$).

3.2. Effect of paclitaxel on thrombin- or TG-induced SOCE

Tubulin microtubules in resting platelets are organized in a core-coiled structure in the subplasmalemmal region maintaining the discoid shape. After platelet stimulation with physiological agonist tubulin structure is disorganized allowing platelets to shape change [10,12]. In order to test whether microtubular reorganization is necessary for SOCE we used paclitaxel, which induces microtubular stabilization and increases the cellular microtubular content [14,33–35]. As shown in Fig. 3A, preincubation of platelets for 30 min at 37 °C with 10 μM paclitaxel increased thrombin-evoked SOCE by 25.0±6.2% ($P<0.01$, $n=6$), without having any effect on thrombin-induced Ca^{2+} release. Consistent with this, in a medium containing 1 mM Ca^{2+} paclitaxel enhanced the initial peak elevation in $[Ca^{2+}]_c$ above basal after treatment with 0.1 U/mL thrombin from 490±12 nM in controls to 620±36 nM in paclitaxel-treated cells (Fig. 3B). Paclitaxel increased the integral of the rise in $[Ca^{2+}]_c$ above basal for 3 min after treatment with thrombin by 16.1±4.6 ($P<0.05$; $n=4$-6).

In contrast, paclitaxel reduced SOCE stimulated by 200 nM TG by 16.3±1.2% (Fig. 3C; $P<0.01$, $n=7$), without modifying the ability of TG to discharge the Ca^{2+} stores (Fig. 3C). Paclitaxel reduced TG-induced SOCE in a concentration-dependent manner reaching a maximal inhibition of 20.3±2.3% at a concentration of 100 μM ($P<0.001$, $n=5$). These findings support the idea that the tubulin microtubules play a dual role in the modulation of SOCE. To confirm this hypothesis we investigated the effect of paclitaxel on SOCE induced by sequential depletion of the Ca^{2+} stores individually as described above. As shown in Fig. 4, treatment of human platelets with paclitaxel significantly increased TBHQ-induced SOCE by 18.4±3.4% but reduced SOCE mediated by depletion of the DTS by 19.0±5.6% ($P<0.05$).

3.3. Effects of colchicine and paclitaxel on Ca^{2+} extrusion in platelets

Since changes in $[Ca^{2+}]_c$ can result from altered rates of Ca^{2+} removal as well as entry, we investigated the possible effect of these drugs on Ca^{2+} extrusion. As previously reported, under our experimental conditions the plasma membrane Ca^{2+}-ATPase (PMCA) is the main mechanism involved in Ca^{2+}

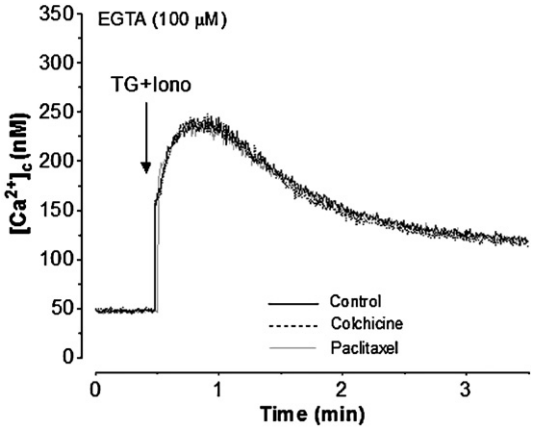

Fig. 5. Effects of colchicine and paclitaxel on restoration of $[Ca^{2+}]_c$ in human platelets. Fura-2-loaded human platelets were incubated at 37 °C for 30 min with 30 μM colchicine or 10 μM paclitaxel. At the time of experiment, 100 μM EGTA was added. Cells were then stimulated with TG (1 μM) combined with Iono (50 nM). Elevations in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Traces shown are representative of four to five independent experiments.

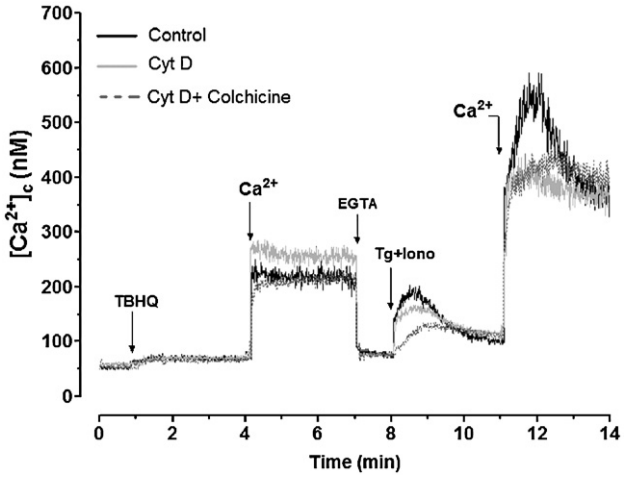

Fig. 6. Effect of colchicine and cytochalasin D on Ca^{2+} entry induced by depletion of TBHQ-sensitive (acidic stores) and insensitive (DTS) stores. Fura-2-loaded human platelets were treated in the absence (Control) or presence 10 μM Cyt D for 40 min alone or in combination with 30 μM colchicine for 30 min and then suspended in a Ca^{2+}-free medium (100 μM EGTA was added). Cells were treated for 3 min with TBHQ (20 μM) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Extracellular Ca^{2+} was chelated again by addition of EGTA (final concentration 1.6 mM) and then platelets were stimulated with TG (10 nM) plus Iono (50 nM) followed by the addition of $CaCl_2$ (2 mM) to allow Ca^{2+} entry. Changes in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$. Traces are representative from nine to twelve independent experiments.

removal from the cytosol by extrusion across the plasma membrane [36]. Following a previously described procedure [36], based on the rate of decay of $[Ca^{2+}]_c$ to basal levels after platelet stimulation with TG (1 μM) in combination with Iono (50 nM), we investigated the effect of colchicine and paclitaxel on PMCA activity. As shown in Fig. 5, in cells not treated with microtubular drugs the decay constant was 0.00646±0.00076. Neither 30 μM colchicine nor 10 μM paclitaxel modified the rate of decay of $[Ca^{2+}]_c$ to basal levels after treatment with TG+ Iono (the decay constants in the presence of colchicine or paclitaxel were 0.00637±0.00083 and 0.00638±0.00056, respectively, P=0.079; n=4-5), which strongly suggest that these compounds did not modify PMCA activity at the concentrations used.

Additionally, we tested the effect of colchicine and paclitaxel on TG-induced Sr^{2+} entry, since Sr^{2+} is transported with lower affinity than Ca^{2+} by PMCA. Our results indicate that the effects of colchicine and paclitaxel on TG-evoked Sr^{2+} entry were similar to those found on Ca^{2+} entry (colchicine enhanced TG-evoked Sr^{2+} entry by 20.2±6.5% and paclitaxel reduced TG-evoked Sr^{2+} entry by 15.38±7.0%; data not shown; n=4–5), which further support the lack of effect of these compounds on PMCA activity.

3.4. Roles of the tubulin- and actin cytoskeletons in SOCE induced by the depletion of specific Ca^{2+} pools in human platelets

We have previously reported that SOCE mediated by depletion of the acidic Ca^{2+} stores using TBHQ is enhanced by disruption of the actin cytoskeleton while this maneuver reduced SOCE regulated by the DTS [26]. Consistent with this and following the protocol depicted in Fig. 2 and previously

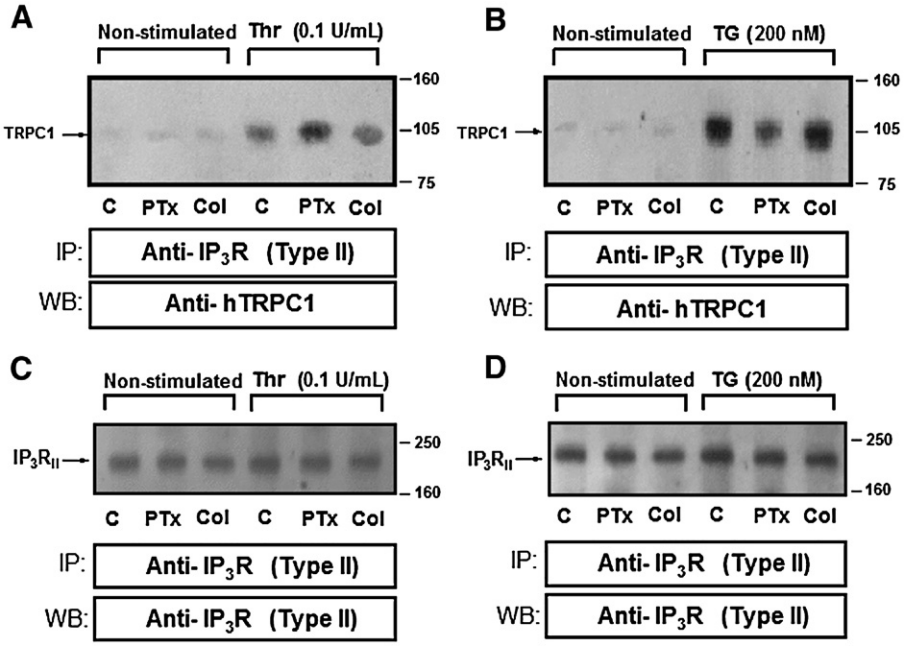

Fig. 7. Effect of colchicine and paclitaxel on thrombin- or TG-induced coupling between hTRPC1 and IP_3RII. Human platelets were incubated at 37 °C for 30 min with 30 μM colchicine or 10 μM paclitaxel and then stimulated in the absence (*non-stimulated*) or presence of thrombin (0.1 U/mL; A and C) or TG (200 nM; B and D) and then lysed. Whole cell lysates were immunoprecipitated (IP) with anti-IP_3RII antibody. Immunoprecipitates were analysed by Western blotting (WB) using anti-hTRPC1 antibody (A and B) and reprobed with anti-IP_3RII antibody (C and D) as described in the Materials and methods section. Positions of molecular mass markers are shown on the right. These results are representative of four to five independent experiments.

described [26] we found that incubation of platelets for 45 min with 10 μM Cyt D enhanced TBHQ-induced SOCE by 26.6±8.9% (Fig. 6; $P<0.01$, $n=9$-12), but reduced SOCE regulated by the DTS by 11.4±7.7%. Interestingly, TBHQ-induced Ca^{2+} entry in cells preincubated with Cyt D and colchicine was not significantly different from that in control (non-preincubated) cells (Fig. 6). Preincubation with Cyt D and colchicine reduced SOCE-regulated by the DTS by 11.0±4.0% ($n=6$–8). This inhibition might be attributed to the reduction in the ability of TG+Iono to deplete the DTS (Fig. 6). In addition, we tested the effect of stabilization of tubulin microtubules with paclitaxel on Cyt D-induced effects on SOCE. As expected preincubation with Cyt D in combination with paclitaxel enhanced TBHQ-induced SOCE by 22.6±9.6% and significantly reduced DTS-regulated SOCE by 20.8±1.1%, (data not shown; $P<0.01$; $n=4$-6).

3.5. Role of tubulin cytoskeleton in the coupling between IP_3RII and hTRPC1 in human platelets

We have reported that a Ca^{2+} store depletion evokes a *de novo conformational coupling* between the IP_3RII and hTRPC1 which may modulate SOCE in human platelets [7,8]. Hence, we have investigated the role of the tubulin-microtubules in this coupling by looking for coimmunoprecipitation in platelet lysates. Cells suspended in a Ca^{2+}-free medium were treated with 30 μM colchicine or 10 μM paclitaxel for 30 min and then stimulated with thrombin (0.1 U/mL) or TG (200 nM). As shown in Fig. 7, and consistent with the $[Ca^{2+}]_c$ determinations described above, treatment of platelets with paclitaxel increased the coupling between IP_3RII and hTRPC1 evoked by thrombin by 31.9±13.4%, while reduced that induced by TG by 24.6±11.6% ($P<0.01$; $n=4$-5). In contrast, colchicine reduced the coupling-evoked by thrombin by 10.0±5.0% but enhanced the TG-induced coupling by 23.4±5.7% ($P<0.01$; $n=4$-5). Reprobing of the membranes with the antibody used for immunoprecipitation revealed that comparable amounts of protein were loaded in all lanes (Fig. 7, lower panels).

4. Discussion

In human platelets and other non-excitable cells, SOCE may be modulated by a physical and reversible interaction between the type II IP_3R located in the membranes of the internal Ca^{2+} stores and the TRPC channels in the plasma membrane [4,8,37]. In platelets, the interaction between IP_3RII and hTRPC1 is allowed by reorganization of the actin cytoskeleton located in the proximity of the plasma membrane which may facilitate the transport of portions of the Ca^{2+} stores to the plasma membrane [3,4]. Although the role of the actin microfilaments in SOCE has been characterized in platelets, the involvement of tubulin microtubules remains unclear. We have recently found that thrombin induces a concentration and time-dependent effect on tubulin microtubules remodeling, which, as for the actin cytoskeleton [38], consists of an initial net depolymerization followed by a sustained increase in the microtubular content [39]. Here we show that disassembly of the tubulin microtubules using colchicine reduced SOCE induced by low concentrations of the physiological agonist thrombin. In addition, colchicine attenuated thrombin-evoked entry of Sr^{2+}, which has been shown to enter through the same store-operated channels as Ca^{2+} [36]. Inhibition of Sr^{2+} entry by colchicine indicates that this agent did not enhance Ca^{2+} extrusion by PMCA, which was further confirmed by estimation of the rate of decay of $[Ca^{2+}]_c$ to basal levels after platelet stimulation in the presence of SERCA inhibitors. Although colchicine reduces thrombin-evoked Ca^{2+} release, which is in agreement with previous studies [40], we believe that colchicine exert a direct effect on the Ca^{2+} entry mechanism since we have found that this agent reduces the coupling between IP_3RII and hTRPC1 stimulated by thrombin. The inhibition of Ca^{2+} entry by colchicine, which agrees with a previous report by Oka *et al*. in mast cells [41], suggest that the integrity of the tubulin cytoskeleton is required in the intracellular transport that facilitates the coupling between IP_3RII and hTRPC1 and, therefore, Ca^{2+} entry. Consistent with this, paclitaxel, which stabilizes the microtubules and induces polymerization enhanced thrombin-evoked Ca^{2+} entry, without altering Ca^{2+} release or extrusion.

Surprisingly, we have observed that colchicine and paclitaxel exerted opposite effects on SOCE and the coupling between IP_3RII and TRPC1 induced by high concentrations of TG. The possible explanation might reside in the activation of different pathways for SOCE by low concentrations of thrombin or TG. We have recently identified two distinct mechanisms for Ca^{2+} entry in human platelets activated by depletion of two independent Ca^{2+} pools, the DTS and the acidic Ca^{2+} stores [26]. The major Ca^{2+} store has long been identified as the DTS and its SERCA shows a high affinity for TG, while the acidic stores have a SERCA with low affinity for TG but which is sensitive to TBHQ [20,32]. Additionally, thrombin-induced platelet stimulation is mediated through the activation of three membrane receptors the protease-activated receptor (PAR) 1 and 4, and the glycoprotein receptor GPIb-IX-V [30,31]. PAR-1 and PAR-4 release Ca^{2+} from both stores, while the high affinity GPIb-IX-V only discharges the acidic stores so that SOCE mediated by low concentrations of thrombin is mostly mediated by full depletion of the acidic stores and only by partial discharge of the DTS [31]. In contrast, the concentration of TG used here inactivates the SERCA isoforms located in both Ca^{2+} stores, and, therefore, activates SOCE by both pathways equally, with most of the Ca^{2+} entry being mediated by depletion of the DTS [26]. This may explain the different extent of Ca^{2+} entry induced by TG or thrombin, even though the latter induces a more rapid release of Ca^{2+} from the stores. Therefore, the different roles of the tubulin microtubules in SOCE induced by thrombin or TG may be explained by the different contributions of the DTS or the acidic Ca^{2+} stores involved. In support of this hypothesis we found that colchicine and paclitaxel induced the same effects on SOCE activated by 0.1 U/mL thrombin and TBHQ, which selectively depletes the acidic stores [23].

The lack of effect of colchicine and paclitaxel on TG-induced Ca^{2+} release and the different effects on thrombin or

TG-evoked Ca^{2+} entry suggest that these compounds did not alter the ability of platelets to accumulate Ca^{2+} into the stores, and are not Ca^{2+} chelators or Ca^{2+} channel blockers.

We have previously shown that disruption of the actin cytoskeleton facilitates TBHQ-induced SOCE, suggesting that actin filaments act as a barrier that prevents Ca^{2+} entry. Now we have found that this process requires microtubular polymerization since colchicine was able to reduce TBHQ-evoked SOCE in the absence or presence of Cyt D. However, the tubulin microtubules act as a negative clamp for SOCE regulated by the DTS since colchicine increases Ca^{2+} entry mediated by depletion of both stores in the absence or presence of Cyt D, even though it inhibits TBHQ-induced SOCE. Therefore, our findings suggest that both cytoskeletal components, actin and tubulin filaments, are involved in the modulation of SOCE in platelets.

In summary, our results indicate that the tubulin microtubules play a dual role in the SOCE mechanism in human platelets, acting as a physical barrier that may prevent the transport of portions of the DTS to the plasma membrane, and also supporting the trafficking of the acidic Ca^{2+} stores to facilitate the coupling between IP_3RII and hTRPC1. Since the intracellular Ca^{2+} stores in platelets show different sensitivity to platelet agonists [23] the functional relevance of the tubulin cytoskeleton on SOCE in platelets depends on the agonist concerned.

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