RESEARCH COMMUNICATION

Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca^{2+} stores are depleted

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In the present study we have investigated the role of inositol 1,4,5-trisphosphate (IP_3), functional IP_3 receptors (IP_3Rs) and the human homologue of the *Drosophila* transient receptor potential (Trp) channel, human Trp1 (hTrp1), in store-mediated Ca^{2+} entry (SMCE) in human platelets. Inhibition of IP_3 recycling using Li^+ , or the inhibition of IP_3Rs using xestospongine C, both resulted in the inhibition of SMCE activation following Ca^{2+} store depletion using thapsigargin. Co-immunoprecipitation experiments indicated that endogenously expressed hTrp1 couples with

IP_3R type II, but not types I or III, in platelets with depleted intracellular Ca^{2+} stores, but not in control, undepleted cells. These results provide strong evidence for the activation of SMCE by conformational coupling involving *de novo* association between IP_3Rs and a plasma membrane channel in normal human cells.

Key words: Trp channels, Ca^{2+} entry, IP_3 receptors.

INTRODUCTION

Cytosolic Ca^{2+} elevation is a ubiquitous signal for cellular activation and the regulation of different processes, such as secretion, cell growth and differentiation [1]. Occupation of membrane receptors elevates the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by releasing Ca^{2+} from intracellular stores and activating the entry of Ca^{2+} through plasma membrane (PM) channels. In many cells, the major pathway for Ca^{2+} entry is activated following depletion of the intracellular Ca^{2+} stores [2]; however, the mechanism of this store-mediated Ca^{2+} entry (SMCE) is poorly understood. The various models proposed to account for communication between the intracellular Ca^{2+} stores and the PM can be divided into two classes: those suggesting a diffusible messenger and those suggesting a physical coupling between elements in the two membranes [2]. Recently, the physical coupling model has received support from studies which show that the activation of SMCE shares properties with the activation of secretion [3–5]. In several cell types including human platelets, the reorganization of the actin cytoskeleton appears to play a key role in the activation of SMCE, apparently by mediating translocation of the endoplasmic reticulum (ER) to the PM to enable coupling to occur [3,5].

For some time the conformational-coupling model has been proposed to involve interaction between the inositol 1,4,5-trisphosphate receptor (IP_3R) in the ER membrane and another protein in the PM [2,6]. The discovery of mammalian homologues of the *Drosophila* transient receptor potential (Trp) channel proteins has focused attention on Trp channels as candidates for the conduction of SMCE [7–11]. A functional interaction between

IP_3Rs and human Trp (hTrp) 3 has been demonstrated in patch-clamp studies of human embryonic kidney (HEK)-293 cells stably expressing hTrp3 [12]. Other studies with transfected cells have demonstrated coupling between the IP_3R type III isoform, IP_3RIII , and hTRPs 1, 3 and 6 [11,13], and that the activation of hTrp3 can be inhibited by the membrane permeant inhibitor of IP_3R function, 2-aminoethoxydiphenyl borate [14]. IP_3Rs have also been shown to be required for the activity of I_{min} , a Ca^{2+} -selective channel activated by store depletion, in excised membrane patches from A431 human carcinoma cells [15].

In the present study we have investigated the dependence of SMCE on IP_3 and IP_3Rs in human platelets. We show for the first time in a normal human cell that IP_3 is required for SMCE and that Ca^{2+} store depletion results in coupling between naturally expressed hTrp1 and the IP_3RII isoform, but not the IP_3RI or IP_3RIII isoforms.

MATERIALS AND METHODS

Materials

Fura 2 acetoxymethyl ester (AM) was obtained from Texas Fluorescence (Austin, TX, U.S.A.). Apyrase (grade V), aspirin, thrombin, *myo*-inositol, BSA and thapsigargin (TG) were purchased from Sigma (Poole, Dorset, U.K.). Ionomycin and xestospongine C (Xest C) were obtained from Calbiochem-Novabiochem (Nottingham, U.K.). Dimethyl-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (dimethyl-BAPTA) acetoxymethyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Anti-hTrp1 polyclonal antibody and control

Abbreviations used: AM, acetoxymethyl ester; dimethyl-BAPTA, dimethyl-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; $[\text{Ca}^{2+}]_i$, cytosolic calcium concentration; ER, endoplasmic reticulum; HA, haemagglutinin; HEK, human embryonic kidney; IP_3 , inositol 1,4,5-trisphosphate; IP_3R , IP_3 receptor; PM, plasma membrane; SMCE, store-mediated calcium entry; TBST, Tris-buffered saline with 0.1% Tween 20; TG, thapsigargin; Trp, transient receptor potential; hTrp, human Trp; Xest C, xestospongine C.

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antigen peptide were obtained from Alomone Laboratories (Jerusalem, Israel). Anti-IP₃R type I, II and III polyclonal antibodies (C-20) and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody was obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). All other reagents were of analytical grade.

Platelet preparation and measurement of [Ca²⁺]_i

Fura 2-loaded platelets were prepared as previously described [16]. Platelet-rich plasma was incubated at 37 °C with 2 µM fura-2/AM for 45 min. For loading with dimethyl-BAPTA, cells were incubated for 30 min at 37 °C with 10 µM dimethyl-BAPTA/AM. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in Hepes-buffered saline, containing 10 mM Hepes (pH 7.45), 145 mM NaCl, 10 mM D-glucose, 5 mM KCl and 1 mM MgSO₄, supplemented with 0.1% BSA and 40 µg/ml apyrase. Fluorescence was recorded from 1.5 ml aliquots of magnetically-stirred platelet suspension (10⁸ 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 an emission wavelength of 500 nm. Changes in [Ca²⁺]_i were monitored using the fura 2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. [17].

Immunoprecipitation and Western blotting

Aliquots (500 µl) of platelet suspension (2 × 10⁹ cells/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% (v/v) sodium deoxycholate, 2% (w/v) Triton X-100, 2 mM Na₃VO₄, 2 mM PMSF, 100 µg/ml leupeptin and 10 mM benzamide. Insoluble cell debris was removed by centrifugation and cell lysates were precleared with Protein A-agarose. Aliquots of platelet lysates (1 ml) were immunoprecipitated by incubation with 3 µg of anti-hTrp1 polyclonal antibody, 3 µg of anti-IP₃RI polyclonal antibody, 3 µg of anti-IP₃RII polyclonal antibody or 3 µg of anti-IP₃RIII polyclonal antibody overnight at 4 °C on a rocking platform, followed by the addition of Protein A-agarose and incubation for 1 h at 4 °C. The immunoprecipitates were resolved by SDS/PAGE [8% (w/v) polyacrylamide], and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm², in a semi-dry blotter (Hoefer Scientific, Newcastle under-Lyme, Staffs., U.K.), on to nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein-binding sites. Immunodetection of hTrp1 or IP₃R types I, II or III was achieved using the anti-hTrp1 antibody diluted 1:200 in TBST or with anti-IP₃RI, anti-IP₃RII or anti-IP₃RIII antibodies diluted 1:500 in TBST for 3 h. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG antibody or horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody diluted 1:10000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to preflashed photographic film.

Statistical analysis

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

RESULTS AND DISCUSSION

Requirement of IP₃ for SMCE in human platelets

The possible requirement of IP₃ for SMCE was investigated by blocking the recycling of IP₃ [18]. This was achieved by incubating fura 2-loaded platelets with Li⁺ (10 mM) for 2 h. In control cells, in the absence of external Ca²⁺, the physiological agonist thrombin evoked a substantial rise in [Ca²⁺]_i due to the release of Ca²⁺ from intracellular stores (Figure 1A). The initial peak in [Ca²⁺]_i above basal levels after treatment with 0.1 unit/ml thrombin was 230 ± 27 nM (mean ± S.E.M.; *n* = 6). The thrombin-evoked rise in [Ca²⁺]_i was almost abolished after pretreatment with Li⁺ (Figure 1B; *n* = 6, *P* < 0.001), although the subsequent addition of sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase inhibitor TG (250 nM) [19] with ionomycin (50 nM) was able to release Ca²⁺ from the stores. This confirmed that Li⁺ treatment was essentially eliminating IP₃ production, while leaving the Ca²⁺ stores intact. Addition of TG (200 nM) alone in the absence of extracellular Ca²⁺ resulted in a small rise in [Ca²⁺]_i as the Ca²⁺ stores depleted, and the subsequent addition of CaCl₂ (300 µM) resulted in a large rise in [Ca²⁺]_i indicative of SMCE (Figure 1D). TG (200 nM) itself evoked a similar rise in [Ca²⁺]_i in Li⁺-treated cells, but SMCE on the addition of Ca²⁺ was essentially abolished (Figure 1E; *n* = 8, *P* < 0.001). To check that the effect of Li⁺ was mediated by blocking the recycling of IP₃ we performed a series of experiments adding *myo*-inositol (10 mM) at the same time as Li⁺. As shown in Figure 1(C), incubation of platelets with *myo*-inositol prevented the inhibitory effect of Li⁺ on thrombin-evoked release of Ca²⁺ from the internal stores (*n* = 5). In addition, incubation with *myo*-inositol overcame the effect of Li⁺ on TG-induced SMCE (Figure 1F; *n* = 5). These findings indicate that the effect of Li⁺ is mediated by interference with IP₃ production.

To further explore the requirement of IP₃ for SMCE we used the cell-permeant inhibitor of IP₃R function, Xest C [20]. Pretreatment of platelets with Xest C (20 µM) for 30 min essentially abolished thrombin-evoked release of Ca²⁺ from intracellular stores (Figures 2A and 2B; *n* = 5, *P* < 0.001). Xest C did not alter TG-evoked release of Ca²⁺ from the intracellular stores, but completely abolished the subsequent SMCE observed on the addition of CaCl₂ (300 µM) (Figures 2C and 2D; *n* = 5, *P* < 0.001).

We also investigated the effect of adding Xest C after SMCE had been activated using TG. Substantial SMCE was detectable on the addition of CaCl₂ (300 µM) 3 min after store depletion was initiated by the addition of TG (200 nM) (Figure 2E, left side). Addition of Xest C 3 min after TG resulted in complete abolition of Ca²⁺ entry when CaCl₂ was added 30 min later, whereas substantial SMCE was seen in vehicle-treated controls (Figure 2E, right side; *n* = 6, *P* < 0.001). Similar results were obtained when Li⁺ was added after store depletion was initiated using TG (results not shown). The effects of Li⁺ and Xest C indicate that basal IP₃ production is essential for both the activation and maintenance of SMCE in human platelets.

Presence of Trp1 in human platelets

The nature of the channels conducting SMCE in platelets is unknown. Human platelets have been shown to contain mRNA for hTrp1 and its splice variant hTrp1A (D. Molin, E. den Dekker, G. Breikers, R. van Oerle, J. W. Akkerman and J. W. A. Heemskerk, personal communication). Both hTrp1 [21] and hTrp1A [22] have been shown to form non-selective cation channels. The transfection of HEK-293 or human

Figure 1 Effects of Li^+ on Ca^{2+} signalling in fura 2-loaded human platelets

Human platelets were loaded with fura 2 and resuspended in a Ca^{2+} -free medium as described in the Materials and methods section. Elevations in $[\text{Ca}^{2+}]_i$ were monitored by using the 340/380 nm ratio and traces were calibrated in terms of $[\text{Ca}^{2+}]_i$. At the time of the experiment 100 μM EGTA was added. (A) Platelets were treated with thrombin (0.1 unit/ml) at the time indicated. (B) After treatment with Li^+ (10 mM) for 2 h platelets were stimulated with thrombin (0.1 unit/ml) followed by the application of TG (250 nM) and ionomycin (IONO; 50 nM), as indicated. (C) Platelets were treated with Li^+ (10 mM) and myo-inositol (10 mM) for 2 h, platelets were then stimulated with thrombin (0.1 unit/ml). The traces shown are representative of five to six separate experiments. Platelets were treated for 2 h in the absence (D) or in the presence (E) of Li^+ (10 mM) or with a combination of Li^+ (10 mM) and myo-inositol (10 mM) (F). Cells were then stimulated with TG (200 nM) and 3 min later CaCl_2 (final concentration 300 μM) was added to the medium to initiate Ca^{2+} entry. The traces shown are representative of five to eight independent experiments.

submandibular-salivary-gland cell lines with hTrp1 has been reported to increase SMCE [10,23], indicating that hTrp1, unlike some other Trp isoforms [24,25] is regulated by the filling state of the intracellular Ca^{2+} stores. SMCE in human pulmonary-artery-endothelial cells has been suggested to be mediated by hTrp1 [26].

We therefore tested for the presence of hTrp1 in human platelet lysates by SDS/PAGE and Western blotting using an anti-hTrp1 antibody, specific for the amino acid residues 557–571 of hTrp1, that has been shown to be effective in Western blotting, immunoprecipitation and immunolocalization studies [10,27]. Immunoblotting of platelet whole-cell lysates with the anti-hTrp1 antibody revealed the presence of hTrp1 in these cells (Figure 3A, lane 1; $n = 4$). Detection of hTrp1 was abolished when the anti-hTrp1 antibody was previously incubated for 1 h with a control antigen peptide, confirming the specificity of the antibody. These data support the reported presence of hTrp1 mRNA in human platelets (D. Molin, E. den Dekker, G. Breikers, R. van Oerle, J. W. Akkerman and J. W. A. Heemskerk, personal communication) and indicate that hTrp1 is expressed in these cells.

Coupling between IP_3 Rs and Trp1

Platelets are reported to express the IP_3 R isoforms I, II and III [28,29]. Isoform I has been located in the intracellular membranes [28,29], in contrast to isoform III that has been found exclusively at the PM [29]. IP_3 RII exhibits a dual distribution [28,29]. We therefore tested for coupling between these IP_3 Rs and hTrp1 by looking for co-immunoprecipitation from platelet lysates. Immunoprecipitation and subsequent SDS/PAGE and

Western blotting were conducted using control platelets and platelets in which the intracellular Ca^{2+} stores had been depleted by 3 min of pretreatment with TG (1 μM) and ionomycin (50 nM) in the absence of extracellular Ca^{2+} (100 μM EGTA). Platelets heavily loaded with the Ca^{2+} -chelator indicator, dimethyl-BAPTA, were used for this study so as to eliminate Ca^{2+} , but not store depletion-dependent responses [30]. After immunoprecipitation with the anti-hTrp1 antibody, Western blotting revealed the presence of IP_3 RII in samples from store-depleted, but not control platelets, while IP_3 RI and IP_3 RIII were undetectable in samples from either control or depleted cells (Figure 3B, top panel; $n = 4$). Western blotting with anti-hTrp1 confirmed a similar content of this protein in all lanes (Figure 3B, bottom panel).

We also conducted converse experiments, immunoprecipitating platelet lysates with anti- IP_3 RI, anti- IP_3 RII or anti- IP_3 RIII antibodies and detecting for the presence of hTrp1. After immunoprecipitation with anti- IP_3 RI or anti- IP_3 RIII, hTrp1 was undetectable in samples from control or Ca^{2+} -store-depleted platelets immunoblotted with anti-hTrp1; however, hTrp1 was detected in samples from store-depleted, but not control cells, after immunoprecipitation with anti- IP_3 RII (Figure 3C, upper panel; $n = 4$). Immunoblot analysis of anti- IP_3 RI, anti- IP_3 RII or anti- IP_3 RIII immunoprecipitates with the same antibodies, as appropriate, revealed a similar content of these proteins in the relevant lanes and furthermore confirmed the presence of all three receptor isoforms in these cells (Figure 3C, lower panels) in agreement with previous studies [29]. The results from the present study indicate that hTrp1 is present in human platelets and that this protein couples with IP_3 R type II, but not type I or type III, when the intracellular Ca^{2+} stores are depleted. Furthermore, our

Figure 2 Effects of the IP₃R antagonist Xest C on platelet Ca²⁺ signalling

Human platelets were loaded with fura 2 and resuspended in a Ca²⁺-free medium as described in the Materials and methods section. Elevations in [Ca²⁺]_i were determined as described in the legend to Figure 1. At the time of the experiment 100 μM EGTA was added. Platelets were treated for 30 min with Xest C (20 μM) (B and D) or the vehicle (DMSO) (A and C). Cells were then stimulated with thrombin (0.1 unit/ml) (A and B) or with TG (200 nM) followed by the addition of CaCl₂ (final concentration 300 μM) 3 min later to initiate Ca²⁺ entry (C and D). (E) Platelets were stimulated with TG (200 nM) and 3 min later Xest C (20 μM) or the vehicle (DMSO; control *t* = 33 min) was added. CaCl₂ (final concentration 300 μM) was added to the medium at the same time, as a control (control *t* = 3 min) or 30 min after Xest C or the vehicle to initiate Ca²⁺ entry. Traces are representative of five to six independent experiments.

results indicate that only homotetramers of IP₃R type II couple with hTrp1 upon store depletion. Our observations, showing that hTrp1 and IP₃RII couple in response to depletion of the intracellular Ca²⁺ stores, in agreement with earlier reports that IP₃RII is the only isoform located both in the PM and in the intracellular membranes in platelets [28,29], suggest this mechanism as a candidate for the mediation of SMCE in normal human cells. The involvement of hTrp1, which has been shown to form non-selective cation channels [21], in SMCE in platelets may explain earlier reports that TG evokes a rise in cytosolic [Na⁺] in these cells [12].

In HEK-293 cells stably expressing haemagglutinin (HA)-tagged hTrp3, anti-HA antibodies co-immunoprecipitated tagged hTrp3 and IP₃RI, suggesting a coupling between hTrp3 and IP₃RI in these cells [12]. Another study using HEK-293 cells stably expressing HA- or c-Myc-tagged Trp3 or Trp6 has demonstrated co-immunoprecipitation of IP₃RIII with anti-

Figure 3 hTrp1 is present in human platelets and co-immunoprecipitates with IP₃RII

(A) Human platelets were lysed and whole-cell lysate was analysed by Western blotting using either anti-hTrp1 antibody (lane 1) or with anti-hTrp1 antibody preincubated with the control antigen peptide (CAP; lane 2) as described in the Materials and methods section. The position of hTrp1 is indicated on the right. (B) Dimethyl-BAPTA-loaded human platelets were treated with no addition (control) or with 1 μM TG and 50 nM ionomycin (store-depleted) and then lysed. Whole-cell lysates were immunoprecipitated (IP) with anti-hTrp1 antibody. Immunoprecipitates were analysed by Western blotting (WB) using either anti-IP₃R type I, II or III antibodies (top panel) or anti-hTrp1 antibody (bottom panel) as described in the Materials and methods section. (C) Dimethyl-BAPTA-loaded human platelets were treated with no addition (control) or with 1 μM TG and 50 nM ionomycin (store-depleted) and then lysed. Whole-cell lysates were immunoprecipitated with anti-IP₃R type I, II or III antibodies. Immunoprecipitates were analysed by Western blotting using either anti-hTrp1 antibody (upper panel) or anti-IP₃R type I, II or III antibodies, as indicated (lower panels). Positions of molecular-mass markers are shown on the left. These results are representative of four independent experiments.

bodies to both of these Trps and co-immunoprecipitation of both Trps with an antibody recognising all three IP₃R isoforms [13]. Recently, co-immunoprecipitation of hTrp1 with IP₃RIII in a human submandibular-salivary-gland cell line stably transfected with hTrp1 has been reported [11]. In these cells, hTrp1 and IP₃RIII appeared to form part of a multimeric signalling complex also including caveolin-1 and Gα_{q/11}. We found no evidence for coupling between hTRP1 and IP₃RIII in human platelets. It is possible that this reflects the reported lower level of expression of IP₃RIII compared with the other isoforms in platelets [29] and variations in antibody sensitivity. However, since IP₃RIII has been reported to be exclusively located in the PM of human platelets [29], it seems unlikely that this isoform could play a role in the coupling mechanism of SMCE.

In all earlier studies [11–13], the reported coupling between IP₃Rs and Trps appeared to be present independently of Ca²⁺ store depletion. These results [11–13] from transfected cell lines thus suggest relatively stable interactions between IP₃Rs and Trps, with channel activity depending on changes in IP₃ concentration and/or store Ca²⁺ content. In contrast, in human platelets

we found coupling between hTrp1 and IP₃R II only in cells with depleted Ca²⁺ stores. This finding is compatible with the secretion-like coupling model for SMCE, where reorganization of the actin cytoskeleton is required for the activation of Ca²⁺ entry, apparently by allowing the translocation of the ER to the PM in order to permit coupling between proteins in the two membranes to occur [3,5].

Our findings shed new light on the mechanisms involved in SMCE in human platelets. We have shown that IP₃ and functional IP₃Rs are required for SMCE in human platelets. These cells contain the putative SMCE channel, hTrp1, and depletion of the intracellular Ca²⁺ stores results in the coupling of this protein to IP₃R II, but not IP₃R I or IP₃R III. These results provide the first direct evidence for the mediation of SMCE by conformational coupling *de novo*, involving IP₃Rs in the ER membrane and Ca²⁺ channels in the PM of normal human cells.

J. A. R. is supported by a Grant of Junta de Extremadura-Consejería de Educación y Juventud and Fondo Social Europeo, Spain. This work was supported in part by the Wellcome Trust (051560).

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Received 19 June 2000/11 July 2000; accepted 1 August 2000