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I. Jardin, L. J. Gómez, G. M. Salido, Juan A. Rosado. Dynamic interaction of hTRPC6 with the Orai1/STIM1 complex or hTRPC3 mediates its role in capacitative or non-capacitative Ca²⁺ entry pathways. *Biochemical Journal*, Portland Press, 2009, 420 (2), pp.267-276. 10.1042/BJ20082179 . hal-00479123

HAL Id: hal-00479123

<https://hal.archives-ouvertes.fr/hal-00479123>

Submitted on 30 Apr 2010

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Dynamic interaction of hTRPC6 with the Orai1/STIM1 complex or hTRPC3 mediates its role in capacitative or non-capacitative Ca²⁺ entry pathways

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Running Title: Role of hTRPC6 in capacitative and non-capacitative Ca²⁺ entry

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Abstract. TRPC channel subunits have been shown to assemble into homo- or heteromeric channel complexes, including different Ca²⁺-handling proteins, required for the activation of capacitative Ca²⁺ entry (CCE) or non-capacitative Ca²⁺ entry (NCCE) pathways. Here we present evidence for the dynamic interaction between endogenously expressed hTRPC6 with either both Orai1 and STIM1 or hTRPC3 to participate in CCE or NCCE. Electrotransfection of cells with anti-hTRPC6 antibody, directed towards the C-terminal region, reduces CCE induced by N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), which reduces intraluminal free Ca²⁺ concentration. Cell stimulation with thrombin or extensive Ca²⁺ store depletion by thapsigargin (TG) + Iono enhanced interaction between hTRPC6 and the CCE proteins Orai1 and STIM1. In contrast, stimulation with the diacylglycerol analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) displaces hTRPC6 from Orai1 and STIM1 and enhances the association between hTRPC6 and hTRPC3. The interaction between hTRPC6 and hTRPC3 was abolished by dimethyl-BAPTA loading, which indicate that this phenomenon is Ca²⁺-dependent. These findings support that hTRPC6 participate both in CCE and NCCE through its interaction with the Orai1/STIM1 complex or hTRPC3, respectively.

Key words. hTRPC6, STIM1, Orai1, hTRPC3, calcium entry, thrombin, platelets.

INTRODUCTION

The canonical transient receptor potential (TRPC) cation channels are mammalian homologues of the photoreceptor TRP channel in *Drosophila melanogaster* [1]. A number of evidences have supported the role of TRPC channels in both capacitative Ca^{2+} entry (CCE) and non-capacitative Ca^{2+} entry (NCCE) pathways [2-5].

The identification of the endoplasmic reticulum (ER) Ca^{2+} sensor STIM1 and the plasma membrane (PM) Ca^{2+} channel Orai1 has shed new light on the mechanism of activation of CCE. Orai1 forms a highly Ca^{2+} selective channel that has been proposed to conduct I_{CRAC} [6]. Orai1 has been reported to participate in the formation of multimeric ion-channel complexes involving hTRPC1 and STIM1 [7, 8]. STIM1 is a low affinity Ca^{2+} -binding protein mostly located in the ER membrane that contains an EF-hand domain in the NH_2 terminus, located in the lumen of the ER, that has been proposed to function as a Ca^{2+} sensor in the ER [9, 10], conferring TRPC1 protein store-operated sensitivity [8].

hTRPC6 has been presented as a component of NCCE, as well as CCE, channels. hTRPC6 was initially presented as a channel activated by diacylglycerol (DAG) generated by receptor-induced phospholipase C activation [11, 12]. Furthermore, in human platelets, heteromeric interaction of hTRPC6 with hTRPC1 forms a complex activated by depletion of the intracellular Ca^{2+} stores [13]. Consistent with this, we have previously reported that hTRPC6 is required for thapsigargin (TG)-mediated Ca^{2+} entry in platelets [4]. In addition, in smooth muscle cells, hTRPC6 channels are activated by mechanical stretch, leading to membrane depolarization and the activation of voltage-dependent Ca^{2+} entry [14]. Therefore, TRPC6-forming channels function as integrators of a number of intracellular signals.

There is significant controversy concerning the mode of activation (capacitative or non-capacitative) of particular TRPC channel subunits [15]. These discrepancies might at least be partially attributed to the tetrameric composition of TRPC channels. Thus, different combinations of TRPC subunits might lead to the formation of channels with different biophysical properties. The assembly of a particular combination of TRPC subunits might be performed *in situ* under an adequate stoichiometric relationship, conditions that might be altered in TRPC-overexpressing cells [15], which supports the importance of experiments in cells endogenously expressing TRPC proteins.

In HEK-293 cells expressing TRPC3, TRPC6 and the three Orai isoforms, interaction of Orai proteins with both TRPC proteins has been reported to confer them the ability to respond to store depletion [16].

In the present study we have investigated the interaction of endogenously expressed hTRPC6 with components of the CCE and NCCE pathways at resting conditions and upon store depletion either by pharmacological tools or the physiological agonist thrombin or after stimulation with the diacylglycerol analogue OAG. Our results indicate that Ca^{2+} store depletion stimulates rapid interaction between hTRPC6 and both, Orai1 and STIM1, while stimulation with the NCCE inducer OAG evokes the displacement of hTRPC6 from the CCE pathway and promotes its interaction with the NCCE Ca^{2+} permeable channel, hTRPC3.

MATERIAL AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) and calcein-AM were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, thapsigargin (TG), thrombin, phenylmethanesulphonyl fluoride (PMSF) and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Ionomycin (Iono), N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were from Calbiochem

(Nottingham, U.K.). Goat anti-hTRPC6 polyclonal antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were from Santa Cruz (Santa Cruz, CA, U.S.A.). Rabbit anti-TRPC1 antibody was from Alomone Labs (Jerusalem, Israel). Rabbit anti-TRPC3 polyclonal antibody was from Abcam (Cambridge, U.K.). Rabbit anti-Orai1 antibody was from ProSci (Poway, CA, U.S.A.). Mouse anti STIM1 antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, U.S.A.). Hyperfilm ECL was from Amersham (Buckinghamshire, U.K.). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Enhanced chemiluminescence detection reagents and rabbit anti-mouse IgG were from Pierce (Cheshire, U. K.). All other reagents were of analytical grade.

Platelet Preparation

Fura-2-loaded platelets were prepared as previously described [17] 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 μ M) and apyrase (40 μ g/mL) were added. Platelet-rich plasma was incubated at 37 °C with 2 μ M fura-2 acetoxymethyl ester for 45 min. Cells were then collected by centrifugation at 350 g for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO₄ and supplemented with 0.1% BSA and 40 μ g/mL apyrase.

Cell viability

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μ M calcein-AM at 37 °C, centrifuged and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 mL aliquots using a Cary Eclipse Spectrophotometer (Varian Ltd, Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique.

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

Fluorescence was recorded from 2 mL aliquots of magnetically stirred platelet suspension (2×10^8 cells/mL) at 37 °C using a fluorescence spectrophotometer with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and, when TPEN was absent, calibrated according to the method of Grynkiewicz et al. [18].

Ca^{2+} entry was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after addition of $CaCl_2$ [19]. Control experiments were performed for all experimental procedures in order to correct the amount of Ca^{2+} entry (integrals) by subtraction of the integral of the $[Ca^{2+}]_c$ elevation due to leakage of the indicator. Ca^{2+} release from the intracellular stores was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after the addition of thrombin.

Reversible Electroporation Procedure

The platelet suspension was transferred to an electroporation chamber containing antibodies at a final concentration of 2 μ g/mL, and the antibodies were transfected according to published methods [4, 20]. Reversible electroporation was performed at 4 kV/cm at a setting of 25-microfarad capacitance and was achieved by 7 pulses using a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, U.S.A.). Following

electroporation, cells were incubated with antibodies for an additional 60 min at 37 °C then were centrifuged at 350 g for 20 min and resuspended in HBS containing 200 μ M CaCl₂. At the time of experiment 250 μ M EGTA was added.

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [21]. Briefly, 500 μ L aliquots of platelet suspension (2×10^9 cell/mL) were lysed with an equal volume of RIPA buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2 % SDS, 2% sodium deoxycholate, 2 % triton X-100, 2 mM Na₃VO₄, 2 mM PMSF, 100 μ g/mL leupeptin and 10 mM benzamide. Aliquots of platelet lysates (1 mL) were immunoprecipitated by incubation with 2 μ g of either anti-Orai1, anti-TRPC3 or anti-TRPC6 antibody and 25 μ L of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10 % SDS-PAGE and separated proteins were electrophoretically transferred onto 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 STIM1, hTRPC3, hTRPC6 and Orai1 was achieved using the anti-STIM1 antibody diluted 1:250 in TBST for 2h or the anti-hTRPC3, anti-hTRPC6 or anti-Orai1 antibody diluted 1:1000 in TBST for 2h, respectively. 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 horseradish peroxidase-conjugated donkey anti-goat IgG antibody, horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody or horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:10000 in TBST and then exposed to enhanced chemiluminescence reagents for 4 min. Blots were then exposed to photographic films. The density of bands on the film was measured using scanning densitometry. Data were normalized to the amount of protein recovered by the antibody used for the immunoprecipitation.

Statistical Analysis

Analysis of statistical significance was performed using one-way analysis of variance. For comparison between two groups Student's *t* test was used. $P < 0.05$ was considered to be significant for a difference.

RESULTS

hTRPC6 co-immunoprecipitates with STIM1 and Orai1 in human platelets

A role for endogenously expressed hTRPC6 in agonist-induced Ca²⁺ entry has been demonstrated in human platelets [4, 12]. We have now investigated the association between hTRPC6 and both, STIM1 and Orai1, by looking for co-immunoprecipitation from platelet lysates. Immunoprecipitation and subsequent SDS-PAGE and Western blotting were conducted using resting platelets and platelets stimulated in the absence of extracellular Ca²⁺ (100 μ M EGTA was added to the extracellular medium) with the physiological agonist thrombin (1U/mL) or with the inhibitor of the sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) TG (1 μ M) plus a low concentration of Iono (50 nM), to induce extensive depletion of the intracellular stores in platelets [22]. After treatment with these agents cell viability in our platelet preparations was estimated as 95%. After immunoprecipitation with anti-hTRPC6 antibody, Western blotting with anti-STIM1 antibody revealed a band between 84 and 90 kDa (the predicted molecular weight of STIM1 [23]) in samples from resting platelets (Figure 1A, *upper panel*). Treatment with thrombin increased the association

between STIM1 and hTRPC6 in a time-dependent manner, reaching a maximal effect after 30 s of platelet stimulation (Figure 1A, *upper panel*; $n=6$). In addition, we have investigated the possible interaction between hTRPC6 and Orai1. After immunoprecipitation with anti-hTRPC6 antibody, Western blotting with anti-Orai1 antibody revealed a band of approximately 45 kDa (the predicted molecular weight of Orai1 [24]) in samples from resting platelets (Figure 1B, *upper panel*). Treatment with thrombin enhanced the co-immunoprecipitation of hTRPC6 with Orai1 in a time-dependent manner (Figure 1B, *upper panel*; $n=6$). Similar results were observed when cells were stimulated with TG+Iono, which increased coimmunoprecipitation of hTRPC6 with STIM1 and Orai1 in a time-dependent manner, reaching a maximum after 10 s of stimulation, and then was sustained for at least 60 s (Figure 2A and B, *upper panel*; $n=6$). We also conducted converse experiments, immunoprecipitating platelet lysates with anti-STIM1 or anti-Orai1 antibodies and detecting for the presence of hTRPC6, a protein of approximately 100 kDa [25]. Under these conditions, treatment with thrombin or TG+Iono enhanced the association of hTRPC6 with STIM1 in a time-dependent manner, with a 148 ± 24 , 195 ± 34 and $179 \pm 37\%$ of control for thrombin and 143 ± 18 , 155 ± 31 and $126 \pm 31\%$ of control for TG+Iono at 10, 30 and 60 s, respectively. Similarly, both agents enhanced the association between hTRPC6 and Orai1 with a 115 ± 6 , 150 ± 22 and $180 \pm 40\%$ of control for thrombin and 135 ± 17 , 158 ± 16 and $131 \pm 21\%$ of control for TG+Iono at 10, 30 and 60 s, respectively (Figures 1 and 2, C and D *upper panels*). Western blotting of the same membranes with the antibody used for immunoprecipitation confirmed similar protein content in all lanes (Figures 1 and 2, *lower panels*). No specific proteins were detected when immunoprecipitation was performed using a non-specific rabbit IgG control. Only the heavy chain of the rabbit IgG used for immunoprecipitation was detected when a horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody in lanes 3 to 5 (Supplementary Figure 1).

hTRPC6 is involved in TPEN-mediated Ca^{2+} entry

TPEN may be a useful tool to investigate the mechanism of CCE due to its ability to reduce intraluminal free Ca^{2+} concentration. The use of TPEN avoids potential confounders, such as changes in cytosolic Ca^{2+} and second messenger levels, secondary to stimulation with agonists, as well as the use of pharmacological inhibitors of SERCA, which has been suggested to play a role in CCE [26]. In a Ca^{2+} -free medium, treatment of fura-2-loaded human platelets with thrombin (1U/mL) in stirred cuvettes at 37 °C evoked a transient elevation in $[\text{Ca}^{2+}]_c$ due to Ca^{2+} release from internal stores. Subsequent addition of 300 μM Ca^{2+} to the external medium induced a sustained increase in $[\text{Ca}^{2+}]_c$ (Figure 3A *upper graph*; $n=5$). TPEN is a membrane-permeant multivalent cation chelator, that shows moderate affinity for Ca^{2+} (K_d for Ca^{2+} ~ 130 μM), which has been used to rapidly and reversibly chelate Ca^{2+} within the intracellular stores [27]. In the absence of extracellular Ca^{2+} , the addition of 500 μM TPEN to human platelets did not modify $[\text{Ca}^{2+}]_c$; subsequent addition of 300 μM Ca^{2+} to the external medium induced a sustained elevation in $[\text{Ca}^{2+}]_c$ indicative of SOCE [28], due to *in situ* chelation of Ca^{2+} in the intracellular stores (Figure 3A *upper graph*; $n=5$). The elevation in Fura-2 fluorescence ratio observed after the addition of Ca^{2+} to the medium in the presence of TPEN was clearly greater than that observed in the absence of TPEN (Figure 3A *lower graph*; $n=5$). In the presence of TPEN, thrombin-evoked Ca^{2+} release and entry were significantly reduced (Figure 3A *upper graph*), a phenomenon that is not likely attributed to a decrease in cell viability (95% of cells were viable in our platelet preparations treated with TPEN, at least during the performance of the experiments).

To investigate the involvement of hTRPC6 in TPEN-mediated CCE we have induced functional knock down of hTRPC6 by electrotransfection of cells with anti-hTRPC6

antibody, directed towards an intracellular C-terminal sequence, following a previously described procedure [4]. Platelet viability after reversible electroporation was 90%; therefore experimental cells and controls were treated in the same way, except for the antibody electrotransfected (anti-hTRPC3 or hTRPC6 antibodies in experimental cells and a non-specific rabbit IgG in controls). We have recently reported that platelet incubation with the anti-hTRPC6 antibody reduced Ca^{2+} and Mn^{2+} entry induced by thrombin, TG or 2,5-di-(tert-butyl)-1,4-hydroquinone, thus suggesting a role for hTRPC6 in CCE [4]. Here we show for the first time that electrotransfection with 1 $\mu\text{g}/\text{mL}$ hTRPC6 significantly reduced TPEN-evoked CCE by $33 \pm 6\%$ compared to controls (cells electrotransfected with 1 $\mu\text{g}/\text{mL}$ goat IgG; Figure 3B *upper graph*; $P < 0.05$; $n = 5$). We have further explored the role of hTRPC3 in CCE induced by TPEN by electrotransfection of cells with anti-hTRPC3 antibody directed towards the intracellular NH_2 -terminal sequence. As shown in Figure 3B *upper graph*, TPEN-evoked CCE was not modified in cells electrotransfected with 1 $\mu\text{g}/\text{mL}$ anti-hTRPC3 antibody compared to platelets electrotransfected with 1 $\mu\text{g}/\text{mL}$ rabbit IgG. Finally, we found that electrotransfection of the anti-hTRPC6 antibody in combination with anti-hTRPC3 antibody reduced CCE by TPEN to a similar extent as the anti-hTRPC6 antibody alone (Figure 3B *upper graph*).

As mentioned in material and methods, the amount of Ca^{2+} entry was corrected by subtraction of the elevation in $[\text{Ca}^{2+}]_c$ observed after the addition of Ca^{2+} in cells treated with vehicle instead of thrombin and/or TPEN (Figure 3A and B *lower graphs*).

Ca^{2+} -dependent association of hTRPC6 and hTRPC3

We have further investigated the interaction between hTRPC6 and hTRPC3 upon stimulation with thrombin or TG + Iono. Immunoprecipitation with anti-hTRPC6 antibody followed by Western blotting with anti-hTRPC3 antibody revealed a band of approximately 100 kDa (the predicted molecular weight of hTRPC3 [29]), which indicates association of both hTRPC proteins in resting platelets (Figure 4 *upper panels*). In the absence of extracellular Ca^{2+} , platelet stimulation with thrombin or extensive Ca^{2+} store depletion, induced by TG + Iono, enhanced co-immunoprecipitation between hTRPC6 and hTRPC3 (Figure 4A and B *upper panels*; $P < 0.05$). This effect was abolished by platelet loading with the intracellular Ca^{2+} chelator dimethyl BAPTA, which prevents thrombin-, as well as TG + Iono-induced Ca^{2+} elevations in $[\text{Ca}^{2+}]_c$ (Figure 4C-F). Similar results were observed when converse experiments were conducted immunoprecipitating platelet lysates with anti-hTRPC3 antibody and detecting for the presence of hTRPC6. Treatment with thrombin or TG+Iono enhanced the association of hTRPC6 with hTRPC3 by 142 ± 8 and $151 \pm 11\%$ of control, respectively (Figure 4A and B, *lower panels*). This event was prevented by BAPTA loading (Figure 4E and F, *lower panels*).

Interestingly, we have found that OAG, a DAG analogue that activates NCCE, attenuates the association between hTRPC6 and both, Orai1 and STIM1, as detected by immunoprecipitation of platelet lysates with anti-hTRPC6 antibody followed by Western blotting with anti-Orai1 or anti-STIM1 antibodies (Figure 5A and B, *upper panels*; $P < 0.05$) as well as by converse experiments (Figure 5A and B, *lower panels*; $P < 0.05$). In the absence of extracellular Ca^{2+} , OAG was unable to induce association between hTRPC6 and hTRPC3 (Figure 5C). In contrast, in the presence of 1 mM extracellular Ca^{2+} OAG significantly enhanced association between both hTRPC proteins (Figure 5D; $P < 0.05$). Western blotting of the same membranes with the anti-hTRPC6 antibody confirmed comparable amounts of protein in all lanes (Figures 4 and 5).

As shown in Figure 5E *upper graph*, treatment of platelets in a Ca^{2+} -free medium with 100

μM OAG did not increase $[\text{Ca}^{2+}]_c$; subsequent addition of Ca^{2+} to the external medium resulted in a rise in $[\text{Ca}^{2+}]_c$, greater than that induced in cells treated with vehicle alone (Figure 5E *lower graph*), indicative of NCCE. Electrotransfection with $1 \mu\text{g}/\text{mL}$ hTRPC3 or hTRPC6 significantly reduced OAG-evoked NCCE by 40 ± 9 and $56 \pm 2\%$, respectively (Figures 5G and H) compared to controls (cells electrotransfected with $1 \mu\text{g}/\text{mL}$ rabbit IgG; Figure 5F(*α -mouse IgG*); $P < 0.05$; $n = 5$). Altogether, these findings suggest that the association of hTRPC6 and hTRPC3 induced by OAG is mediated by a Ca^{2+} -dependent mechanism, as previously described for the association between both proteins stimulated by thrombin or TG + Iono.

Treatment with TG + ionomycin is unable to induce association of hTRPC3 with hTRPC1, STIM1 or Orai1

We have further investigated the possible interaction between hTRPC3 and hTRPC1, STIM1 and Orai1, which form a dynamic complex involved in CCE [30], upon stimulation with TG + Iono. Immunoprecipitation with anti-hTRPC3 antibody followed by Western blotting with anti-hTRPC1 antibody revealed a faint band of approximately 100 kDa (the predicted molecular weight of hTRPC1 [31, 32]), which indicates certain association of both hTRPC proteins in resting platelets (Figure 6A *upper panel*; $n = 5$). After platelet stimulation with TG + Iono the amount of hTRPC1 immunoprecipitated with the anti-hTRPC3 antibody was not significantly different from that observed in resting cells (Figure 6A *upper panel*). No protein with the predicted molecular weight of STIM1 or Orai1 were detected in hTRPC3 immunoprecipitates either in resting or in TG+Iono-stimulated cells (Figure 6B and C, *upper panels*; $n = 5$). Similar results were observed when converse experiments were conducted immunoprecipitating platelet lysates with anti-hTRPC1, anti-STIM1 or anti-Orai1 antibody and detecting for the presence of hTRPC3 (Figure 6 *lower panels*).

DISCUSSION

Canonical TRP proteins have been shown to participate both in capacitative and non-capacitative Ca^{2+} influx [15]. hTRPC6 has long been proposed as a candidate to mediate NCCE in different cell types [11, 33, 34], including human platelets [12]. In these cells, we have recently suggested that hTRPC6 might also be involved in the conduction of CCE on the base of the inhibitory effect of electrotransfection of the anti-hTRPC6 antibody on TG-induced Ca^{2+} entry [4]. CCE is a mechanism for Ca^{2+} influx regulated by the Ca^{2+} content of the intracellular stores rather than by the Ca^{2+} signals generated by agonists or pharmacological tools like TG [35]. Here we have further observed the involvement of hTRPC6 in CCE by using TPEN, an intracellular Ca^{2+} chelator that reduces free Ca^{2+} concentration in the intracellular stores ($[\text{Ca}^{2+}]_s$) without changes in $[\text{Ca}^{2+}]_c$ secondary to stimulation with agonists or TG, which confirms previous results [4]. We have found that hTRPC3, which has been shown to conduct CCE in a number of cell types [36, 37] but NCCE in others [38, 39], is not required for Ca^{2+} entry induced by TPEN, as demonstrated by electrotransfection of the anti-hTRPC3 antibody, which further suggest that treatment with TPEN induces CCE in human platelets. The specificity of electrotransfection with antibodies has been explored by testing the effect of incubation with an antibody directed to a protein not related to hTRPC6 or hTRPC3 proteins or any other platelet protein. We have used goat or rabbit IgG since these are the natures of the anti-hTRPC6 and anti-hTRPC3 antibodies, respectively.

Thrombin-evoked Ca^{2+} release and entry were significantly reduced in the presence of TPEN. TPEN has been shown to cause a rapid decrease in $[\text{Ca}^{2+}]_s$ [40], where Ca^{2+} concentration is elevated (~ 0.2 - 1 mM [41]), which might explain the significant reduction of the extent of Ca^{2+} release induced thrombin. The inhibitory effect of TPEN on Ca^{2+} entry mediated by thrombin might be partially attributed to its chelating effects and to the known activation of Ca^{2+} extrusion through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [42].

CCE has been shown to require a dynamic interplay between the ER Ca^{2+} sensor STIM1 and the PM channels Orai1 and hTRPC1 [7]. In human platelets we have recently reported that Orai1 mediates the communication between STIM1 and hTRPC1, which is essential to confer hTRPC1 properties of capacitative Ca^{2+} entry channel [8]. Here we present for the first time evidence for the interaction between endogenously expressed hTRPC6 and both Orai1 and STIM1 in platelets. The association between these proteins was detected in resting platelets and enhanced by the physiological agonist thrombin or extensive store depletion using TG + Iono. The interaction between hTRPC6 and both Orai1 and STIM1 was found to be independent on extracellular Ca^{2+} and might mediate the involvement of hTRPC6 in CCE in these cells.

On the basis that hTRPC6 and hTRPC3 have been shown to be involved in NCCE and that both channels have been reported to be associated to the same platelet membrane regions [43], we have explored the interaction between both proteins upon stimulation with thrombin or TG + Iono and OAG, a DAG analogue that activates NCCE [4]. Platelet treatment with thrombin or TG + Iono enhances interaction between hTRPC6 and hTRPC3 only when rises in $[\text{Ca}^{2+}]_c$ are allowed, a response that was abolished by cell loading with dimethyl-BAPTA. Since capacitative Ca^{2+} entry was first proposed as a mechanism for Ca^{2+} influx regulated by the Ca^{2+} content of the intracellular stores rather than by the Ca^{2+} signals generated by agonists [35], our findings suggest that this interaction is non-capacitative in nature due to its dependence on rises in $[\text{Ca}^{2+}]_c$. Similarly, stimulation with OAG increases the association of hTRPC6 with hTRPC3 when Ca^{2+} entry is allowed, suggesting that this interaction is Ca^{2+} -dependent. In addition, this finding suggests that a different mechanism mediates the initial Ca^{2+} entry step activated by OAG. Interestingly, treatment with OAG decreased the interaction of hTRPC6 with Orai1 and STIM1, which indicates that activation of NCCE pathways results in displacement of hTRPC6 from the CCE protein complex to the NCCE mechanism. To further support in role of hTRPC3 in NCCE we have found that store depletion with TG + Iono did not induce association between hTRPC3 with hTRPC1, STIM1 or Orai1.

Our findings suggest that hTRPC6 interacts with the CCE channel protein Orai1 and the ER Ca^{2+} sensor STIM1 upon stimulation with the physiological agonist thrombin or extensive Ca^{2+} store depletion. hTRPC6 is required for CCE induced either by TG [4] or TPEN, which induces a decrease in $[\text{Ca}^{2+}]_s$ but avoiding rises in $[\text{Ca}^{2+}]_c$. The NCCE activator OAG displaces hTRPC6 from Orai1 and STIM1 and induces its association with hTRPC3. We propose that depletion of $[\text{Ca}^{2+}]_s$ induces the formation of a macromolecular complex involving plasma membrane Ca^{2+} -permeable channels like Orai1, hTRPC6 and hTRPC1 [8]. In contrast, activation of NCCE results in displacement of hTRPC6 from the CCE pathway and favors Ca^{2+} -dependent association between hTRPC6 and hTRPC3 (a schematic diagram of the proposed model is depicted in Figure 7). These data support that hTRPC6 participates in CCE and NCCE pathways in human platelets by association to different Ca^{2+} -handling proteins.

ACKNOWLEDGEMENTS

This work was supported by MEC grant BFU2007-60104. I.J. was supported by a MEC fellowship (BFU2007-60104).

FOOTNOTES

The abbreviations used are: $[Ca^{2+}]_c$, cytosolic free calcium concentration; $[Ca^{2+}]_s$, free Ca^{2+} concentration in the intracellular stores; BSA, bovine serum albumin; CCE, capacitative Ca^{2+} entry; DAG, diacylglycerol; ER, endoplasmic reticulum; HBS, HEPES-buffered saline; hTRPC6, human canonical TRP6; Iono, ionomycin; IP_3R , inositol 1,4,5-trisphosphate receptor; NCCE, non-capacitative Ca^{2+} entry; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PBS, phosphate-buffered saline; PLC, phospholipase C; PM, plasma membrane; PMSF, phenyl methyl sulphonyl fluoride; STIM1, stromal interaction molecule 1; TBST, tris-buffered saline with 0.1 % Tween 20; TG, thapsigargin; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylene diamine; TRP, transient receptor potential.

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REFERENCES

- 1 Montell, C. (2003) The venerable inveterate invertebrate TRP channels. *Cell Calcium*. **33**, 409-417
- 2 Cheng, K. T., Liu, X., Ong, H. L. and Ambudkar, I. S. (2008) Functional requirement for Orai1 in store-operated TRPC1-STIM1 channels. *J Biol Chem*. **283**, 12935-12940
- 3 Liao, Y., Erxleben, C., Abramowitz, J., Flockerzi, V., Zhu, M. X., Armstrong, D. L. and Birnbaumer, L. (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. *Proc Natl Acad Sci U S A*. **105**, 2895-2900
- 4 Jardin, I., Redondo, P. C., Salido, G. M. and Rosado, J. A. (2008) Phosphatidylinositol 4,5-bisphosphate enhances store-operated calcium entry through hTRPC6 channel in human platelets. *Biochim Biophys Acta*. **1783**, 84-97
- 5 Vazquez, G., Wedel, B. J., Kawasaki, B. T., Bird, G. S. and Putney, J. W., Jr. (2004) Obligatory role of Src kinase in the signaling mechanism for TRPC3 cation channels. *J Biol Chem*. **279**, 40521-40528
- 6 Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R. and Kinet, J. P. (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. *Science*. **312**, 1220-1223
- 7 Ong, H. L., Cheng, K. T., Liu, X., Bandyopadhyay, B. C., Paria, B. C., Soboloff, J., Pani, B., Gwack, Y., Srikanth, S., Singh, B. B., Gill, D. L. and Ambudkar, I. S. (2007) Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem*. **282**, 9105-9116
- 8 Jardin, I., Lopez, J. J., Salido, G. M. and Rosado, J. A. (2008) Orai1 Mediates the Interaction between STIM1 and hTRPC1 and Regulates the Mode of Activation of hTRPC1-forming Ca^{2+} Channels. *J Biol Chem*. **283**, 25296-25304
- 9 Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Liudyno, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Velicelebi, G. and Stauderman, K. A. (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol*. **169**, 435-445
- 10 Mercer, J. C., Dehaven, W. I., Smyth, J. T., Wedel, B., Boyles, R. R., Bird, G. S. and Putney, J. W., Jr. (2006) Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *J Biol Chem*. **281**, 24979-24990
- 11 Thebault, S., Zholos, A., Enfissi, A., Slomianny, C., Dewailly, E., Roudbaraki, M., Parys, J. and Prevarskaya, N. (2005) Receptor-operated Ca^{2+} entry mediated by TRPC3/TRPC6 proteins in rat prostate smooth muscle (PS1) cell line. *J Cell Physiol*. **204**, 320-328
- 12 Hassock, S. R., Zhu, M. X., Trost, C., Flockerzi, V. and Authi, K. S. (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood*. **100**, 2801-2811
- 13 Redondo, P. C., Jardin, I., Lopez, J. J., Salido, G. M. and Rosado, J. A. (2008) Intracellular Ca^{2+} store depletion induces the formation of macromolecular complexes involving hTRPC1, hTRPC6, the type II IP_3 receptor and SERCA3 in human platelets. *Biochim Biophys Acta*. **1783**, 1163-1176

- 14 Wang, Y., Deng, X., Hewavitharana, T., Soboloff, J. and Gill, D. L. (2008) Stim, orai and trpc channels in the control of calcium entry signals in smooth muscle. *Clin Exp Pharmacol Physiol.* **35**, 1127-1133
- 15 Parekh, A. B. and Putney, J. W., Jr. (2005) Store-operated calcium channels. *Physiol Rev.* **85**, 757-810
- 16 Liao, Y., Erxleben, C., Yildirim, E., Abramowitz, J., Armstrong, D. L. and Birnbaumer, L. (2007) Orai proteins interact with TRPC channels and confer responsiveness to store depletion. *Proc Natl Acad Sci U S A.* **104**, 4682-4687
- 17 Redondo, P. C., Jardin, I., Hernandez-Cruz, J. M., Pariente, J. A., Salido, G. M. and Rosado, J. A. (2005) Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. *Biochem Biophys Res Commun.* **333**, 794-802
- 18 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem.* **260**, 3440-3450
- 19 Redondo, P. C., Harper, M. T., Rosado, J. A. and Sage, S. O. (2006) A role for cofilin in the activation of store-operated calcium entry by de novo conformational coupling in human platelets. *Blood.* **107**, 973-979
- 20 Watson, S. P. and Authi, K. S. (1996) Platelets : a practical approach. IRL Press at Oxford University Press, Oxford
- 21 Woodard, G. E., Salido, G. M. and Rosado, J. A. (2008) Enhanced exocytotic-like insertion of Orai1 into the plasma membrane upon intracellular Ca^{2+} store depletion. *Am J Physiol Cell Physiol.* **294**, C1323-1331
- 22 Rosado, J. A., Lopez, J. J., Harper, A. G., Harper, M. T., Redondo, P. C., Pariente, J. A., Sage, S. O. and Salido, G. M. (2004) Two pathways for store-mediated calcium entry differentially dependent on the actin cytoskeleton in human platelets. *J Biol Chem.* **279**, 29231-29235
- 23 Manji, S. S., Parker, N. J., Williams, R. T., van Stekelenburg, L., Pearson, R. B., Dziadek, M. and Smith, P. J. (2000) STIM1: a novel phosphoprotein located at the cell surface. *Biochim Biophys Acta.* **1481**, 147-155
- 24 Gwack, Y., Srikanth, S., Feske, S., Cruz-Guilloty, F., Oh-hora, M., Neems, D. S., Hogan, P. G. and Rao, A. (2007) Biochemical and functional characterization of Orai proteins. *J Biol Chem.* **282**, 16232-16243
- 25 Zhang, W., Chu, X., Tong, Q., Cheung, J. Y., Conrad, K., Masker, K. and Miller, B. A. (2003) A novel TRPM2 isoform inhibits calcium influx and susceptibility to cell death. *J Biol Chem.* **278**, 16222-16229
- 26 Redondo, P. C., Salido, G. M., Pariente, J. A., Sage, S. O. and Rosado, J. A. (2008) SERCA2b and 3 play a regulatory role in store-operated calcium entry in human platelets. *Cell Signal.* **20**, 337-346
- 27 Hofer, A. M., Fasolato, C. and Pozzan, T. (1998) Capacitative Ca^{2+} entry is closely linked to the filling state of internal Ca^{2+} stores: a study using simultaneous measurements of ICRAC and intraluminal $[\text{Ca}^{2+}]$. *J Cell Biol.* **140**, 325-334
- 28 Iwasaki, H., Mori, Y., Hara, Y., Uchida, K., Zhou, H. and Mikoshiba, K. (2001) 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels.* **7**, 429-439
- 29 Brenner, J. S. and Dolmetsch, R. E. (2007) TrpC3 regulates hypertrophy-associated gene expression without affecting myocyte beating or cell size. *PLoS ONE.* **2**, e802
- 30 Jardin, I., Lopez, J. J., Salido, G. M. and Rosado, J. A. (2008) Orai1 mediates the interaction between STIM1 and hTRPC1 and regulates the mode of activation of hTRPC1-forming Ca^{2+} channels. *J Biol Chem*

- 31 Xu, S. Z. and Beech, D. J. (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca(2+) channels in native vascular smooth muscle cells. *Circ Res.* **88**, 84-87
- 32 Rosado, J. A., Brownlow, S. L. and Sage, S. O. (2002) Endogenously expressed Trp1 is involved in store-mediated Ca²⁺ entry by conformational coupling in human platelets. *J Biol Chem.* **277**, 42157-42163
- 33 Boulay, G., Zhu, X., Peyton, M., Jiang, M., Hurst, R., Stefani, E. and Birnbaumer, L. (1997) Cloning and expression of a novel mammalian homolog of *Drosophila* transient receptor potential (Trp) involved in calcium entry secondary to activation of receptors coupled by the Gq class of G protein. *J Biol Chem.* **272**, 29672-29680
- 34 Trebak, M., Vazquez, G., Bird, G. S. and Putney, J. W., Jr. (2003) The TRPC3/6/7 subfamily of cation channels. *Cell Calcium.* **33**, 451-461
- 35 Putney, J. W., Jr. (1986) A model for receptor-regulated calcium entry. *Cell Calcium.* **7**, 1-12
- 36 Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L. and Muallem, S. (1998) Functional interaction between InsP3 receptors and store-operated Htrp3 channels. *Nature.* **396**, 478-482
- 37 Vazquez, G., Lievremon, J. P., St, J. B. G. and Putney, J. W., Jr. (2001) Human Trp3 forms both inositol trisphosphate receptor-dependent and receptor-independent store-operated cation channels in DT40 avian B lymphocytes. *Proc Natl Acad Sci U S A.* **98**, 11777-11782
- 38 Zitt, C., Obukhov, A. G., Strubing, C., Zobel, A., Kalkbrenner, F., Luckhoff, A. and Schultz, G. (1997) Expression of TRPC3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. *J Cell Biol.* **138**, 1333-1341
- 39 Zhu, X., Jiang, M. and Birnbaumer, L. (1998) Receptor-activated Ca²⁺ influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. Evidence for a non-capacitative Ca²⁺ entry. *J Biol Chem.* **273**, 133-142
- 40 Caroppo, R., Colella, M., Colasuonno, A., DeLuisi, A., Debellis, L., Curci, S. and Hofer, A. M. (2003) A reassessment of the effects of luminal [Ca²⁺] on inositol 1,4,5-trisphosphate-induced Ca²⁺ release from internal stores. *J Biol Chem.* **278**, 39503-39508
- 41 Verkhatsky, A. (2005) Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol Rev.* **85**, 201-279
- 42 Shmist, Y. A., Kamburg, R., Ophir, G., Kozak, A., Shneyvays, V., Appelbaum, Y. J. and Shainberg, A. (2005) N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine improves myocardial protection against ischemia by modulation of intracellular Ca²⁺ homeostasis. *J Pharmacol Exp Ther.* **313**, 1046-1057
- 43 Brownlow, S. L. and Sage, S. O. (2005) Transient receptor potential protein subunit assembly and membrane distribution in human platelets. *Thromb Haemost.* **94**, 839-845

FIGURE LEGENDS

Figure 1. Time-course of thrombin-induced hTRPC6 co-immunoprecipitation with STIM1 and Orai1 in human platelets.

A-D, Human platelets (2×10^9 cell/mL) were suspended in a Ca^{2+} -free medium (100 μM EGTA added) and then stimulated for various periods of time (10-60 s) with thrombin (1 U/mL) and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-hTRPC6 (**A** and **B**), anti-STIM1 (**C**) or anti-Orai1 antibody (**D**). Immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western blotting with a specific anti-STIM1 (**A**), anti-Orai1 (**B**) or anti-hTRPC6 antibody (**C** and **D**). Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panel shows results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of hTRPC6-STIM1 association (**A**) or hTRPC6-Orai1 association (**B**) in resting (control) and thrombin-treated cells. Results are presented as arbitrary optical density units and expressed as mean \pm S.E.M. * $P < 0.05$ vs. control.

Figure 2. Time-course of TG + ionomycin-induced hTRPC6 co-immunoprecipitation with STIM1 and Orai1 in human platelets.

Human platelets (2×10^9 cell/mL) were suspended in a Ca^{2+} -free medium (100 μM EGTA added) and then stimulated for various periods of time (10-60 s) with TG (1 μM) + Iono (50 nM) and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-hTRPC6 (**A** and **B**), anti-STIM1 (**C**) or anti-Orai1 antibody (**D**) and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western blotting with a specific anti-STIM1 (**A**) anti-Orai1 (**B**) or anti-hTRPC6 antibody (**C** and **D**). Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panel shows results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of hTRPC6-STIM1 association (**A**) or hTRPC6-Orai1 association (**B**) in resting (control) and thrombin-treated cells. Results are presented as arbitrary optical density units and expressed as mean \pm S.E.M. * $P < 0.05$ vs. control.

Figure 3. TPEN-mediated Ca^{2+} entry is attenuated by electroporation of the anti-hTRPC6 antibody.

(**A**) Fura-2-loaded human platelets were suspended in a Ca^{2+} -free medium (100 μM EGTA was added) and treated with TPEN (500 μM ; *upper graph*) or the vehicle (DMSO, *lower graph*), as control. Platelets were stimulated with 1 U/mL thrombin (*upper graph*) or the vehicle (HBS, *lower graph*) 1 min later followed by the addition of CaCl_2 (final concentration 300 μM) 3 min later to initiate Ca^{2+} entry. (**B**) Human platelets were electroporated in a Gene Pulser as described in "material and methods" and then were incubated in the presence of 1 $\mu\text{g}/\text{mL}$ anti-hTRPC6 antibody, anti-hTRPC3 antibody, both or 1 $\mu\text{g}/\text{mL}$ rabbit or goat IgG (*Control upper graph* and *lower graph*) for 60 min as indicated. Cells were then stimulated in a Ca^{2+} -free medium (250 μM EGTA was required since electroporated cells were suspended in medium containing 200 μM CaCl_2 , see material and

methods) with 500 μM TPEN (*upper graph*) or the vehicle (DMSO, *lower graph*) and 3 min later CaCl_2 (1 mM) was added to the medium to initiate Ca^{2+} entry. The traces are representative of five independent experiments.

Figure 4. Thrombin and extensive Ca^{2+} store depletion induced by TG + ionomycin induce Ca^{2+} -dependent co-immunoprecipitation of hTRPC6 with hTRPC3.

Control (**A** and **B**) and dimethyl BAPTA-loaded (**E** and **F**) human platelets (2×10^9 cell/mL) were suspended in a Ca^{2+} -free medium (100 μM EGTA added) and then stimulated for 30 s with thrombin (1U/mL; **A** and **E**) or TG (1 μM) + Iono (50 nM; **B** and **F**) and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-hTRPC6 antibody (**A**, **B**, **E** and **F** *upper panels*) or anti-hTRPC3 antibody (**A**, **B**, **E** and **F** *lower panels*) and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western blotting with a specific anti-hTRPC6 (*upper panels*) or anti-hTRPC3 (*lower panels*) antibody. Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of hTRPC6-hTRPC3 association in resting (control) and stimulated cells. Results are presented as arbitrary optical density units and expressed as mean \pm S.E.M. * $P < 0.05$ vs. control. (**C** and **D**) Human platelets were incubated at 37 °C for 30 min in the presence or absence of 10 μM dimethyl BAPTA, as indicated. At the time of experiment 100 μM EGTA was added. Cells were then stimulated with 1 U/mL thrombin (**C**) or 1 μM TG + 50 nM Iono (**D**). Elevations in $[\text{Ca}^{2+}]_c$ were monitored using the 340/380 nm ratio as described in the materials and methods section. Traces shown are representative of 5 independent experiments.

Figure 5. OAG enhances co-immunoprecipitation of hTRPC6 with hTRPC3 in the presence of extracellular Ca^{2+} .

A-D, Human platelets (2×10^9 cell/mL) were suspended in a Ca^{2+} -free medium (100 μM EGTA added; **A-C**) or in a medium containing 1 mM CaCl_2 (**D**) and then stimulated for 30 s with OAG (100 μM) and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-hTRPC6 antibody and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western blotting with a specific anti-hTRPC6 (**A-D**, *upper panels*), anti-Orai1 (**A**, *lower panel*), anti-STIM1 (**B**, *lower panel*) or anti-hTRPC3 antibody (**C** and **D**, *lower panels*). Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panel shows results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms represent the quantification of the association of hTRPC6 with Orai1 (**A**), STIM1 (**B**) and hTRPC3 (**C** and **D**) in resting (control) and OAG-stimulated cells. Results are presented as arbitrary optical density units and expressed as mean \pm S.E.M. * $P < 0.05$ vs. control. (**E-H**) Human platelets were electropermeabilized in a Gene Pulser as described in “material and methods” and then were incubated in the absence (**E**) or presence of 1 $\mu\text{g}/\text{mL}$ anti-hTRPC6 antibody (**G**), anti-hTRPC3 antibody (**H**), or rabbit anti-mouse IgG (**F**) for 60 min as indicated. Platelets were suspended in a Ca^{2+} -free medium (250 μM EGTA was added) and stimulated with OAG (100 μM ; **E** *upper graph*, **F**, **G** and **H**) or the vehicle (DMSO; **E** *lower graph*), as control, followed by the addition of CaCl_2

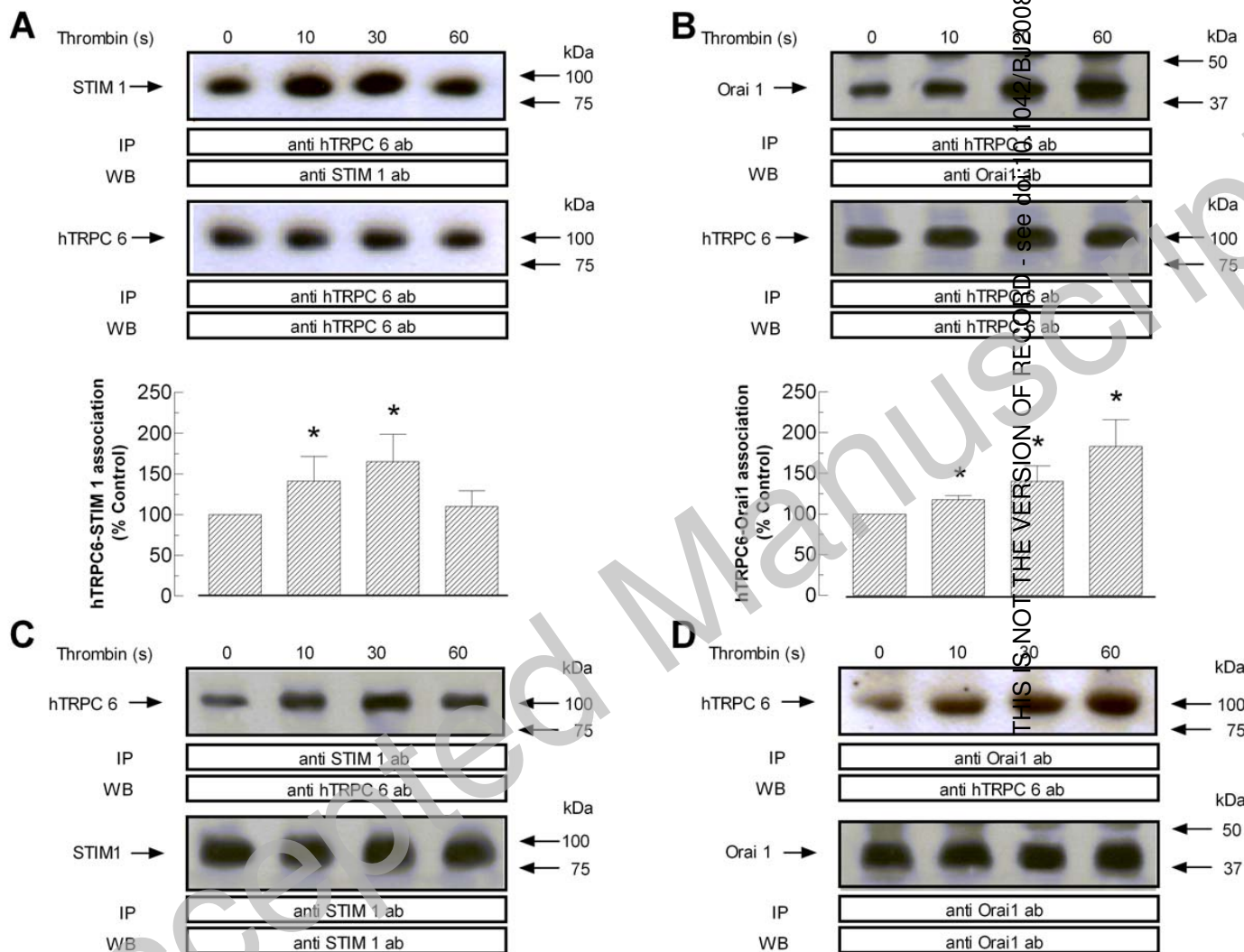
(final concentration 1 mM) 3 min later to initiate Ca^{2+} entry.

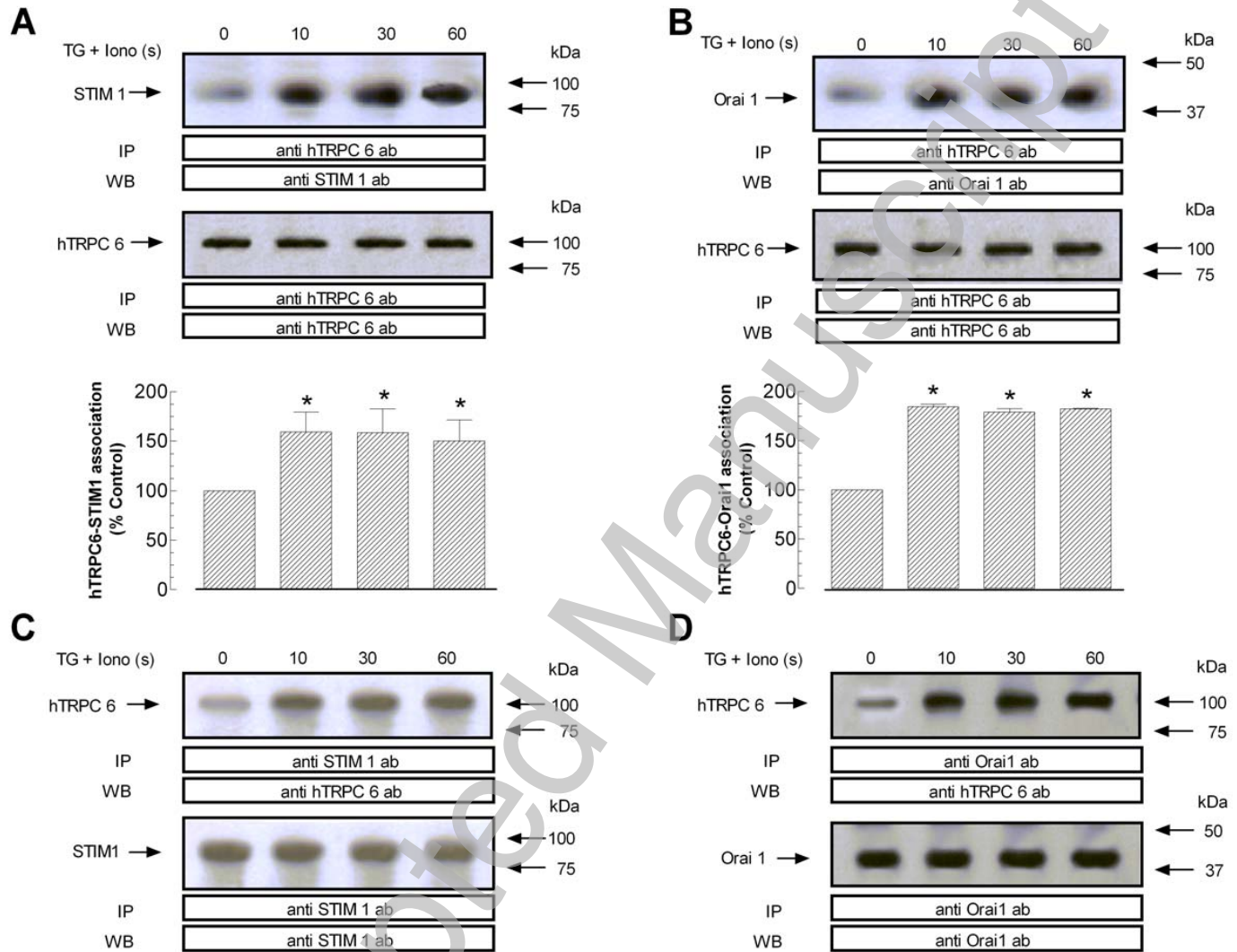
Figure 6. Treatment with TG + ionomycin is unable to induce co-immunoprecipitation of hTRPC3 with hTRPC1, STIM1 or Orai1.

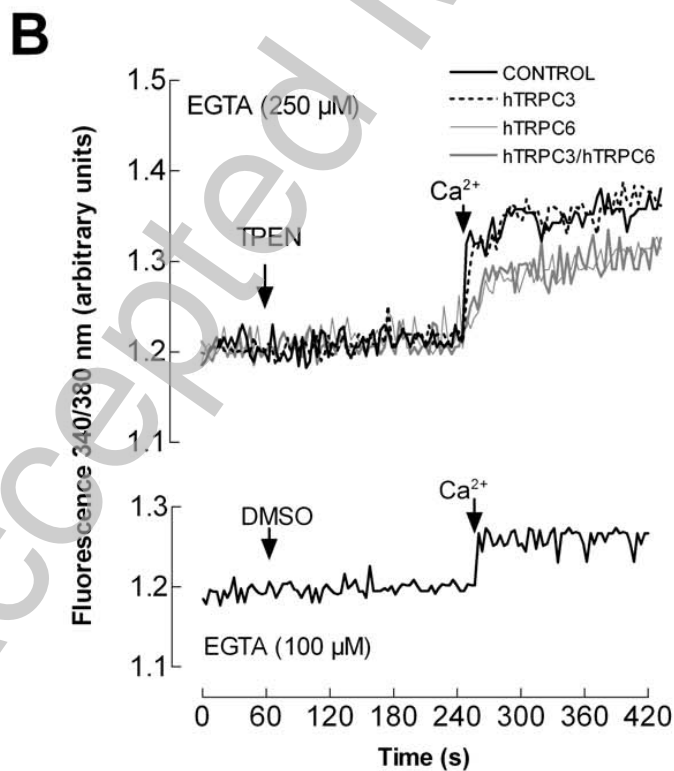
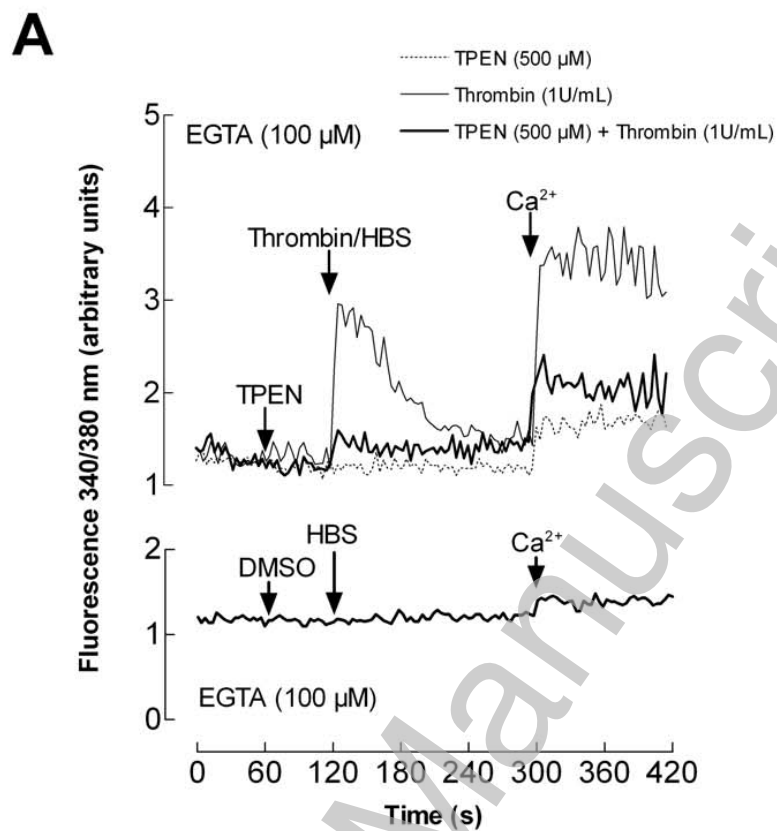
Human platelets (2×10^9 cell/mL) were suspended in a Ca^{2+} -free medium (100 μM EGTA added) and then stimulated for 30 s with TG (1 μM) + Iono (50 nM) and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-hTRPC3 (*upper panels*), anti-hTRPC1 (**A**, *lower panel*), anti-STIM1 (**B**, *lower panel*) or anti-Orai1 antibody (**C**, *lower panel*) and immunoprecipitates were subjected to 10 % SDS-PAGE and subsequent Western blotting with a specific anti-hTRPC1 (**A**, *upper panel*), anti-STIM1 (**B**, *upper panel*), anti-Orai1 (**C**, *upper panel*) or anti-hTRPC3 antibody (*lower panels*). Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panel shows results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Histograms (**A**) represent the quantification of the association of hTRPC1 with hTRPC3 in resting (control) and TG+Iono-stimulated cells. Results are presented as arbitrary optical density units and expressed as mean \pm S.E.M.

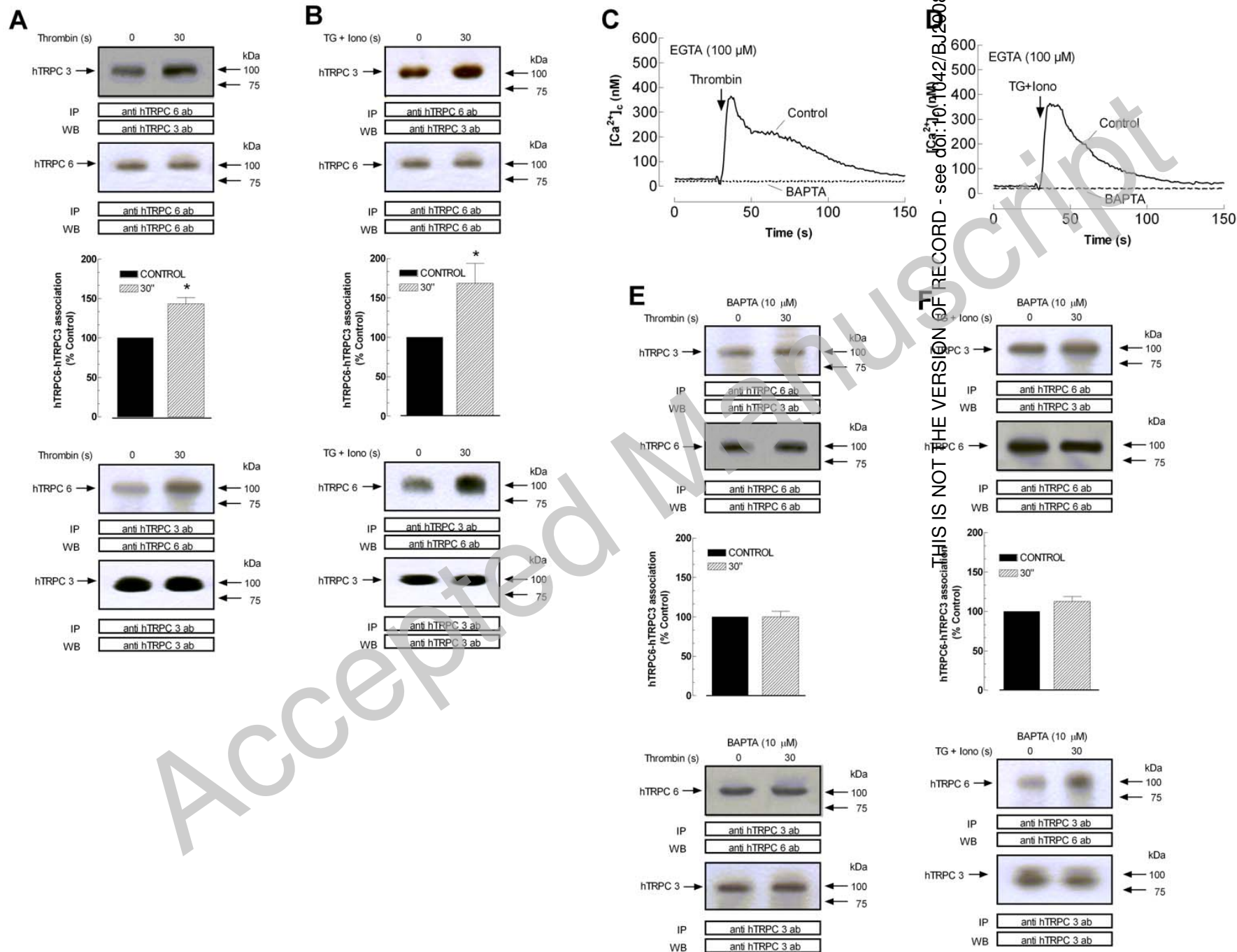
Figure 7. Speculative model of the involvement of hTRPC6 in capacitative and non-capacitative Ca^{2+} entry in platelets.

Activation of phospholipase C (PLC), via G protein-coupled receptors, results in the synthesis of IP_3 , which, in turn, activates IP_3 receptors (IP_3R) in the endoplasmic reticulum (ER). Depletion of the ER leads to the association of hTRPC6 to Orai1 and STIM1 (presented as the ER Ca^{2+} sensor) to participate in store-operated or capacitative Ca^{2+} entry (CCE). In contrast, activation of a non-capacitative Ca^{2+} entry (NCCE) pathway (i.e. via generation of DAG) displaces hTRPC6 from Orai1 and STIM1 and induces its association with hTRPC3.

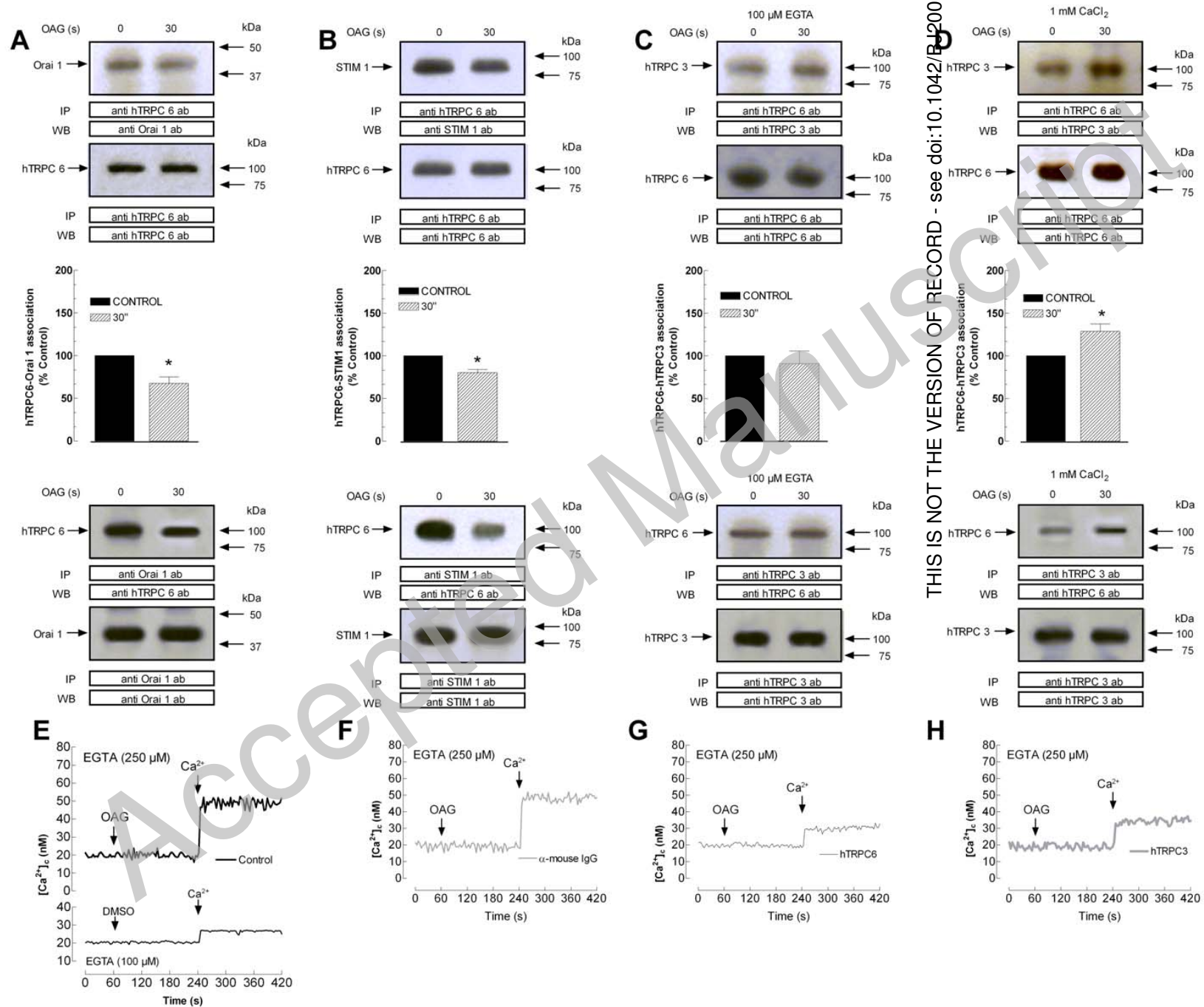








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