Transient receptor potential ankyrin-1 (TRPA1) modulates store-operated Ca²⁺ entry by regulation of STIM1-Orai1 association

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ABSTRACT

TRPA1 is a non-selective Ca²⁺ permeable channel located in the plasma membrane that functions as a cellular sensor detecting mechanical, chemical and thermal stimuli, being a component of neuronal, epithelial, blood and smooth muscle tissues. TRPA1 has been shown to influence a broad range of physiological processes that involve Ca²⁺-dependent signaling pathways. Here we report that TRPA1 is expressed in MEG01 but not in platelets at the protein level. MEG01 cells maturation induced by PMA results in attenuation of TRPA1 protein expression and enhances thapsigargin-evoked Ca²⁺ entry without altering the release of Ca²⁺ from intracellular stores. Inhibition of TRPA1 by HC-030031 results in enhancement of both thrombin- and thapsigargin-stimulated Ca²⁺ entry. Co-immunoprecipitation experiments revealed that TRPA1 associates with STIM1, as well as Orai1, TRPC1 and TRPC6. Downregulation of TRPA1 expression by MEG01 maturation, as well as pharmacological inhibition of TRPA1 by HC-030031, results in enhancement of the association between STIM1 and Orai1. Altogether, these findings provide evidence for a new and interesting function of TRPA1 in cellular function associated to the regulation of agonist-induced Ca²⁺ entry by the modulation of STIM1/Orai1 interaction.

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1. Introduction

Transient receptor potential (TRP) ankyrin 1 (TRPA1) is a non-selective Ca²⁺ permeable channel that belongs to the TRP superfamily [1]. Like other TRP channels, the architecture of TRPA1 includes six transmembrane domains (TMD) with the presumed pore loop between TMD5 and TMD6 [2]. TRPA1 is structurally differentiated by a large number of ankyrin repeats, which vary among species (up to 18 repeats in humans), located in the intracellular N-terminal region of the protein [3]. Ankyrin repeats are proposed as a key integration site for multiple physiological signals that activates the channel [4]. Furthermore, the C-terminal region of the channel comprises basic residues that confer both chemical and voltage sensitivity to TRPA1 channel [5].

Activation of TRPA1 by physical stimuli (i.e., cold temperature, ultraviolet light) or endogenous (i.e., Ca²⁺, reactive oxygen species, nitric oxide) and exogenous (i.e., allyl isothiocyanates) ligands results in a massive influx of Ca²⁺ into the stimulated cells [6] which actives a wide range of physiological events in different cell types [7–12]. Ca²⁺ is considered the most important physiological activator of TRPA1 [6]. A recent model proposes that ankyrin repeat structure includes a Ca²⁺-binding EF-hand domain which changes the overall flexibility of the channel structure and modulates the open probability by acting as a soft spring [13]. Another novel study proposes that the acidic cytoplasmic C-terminal tail is also involved in the potentiation and inactivation of TRPA1 channel [6].

Store-operated Ca²⁺ entry (SOCE), the most important mechanism for Ca²⁺ influx in non-excitable mammalian cells, is controlled by the filling state of the intracellular Ca²⁺ stores [14]. Different molecules have been reported to be involved in SOCE. Stromal interaction molecule 1 (STIM1) is a Ca²⁺-binding protein identified both in the plasma membrane and in the membrane of intracellular Ca²⁺ stores, the endoplasmic reticulum (RE) and acidic stores [15–17]. Depletion of these intracellular Ca²⁺ stores is detected by STIM1, which re-localizes at sites of close proximity to the plasma membrane and the ER membrane allowing its association with two types of Ca²⁺ influx SOC channels: the Orai family and TRP canonical (TRPC) family channels [18,19].

SOCE has been reported to be modulated by different cell parameters such as temperature [20] and redox status [21] and by intracellular mechanisms like STIM1 phosphorylation [22] and adaptor proteins like Homer family proteins [23]. Recent studies have demonstrated that proteins implicated in the regulation of the intracellular Ca²⁺ homeostais like the two-pore Ca²⁺ channel 2 (TPC2) [24], the sarco/endoplasmic reticulum Ca²⁺-ATPases (SERCA) pump family [25,26], and the TRPC3 [27,28] modulates SOCE in different type cells by the regulation of the association of STIM1 with Orai1.
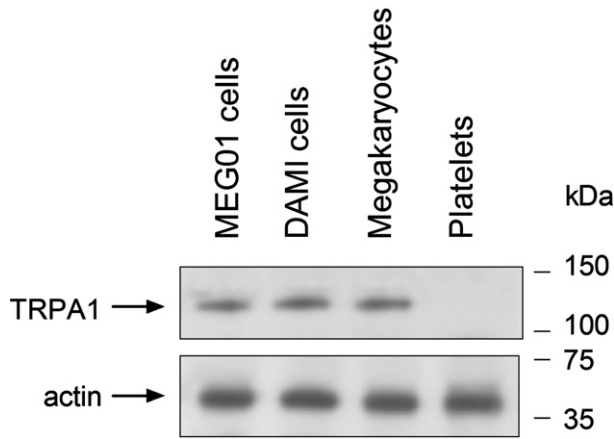
Fig. 1. TRPA1 expression in the human megakaryoblastic cell line MEG01 cells, DAMI cells, megakaryocytes and human platelets. MEG01, DAMI, megakaryocytes and platelet protein lysates were immunoblotted, as described in Materials and methods section, to detect the presence of TRPA1 channel. Membranes were probed with anti-actin antibody for protein loading control. The results are representative of 3 independent experiments.

In the present study we have investigated the possible role of TRPA1 channels in the regulation of SOCE in the megakaryoblastic lineage. Our results indicate that TRPA1 expression decreases during PMA-induced MEG-01 maturation and it is correlated with an increase of SOCE mediated by an enhanced interaction of STIM1 and Orai1, which suggests a functional role for TRPA1 in the modulation of SOCE in these cells.
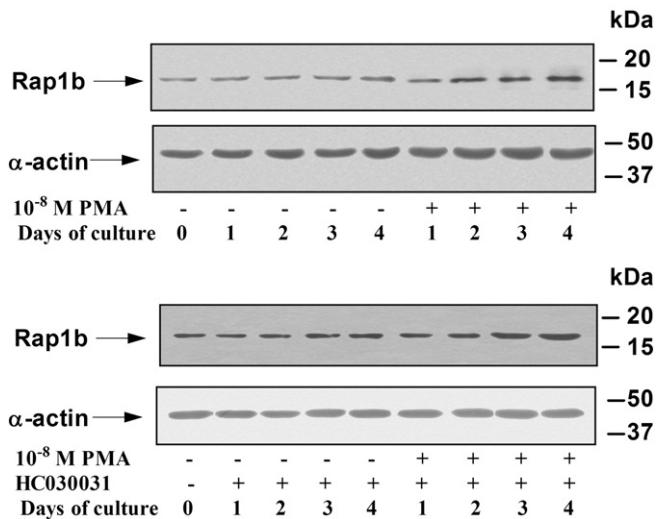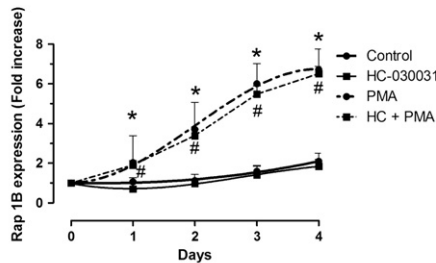

Fig. 2. Enhanced Rap1b expression during maturation of MEG-01 megakaryocytic cell lines induced by PMA treatment. MEG01 cells were cultured in the absence or presence of 10 nM PMA for up to 0 to 4 days and treated or not with 60 μM HC-030031. Protein lysates were immunoblotted for detect the presence of Rap1b. The bottom panels show representatives images of Western blotting experiments. The mean value of the cells treated for day 0 was arbitrarily taken as 100%. The results are representative of 6 independent experiments. $^*p < 0.05$ compared with their control. $^\#p < 0.05$ compared with their control (cells treated with HC-030031).

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM) and calcein were from Invitrogen (Madrid, Spain). Thrombin, thapsigargin (TG), apyrase (grade VII), dimethyl BAPTA/AM, aspirin, leupeptin, benzamidine, phenyl methyl sulphonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), rabbit anti-Orai1 antibody (C-terminal), 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide (HC-030031), allyl isothiocyanate (AITC), nonidet P-40, phorbol 12-myristate 13-acetate (PMA) and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Mouse anti-STIM1 antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, U.S.A.). Rabbit anti-hTRPC1 antibody, goat anti-hTRPC6 antibody and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were from Jackson Immunoresearch (Suffolk, U.K.). Hyperfilm ECL was from Amersham (Buckinghamshire, U.K.). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U.K.). All other reagents were of analytical grade.

2.2. Platelet preparation

Platelets were prepared as previously described [29], as approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Briefly, blood was obtained from healthy drug-free 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 (PRP) was then prepared by centrifugation for 5 min at 700 ×g and aspirin (100 μM) and apyrase (40 μg/mL) were added. Cells were then collected by centrifugation at 350 ×g for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO₄ and supplemented with 0.1% (w/v) BSA and 40 μg/mL apyrase. For BAPTA loading, cells were incubated with 10 μM dimethyl BAPTA for 30 min and then resuspended in HBS.

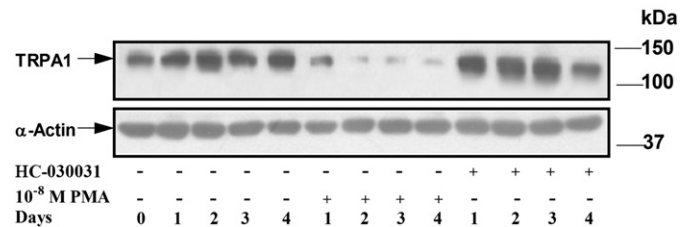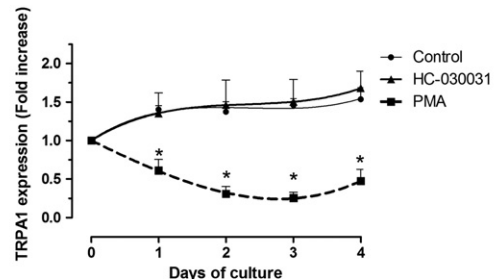**Fig. 3.** Abolished TRPA1 expression during PMA-induced maturation of MEG-01 cells. MEG01 cells were cultured in the absence or presence of 10 nM PMA and/or 60 μM HC-030031 for 0 to 4 days. Protein lysates were inmunoblotted to detect TRPA1 expression. The bottom panels show images representative of five others. The mean value of the cells at day 0 was arbitrarily taken as 100%. *, $p < 0.05$ compared with control.
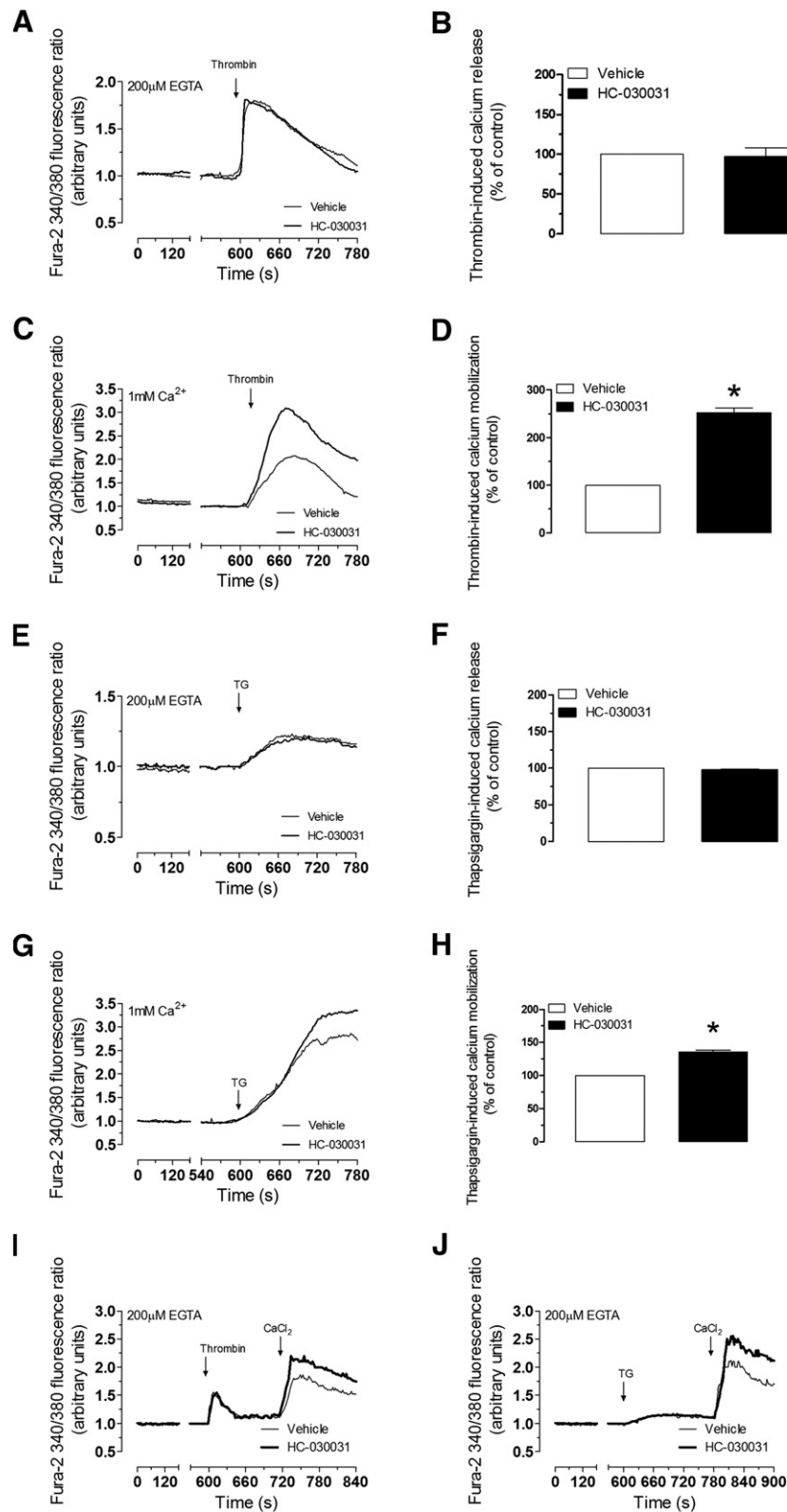
Fig. 4. Effect of HC-030031 on thrombin and thapsigargin-evoked Ca^{2+} entry in MEG01 cells and megakaryocytes. Fura 2-loaded MEG01 cells were incubated at 37 °C for 10 min in the presence of 60 µM HC-030031 or the vehicle (control) and then stimulated with 0.1 U/mL thrombin (A and C) or 1 µM thapsigargin (E and G) in a Ca^{2+} free medium (200 µM EGTA) (A and E) or in the presence of 1 mM extracellular Ca^{2+} (C and G). Traces shown are representative of 8 separate experiments. Histograms represent the amount of Ca^{2+} mobilization induced by agonists in the presence of HC-030031 as compared with their respective controls. Values are means ± S.E.M. *, $p < 0.05$ compared with control. I and J, fura 2-loaded megakaryocytes were incubated at 37 °C for 10 min in the presence of 60 µM HC-030031 or the vehicle (control) and then stimulated with 0.1 U/mL thrombin (I) or 1 µM thapsigargin (J) in a Ca^{2+} free medium (200 µM EGTA), subsequently, $CaCl_2$ (1 mM) was added to the extracellular medium to initiate Ca^{2+} entry. Traces shown are representative of 3 separate experiments.

2.3. Cell culture

The human megakaryoblastic cell lines MEG01 and DAMI were obtained from ATCC (Manassas, VA, USA) and cultured at 37 °C with a 5% CO_2 in RPMI media, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg/mL gentamycin.

At the time of the experiments cells were suspended in basic salt solution (BSS) containing (in mM): 125 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 25 HEPES, and pH 7.3. This is supplemented with 0.1% (w/v) BSA and apyrase (0.02 U/mL).

Megakaryocytic cell-line maturation was induced using PMA, a protein kinase C activator [30]. For this purpose, MEG01 cells were cultured in RPMI media containing 10^{-8} M PMA for up to 4 days of culture.

2.4. Isolation of human megakaryocytes

Human megakaryocytes were isolated as described previously [31]. Briefly, umbilical cord blood was collected under informed consent during normal full-term deliveries and used within 48 h. CD34+ stem cells were isolated using the miniMACS CD34 progenitor cell isolation system from Miltenyi Biotec (Bergisch Gladbach, Germany). Cells were cultured for either 7 or 14 days. Day 7 cells were labeled with anti-CD61 antibody (7F12) and day 14 cells were labeled with anti-CD42b antibody, for 20 min at 4 °C. Cells were then labeled for 20 min (4 °C) with goat-anti-mouse magnetic microbeads. The cell suspension was brought on a ferromagnetic column, type MS+ (Miltenyi Biotec), and after three washing steps with 700 μl buffer A (PBS containing 0.5% BSA and 2.5 mM EDTA) each, the retained cells were eluted with buffer A.

2.5. Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [32]. Briefly, 500 μL aliquots of MEG01 suspension

(1×10^7 cell/mL) were lysed with an equal volume of NP-40 buffer, pH 8, containing 274 mM NaCl, 40 mM Tris, 4 mM EGTA, 20% glycerol, 2% nonidet P-40, 2 mM Na_3VO_4, 2 mM PMSF, 100 μg/mL leupeptin and 10 mM benzamidine.

Aliquots of MEG01 lysates (1 mL) were immunoprecipitated by incubation with 2 μg of either anti-STIM1, anti-Orai1, anti-hTRPC1 or anti-hTRPC6 and 25 μL of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 8% SDS–PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated for 1 h at room temperature with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of STIM1, Orai1, hTRPC1 and hTRPC6 was achieved using the anti-STIM1 antibody or the anti-Orai1 antibody diluted 1:250 in TBST for 2 h and the anti-hTRPC1 or anti-hTRPC6 diluted 1:200 in TBST for 2 h respectively at room temperature. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated for 45 min with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:10000 in TBST and then exposed to enhanced chemiluminiscence reagents for 4 min. Blots were then exposed to photographic films. The density of bands on the film was measured using scanning densitometry and analyzed using ImageJ software for gel analysis from NIH. Data were normalized to the amount of actin protein or the amount of protein recovered by the antibody used for the immunoprecipitation.

For each antibody-based immunoprecipitation we performed an antibody-free, protein A-agarose only control in order to ensure that the proteins immunoprecipitated are not pulled down by protein A-agarose itself. In addition, for each antibody used in Western blotting a primary antibody-free control was carried out to confirm that the detection is specific and not due to non-specific binding by the secondary antibody (data not shown).
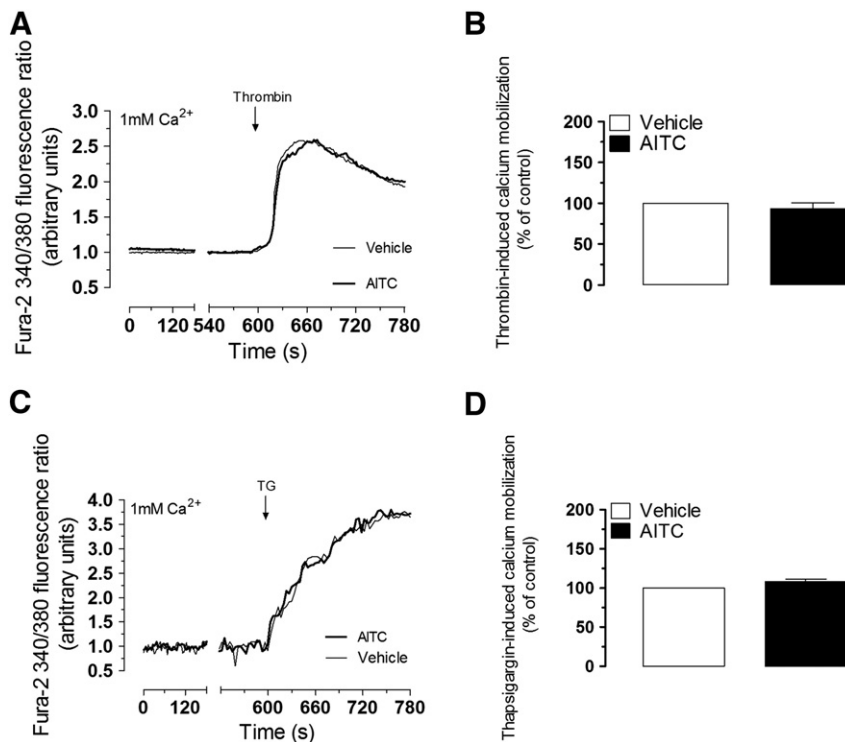
Fig. 5. Effect of allyl isothiocyanate (AITC) on thapsigargin and thrombin-evoked Ca^{2+} entry in MEG01 cells. Fura 2-loaded MEG01 cells were incubated at 37 °C for 10 min in the presence of 5 μM AITC or the vehicle (control) and then stimulated with 0.1 U/mL thrombin (A) or 1 μM thapsigargin (C) in the presence of 1 mM extracellular Ca^{2+}. Traces shown are representative of 8 separate experiments. B and D, Histograms indicating the amount of Ca^{2+} mobilization induced by 0.1 U/mL thrombin (B) and 1 μM thapsigargin (D). Values are means ± S.E.M.

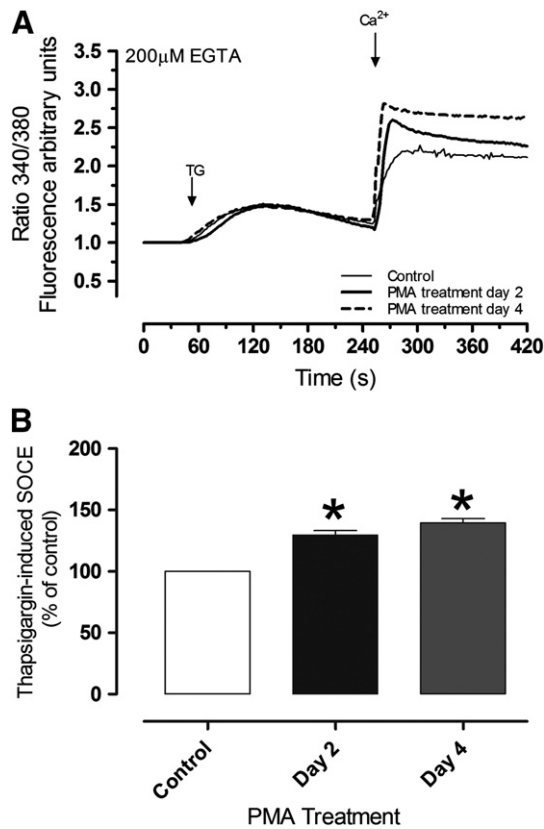**Fig. 6.** Effect of PMA-induced maturation on thapsigargin-evoked Ca^{2+} entry in MEG01 cells. MEG01 cells were cultured in the absence (control) or presence of 10 nM PMA during 2 and 4 days. Afterwards, fura 2-loaded MEG01 cells were stimulated with 1 μM thapsigargin in a Ca^{2+} free medium, and 3 min later 600 μM Ca^{2+} was added to initiate Ca^{2+} entry. (A) The traces shown are representative of 5 separate experiments. B, Histograms indicating the amount of thapsigargin-induced Ca^{2+} entry after 2 and 4 days of treatment with PMA as compared to control. Values are means ± S.E.M. *, $p < 0.05$ compared with control.

2.6. Measurement of cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells were loaded with fura-2 by incubation with 2 μM fura 2/AM for 45 min at 37 °C. Coverslips with cultured cells were mounted on a perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA) with image acquisition and analysis system for videomicroscopy (Hamamatsu Photonics, Hamamatsu, Japan). Cells were continuously superfused with BSS. Cells were alternatively excited with light from a xenon lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using Aquacosmos 2.5 software (Hamamatsu Photonics, Hamamatsu, Japan). Ca^{2+} entry was estimated using the integral of the rise in fura-2 fluorescence 340/380 nm ratio for 2 min after addition of $CaCl_2$. Ca^{2+} release was estimated using the integral of the rise in fura-2 fluorescence 340/380 nm ratio for 2 min after the addition of thrombin or TG, in presence or absence of 60 μM HC-030031 or 5 μM AITC.

To calculate the initial rate of Ca^{2+} elevation after the addition of Ca^{2+} to the medium, the traces were fitted to the equation $y = A + Kx$ where K is the slope.

2.7. Statistical analysis

Analysis of statistical significance was performed using Student's t-test for comparison between two groups. $p < 0.05$ was considered to be significant for a difference.

3. Results

3.1. TRPA1 is expressed in human MEG01 and DAMI cells and megakaryocytes but not in platelets

Even though TRPA1 mRNA expression has been reported in human platelets [33], we have further explored the expression of TRPA1 at the protein level in human platelets by using Western blotting. As shown in Fig. 1, Western blotting of whole cell platelet lysates using a specific anti-TRPA1 antibody failed to detect TRPA1 in human platelets. By contrast, under the same experimental conditions, the anti-TRPA1 antibody was able to detect a single band, at 140 kDa (the predicted TRPA1 size), in the human megakaryoblastic cell lines MEG01 and DAMI and human megakaryocytes (Fig. 1A).

3.2. Effect of MEG01 maturation in TRPA1 expression

Since we have observed that TRPA1 protein is not expressed in human platelets but it was found in the megakaryoblastic cell line MEG01 we explored whether the expression of this channel is modified during the process of maturation. Maturation was induced using PMA, an activator of protein kinase C [30]. Rap1b protein, a ubiquitous Ras-related GTPase that increased with megakaryocytic maturation, was used as a maturation marker [34–37]. Treatment of MEG01 cells with 10 nM PMA resulted in a significant and time-dependent increase in Rap1b expression as compared to control cells treated with vehicle, where the expression of Rap1b was not modified during the 4 days of the experiment (Fig. 2, top panel and graph; $p < 0.05$; $n = 6$). In order to investigate the possible role of TRPA1 in maturation, cells were treated with the TRPA1 specific inhibitor HC-030031 [38–40]. MEG01 cells were treated with 60 μM HC-030031 and the expression of Rap1b was analyzed every 24 h during 4 days. As shown in Fig. 2, treatment with HC-030031 neither modified the expression of Rap1b per se nor altered PMA-induced Rap1b expression increase; thus demonstrating that TRPA1 activity has a negligible effect, if any, in MEG01 cell maturation. Reprobing of the same membranes with anti-actin antibody was performed for protein loading control and revealed a similar amount of protein loaded in all lanes (Fig. 2, bottom panels).

As reported in Fig. 3, treatment of MEG01 cells with 10 nM PMA significantly reduced TRPA1 expression in a time-dependent manner, with a detectable attenuation of TRPA1 protein expression after 24 h of treatment with PMA ($p < 0.05$; $n = 6$). Reprobing of the same samples with anti-actin antibody revealed a comparable amount of protein loaded in all lanes (Fig. 3, bottom panel). By contrast, MEG01 cells treatment with the TRPA1 inhibitor did not alter TRPA1 protein expression at least during the first 4 days of treatment as compared to control (vehicle added); thus confirming that this inhibitor does not modify the expression of TRPA1. These findings indicate that, while TRPA1 function is not relevant for MEG01 maturation induced by PMA, maturation itself significantly attenuates TRPA1 protein expression, thus explaining the expression of TRPA1 in megakaryoblastic cells but not in mature human platelets.

3.3. Role of TRPA1 in Ca^{2+} mobilization

Since TRPA1 is expressed in MEG01 cells we have investigated its involvement in Ca^{2+} mobilization induced by physiological agonists. As shown in Fig. 4A and B, treatment of MEG01 cells with thrombin in a Ca^{2+}-free medium resulted in a transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from intracellular Ca^{2+} stores [41]. Pretreatment of the cells for 10 min with 60 μM HC-030031, a TRPA1 inhibitor [38], did not significantly alter either resting $[Ca^{2+}]_i$ or thrombin-induced Ca^{2+} release (the integral of the rise in fura-2 fluorescence 340/380 nm ratio for 2 min after the addition of thrombin in the absence or presence of HC-030031 were 3087 ± 504 and 2994 ± 412, respectively; $n = 8$). In the presence of 1 mM extracellular Ca^{2+}, thrombin induced a greater elevation in $[Ca^{2+}]_i$ than that obtained in a Ca^{2+}-free medium as a

result of Ca^{2+} entry (Fig. 4C). Interestingly, treatment of MEG01 cells with HC-030031 significantly enhanced thrombin-evoked Ca^{2+} mobilization in the presence of 1 mM extracellular Ca^{2+}, thus, suggesting that inhibition of TRPA1 function enhances Ca^{2+} entry evoked by thrombin (Fig. 4C and D, $p < 0.05$; $n = 8$).

Thrombin has been reported to induce both capacitative (store-operated) and non-capacitative Ca^{2+} entry [42]. Hence, we have investigated the role of TRPA1 in store-operated Ca^{2+} entry (SOCE) in MEG01 cells. In a Ca^{2+}-free medium, TG, a specific inhibitor of the sarco/endoplasmic-reticulum Ca^{2+}-ATPase [43], evoked a sustained elevation of $[Ca^{2+}]_i$ in MEG01 cells owing to the release of Ca^{2+} from intracellular pools, which was insensitive to treatment of MEG01 cells

with the TRPA1 inhibitor HC-030031 (the integral of the rise in fura-2 fluorescence 340/380 nm ratio for 2 min after the addition of TG in the absence or presence of HC-030031 was 1207 ± 201 and 1170 ± 112, respectively; Fig. 4E and F). In the presence of 1 mM extracellular Ca^{2+} TG-evoked a prolonged and greater increase in $[Ca^{2+}]_i$ due to both Ca^{2+} release from internal stores and the subsequent activation of SOCE. As for thrombin, treatment with HC-030031 significantly enhanced TG-evoked Ca^{2+} mobilization in the presence of extracellular Ca^{2+}, which is entirely dependent on the effect of TRPA1 on the activation of SOCE (Fig. 4G and H, $p < 0.05$; $n = 8$). Similar results were observed in human megakaryocytes. As shown in Fig. 4I and J, treatment of megakaryocytes with thrombin or TG in a Ca^{2+}-free medium
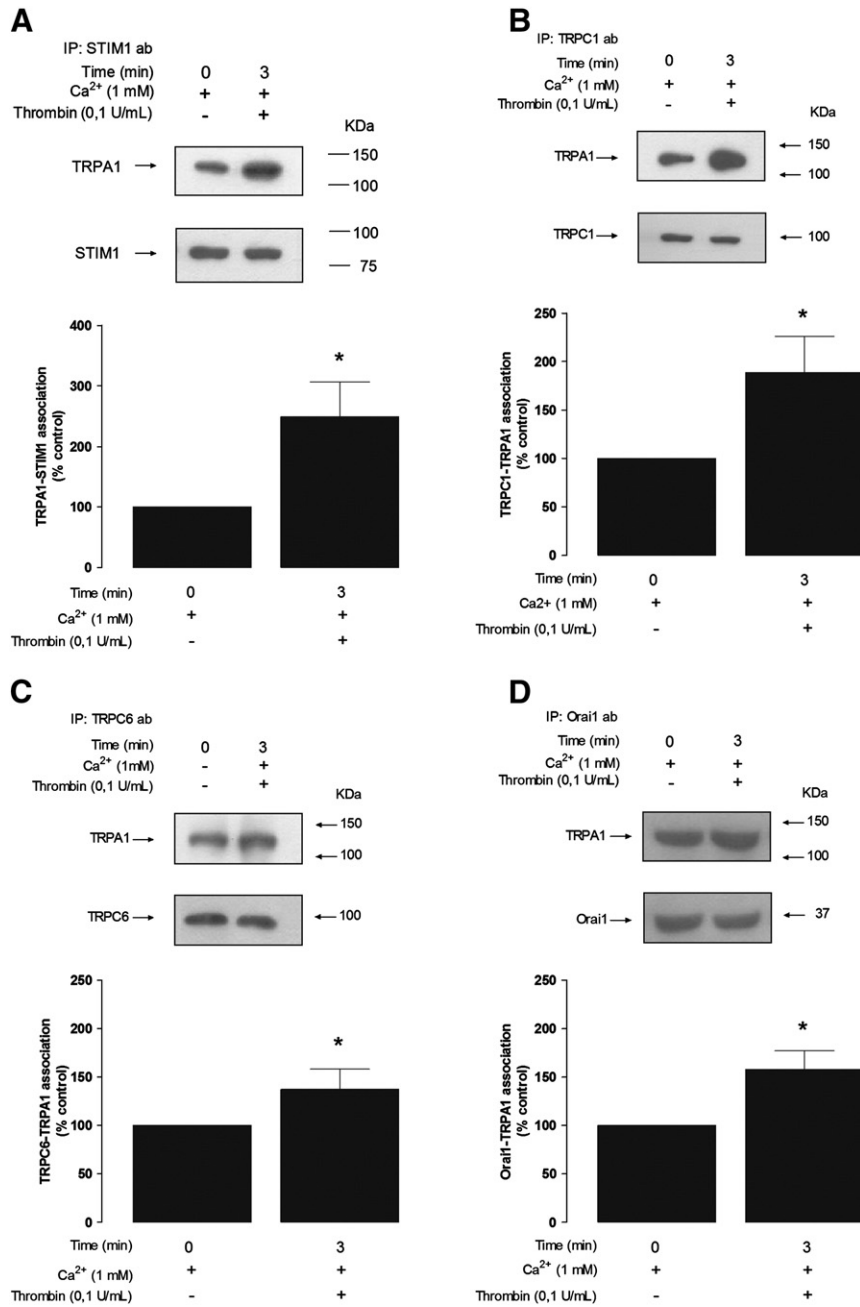

Fig. 7. Thrombin stimulates the association of TRPA1 with STIM1 and the Ca^{2+}-permeable channels Orai1, TRPC1 and TRPC6. Dimethyl BAPTA-loaded MEG01 cells were suspended in Ca^{2+}-free BSS. Cells were stimulated with 0.1 U/mL thrombin for 3 min or left untreated (control) and lysed. Cell lysates were immunoprecipitated with anti-STIM1 antibody (A), anti-TRPC1 antibody (B), anti-TRPC6 antibody (C) or anti-Orai1 antibody (D), followed by Western blotting using an anti-TRPA1 antibody. Membranes were reprobed with the immunoprecipitating antibody for protein loading control. Histograms represent the interaction of TRPA1 with the different proteins in resting and thrombin-stimulated cells. Data shown are representative of 4–5 independent experiments. Values are means ± S.E.M.. *, $p < 0.05$ compared with control.

resulted in elevation of $[Ca^{2+}]_i$ due to Ca^{2+} release from the intracellular stores. Subsequent addition of Ca^{2+} resulted in a further elevation in $[Ca^{2+}]_i$ indicative of Ca^{2+} entry. Treatment for 10 min with 60 μM HC-030031 significantly enhanced thrombin and TG-induced Ca^{2+} entry by 22 and 27%, respectively, without having any effect on Ca^{2+} release from the intracellular stores. These findings demonstrate that TRPA1 is a regulator of SOCE in MEG01 cells and megakaryocytes.

We have further explored whether TRPA1 activation results in any change in agonist-induced Ca^{2+} mobilization. As depicted in Fig. 5, treatment of MEG01 cells with 5 μM AITC did not modify $[Ca^{2+}]_i$.

Pharmacological activation of TRPA1 using AITC did not alter either thrombin- or TG-evoked Ca^{2+} mobilization ($n = 8$).

We have further investigated the role of TRPA1 in SOCE in MEG01 cells by using an approach based on the downregulation of TRPA1 expression during MEG01 maturation. As depicted in Fig. 3, the expression of TRPA1 is time-dependently attenuated during maturation; therefore, we have tested TG-evoked Ca^{2+} entry in control MEG01 cells and cells treated with PMA for 2 and 4 days. As shown in Fig. 6, top panel, treatment with PMA did not alter TG-evoked Ca^{2+} release from the intracellular stores but significantly enhanced TG-evoked SOCE ($p < 0.05$; $n = 5$).
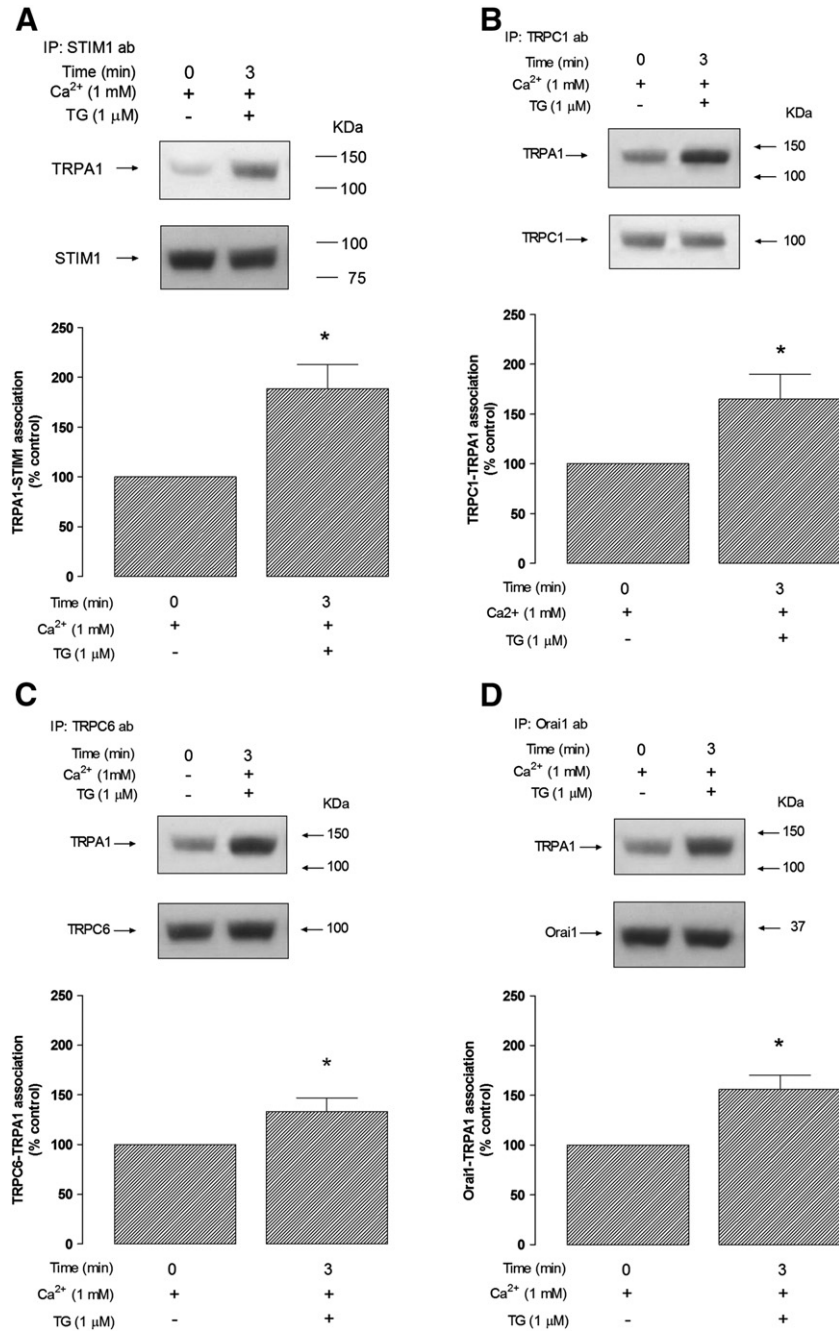

Fig. 8. Thapsigargin evokes the association of TRPA1 with STIM1 and the Ca^{2+}-permeable channels Orai1, TRPC1 and TRPC6. Dimethyl BAPTA-loaded MEG01 cells were suspended in Ca^{2+}-free BSS. Cells were stimulated with 1 μM thapsigargin for 3 min or left untreated (control) and lysed. Cell lysates were immunoprecipitated with anti-STIM1 antibody (A), anti-TRPC1 antibody (BA), anti-TRPC6 antibody (C) or anti-Orai1 antibody (DE), followed by Western blotting using an anti-TRPA1 antibody. Membranes were reprobed with the immunoprecipitating antibody for protein loading control. Histograms represent the interaction of TRPA1 with the different proteins in resting and thapsigargin-stimulated cells. Data shown are representative of 4 independent experiments. Values are means ± S.E.M.. *, $p < 0.05$ compared with control.

Although we cannot rule out other possible mechanisms involved in the enhancement of Ca^{2+} entry, these findings indicate that maturation results in an increase in SOCE in these cells, probably due to the attenuation of TRPA1 protein expression, consistent with the results found using the TRPA1 inhibitor HC-030031 (Fig. 4).

As expected, treatment of human platelets with AITC or HC-030031 did not result in any change in $[Ca^{2+}]_i$ or alter agonist-evoked Ca^{2+} mobilization (data not shown), which is consistent with the lack of TRPA1 protein expression in these cells (Fig. 1).

3.4. TRPA1 associates with SOCE elements in MEG01 cells

In human platelets, we have reported that SOCE requires the association of STIM1 and the Ca^{2+} permeable channels Orai1, TRPC1 and TRPC6 [15,44–46]. Hence we have investigated the association of TRPA1 with these proteins by looking for co-immunoprecipitation from MEG01 lysates. Cells loaded with dimethyl BAPTA were used in order to eliminate Ca^{2+}-dependent responses. Immunoprecipitation and subsequent SDS–PAGE and Western blotting were conducted using control cells and cells stimulated with the physiological agonist thrombin. After immunoprecipitation of whole cell lysates with the anti-STIM1 antibody, Western blotting revealed the presence of TRPA1 in samples from cells stimulated by thrombin and control. Interestingly, treatment with thrombin significantly enhanced the association of TRPA1 with STIM1 (Fig. 7A, top panel; $p < 0.05$; $n = 5$). Similar results were observed when cells were stimulated with TG (Fig. 8; $p < 0.05$; $n = 4$). Western blotting with anti-STIM1 antibody revealed a similar content of this protein in all lanes (Figs. 7A and 8A, bottom panel).

Furthermore, we found association of TRPA1 with TRPC1, TRPC6 and Orai1 both in resting and thrombin-stimulated MEG01 cells (Fig. 7B–D, top panel). Thrombin-evoked a significant increase in the association of TRPA1 with these channels as compared to controls ($p < 0.05$; $n = 5$), and similar results were found when cells were stimulated with TG

(Fig. 8; $p < 0.05$; $n = 5$), thus, suggesting that TRPA1 takes part in the macromolecular complex regulating SOCE in these cells.

3.5. TRPA1 regulates the association between STIM1 and Orai1 in MEG01 cells

Finally, we have investigated the mechanism underlying the regulation of SOCE by TRPA1 in MEG01 cells. The association between STIM1 and Orai1 has been reported to be crucial for the activation of SOCE [46–48]. Hence we have investigated whether TRPA1 might regulate the association between these proteins in dimethyl BAPTA-loaded cells. We have tested this possibility by using two different approaches. First of all, we tested the effect of maturation in the association between STIM1 and Orai1 induced by agonist, taking into account that maturation downregulates TRPA1 expression. Alternatively, we tested the effect of the TRPA1 inhibitor, HC-030031, in the association between STIM1 and Orai1 induced by agonists. Concerning the first approach, we have tested the association between STIM1 and Orai1 in control MEG01 cells and cells treated with PMA for 2 and 4 days. As shown in Fig. 9A, top panel, treatment with PMA for 2 or 4 days significantly enhanced the association between STIM1 and Orai1 in these cells induced by thrombin ($p < 0.05$; $n = 4$). Consistent with a role for TRPA1 in the STIM1/Orai1 association we found that HC-030031 significantly increased thrombin-induced association between STIM1 and Orai1, without having any effect on the association between these proteins in resting cells ($p < 0.05$; $n = 4$) (Fig. 9B, top panel). These findings strongly indicate that TRPA1 modulates SOCE in MEG01 cells by attenuation of the association between STIM1 and Orai1.

4. Discussion

TRP channels have been reported to play a relevant role in cell function, mostly due to the conduction of cation currents through membranes.
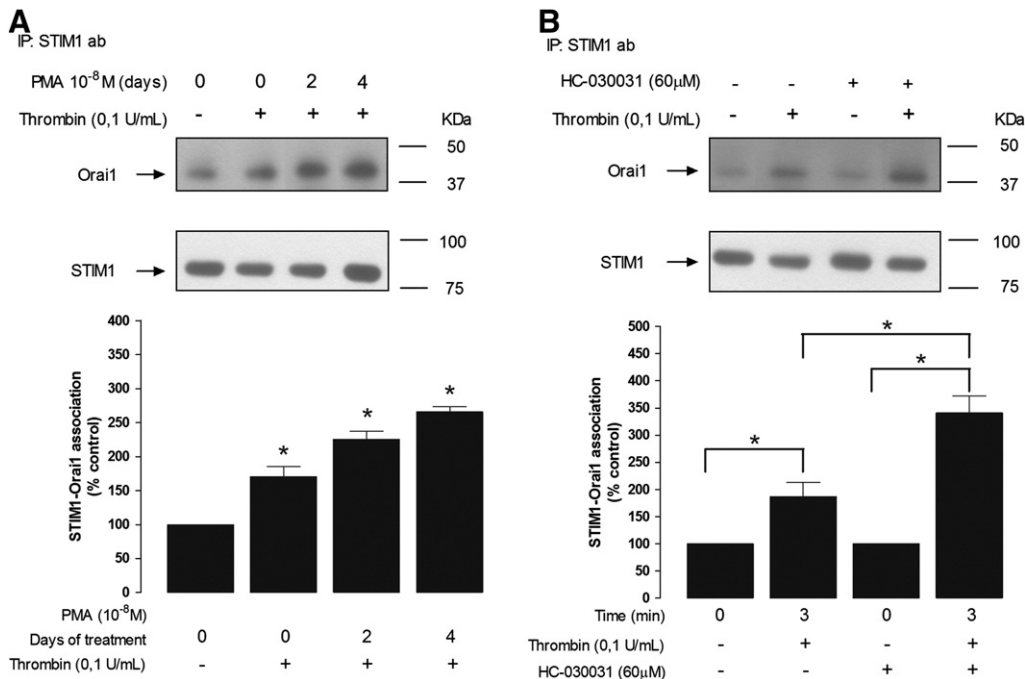

Fig. 9. TRPA1 negatively regulates the association of STIM1 with Orai1 in MEG01 cells. A, MEG01 cells were cultured in the absence (control) or presence of 10 nM PMA during 2 and 4 days. Dimethyl BAPTA-loaded cells were stimulated with 0.1 U/mL thrombin for 3 min or left untreated and lysed. Cell lysates were immunoprecipitated with anti-STIM1 antibody, followed by Western blotting using an anti-Orai1 antibody. B, Dimethyl BAPTA-loaded MEG01 cells were treated with 60 μM HC-030031 or the vehicle (as control) and then stimulated with 0.1 U/mL thrombin for 3 min of left untreated and lysed. Cell lysates were immunoprecipitated with anti-STIM1 antibody, followed by Western blotting using an anti-Orai1 antibody. Histograms represent the interaction of STIM1 with Orai1. Data shown are representative of 4 independent experiments. Values are means ± S.E.M. *, $p < 0.05$ compared with control.

Especially relevant is the role of TRP channels in Ca^{2+} influx from the extracellular medium [49]. In addition, a new role for TRP channels in the regulation of Ca^{2+} movements through other channels is emerging. To illustrate this statement, TRPC1 has been recently reported as a negative regulator of TRPV6-mediated Ca^{2+} influx through the attenuation of TRPV6 expression in the plasma membrane [50]. In the present study we report for the first time the role of TRPA1 as a negative regulator of SOCE in MEG01 cells and megakaryocytes. This finding has been demonstrated using two different approaches: channel silencing expression as a result of PMA-induced maturation of MEG01 cells, as well as inactivation of TRPA1 function using a pharmacological tool. Although we cannot rule out that MEG01 cell maturation results in enhancement of SOCE also by mechanisms different from TRPA1 expression downregulation, inactivation of TRPA1 by using HC-030031 produced basically the same effects, which strongly suggests that SOCE enhancement during maturation is, at least partially, dependent on TRPA1. Treatment of MEG01 cells with HC-030031 did not significantly alter Ca^{2+} release stimulated by thrombin or passive Ca^{2+} efflux induced by TG, which indicates that this inhibitor does not alter the ability of cells to accumulate Ca^{2+} into the intracellular stores or the ability of thrombin to induce Ca^{2+} release from the stores. Furthermore, these findings suggest that TRPA1 is not involved in agonist-induced Ca^{2+} release or Ca^{2+} leakage from the intracellular Ca^{2+} compartments.

TRPA1 negatively regulates Ca^{2+}-independent association of STIM1 with Orai1, which might be the mechanism underlying the modulation of SOCE in MEG01 cells. The regulation of signaling complexes by TRPA1 resembles that previously reported to TRPC3, which recruits Orai1, STIM1, RACK1 and the type I IP_3 receptor into a TRPC3-dependent signalplex that modulates agonist-induced Ca^{2+} release and entry in HEK-293 cells [27,28]. We have found that TRPA1 is constitutively associated to STIM1 and Orai1, although these interactions are enhanced by stimulation with agonists. Furthermore, our results indicate that TRPA1 is constitutively active, since cell treatment with AITC, a TRPA1 activator, did not result in further inhibition of agonist-induced Ca^{2+} entry and SOCE.

Interestingly, the role of TRPA1 as a SOCE modulator disappears with maturation so that mature human platelets do not express TRPA1 at the protein level, thus resulting in a significantly greater SOCE in platelets upon depletion of the intracellular Ca^{2+} stores by TG, as demonstrated in Fig. 10, which depicts the determination of SOCE in MEG01 and platelets performed under comparable conditions. The present study presents a new and interesting function of TRPA1 channels in cellular function not associated with the previously reported thermal and noxious sensitivity [51]. This new feature described here should be taken into account in the design of therapeutic drugs aimed at the modification of calcium entry.
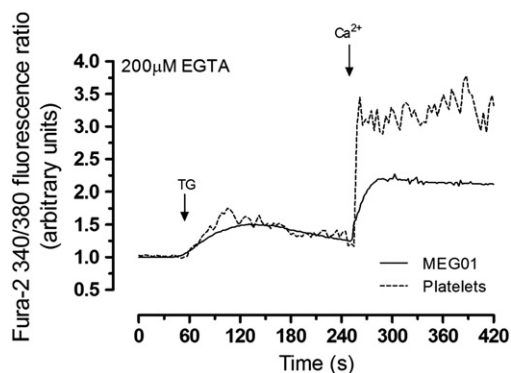

Fig. 10. Comparative analysis of TG-evoked store-operated Ca^{2+} entry in MEG01 cells and platelets. MEG01 cells and human platelets were suspended in BSS and HBS, respectively. Fura 2-loaded cells were stimulated with 1 μM TG in a Ca^{2+}-free medium (200 μM EGTA added) and 3 min later $CaCl_2$ (final concentration 600 μM) was added to initiate Ca^{2+} entry. Traces shown are representative of 5 separate experiments.

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