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FKBP52 is involved in the regulation of SOCE channels in the human platelets and MEG 01 cells

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ABSTRACT

Immunophilins are FK506-binding proteins that have been involved in the regulation of calcium homeostasis, either by modulating Ca^{2+} channels located in the plasma membrane or in the rough endoplasmic reticulum (RE). We have investigated whether immunophilins would participate in the regulation of stored-operated Ca^{2+} entry (SOCE) in human platelets and MEG 01. Both cell types were loaded with fura-2 for determining cytosolic calcium concentration changes ($[Ca^{2+}]_c$), or stimulated and fixed to evaluate the protein interaction profile by performing immunoprecipitation and western blotting. We have found that incubation of platelets with FK506 increases Ca^{2+} mobilization. Thapsigargin (TG)-evoked, Thr-evoked SOCE and TG-evoked Mn^{2+} entry resulted in significant reduction by treatment of platelets with immunophilin antagonists. We confirmed by immunoprecipitation that immunophilins interact with transient receptor potential channel 1 (TRPC1) and Orai1 in human platelets. FK506 and rapamycin reduced the association between TRPC1 and Orai1 with FK506 binding protein (52) (FKBP52) in human platelets, and between TRPC1 and the type II IP_3R, which association is known to be crucial for the maintenance of SOCE in human platelets. FKBP52 role in SOCE activation was confirmed by silencing FKBP52 using SiRNA FKBP52 in MEG 01 as demonstrated by single cell configuration imaging technique. TRPC1 silencing and depletion of cell of TRPC1 and FKBP52 simultaneously, impair activation of SOCE evoked by TG in MEG 01. Finally, in MEG 01 incubated with FK506 we observed a reduction in TRPC1/FKBP52 coupling, and similarly, FKBP52 silencing reduced the association between IP3R type II and TRPC1 during SOCE. All together, these results demonstrate that immunophilins participate in the regulation of SOCE in human platelets.

1. Introduction

Stored-operated Ca^{2+} entry (SOCE) is a major mechanism for conducting Ca^{2+} influx in human platelets, as well as in other non-excitable cell types [1–4]. In platelets, association of STIM1 and IP_3RII located in the membranes of internal stores, with Ca^{2+} channels expressed in the plasma membrane, like Orai1 and TRPC1, promotes SOCE [4–8]. In human platelets, several members of the canonical transient receptor potential channels, such as TRPC1, TRPC4 [6,8,9], TRPC3 and TRPC6 [6,10] have been reported to associate to Orai1; thus conforming heteromultimeric channel complexes, which have been shown to present different sensitivity to store depletion, which relies on STIM1 function [11–13].

Immunophilin family groups proteins with chaperone activity (PPIase-activity) have been classified into three main subfamilies: cyclophilins (Cyps) [14,15], that bind cyclosporin A (CsA) [14,16,17]; immunophilins (FK506-binding proteins or FKBPs) inhibited by FK506 (tacrolimus) and rapamycin (sirolimus), both structurally unrelated to cyclosporin A (CsA); and FCBPs (FK506- and cyclosporin-binding proteins) that bind both macrolides [16]. In human lymphocytes and other cell types, including human platelets, impairment of immunophilin activity evokes deregulation of intracellular Ca^{2+} homeostasis. In this sense, calstabin 12 (FKBP12.6) silencing in the mdx mice model evoked alteration of Ca^{2+} leak from the endoplasmic reticulum (ER), which resulted in a weak myocyte contraction that is characteristic of Duchene's disease [18,19]. In addition, several Ca^{2+}-handling proteins, which activity is crucial for cellular processes, have been shown to be compromised in the presence of antagonists of the immunophilin subfamily, such as cyclophilins. Treatment of cells with these antagonists impaired, for instance, the activity of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) in human platelets

Abbreviations: Cyps, cyclophilins; FKBPs, immunophilin; FKBP52, FK506 binding protein (52); ER, rough endoplasmic reticulum; SERCA2b, sarco-endoplasmic Ca^{2+}-ATPase isotype 2b; IP_3R II, inositol 1,4,5-trisphosphate receptor; TRPC, transient receptor potential channel; CsA, cyclosporin A; FK506, tacrolimus; PPIase, peptidyl-prolyl isomerase activity; CN, calcineurin; CaM, calmodulin; Rapa, rapamycin; Cyp, cypermethrin; $[Ca^{2+}]_c$, cytosolic calcium concentration; TG, thapsigargin; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; BSA, bovine serum albumin; Thr, thrombin; SiRNA FKBP52, small interfering RNA of FKBP52; ShRNA TRPC1, short hairpin RNA of TRPC1

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[20] and other cell types [21], and PMCA4C or calcineurin (CN) [22,23]. Finally, there is scarce information regarding the participation of immunophilins in SOCE, or whether they associate with SOC channels [24,25]. Hence, taking into account that some FKBP subfamily members regulate the permeability of intracellular Ca^{2+} channels, such as ryanodine receptors and IP_3 receptors [19,23], here we address the role of FKBPs in SOCE in human platelets and MEG01.

2. Material and methods

2.1. Materials

Apyrase (grade VII), aspirin, thrombin (Thr), dithiothreitol (DTT), thapsigargin (TG), sodium dodecyl sulphate (SDS), ionic detergent tween 20 (Tween-20), cypermethrin, ECL reagents and bovine serum albumin (BSA), rabbit anti-Orai1 antibody (c-terminal) were from Sigma (Madrid, Spain). FK506, cyclosporin A and rapamycin were from Selleck Chemical® (Huston, Texas, U. S. A). Protein A-agarose was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-hTRPC1 antibody, Anti-CN (PP2B) antibody, anti-FKBP52 antibody, anti-IP_3R type II antibody and SiRNA against FKBP52 were from (SantaCruz biotechnology®). ShRNA against TRPC1 was kindly provided by Dr. Ambudkar. MEG 01 culture medium RPMI, streptomycin/penicillin and other elements required for MEG 01 cell culture and protein silencing (KIT-C Amaxa) were purchased from LONZA®. Horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibodies, hyperfilm ECL and molecular weight markers were from GE Healthcare UK Ltd (Chalfont St. Giles, UK). All other reagents were of analytical grade.

2.2. Platelet preparation

Fura-2-loaded platelets were prepared as described previously [2–4]. 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 was then prepared by centrifugation for 5 min at 700×*g* and then aspirin (100 μM) and apyrase (40 μg/mL) were added. For intracellular Ca^{2+} concentration measurement, the platelet-rich plasma was incubated at 37°C with 2 μM of fura-2/AM for 45 min. Platelets were collected by centrifugation at 350×*g* for 20 min, then resuspended in HEPES-buffered saline (HBS), and finally subjected to the appropriate stimulation protocol as required. HBS contains (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase.

2.3. Cell culture and protein silencing procedure

The human megakaryoblastic cell line, MEG 01 cells, was obtained from ATCC (Manassas, VA, USA) and cultured at 37 °C with a 5% CO_2 in RPMI media, supplemented with 10% fetal calf serum, 2 mM L-glutamine and a cocktail of penicillin and streptomycin as recommended by the supplier. Cells were transiently transfected with SiRNA against FKBP52, or with ShRNA against TRPC1, and with both ShTRPC1 and SiFKBP52 simultaneously, using the kit-C and Amaxa Nucleofection system®, and following the manufacturer's instructions which in our hand have been previously shown to obtain efficient results [26]. Transfected MEG 01 cells were used 72 h after transfection upon ensuring by western blotting that targeted protein expression was efficiently reduced.

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

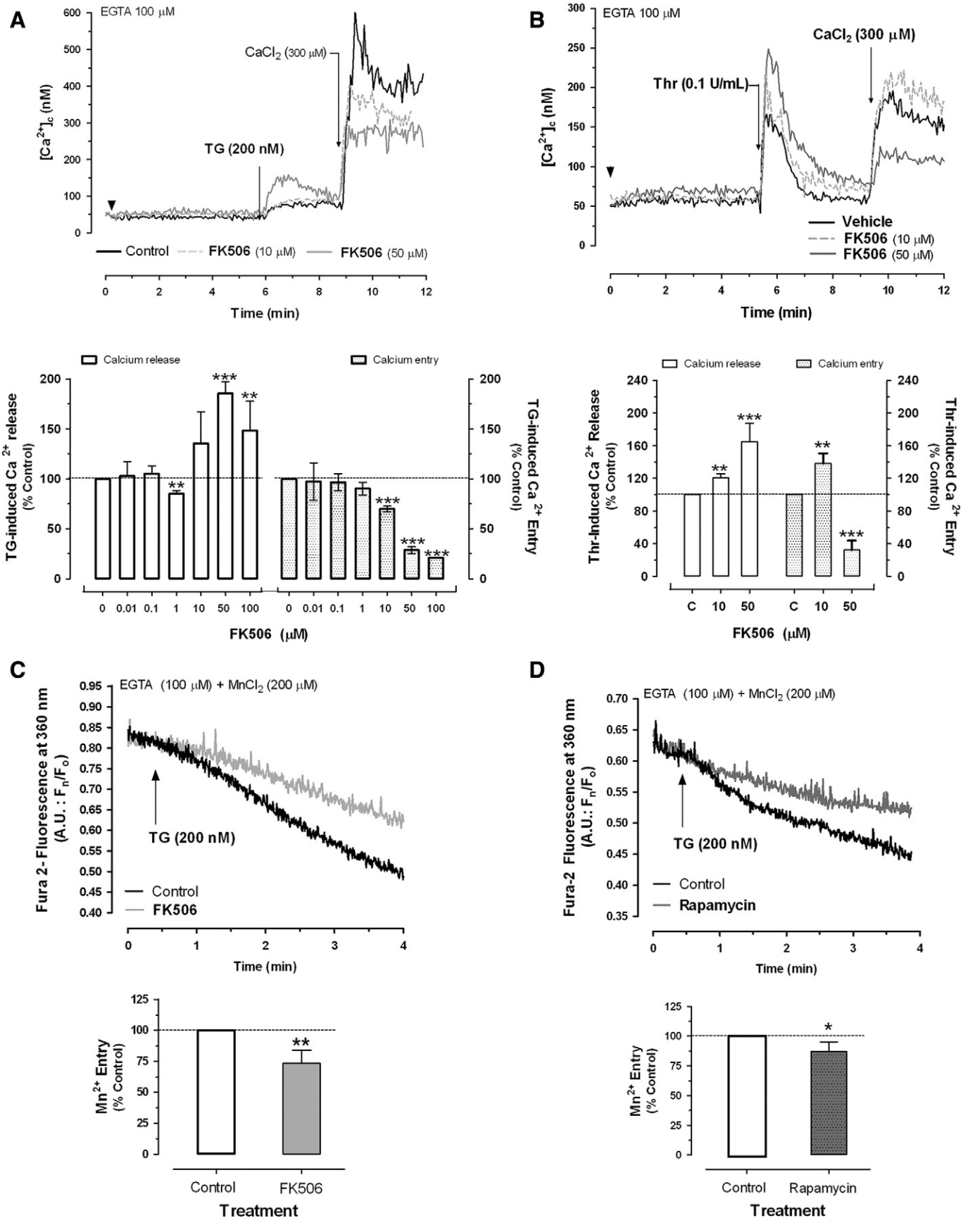
Fluorescence was recorded from 2 mL aliquots of magnetically stirred platelet suspension (10^8 cells/mL) at 37 °C using a spectrophotometer (Cary Eclipse, Varian, Madrid) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz [27]. Alternatively, MEG 01 cells were incubated for 30 min with 2 μM of fura-2/AM at room temperature and then centrifuged for 2 min at 100×*g*, and resuspended in fresh HBS medium supplemented with 50 μM of $CaCl_2$. MEG 01 cells were then transferred to a perfusion chamber that was placed under an inverted microscope. Changes in fura-2 fluorescence as result of changes in the $[Ca^{2+}]_c$ in MEG 01 cells were monitored using a single-cell configuration equipment and were processed using Aquacosmos software (Hamamatsu ®). Data are expressed as change in fluorescence after the addition of different stimulus (F_n) divided by fluorescence emitted by the cells under resting conditions (F_0). Additionally, Mn^{2+}-induced quenching of fura-2 fluorescence excited at 360 nm was used as a surrogate for monitoring Ca^{2+} entry, since both cations share the same channels and mechanisms for entering platelets, as previously described [28]. Data in the histogram are expressed as percentage of the changes in the decay rate at 360 nm evoked by fura-2-fluorescence quenching of Mn^{2+}, after addition of the stimulus (F_n) and compared to control non-stimulated cells (F_0).

2.5. Immunoprecipitation and western blotting

The immunoprecipitation and western blotting were performed as described previously [2–4]. Briefly, 250 μL aliquots of platelet suspension (10^9 cells/mL), MEG 01 cells wt (10×10^6 cells/mL) and MEG 01 cells transfected with the FKBP52 Si RNA or TRPC1 Sh RNA, or both RNAs, were then stimulated and lysed by mixing with an equal volume of lysis buffer, RIPA at pH 7.2, that contains; 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% triton X-100, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 100 μg/mL leupeptin and 10 mM benzamidine. Aliquots of platelet and MEG 01 cell lysates (500 μL) were immunoprecipitated by incubating cells with 2 μg/mL of anti-IP_3R type II, anti-FKBP52, anti-CN and 25 μL of protein A-agarose, overnight at 4 °C and placed in 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 or skimmed milk in tris-buffered saline with 0.1 % Tween 20 (TBST) to block residual protein binding sites. Immunodetection was achieved using the anti-TRPC1 polyclonal antibody diluted 1:200 in TBST (BSA), anti-FKBP52 diluted 1:200 in TBST (skimmed milk) and incubated for 2 h, or using an anti-Orai1 antibody incubated overnight at 4 °C and diluted 1:1000 in TBST (BSA). 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 with the appropriate horseradish peroxidase-conjugated secondary antibody diluted from 1:2500 to 1:10000 in TBST (BSA or skimmed milk), depending of the primary antibody used, and then exposed to enhanced chemiluminescence reagents for 4 min. Blots were then exposed to a photographic film. The density of bands on the film was measured using the Image J free software from NIH. Stripping of the membranes and reprobing using anti-IP_3RII, anti-CN, and anti-FKBP52, was done to corroborate that similar amount of protein was loaded in each gel lane.

2.6. Statistical analysis

Analysis of statistical significance was performed using Student's unpaired *t*-test and only values with $p<0.05$ were accepted as significant.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CaCl2 (300 μM)
TG (200 nM)
[Ca2+]c (nM)
Control
FK506 (10 μM)
FK506 (50 μM)
Time (min)
B
EGTA 100 μM
CaCl2 (300 μM)
Thr (0.1 U/mL)
[Ca2+]c (nM)
Vehicle
FK506 (10 μM)
FK506 (50 μM)
Time (min)
Calcium release
Calcium entry
TG-induced Ca 2+ release (% Control)
TG-induced Ca 2+ Entry (% Control)
FK506 (μM)
Calcium release
Calcium entry
Thr-Induced Ca 2+ Release (% Control)
Thr-induced Ca 2+ Entry (% Control)
FK506 (μM)
C
EGTA (100 μM) + MnCl2 (200 μM)
Fura 2- Fluorescence at 360 nm (A.U. : Fn/Fo)
TG (200 nM)
Control
FK506
Time (min)
Mn2+ Entry (% Control)
Control
FK506
Treatment
D
EGTA (100 μM) + MnCl2 (200 μM)
Fura-2 Fluorescence at 360 nm (A.U.: Fn/Fo)
TG (200 nM)
Control
Rapamycin
Time (min)
Mn2+ Entry (% Control)
Control
Rapamycin
Treatment

3. Results

3.1. FK506 alters Ca^{2+} leakage and active Ca^{2+} release from intracellular stores induced by agonists in human platelets

Treatment of human platelets in a Ca^{2+}-free medium with the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker, thapsigargin (TG) or with the physiological agonist thrombin (Thr), resulted in sustained or transient increase in $[Ca^{2+}]_c$, respectively (Fig. 1A–B). As shown in Fig. 1A, incubation of platelets with the immunophilin antagonist, FK506, increased TG- and Thr-evoked Ca^{2+} release from the intracellular pools. The effect of FK506 pretreatment for 5 min was dose-dependent. Thus, we found that TG-evoked Ca^{2+} release was increased by 36.6 ± 17.8% using 10 μM of FK506 (Fig. 1A, dark-gray dotted line; $p<0.05$; n = 6) and 85.4 ± 11.6% in cells incubated with 50 μM (Fig. 1A, dark-gray solid trace; $p<0.001$; n = 6). We have also found that in human platelets stimulated with TG, EC_{50} of FK506 was 9.75 μM. Similarly, active Ca^{2+} release from the stores evoked by thrombin (Thr, 0.1 U/mL) was enhanced in the presence of FK506 (20.63 ± 4.9% using 10 μM; Fig. 1B, dark-gray dotted line; $p<0.05$; n = 6; and 65.02 ± 22.4% in the presence of 50 μM; Fig. 1B, dark-gray solid line; $p<0.01$; n = 6).

3.2. FK506 alters TG- and Thr-induced Ca^{2+} entry in human platelets

As shown in Fig. 1A–B, Ca^{2+} entry induced by incubating human platelets either with TG or Thr, was altered in platelets previously incubated for 5 min with FK506. Incubation of human platelets with increased concentration of FK506 (0.01–100 μM) evoked a dose-dependent reduction in TG-evoked SOCE. IC_{50} of FK506 in SOCE observed was 18.5 μM. Furthermore, FK506 also altered Thr-evoked SOCE. In this case, different results were obtained depending of the FK506 dose used, thus platelets incubation with 10 μM of FK506 enhanced by 38.2 ± 12.5 % in Thr-evoked SOCE ($p<0.01$; n = 6), while 50 μM FK506 induced a significant attenuation of 67.8 ± 11.2% in Thr-evoked Ca^{2+}-entry ($p<0.001$; n = 8).

In order to further explore whether FK506 alters SOCE, experiments in the absence of extracellular Ca^{2+} (EGTA 100 μM was added), but in the presence of extracellular $MnCl_2$ (200 μM) were performed. Mn^{2+} extrusion from the cellular cytosol through PMCA and Na^+/Ca^{2+} is very low, as previously reported, therefore by using this cation we excluded possible interferences with Ca^{2+} extrusion mechanisms during cation entry monitoring [29,30]. As observed in Fig. 1C, incubation of platelets with 50 μM of FK506 significantly reduced TG-evoked Mn^{2+} entry by 26.7 ± 10.7% ($p<0.01$; n = 6).

3.3. Immunophilins regulate SOCE by a calcineurin (CN)-independent signaling pathway

Immunophilin inhibitors have been commonly referred in the literature as specific inhibitors of calcineurin, moreover their actual inhibitory effect that is based on a previous binding and subsequently inhibition of FKBPs, like FKBP12 or FKBP52, has remained unconsidered [22,23] [31]. However, it is widely accepted in the literature that FK506, upon binding to its target immunophilins, acquire a three-dimensional conformation that adapts inside the functional domain of CN inhibiting its activity [32]. Rapamycin is another immunophilin subfamily antagonist that upon complexing to FBKPs, reduces mTOR activity, without affecting CN activity; therefore, we have used rapamycin to ascertain whether CN is involved in SOCE. As shown in Fig. 1D, rapamycin treatment reduced Mn^{2+} entry by 13.1 ± 8.2 % ($p<0.05$; n = 6). Impairment of SOCE by rapamycin was then corroborated by performing experiments in the presence of 300 μM of $CaCl_2$. Under these experimental conditions, rapamycin administration significantly reduced TG-evoked Ca^{2+}-entry by 19.5 ± 9.4% (Fig. 2A; $p<0.05$; n = 6), and contrary to FK506 the Ca^{2+} leak from stores resulted in inhibition by 11.1 ± 4.8% ($p<0.05$; n = 6).

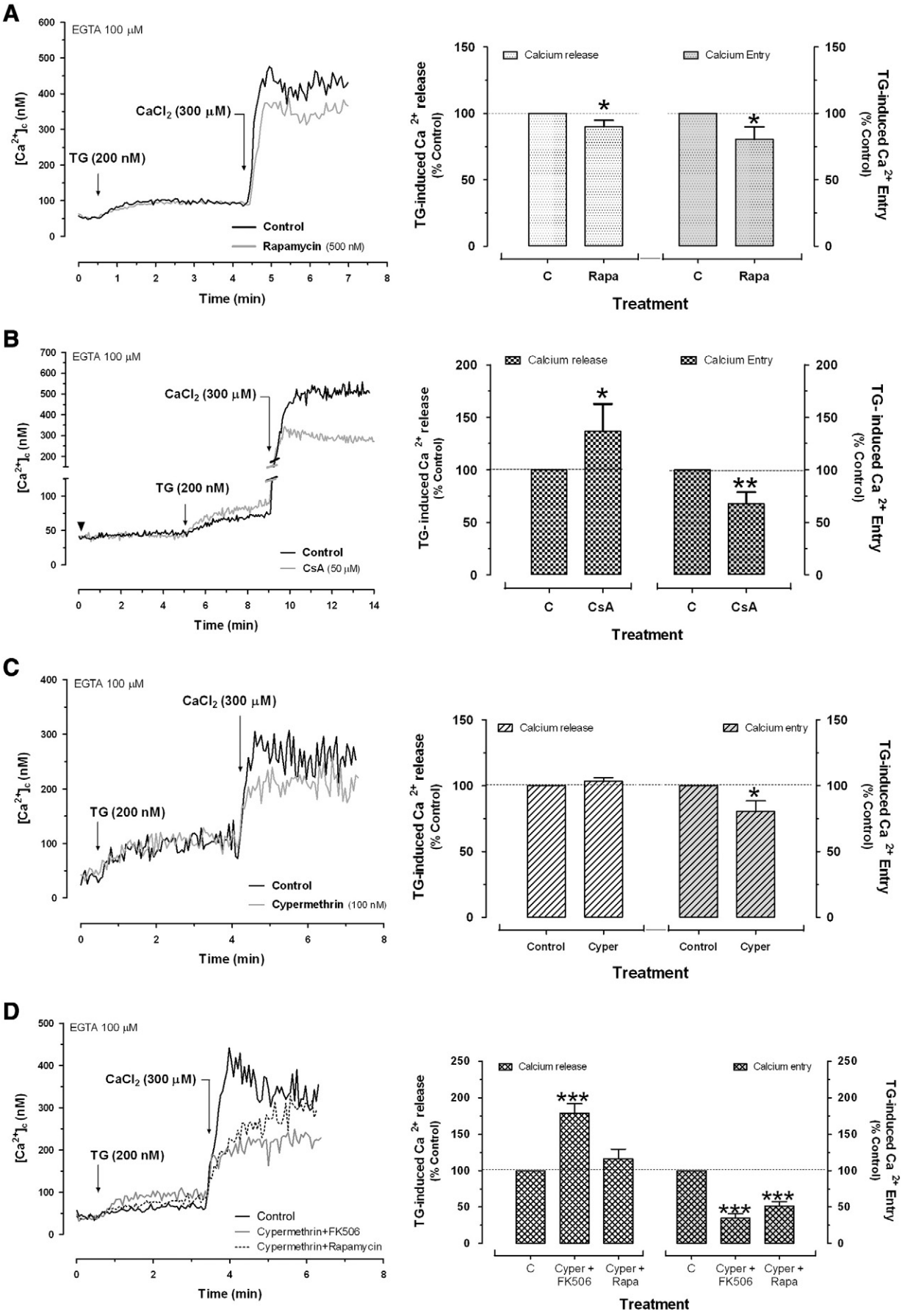
Inhibition of the other main immunophilin subfamily, cyclophilins, by incubating platelets with CsA (50 μM) for 5 min at 37 °C, also reduced TG-activated SOCE by 32.4 ± 11.0% (Fig. 2B; $p<0.01$; n = 6), while an increase on TG-evoked Ca^{2+} release of 36.6 ± 25.9% was observed ($p<0.05$; n = 6), perhaps due to more rapid inhibition of SERCA2b activity, as previously described [20].

Alternatively, incubation of human platelets with the specific CN inhibitor, cypermethrin (100 nM) for 30 min, whose action mechanism is different from that reported for immunophilin antagonist, reduced TG-induced SOCE by 14.0 ± 7.8% (Fig. 2C; $p<0.05$; n = 6), but without altering Ca^{2+} release from the stores. Finally, demonstration of a CN-independent role of immunophilins in SOCE activation is presented in Fig. 2D, where human platelets were incubated for 30 min with cypermethrin (100 nM) + rapamycin (500 nM), or with cypermethrin (100 nM) + FK506 (50 μM; for the latest 5 min previous to $[Ca^{2+}]_c$ determination). As demonstrated by comparing Fig. 2C and D, combined treatment with CN antagonist and immunophilin antagonists evoked bigger SOCE inhibition than cypermethrin alone (51.7 ± 5.8 % and 35.0 ± 5.9%, respectively; $p<0.001$; n = 4). As shown in Fig. 2D, only in the presence of FK506 a significant increase in TG Ca^{2+} release was observed (212.5 ± 20.8%, light-gray traces; $p<0.001$; n = 4). Hence, our results indicate that despite CN is regulating SOCE, an alternative regulatory pathway that relies on immunophilin activity is also present in human platelets.

3.4. Immunophilins are required for complexing Ca^{2+} channels during SOCE activation in platelets

Studies on the role of TRPC1 in SOCE are controversial. While studies in platelets from KO mice where TRPC1 reported no differences in SOCE activation [33], several others independent groups have demonstrated that TRPC1, alone or coupled to Orai1 and IP_3R type II, is an important elements for the activation and maintenance of SOCE in platelets and other cell types [34–36]. Here, we have evaluated the role of FKBPs in the formation of these proteins complexes by performing immunoprecipitations. Briefly, human platelets were treated at 37 °C for 5 min with FK506 (50 μM) or left untreated, and then stimulated in a Ca^{2+}-free medium for 1 min with Thr (0.1 U/mL). As shown in Fig. 3A, stimulation of human platelets with Thr (0.1 U/mL) evokes the formation of TRPC1/type II IP_3R complexes. The generation of this complex was attenuated by 40.2 ± 21.7% ($p<0.05$; n = 4) in platelets previously incubated for 5 min with FK506 (50 μM; Fig. 3A and histogram below). Additionally, in platelets treated for 30 min with the vehicle or rapamycin (500 nM) and then stimulated with Thr (Fig. 3B), the coupling between both elements was reduced by 27.9 ± 2.5% ($p<0.01$; n = 4). Furthermore, incubation of human platelets with FK506 (50 μM) equally impaired the association between TRPC1 and CN

Fig. 1. Effect of FK506 on cytosolic calcium homeostasis induced by TG and thrombin in human platelets. (A–B) Human platelets were suspended in a Ca^{2+}-free medium (100 μM of EGTA was added as indicated by arrowhead), and preincubated for 5 min at 37 °C in the absence (solid black traces) or presence of increased concentrations (0.01–100 μM) of FK506 (light-doted and dark solid-gray traces, respectively). Cells were then stimulated with TG (200 nM; A) or Thr (0.1 U/mL; B) and 3 min later 300 μM of $CaCl_2$ was added to extracellular medium to visualize calcium entry. (C–D) Platelets were suspended in calcium free-HBS and incubated for 5 min with FK506 (50 μM; C, gray trace) or for 30 min with rapamycin (500 nM; D, gray trace) or their respective vehicles (black traces) and upon addition of $MnCl_2$ (200 μM) platelets were stimulated with TG to enhance the opening of SOCE channels. Changes in fura-2 fluorescence were monitored using the 340/380 nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Alternatively, Mn^{2+} quenching properties over 360 nm wavelength of fura-2 is used as surrogated of Ca^{2+} to monitor SOCE activation which is represented as fluorescence initial (F_0) divided by fluorescence upon TG stimulation. Histograms represent mean ± SEM of six to eight separate experiments and they are presented as percentage of control. *, **, and *** represent $p<0.05$, $p<0.01$ and $p<0.001$, with respect to control platelets.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TG (200 nM)
[Ca2+]c (nM)
Control
Rapamycin (500 nM)
Time (min)
Calcium release
Calcium Entry
TG-induced Ca 2+ release (% Control)
TG-induced Ca 2+ Entry (% Control)
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TG (200 nM)
Control
CsA (50 μM)
Time (min)
Calcium release
Calcium Entry
C
CsA
Treatment
C
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
Control
Cypermethrin (100 nM)
Time (min)
Calcium release
Calcium entry
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Cyper
Treatment
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CaCl2 (300 μM)
TG (200 nM)
Control
Cypermethrin+FK506
Cypermethrin+Rapamycin
Time (min)
Calcium release
Calcium entry
C
Cyper + FK506
Cyper + Rapa
Treatment

induced by Thr in a 53.9 ± 7.7%, respect to platelets non-incubated with FK506 but stimulated with Thr (Fig. 3C and histogram below; $p<0.05$; n = 4). As expected, treatment with rapamycin (500 nM) for 30 min was without effect (Fig. 3D and histogram below; $p<0.05$; n = 4). Furthermore, we have found that treatment of platelets with FK506 and rapamycin prevented the increase in the association between FKBP52 and TRPC1 in platelets stimulated with Thr (0.1 U/mL), which in cells non-treated with FK506 was enhanced by 22.8 ± 16.9% (Fig. 3E;*, $p<0.05$; n = 4).

On the other hand, Orai1 have recently been described in human platelets and megakaryocytes as a key element during the activation of SOCE [11,37–39]. Hence, we have evaluated whether immunophilins might be associated to Orai1 during calcium entry in human platelets. As shown in Fig. 4A, we have found that in resting conditions FKBP52 complexed to Orai1, and upon platelet stimulation with Thr, FKBP52/Orai1 interaction was enhanced by 57.2 ± 34.7% ($p<0.05$; n = 4). As reported above for the TRPC1/FKBP52 complex, platelets preincubation for 5 min with FK506 (50 μM) evoked a significant reduction in FKBP52/Orai1 complex, both in resting conditions (25.5 ± 10.1%; ***, $p<0.001$; n = 4), and upon stimulation with Thr (98.3 ± 17.6%; $$$, $p<0.001$; n = 4). Similarly, incubation with rapamycin (500 nM) for 30 min reduced by 86.3 ± 9.5% the coupling between FKBP52 and Orai1 induced by Thr in these cells ($$$, $p<0.001$; n = 4).

3.5. FKBP52 is required for SOCE activation in human platelets and MEG 01 cells

In order to corroborate the involvement of FKBP52 in SOCE, we have silenced FKBP52 in MEG 01 cells, which is a megakaryoblastic cell line that in presence of thrombopoietin and under the adequate experimental conditions have been derived into platelets [40]. It has been reported that MEG 01 cells express most of the elements that participates in calcium homeostasis and in particular that have been previously described participating in SOCE activation mechanism in human platelets [41–45]. Hence, by using Amaxa® nucleofector and Si RNA FKBP52 (2 μg/mL), we were able to reduce efficiently the expression of FKBP52 upon 72 h of transfection procedure, as revealed Western blotting (see Fig. 5A left-hand side image). Furthermore, we have also silenced TRPC1 alone using a Sh RNA TRPC1 (see Fig. 5A right-hand side image). As shown in Fig. 5B, single-cell imaging experiments using MEG 01 cells with fura-2 revealed that silencing of FKBP52, TRPC1 or both reduced significantly TG-evoked SOCE by 25.4 ± 10.1%; $p<0.05$; n = 4), 33.0 ± 4.0% ($p<0.01$; n = 4) and 46.2 ± 18.3% ($p<0.01$; n = 4), respectively. Transfection itself was without effect on Ca^{2+} accumulation inside the intracellular stores, as demonstrated by the fact that irrelevant alteration in calcium leak in response to TG is found upon comparing the areas under the curves as described in the Material and methods section (Fig. 5B and C).

Additionally, MEG 01 cells transfected with Si RNA FKBP52 and incubated for 5 min with FK506 showed reduced TG-evoked Ca^{2+}-release (61.6 ± 14.1%; $p<0,001$; n = 4; Fig. 5C) and SOCE (32,3 ± 21.3%; $p<0,01$; n = 4; Fig. 5C).

Finally, we have observed that in MEG 01 cells, FKBP52/Orai1 complex is detected under resting conditions and it is significantly enhanced by 35.1 ± 15.1% upon stimulation with TG for 1 min (Fig. 4B; $p<0.01$; n = 6), and as occurred in platelets, preincubation with FK506 (50 uM for 5 min) significantly reduces FKBP52/Orai 1 complex generated by stimulating with TG (200 nM) (61.9 ± 28.4%; $p<0.01$; n = 6). Additionally, we have observed lack or very low association between TRPC1-FKBP52 in MEG 01 cells under resting condition, but a very significant TRPC1/FKBP52 coupling was found upon SOCE stimulation with TG (51.5 ± 20.2%; $p<0.05$; n = 4; Fig. 5D). Treatment of MEG 01 cells with 50 μM FK506 for 5 min reduced by 72.8 ± 17.1% the TRPC1/FKBP52 association ($p<0.001$; n = 4). We further explore the role of FKBP52 in generation of SOCE-associated complexes, by silencing FKBP52, which reduced by 127.2 ± 40.7% TG-evoked type II IP_3R/TRPC1 coupling as compared to controls (Fig. 5E; $p<0.001$; n = 4), thus revealing a direct role in the regulation of TRPC1 function in MEG 01, as occurred in platelets.

4. Discussion

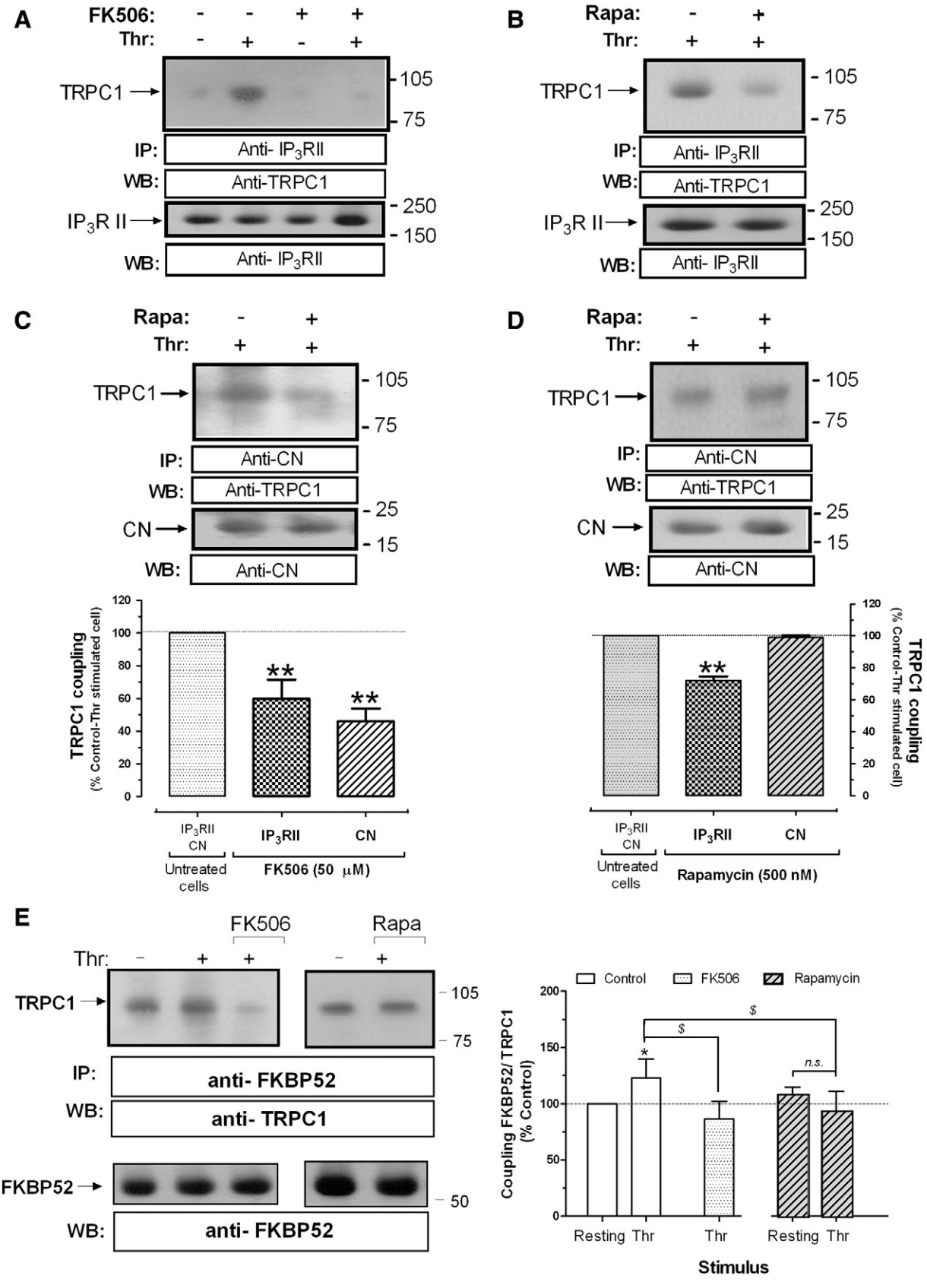
Immunophilin inhibitors have been often considered as specific CN activity antagonists [22,23,31], since immunophilins-FK506 and CsA-immunophilins complexes inhibits CN [46], despite the fact that FK506 or CsA specifically target the peptidyl-prolyl isomerase active domain of immunophilins. Hence, in the present study we have evaluated the possible participation of immunophilins in intracellular Ca^{2+} homeostasis in human platelets independently of CN.

The increasing effect induced by FK506 on Ca^{2+} release may be attributed to the activity of CaM- and CN-dependent phosphorylation/dephosphorylation mechanisms of ER-resident Ca^{2+} channels as previously reported in other cells types [47–50]. However in human platelets, as observed in Fig. 3D, despite CN might participate in the regulation of Ca^{2+} leak from the stores, a CN independent Ca^{2+} leak evoked by FK506 is demonstrated, by using cypermethrin, a CN antagonist, which was reported without effect in Ca^{2+} leak. Under these experimental conditions, FK506 was still able to further increase Ca^{2+} leakage in platelets stimulated with TG. Nevertheless, a regulatory role for CN on Ca^{2+} leakage is probably present in human platelets, due to the significant differences observed in Ca^{2+} leakage between cell treated with cypermethrin and rapamycin and compared with platelets treated with rapamycin alone (see Fig. 3A vs D). In this sense, CN-dependent mechanisms might be also present in MEG 01 cells, where treatments of Si RNA FKBP52 transfected cells with FK506 showed a significant reduction in the Ca^{2+} leak upon stimulation with TG, indicative of a role for FKBP52 Ca^{2+} leak. Furthermore, participation of other immunophilins cannot be rule out.

Our results concerning SOCE regulation by immunophilins are consistent with previous observations in other cell models [31,51]. Nevertheless, other authors have considered a unique retrograde CN-dependent mechanism for regulating Ca^{2+} entry activation. The mechanisms currently proposed suggest that only in conditions where $[Ca^{2+}]_c$ reaches a critical value, CN may evoke the activation of non-capacitative arachidonic acid-dependent Ca^{2+} channels (ARC). Furthermore, these authors contemplate the possibility of an alternative participation of immunophilins, different from the CN pathway in HEK293 cells [31]. In human platelets, as shown in Fig. 2, an alternative CN-independent pathway might also coexist, since as we have shown, FK506 has a dual effect in SOCE depending on the concentration used (see Fig. 1), while rapamycin incubation also inhibits SOCE, and this effect has been linked to inhibition of the mTOR (AKT/PKB) pathway instead of the classically proposed CN mechanism [17,52]. The latest observations in MEG 01 and platelets suggest that FKBP52, or other immunophilin activities, like FKBP12, are impaired by complexing with their target drugs, such as FK506 or rapamycin, being enough to alter SOCE in our cellular lineage.

Fig. 2. Immunophilins participate in SOCE activation by CN-dependent but also CN-independent signaling pathways. Fura-2 loaded platelets were suspended in HBS and subsequently incubated at 37 °C for 30 min with either rapamycin (500 nM; A) or cypermethin (100 nM; C); or a combination of cypermethrin + rapamycin (D, dark-doted trace) and cypermethrin + FK506 (50 μM; D, light-solid gray trace). Alternatively, platelets are incubated for 5 min with cyclosporin A (CsA, 50 μM; B). Once incubation times with the different conditions was over, platelets were stimulated with TG (200 nM) in a calcium free-HBS (EGTA 100 μM was added as indicated the by arrowhead) and 4 min later $CaCl_2$ (300 μM) was added to visualized calcium entry. Changes in fura-2 fluorescence were monitored using the 340/380 nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Traces are representative of four to six independent experiments. *, **, and *** represents $p<0.05$, $p<0.01$ and $p<0.001$, with respect to control platelets.

Despite that other groups have presented evidences against the participation of TRPCs and in particular TRPC1 in SOCE, using protocols that include TRPC1-knockout mice model [33], here we show that in MEG 01 cells TRPC1 silencing has a clear negative effect on SOCE, thus demonstrating that, as previously reported in human platelets and in several other cell types, TRPC1 has an important



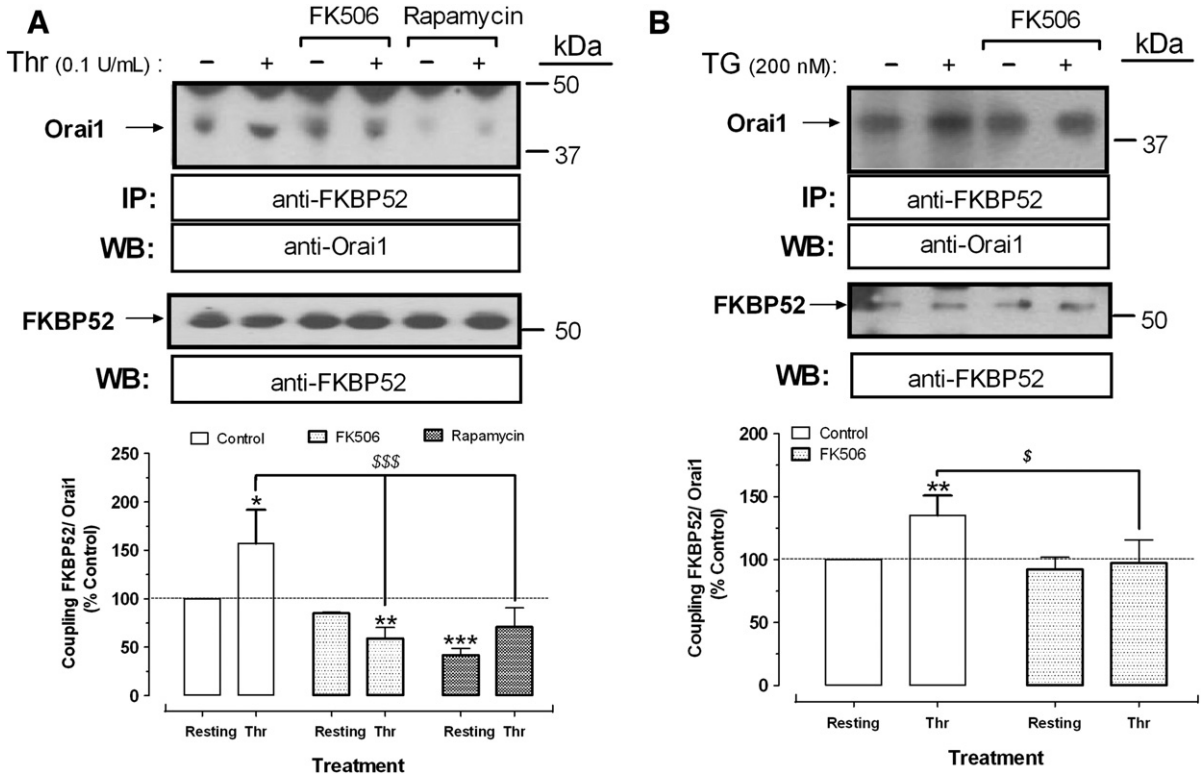


Fig. 4. Platelets treatment with FK506 and rapamycin impairs FKBP52 association to Orai1 in human platelets and MEG 01 cells. Human platelets (A) and MEG 01 (B) suspended in free-Ca^{2+} medium (EGTA, 100 μM) were incubated for 5 min at 37 °C with FK506 (50 μM) or for 30 min with rapamycin (500 nM) as indicated. Cells were then stimulated for 1 min with Thr (0.1 U/mL, A) or TG (200 nM; B) to induce Ca^{2+} entry, and then they were fixed by mixing for 10 min with equal volume of ice-cold RIPA. FKBP52 was immunoprecipitated from platelet lysates by incubating overnight at 4 °C with 2 μgr/mL of specific anti-FKBP52 antibody as described under Material and methods. Western blotting was done by incubation with a specific anti-Orai1 antibody diluted 1:1000 in blocking buffer overnight. Reproving of the membranes was done using the specific anti-FKBP52 antibody for 2 h diluted 1:200 in blocking buffer containing skimmed milk. Images shown are representative of four independent experiment and histograms represents the percentage of coupling as compared to control and expressed as means and standard errors. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, as compared to untreated resting cells.

role in SOCE [34–36]. Experimental data, obtained by our group and others, suggest that TRPC1 is a relevant element of the macromolecular complex newly generated during SOCE [6,11,53].

Recently studies have described by using nuclear magnetic resonance (NMR) exchange spectroscopy, a region within TRPC1 structure, which has been designated as TRP box 2. This region has been described as a suitable target region for the immunophilin-PPIase activity. It has been also suggested that after conformational changes induced by FKBP52 or FKBP12, consisting on isomerization of the proline residues found within this target region, TRPC1 would be unrecognized by Homer. Homer has recently been described as a structural element that would support the complexes of TRPC1 during SOCE activation. On the other hand, it has also being involved in SOCE inhibition due to its complexing to an extra homer-binding domain identified in TRPC1 structure [54]. Hence, we suggest that removal of immunophilins from TRPC1 heteromultimeric complex disturbs the fragile stoichiometry of the macromolecular complex generated during activation of SOCE, being this role independently of calcineurin activity. Evidences of this role of immunophilin in the generation of the macromolecular complexes are presented in Fig. 4, where both, FK506 and rapamycin significantly block the coupling between TRPC1 and type II IP_3R. Similarly in MEG 01 cells transfected with Si RNA FKBP52, a reduction in TRPC1/type II IP_3R was also observed. Furthermore, as mentioned above, TRP regulation by physical interaction of immunophilin members has already been reported mainly linked to non-capacitative Ca^{2+} channels, such as TRPL, TRPV, and TRPC6. Here, we have shown for the very first time that FKBP52 and TRPC1 interaction occurs in human platelets and in MEG 01 cells, and how these complexes are impaired by treating human platelets with the FKBP inhibitors, FK506 and rapamycin. In other cell models, FKBP12 associates with TRPC3 and TRPC6, and FKBP52 binds to TRPC1, TRPC4 and TRPC5, hence participation of additional FKBPs during SOCE activation in human platelets cannot be excluded, since in MEG 01 where FKBP52 was silenced FK506 was still able to evoke a significant reduction of SOCE.

To our knowledge this is the first description that Orai1 interacts with immunophilin members, and this mechanism is found not only in the platelet but also in megakaryoblastic cell line, MEG 01 hence, indicating that this mechanism is not exclusive of differentiated cells like platelets.

Finally, alternative explanation for FKBP52-dependent regulatory mechanism of SOCE would involve a competitive association with the scaffolding protein homer, that has been recently shown to

Fig. 3. Immunophilins regulate the generation of the macromolecular TRPC1-complex during Thr-evoked calcium entry in human platelets. Human platelets suspended in calcium-free medium (EGTA, 100 μM) were incubated for 5 min at 37 °C with FK506 (50 μM; A, C and E) or for 30 min with rapamycin (500 nM; B, D and E), as indicated. Platelets were then stimulated for 1 min with Thr (0.1 U/mL) and lysed by mixing for 10 min with equal volume of ice-cold RIPA. Immunoprecipitation of either the type II IP_3R, CN and FKBP52 was achieved using 2 μgr/mL of specific antibodies as described under Material and methods. Western blotting using an anti-TRPC1 antibody diluted in TBST containing BSA (1:200) for 2 h was done to analyze coupling formation. Membrane reproving with the antibody used for immunoprecipitation confirmed that similar amount of protein was loaded in all gel lanes. Histograms below show the percentage of association of the different proteins with TRPC1 in Thr-stimulated platelets incubated under the different experimental conditions, and compared to platelets left untreated but stimulated with Thr. *, $p<0.05$ and **, $p<0.01$ with respect to resting untreated platelets and $, $p<0.05$ with respect to untreated but Thr-stimulated platelets. Images are representative of additional four independent experiments.

interact with TRPC1 and Orai1 and regulates SOCE in several cell types including platelets [55–58]. In this sense, in neurons it has recently been shown that homer shares a binding region with FKBP52 that is localized within the TRPC1 C-terminal domain, however the existence of such a competence has not been confirmed in human platelets yet and to our knowledge would deserved future investigation according to the relevance of the result concerning FKBP52 presented here [59].

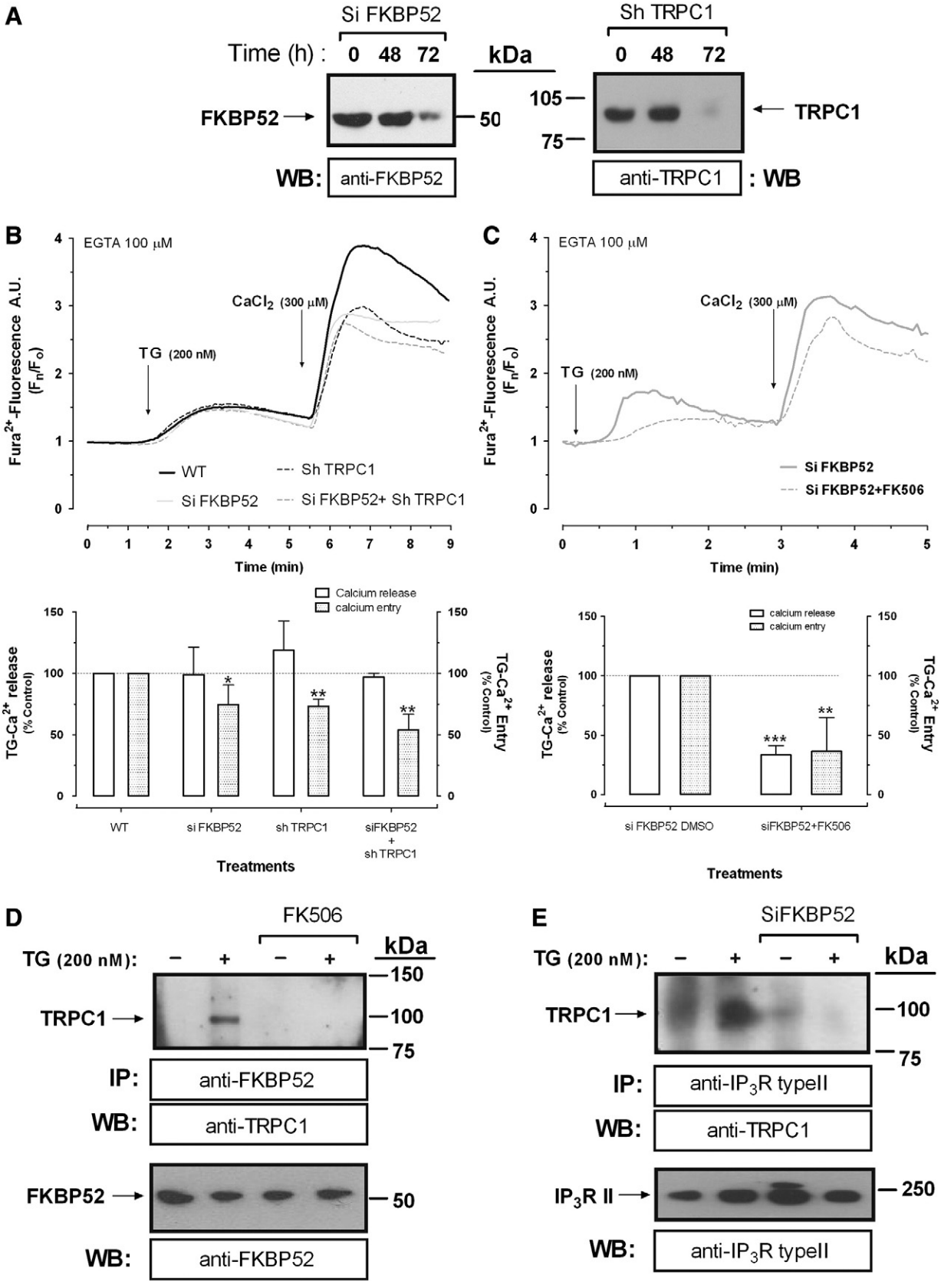


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Fig. 5. Effect of FKBP52 and TRPC1 silencing in SOCE in MEG 01 cells. (A–C, E) MEG 01 cells were transfected either with empty plasmid (WT), or with Si RNA FKBP52, Sh RNA TRPC1 or both for 72 h, or left untreated (D). (A) Evaluation of the silencing efficiency was corroborated after 72 h by Western blotting using anti-FKBP52 and anti-TRPC1 antibodies as described under Material and methods (B–C). MEG 01 cells transfected as indicated were loaded with fura-2 and additionally incubated for 5 min in absence (B) or presence of FK506 (50 μM; C). TG-evoked Ca^{2+} release and entry were monitored by using a flow chamber and single-cell imaging configuration in cells alternatively excited at 340/380. Additionally, MEG 01 cells non transfected (D) or transfected with siRNA FKBP52 (E), were left under resting condition or incubated for 5 min with FK506 (50 μM) as indicated. Cells were then stimulated with TG (200 nM) for 1 min and then lysed. Immunoprecipitation of either FKBP52 (D) or type II IP_3R (E) was achieved using the appropriate antibody, and subsequent Western blotting was performed using an anti-TRPC1 antibody as described under Material and methods section. Histograms represent means and standard errors of four to six independent experiments, while images are representatives of four independent coimmunoprecipitations. *, $p<0.05$, **, $p<0.01$. and ***, $p<0.001$ compared to MEG 01 WT, or siRNA-FKBP52 transfected MEG 01 cells treated with vehicle.

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