Rapid agonist-evoked coupling of type II Ins(1,4,5)P_3 receptor with human transient receptor potential (hTRPC1) channels in human platelets

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Depletion of intracellular Ca^{2+} stores results in the activation of SMCE (store-mediated Ca^{2+} entry) in many cells. The mechanism of activation of SMCE is poorly understood. In human platelets, a secretion-like coupling model may be involved. This proposes that store depletion results in trafficking of portions of the endoplasmic reticulum to the plasma membrane, enabling coupling between proteins in the two membranes. In support of this, we have shown that, in human platelets, agonist-evoked Ca^{2+} store depletion results in *de novo* and reversible coupling of the InsP_3RII [type II inositol (1,4,5)trisphosphate receptor] with the putative Ca^{2+} entry channel hTRPC1 [human canonical transient receptor potential 1 (protein); Rosado, Brownlow and Sage (2002) J. Biol. Chem. **277**, 42157–42163]. A crucial test of the hypothesis that this coupling activates SMCE is that it should occur rapidly enough to account for agonist-evoked Ca^{2+} entry. In the present study, we have used quenched- and stopped-flow approaches to determine the latencies of thrombin-evoked coupling of InsP_3RII with hTRPC1 and of thrombin-evoked bivalent cation entry using Mn^{2+} quenching of fura 2 fluorescence. Thrombin-evoked Mn^{2+} entry was detected with a latency of 0.81 ± 0.07 s (S.E.M., $n = 7$) or 1.36 ± 0.09 s (S.E.M., $n = 7$) at a concentration of 1.0 or 0.1 unit/ml respectively. Coupling between InsP_3RII and hTRPC1, assessed at 100 ms intervals, was first detected with a latency of 0.9 or 1.4 s after stimulation with thrombin at a concentration of 1.0 or 0.1 unit/ml respectively. These results support the hypothesis that *de novo* coupling of InsP_3RII with hTRPC1 could activate SMCE in human platelets.

Key words: agonist-evoked Ca^{2+} entry, Ca^{2+} influx, human transient receptor potential, quenched flow, rapid coupling, stopped flow.

INTRODUCTION

The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) regulates many cellular processes, including contraction, secretion, cell growth and cell death (see e.g. [1]). Increases in $[Ca^{2+}]_i$ are generated by the release of Ca^{2+} from intracellular Ca^{2+} stores and the entry of Ca^{2+} across the plasma membrane [2,3]. In non-excitable cells, SMCE (store-mediated Ca^{2+} entry), a mechanism regulated by the filling state of the intracellular Ca^{2+} stores, is believed to be an important pathway for Ca^{2+} influx [4,5].

The mechanism of activation of SMCE is not fully understood. Several hypotheses have been put forward, which fall into two main categories: those suggesting a diffusible messenger that gates plasma membrane channels and those suggesting a conformational coupling between elements in the endoplasmic reticulum, possibly InsP_3Rs [inositol (1,4,5)trisphosphate receptors], and the plasma membrane [3,6]. A modification of the conformational coupling model has been proposed to operate in several cell types. This 'secretion-like coupling model' is based on the trafficking of portions of the endoplasmic reticulum towards the plasma membrane to allow the coupling of proteins in the endoplasmic reticulum and the plasma membrane [7–9]. In some cells, such a process would require remodelling of cortical actin. Agents that disrupt this actin remodelling have been shown to inhibit both the activation and maintenance of SMCE in human platelets [8,9] and other cells [10].

The nature of the channels that conduct SMCE is uncertain. Much attention has focused on human homologues of *Drosophila* TRP (transient receptor potential) channels (see [6]). TRP proteins are divided into three main subfamilies: the TRPC (canonical) group, the TRPV (vanilloid) group and the TRPM (melastatin) group [11]. Functional expression of TRPC proteins enhanced SMCE in several types of mammalian cells [12,13], and disruption of the expression of TRP sequences in antisense studies was found to affect the activation of SMCE [12]. Human platelets express mRNA for TRPC1 and its splice variant TRPC1A [14], and TRPC1 proteins have been detected in platelets using anti-hTRPC1 (h refers to human) antibodies specific for the amino acid residues 557–571 or 571–590 [15,16]. Residues 557–571 of TRPC1 are predicted to lie in the pore-forming region of the protein [17,18]. An antibody raised against this sequence has been shown to inhibit SMCE in human platelets [16].

In human platelets, we have shown that depletion of the intracellular Ca^{2+} stores using TG (thapsigargin) or the physiological agonist thrombin results in *de novo* coupling of hTRPC1 with InsP_3RII (type II InsP_3R) [15,16,19]. Agents that enhanced or inhibited SMCE were found to increase or decrease the coupling of hTRPC1 with InsP_3RII [19]. Therefore we have proposed that this coupling event could be responsible for the activation of SMCE in human platelets.

A critical prerequisite of this hypothesis is that the coupling of hTRPC1 with InsP_3RII should occur on the same time scale as Ca^{2+} entry, which agonists can activate with latencies of < 500 ms in human platelets [20]. In the present study, we have addressed this issue with respect to responses evoked by thrombin.

A potential complication comes from the fact that SMCE is not the only mechanism by which thrombin has been shown to activate Ca^{2+} entry in platelets. We have shown that, at higher

Abbreviations used: AA, arachidonic acid; ARC, arachidonate-regulated Ca^{2+} conductance; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; DAG, diacylglycerol; fura 2/AM, fura 2 acetoxymethyl ester; HBS, Hepes-buffered saline; InsP_3, inositol (1,4,5)trisphosphate; InsP_3R, InsP_3 receptor; InsP_3RII, type II InsP_3R; IONO, ionomycin; PKC, protein kinase C; PMCA, plasma-membrane Ca^{2+}-ATPase; SMCE, store-mediated calcium entry; TG, thapsigargin; TRP, transient receptor potential (protein); TRPC, canonical TRP; hTRPC1, human TRPC1.

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concentrations, thrombin also activates Ca^{2+} entry independent of the release of Ca^{2+} from intracellular stores. This was demonstrated by strongly depleting the intracellular stores using a high concentration of TG (1 μM) together with a low concentration (50 nM) of the Ca^{2+} ionophore IONO (ionomycin) in the absence of extracellular Ca^{2+} [21]. Bivalent cation entry was then initiated by the addition of $SrCl_2$ {Sr^{2+} was used, since it is a poor substrate for the PMCA (plasma-membrane Ca^{2+}-ATPase), allowing assessment of unidirectional bivalent cation flux [22]}. The subsequent addition of a high concentration of thrombin (10 units/ml) evoked further bivalent cation entry. The mechanism of this store-independent bivalent cation entry appears to involve the formation of DAG (diacylglycerol) and activation of PKC (protein kinase C), since the pathway can also be activated by 1-oleoyl-2-acetyl-*sn*-glycerol or PMA and is inhibited by agents that inhibit PKC [21]. The store-mediated and -independent pathways appear distinct. An anti-hTRPC1 antibody inhibits the former without affecting the latter [16], whereas PKC inhibitors abolish the latter without affecting the former [21].

In the present study, we have investigated the contribution of the store-independent, PKC-dependent pathway to the entry of Ca^{2+} evoked by thrombin over a range of concentrations. We then determined the time course of thrombin-evoked coupling of hTRPC1 with InsP_3RII using a quenched-flow procedure in parallel with stopped-flow fluorimetric analysis of thrombin-evoked bivalent cation entry using conditions under which the PKC-dependent pathway was not operative. We show that the coupling of hTRPC1 with InsP_3RII occurs rapidly to account for thrombin-evoked SMCE as assessed from Mn^{2+} quenching of fura 2 fluorescence [23].

MATERIALS AND METHODS

Materials

Fura 2/AM (fura 2 acetoxymethyl ester) was obtained from Texas Fluorescence (Austin, TX, U.S.A.). Apyrase (grade VII), aspirin, BSA, strontium chloride, thrombin and TG were obtained from Sigma (Poole, Dorset, U.K.). IONO, PMA and the PKC inhibitor Ro-31-8220 were obtained from Calbiochem (Nottingham, U.K.). Anti-hTRPC1 polyclonal antibody was obtained from Alomone Laboratories (Jerusalem, Israel). Anti-InsP_3RII polyclonal antibody (C-20) and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other reagents were of analytical grade.

Platelet preparation

Experiments were performed on human blood platelets, obtained from healthy drug-free volunteers with the approval of the local ethical committee. Fura 2-loaded platelets were prepared as described previously [9]. Briefly, blood was mixed with a 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 at 700 ***g*** for 5 min, and aspirin (100 μM) and apyrase (40 μg/ml) were added. The platelet-rich plasma was incubated at 37 °C with 2.5 μM fura 2/AM for 45 min. Cells were then collected by centrifugation at 350 ***g*** for 20 min and resuspended in HBS (Hepes-buffered saline, 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 μg/ml apyrase.

Measurement of $[Ca^{2+}]_i$

Fluorescence was recorded from 1.5 ml aliquots of platelet suspension (10^8 cells/ml) at 37 °C using a Cairn Research Spectrophotometer (Cairn Research, 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 fluorescence ratio of 340:380 and calibrated in terms of $[Ca^{2+}]_i$ by the method of Grynkiewicz et al. [24].

Stopped-flow kinetic measurements

The kinetics of fluorescence change from fura 2-loaded platelets was investigated by stopped-flow fluorimetry at 37 °C using a Hi-Tech Scientific SF-61 DX2 Double Mixing Stopped-Flow System (Hi-Tech Ltd., Salisbury, Wilts., U.K.) with an excitation wavelength of 340 or 360 nm and emission at 500 nm. Dye-loaded cells (100 μl) and an agonist solution (100 μl) were introduced into the sample flow circuit via separate reservoirs at the top of the sample-handling unit. Mn^{2+} influx was monitored as a quenching of fura 2 fluorescence at the isoemissive wavelength of 360 nm, presented on an arbitrary linear scale [23]. Results were corrected for the effects of quenching extracellular fura 2 and basal leak of Mn^{2+} into the cells by subtraction of agonist-free control runs [23]. To reduce leakage of Mn^{2+} into the cells before the experiment, 50 μM $MnCl_2$ was added to the cell suspension and 350 μM $MnCl_2$ was added to the agonist solution, giving a final concentration of 200 μM extracellular Mn^{2+} after mixing.

Monitoring of Sr^{2+} entry

Sr^{2+} was used to monitor non-capacitative cation entry to avoid complications arising from stimulation of the platelet PMCA by thrombin [25], since Sr^{2+} is carried with lower affinity than Ca^{2+} by the PMCA [22]. If Ca^{2+} was used, PKC-dependent, thrombin-evoked Ca^{2+} entry might be masked by stimulated Ca^{2+} removal. Sr^{2+} entry was determined in Ca^{2+}-free HBS containing EGTA (100 μM) to minimize the effects of contamination by Ca^{2+}. Cytosolic Sr^{2+} was monitored using the fura 2 fluorescence ratio of 340:380.

Quenched flow, immunoprecipitation and Western-blot analysis

Aliquots of platelet suspension (75 μl, 2×10^9 cells/ml) were rapidly mixed with 75 μl of either agonist-free HBS (as control) or HBS containing agonist, and incubated for various time periods at 37 °C before lysis with 75 μl of lysis buffer [316 mM NaCl/20 mM Tris/2 mM EGTA/0.2 % SDS/2 % (w/v) sodium deoxycholate/2 % (v/v) Triton X-100/2 mM Na_3VO_4/2 mM PMSF/100 μg/ml leupeptin/10 mM benzamidine; pH 7.2] in a Hi-Tech Scientific RQF-63 Double Mixing Quench-Flow System (Hi-Tech Ltd.). The syringes containing the required aliquots of each of the three solutions as well as the mixing loop were thermostatically controlled at 37 °C. The apparatus employed a precision stepper motor to apply two pushes to a common push plate that acted on the syringe pistons. The first push injected cell suspension and buffer with or without thrombin through a mixing valve into an incubation loop. After a preset time period, a second push was applied, which pushed the cell mixture from the incubation loop through a mixing valve where it mixed with the lysis buffer. Temporal calibration of the instrument was conducted by the manufacturer by following the alkaline hydrolysis of 2,4-dinitrophenol acetate and monitoring the formation of dinitrophenol by UV spectrometry. The reaction was conducted with OH^- in excess to achieve pseudo-first-order conditions. A second-order rate constant of 56 $M^{-1} \cdot s^{-1}$ at 20 °C was assumed.

The total platelet lysate (225 μl) was collected from the collection loop and stored on ice. Insoluble cell debris was removed by centrifugation. Aliquots of platelet lysates (200 μl) were then immunoprecipitated by incubation overnight at 4 °C on a rocking platform using either 2 μg of anti-hTRPC1 polyclonal antibody (Alomone Laboratories) or 3 μg of anti-InsP_3RII polyclonal antibody and 25 μl of Protein A–agarose. Immunoprecipitates were then collected by centrifugation and resuspended in lysis-wash buffer [158 mM NaCl/10 mM Tris/1 mM EGTA/0.1 % SDS/1 % sodium deoxycholate/1 % Triton X-100/2 mM Na_3VO_4/2 mM PMSF, pH 7.2]. This wash procedure was repeated twice. The pelleted immunoprecipitates were finally resuspended in 80 μl of Laemmli's buffer [70 mM Tris/10 % (v/v) glycerol/0.002 % (w/v) Bromophenol Blue/2 % (w/v) SDS, pH 7.4] and boiled for 5 min. Each quenched-flow run provided sufficient protein for analysis on the two gels. Immunoprecipitates were resolved by SDS/PAGE (8 % gel) and the separated proteins were electophoretically transferred for 2 h at 0.8 mA/cm^2 using a semi-dry blotter (Hoefer Scientific, Newcastle-under-Lyme, Staffs., U.K.) on to nitrocellulose membranes for subsequent probing. The blots were incubated overnight with 5 % (w/v) BSA in TBST (Tris-buffered saline/0.1 % Tween 20) to block residual protein-binding sites. Immunodetection of hTRPC1 or InsP_3RII was achieved using anti-hTRPC1 diluted 1:200 in TBST or with anti-InsP_3RII diluted 1:500 in TBST for 2 h. The primary antibody was removed and each blot was washed four times with TBST (2 × 5 min followed by 2 × 20 min). To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-goat IgG antibody diluted 1:5000 in TBST, washed four times with TBST as mentioned above, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to a preflashed photographic film.

We have tested previously the specificity of the commercial anti-TRPC1 antibody (Alomone Laboratories) used in these experiments in comparison with the anti-hTRPC1 antibody T1E3 [26]. After immunoprecipitation with the commercial antibody, T1E3 displayed reactivity towards exactly the same protein band [16]. To investigate further whether both antibodies recognized the same protein, both were used on the same samples. After immunoprecipitation with the commercial antibody, probing with the same antibody, revealed reactivity against a protein band of approx. 100 kDa. Stripping and reprobing the blot with the T1E3 antibody revealed reactivity towards the same protein [16]. Furthermore, the commercial antibody did not detect any protein in human peripheral blood leucocytes [16] in which hTRPC1 is not expressed, as reported by Wes et al. [17].

Statistical analysis

Analysis of statistical significance was performed using Student's paired *t* test.

RESULTS

Bivalent cation entry pathways activated by thrombin at different concentrations

We first determined conditions under which thrombin activated SMCE but not store-independent, PKC-dependent bivalent cation entry. This was done by assessing the ability of various concentrations of thrombin to evoke bivalent cation entry after depletion of the intracellular stores, and by confirming the ability of the PKC inhibitor Ro-31-8220 to inhibit any detected store-independent entry.

The effectiveness of Ro-31-8220 was checked against the ability of PMA (1 μM) to evoke an increase in $[Ca^{2+}]_i$ in the presence of 1 mM external Ca^{2+}. At a concentration of 5 μM, Ro-31-8220 completely inhibited the PMA-evoked increase in $[Ca^{2+}]_i$ (Figure 1A). The ability of thrombin to evoke bivalent cation entry after the Ca^{2+} stores had been depleted using a combination of TG and the Ca^{2+} ionophore IONO was assessed using Sr^{2+} (see the Materials and methods section). As shown in Figure 1, the addition of 300 μM $SrCl_2$ to platelets after 3 min treatment with TG (1 μM) and IONO (50 nM) resulted in an increase in the fura 2 fluorescence ratio, indicating store-mediated Sr^{2+} entry. The subsequent addition of thrombin at a concentration of 0.1 unit/ml evoked no further change in the fluorescence ratio in the presence or absence of Ro-31-8220 (5 μM; Figure 1B). As shown in Figure 1(C), at a concentration of 1 unit/ml, thrombin evoked a very modest further increase in the fluorescence ratio, and this was abolished after a 5 min pretreatment with Ro-31-8220 (5 μM). As reported previously [21], only at a concentration of 10 units/ml did thrombin evoke a pronounced further Sr^{2+} entry after prior activation of the store-mediated pathway (Figure 1D), an effect that was abolished by Ro-31-8220 (5 μM).

These results indicate that store-independent, PKC-dependent bivalent cation entry does not contribute to responses evoked by thrombin at a concentration of 0.1 unit/ml and makes only a marginal contribution to responses evoked by thrombin at a concentration of 1 unit/ml. Therefore we conducted latency studies using thrombin at these two concentrations, repeating those at the higher concentration in the presence of a PKC inhibitor.

Latencies of Ca^{2+} release from intracellular stores and of Mn^{2+} entry evoked by thrombin

Stopped-flow fluorimetry was used to determine the latencies of thrombin-evoked release of Ca^{2+} from intracellular stores and of thrombin-evoked Mn^{2+} entry at two different agonist concentrations. Fura 2-loaded platelets were rapidly mixed with thrombin at a final concentration of 1.0 or 0.1 unit/ml in the presence of 1 mM EGTA and 200 nM $MnCl_2$. Recording fura 2 fluorescence at an excitation wavelength of 340 nm indicated that the delays in onset of thrombin-evoked release of Ca^{2+} from intracellular stores were 0.81 ± 0.07 s (S.E.M., $n = 7$) or 1.36 ± 0.09 s (S.E.M., $n = 7$) at 1.0 or 0.1 unit/ml respectively (Figure 2A). At the isoemissive wavelength of 360 nm, Mn^{2+} quench of fura 2 fluorescence was first detected with latencies of 0.82 ± 0.07 s (S.E.M., $n = 7$) or 1.36 ± 0.09 s (S.E.M., $n = 7$; Figure 2B). Mixing cells with agonist-free HBS containing 1 mM EGTA and 200 nM $MnCl_2$ evoked no detectable change in fura 2 fluorescence at either excitation wavelength (results not shown), indicating that the mixing procedure alone did not activate the cells.

Latency of thrombin-evoked coupling between hTRPC1 and InsP_3RII

To determine the time course of coupling between hTRPC1 and InsP_3RII, platelet samples were prepared by quenched flow for subsequent immunoprecipitation, SDS/PAGE and Western-blot analysis. The delay times between the rapid mixing of cells with thrombin at final concentrations of 1.0 or 0.1 unit/ml and the subsequent mixing with lysis buffer (see the Materials and methods section) were set at 100 ms intervals, commencing approx. 100 ms before the first detected Mn^{2+} entry evoked by thrombin at the same concentration. Co-immunoprecipitation of hTRPC1 with InsP_3RII (Figure 3A) or of InsP_3RII with hTRPC1 (Figure 3C) was not detected 0.7 or 0.8 s after stimulation with thrombin at

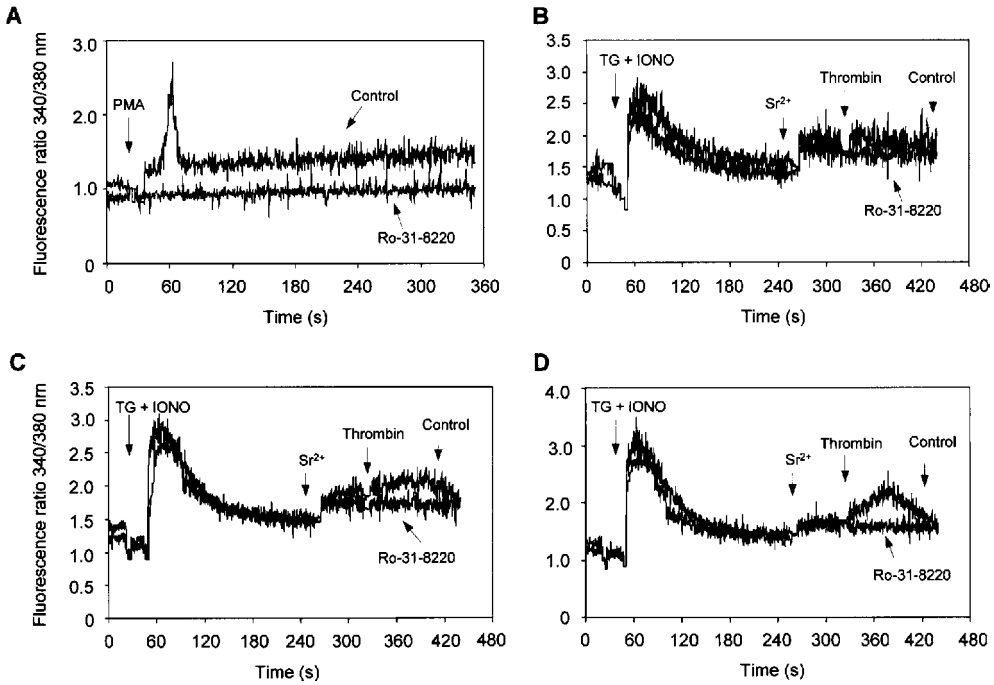

Figure 1 Effects of PKC inhibition on store-independent Sr^{2+} entry in human platelets

(**A**) Cells were pretreated for 5 min at 37 °C with 5 μM Ro-31-8220 or the vehicle (DMSO) alone ('Control'). The platelets were then stimulated with 1 μM PMA in HBS containing 1 mM Sr^{2+}. (**B**–**D**) Cells were pretreated for 5 min at 37 °C with 5 μM Ro-31-8220 or the vehicle ('Control'). The platelets were then stimulated with TG (1 μM) and IONO (50 nM) in Ca^{2+}-free HBS containing 100 μM EGTA. After 3 min, 300 μM $SrCl_2$ was added, followed by the addition of (**B**) 0.1 unit/ml thrombin, (**C**) 1 unit/ml thrombin or (**D**) 10 units/ml thrombin. Results are representative of four independent experiments performed on cell preparations from different donors.

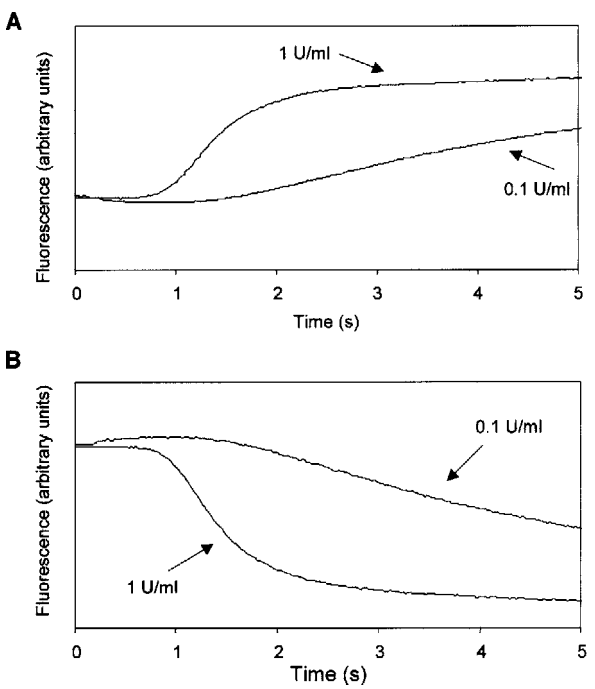

Figure 2 Latency of thrombin-evoked release of Ca^{2+} from intracellular stores and thrombin-evoked Mn^{2+} entry

Human platelets were loaded with fura 2 and rapidly mixed with thrombin at a final concentration of either 0.1 or 1 unit/ml in the presence of 1 mM EGTA and 200 nM $MnCl_2$ and in the absence of added Ca^{2+}. Fura 2 fluorescence was recorded at excitation wavelengths of 340 nm (**A**) and 360 nm (**B**) and emission was recorded at 500 nm. Traces are representative of three or four runs made on cell preparations from two donors.

a concentration of 1.0 unit/ml, whereas co-immunoprecipitation was clearly detected 0.9 s after stimulation. With thrombin at a concentration of 0.1 unit/ml, co-immunoprecipitation of hTRPC1 with InsP_3RII (Figure 3E) or of InsP_3RII with hTRPC1 (Figure 3G) was not detected 1.2 or 1.3 s after stimulation, whereas co-immunoprecipitation was clearly detected 1.4 s after stimulation. In all experiments, successful immunoprecipitation was confirmed by Western-blot analysis with the same antibody used for immunoprecipitation (Figures 3B, 3D, 3F and 3H). Coupling of hTRPC1 with InsP_3RII was not detected in all resting platelet preparations [Figures 3A, 3C, 3E and 3G, lanes marked 'Control (a)']. Coupling was also not detected after cells had been mixed with agonist-free HBS for 1.2 s [Figures 2A and 2C, lanes marked 'Control (b)'] or 1.7 s [Figures 3E and 3G, lanes marked 'Control (b)'], indicating that the mixing and quenching procedures alone did not induce coupling of hTRPC1 with InsP_3RII.

Latencies of release of Ca^{2+} from intracellular stores and of Mn^{2+} entry evoked by thrombin after blockade of PKC

In view of the potential small contribution of the store-independent, PKC-dependent bivalent cation entry pathway to the response evoked by thrombin at a concentration of 1 unit/ml (see above), the latency experiments with thrombin at this concentration were repeated after blockade of PKC. In our previous work (see e.g. [25]), we found that the latency of Mn^{2+} entry is modulated by the presence or absence of extracellular Ca^{2+}. Therefore these experiments were conducted under more physiological conditions (i.e. with 1 mM $CaCl_2$ as well as 200 μM $MnCl_2$ in the medium). Treatment of the cells for 5 min with 5 μM Ro-31-8220 did not affect the latency of the thrombin-evoked

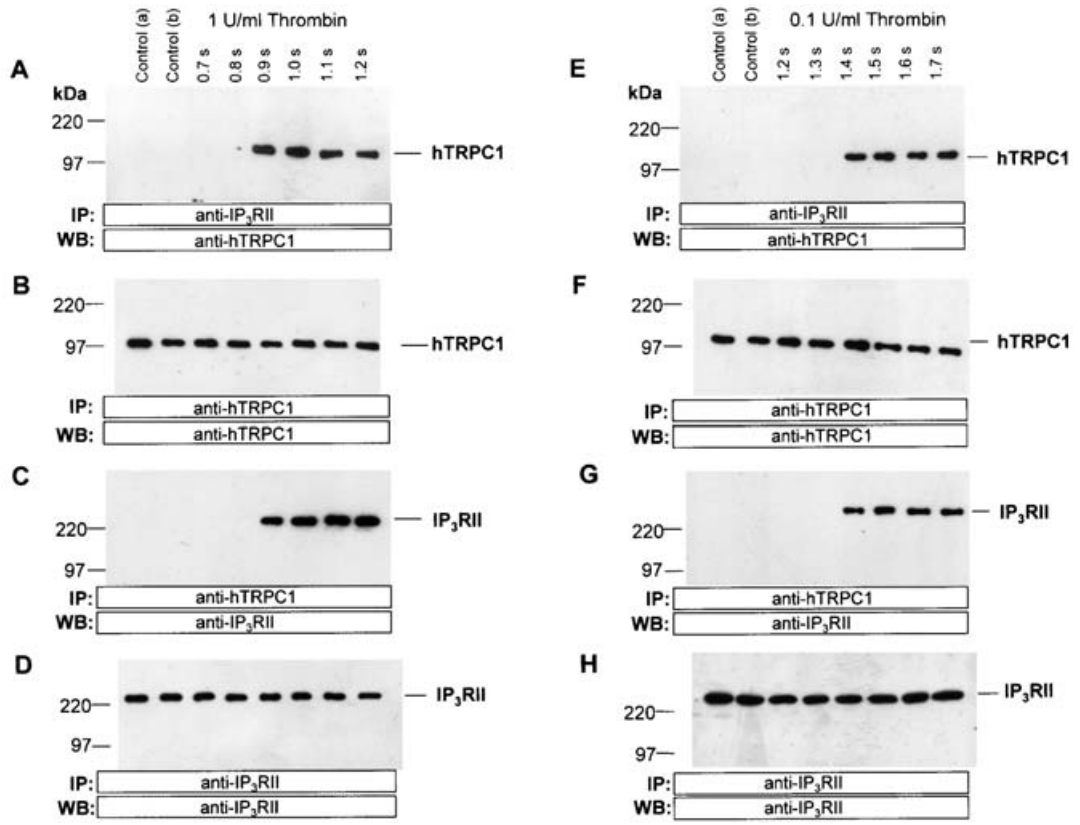

Figure 3 Latency of thrombin-evoked coupling between hTRPC1 and InsP_3RII in human platelets

Platelets were rapidly mixed with thrombin at a final concentration of 1.0 unit/ml (**A**–**D**) or 0.1 unit/ml (**E**–**H**) or with agonist-free HBS ['Control (b)'], and incubated at 37 °C for various time periods before mixing with lysis buffer. Samples were also taken from suspensions that were not subjected to quenched flow ['Control (a)']. Whole-cell lysates were immunoprecipitated (IP) with either anti-InsP_3RII (**A**, **D**, **E**, **H**) or anti-hTRPC1 (**B**, **C**, **F**, **G**) antibody and then subjected to SDS/PAGE followed by Western-blot analysis (WB) using either an anti-hTRPC1 (**A**, **B**, **E**, **F**) or an anti-InsP_3RII (**C**, **D**, **G**, **H**) antibody. Both antibodies detected only a single band (see [16]) and, hence, only partial gels are shown. Positions of molecular-mass standards (in kDa) are shown on the left. Results are representative of duplicate experiments performed on cell preparations from three different donors.

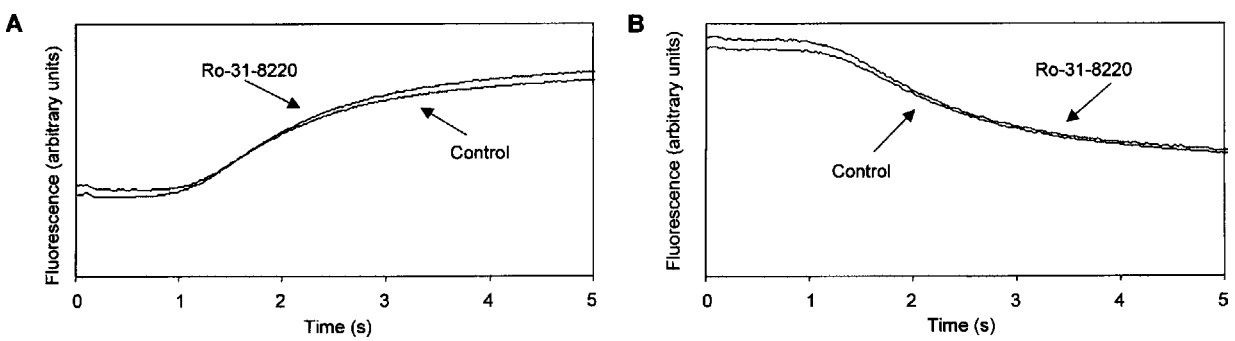

Figure 4 Effects of Ro-31-8220 on the latency of changes in $[Ca^{2+}]_i$ and Mn^{2+} entry evoked by thrombin

Human platelets were loaded with fura 2 and pretreated for 5 min at 37 °C in the absence or presence of 5 μM Ro-31-8220 and then rapidly mixed with thrombin at a final concentration of 1 unit/ml in the presence of 1 mM $CaCl_2$ and 200 nM $MnCl_2$. Fura 2 fluorescence was recorded at excitation wavelengths of 340 nm (**A**) and 360 nm (**B**) and emission was recorded at 500 nm. Traces are representative of three or four runs performed on cell preparations from three donors.

increase in $[Ca^{2+}]_i$ (Figure 4A). The thrombin-evoked increase in $[Ca^{2+}]_i$ was first detected after a delay of 0.92 ± 0.01 s (S.E.M., $n = 10$) after a 5 min pretreatment with 5 μM Ro-31-8220, compared with 0.88 ± 0.01 s (S.E.M., $n = 10$) after treatment with the vehicle (DMSO) alone. The difference was not significant ($P = 0.064$). Similarly, the latency of thrombin-evoked Mn^{2+} entry was not affected by the PKC inhibitor (Figure 4B). Mn^{2+} entry was first detected 0.85 ± 0.02 s (S.E.M., $n = 10$) after agonist addition following a 5 min pretreatment with 5 μM Ro-31-8220, compared with 0.83 ± 0.02 s (S.E.M., $n = 10$) after treatment with the vehicle (DMSO) alone. The difference was not significant ($P = 0.39$).

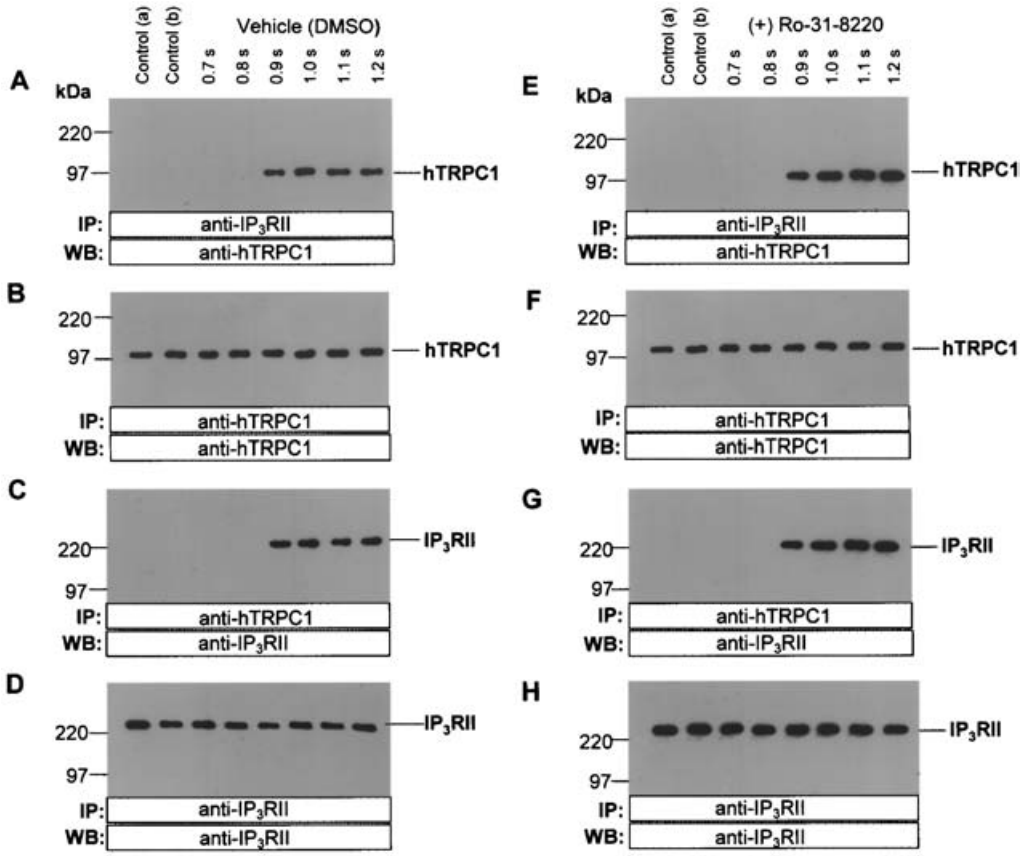

Figure 5 Effects of Ro-31-8220 on the latency of thrombin-evoked coupling between hTRPC1 and InsP_3RII in human platelets

After pretreatment for 5 min at 37 °C with the vehicle (DMSO; **A**–**D**) or 5 μM Ro-31-8220 (**E**–**H**), platelets were rapidly mixed with thrombin at a final concentration of 1.0 unit/ml or with agonist-free HBS ['Control (b)'] and incubated at 37 °C for various time periods before mixing with lysis buffer. Samples were also taken from suspensions that were not subjected to quenched flow ['Control (a)']. Whole-cell lysates were immunoprecipitated (IP) with either an anti-InsP_3RII (**A**, **D**, **E**, **H**) or an anti-hTRPC1 (**B**, **C**, **F**, **G**) antibody and then subjected to SDS/PAGE followed by Western-blot analysis (WB) using either an anti-hTRPC1 (**A**, **B**, **E**, **F**) or an anti-InsP_3RII (**C**, **D**, **G**, **H**) antibody. Positions of molecular-mass standards (in kDa) are shown on the left. Results are representative of duplicate experiments performed on cell preparations from three different donors.

Latency of thrombin-evoked coupling between hTRPC1 and InsP_3RII after blockade of PKC

Finally, we investigated whether the activation of PKC influenced the latency of coupling of hTRPC1 with InsP_3RII evoked by thrombin at a concentration of 1 unit/ml. As shown in Figure 5, pretreatment of the cells with 5 μM Ro-31-8220 for 5 min did not affect the time at which coupling was first detected after stimulation with 1 unit/ml thrombin. In cells treated with the vehicle (DMSO) alone, co-immunoprecipitation of hTRPC1 with InsP_3RII (Figure 5A) or of InsP_3RII with hTRPC1 (Figure 5C) was not detected 0.7 or 0.8 s after agonist addition, whereas co-immunoprecipitation was clearly detected 0.9 s after stimulation. Co-immunoprecipitation was first detected at the same time point (0.9 s) in cells pretreated with Ro-31-8220 (Figures 5E and 5G). In all experiments, successful immunoprecipitation was confirmed by Western-blot analysis with the same antibody used for immunoprecipitation (Figures 5B, 5D, 5F and 5H). Coupling of hTRPC1 with InsP_3RII was not detected in resting preparations of vehicle- and Ro-31-8220-treated cells [Figure 5, lanes marked 'Control (a)']. Coupling was also not detected after vehicle- and Ro-31-8220-treated cells had been mixed with agonist-free HBS for 1.2 s [Figures 5A, 5C, 5E and 5F, lanes marked 'Control (b)'].

DISCUSSION

In several cell types, SMCE has been proposed to be activated by a secretion-like coupling process, in which the trafficking of portions of the endoplasmic reticulum towards the plasma membrane allows coupling between proteins in these two membranes [7–9]. In support of such a model in platelets, we have reported that agents which interfere with remodelling of the cortical actin cytoskeleton also interfere with SMCE. The activation of SMCE after depletion of the intracellular Ca^{2+} stores using TG is decreased if actin polymerization is inhibited using cytochalasin D or latrunculin A, if actin polymerization is induced and stabilized using jasplakinolide or if condensation of F-actin under the plasma membrane occurred after treatment with calyculin A [8,9].

Since the conformational coupling model was first proposed, it has been envisaged that the luminal Ca^{2+} content of the intracellular stores is sensed by InsP_3Rs and that these receptors thus play a role in the activation of SMCE [27]. In support of such a role for InsP_3Rs in human platelets, we have shown that the activation and maintenance of SMCE evoked by TG is completely inhibited if InsP_3 generation is inhibited after treatment with Li^+ or if InsP_3R function is inhibited using xestospongin C [15].

The nature of the channels responsible for SMCE is unclear. In many cells, roles for members of the TRP family have been

proposed [6,11]. We have shown that, in human platelets, antibodies raised against an amino acid sequence (557–571) in the pore-forming region of hTRPC1 block TG and thrombin-evoked Ca^{2+} entry [16], suggesting that TRPC1 contributes to the channel responsible for SMCE in these cells.

We have shown that depletion of the intracellular Ca^{2+} stores with TG results in *de novo* coupling of hTRPC1 with InsP_3RII [15,16,19], which, in platelets, is reported to be associated with both intracellular membranes and the plasma membrane [28]. Reversible coupling between these two proteins is also stimulated by thrombin [15,16]. Therefore we have suggested that the coupling of hTRPC1 with InsP_3RII after Ca^{2+} store depletion may be responsible for the activation of SMCE in human platelets.

A critical requirement for this hypothesis is that the coupling of hTRPC1 with InsP_3RII should occur with the same time course as SMCE. In the present study, we have shown that thrombin-evoked coupling of hTRPC1 with InsP_3RII occurs with a similar latency as that for thrombin-evoked bivalent cation entry, as assessed using Mn^{2+} quench of fura 2 fluorescence, at two concentrations of the agonist.

In addition to SMCE, thrombin has been reported to evoke Ca^{2+} entry by a mechanism independent of Ca^{2+}-store depletion but dependent on the formation of DAG after activation of phospholipase C [21,29]. In our experiments, this pathway appears to be dependent on the activation of PKC, since it can be activated using 1-oleoyl-2-acetyl-*sn*-glycerol or PMA and is inhibited by the PKC inhibitor Ro-31-8220 [21]. Others have reported that DAG activates Ca^{2+} entry in platelets independent of PKC [29]. In the present study, we have shown that after depletion of the intracellular Ca^{2+} stores and the activation of SMCE, thrombin, at a concentration of 0.1 unit/ml, evokes no detectable bivalent cation entry (assessed using Sr^{2+}). After activation of SMCE using TG, thrombin at a concentration of 1.0 unit/ml evoked only a very small further increase in bivalent cation entry, whereas 10 units/ml thrombin evoked substantial further entry. The Sr^{2+} entry evoked by thrombin at 1.0 or 10 units/ml was completely abolished by Ro-31-8220, supporting our earlier evidence that the store-independent Ca^{2+} entry pathway activated by thrombin is PKC-dependent. Inhibition of PKC using Ro-31-8220 had no significant effect on the latency of bivalent cation entry evoked by 1.0 unit/ml thrombin, indicating that the PKC-dependent pathway did not contribute to the observed entry. The latency of coupling of hTRPC1 with InsP_3RII was also unaffected by the inhibition of PKC.

Recently, there have been challenges to the dogma that SMCE is the major Ca^{2+} entry pathway in non-excitable cells. In several cell types, it has been suggested that, at low agonist concentrations, SMCE is not operative; instead, it has been suggested that the major Ca^{2+} entry pathway is activated by AA [30–34]. This ARC (arachidonate-regulated Ca^{2+} conductance) was found to be inhibited by the high $[Ca^{2+}]_i$ generated by SMCE [32]. In A7r5 vascular smooth-muscle cells, the ARC and SMCE pathways appear to be reciprocally regulated by AA, with AA stimulating ARC and inhibiting SMCE [34]. In these cells, only ARC appeared to operate during agonist exposure, with SMCE occurring after agonist removal. Existing evidence indicates that the situation in platelets is different. We have shown that the increase in $[Ca^{2+}]_i$ evoked by exogenous AA in human platelets was completely inhibited if conversion into thromboxane A_2 was blocked using inhibitors of cyclo-oxygenase [35]. Hence, AA itself does not appear to activate Ca^{2+} entry in these cells.

Contrary to the situation in some non-excitable cells, SMCE does appear to contribute significantly to the Ca^{2+} entry evoked by low concentrations of agonists in human platelets. We have shown that the SMCE evoked by TG in platelets is blocked by an antibody to hTRPC1, whereas the store-independent Ca^{2+} entry evoked by activators of PKC or high (10 units/ml) concentrations of thrombin is not [16]. The Ca^{2+} influx evoked by a low (0.05 unit/ml) concentration of thrombin was inhibited up to 80 % by the anti-hTRPC1 antibody at the highest concentration used. This suggests that SMCE is a major contributor to thrombin-evoked Ca^{2+} entry.

The above observations suggest that SMCE is the only known pathway which can account for the thrombin-evoked bivalent cation entry observed in the present study using low concentrations of the agonist in the presence of an inhibitor of PKC. Since the release of Ca^{2+} from intracellular stores, the first detected bivalent cation entry and the first detected coupling of hTRPC1 with InsP_3RII occur with similar latencies, these observations support the hypothesis that *de novo* coupling of hTRPC1 with InsP_3RII when the intracellular Ca^{2+} stores are depleted may be responsible for the activation of SMCE in human platelets.

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