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FKBP25 and FKBP38 regulate non-capacitative calcium entry through TRPC6

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ABSTRACT

Non-capacitative calcium entry (NCCE) contributes to cell activation in response to the occupation of G protein-coupled membrane receptors. Thrombin administration to platelets evokes the synthesis of diacylglycerol downstream of PAR receptor activation. Diacylglycerol evokes NCCE through activating TRPC3 and TRPC6 in human platelets. Although it is known that immunophilins interact with TRPCs, the role of immunophilins in the regulation of NCCE remains unknown. Platelet incubation with FK506, an immunophilin antagonist, reduced OAG-evoked NCCE in a concentration-dependent manner, an effect that was independent on the inactivation of calcineurin (CaN). FK506 was unable to reduce NCCE evoked by OAG in platelets from TRPC6$^{-/-}$ mice. In HEK-293 cells overexpressing TRPC6, currents through TRPC6 were altered in the presence of FK506. We have found interaction between FKBP38 and other FKBPs, like FKBP25, FKBP12, and FKBP52 that were not affected by FK506, as well as with calmodulin (CaM). FK506 modified the pattern of association between FKBP25 and TRPCs as well as impaired OAG-evoked TRPC3 and TRPC6 coupling in both human and mouse platelets. By performing biotinylation experiments we have elucidated that FKBP25 and FKBP38 might be found at different cellular location, the plasma membrane and the already described intracellular locations. Finally, FKBP25 and FKBP38 silencing significantly inhibits OAG-evoked NCCE in MEG-01 and HEK293 cells, while overexpression of FKBP38 does not modify NCCE in HEK293 cells. All together, these findings provide strong evidence for a role of immunophilins, including FKBP25 and FKBP38, in NCCE mediated by TRPC6.

1. Introduction

In human platelets, Ca^{2+} entry from the extracellular medium is a key signaling event for both secretion and aggregation upon occupation of the plasma membrane receptors. Two major receptor-operated Ca^{2+} influx mechanisms have been described in these cells: store-operated or capacitative Ca^{2+} entry (SOCE) and non-capacitative Ca^{2+} entry (NCCE) [1–4]. In human platelets, and other cell types, both mechanisms would occur simultaneously upon the activation of G protein-coupled receptors and, subsequently, phospholipase C, which, in turn, generates diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) [5]. IP_3 mobilizes Ca^{2+} from the internal stores, leading to the activation of the stromal interaction molecule 1 (STIM1) [6–8], which regulates the activation of the store-operated Ca^{2+} (SOC) channels in the plasma membrane. Simultaneously, diacylglycerol either directly or indirectly gates non-capacitative Ca^{2+} channels [5,9].

It has been claimed that certain TRPC and Orai subunits form the channels involved in NCCE [4,10–12]. In human platelets, it has been described the generation of the TRPC1/4/5 and TRPC3/6 heteromultimeric complexes in response to physiological agonists [13]. In this cell type, it has been generally accepted that NCCE mechanism is conducted by heteromultimeric channels resulting from the association between TRPC3 and TRPC6 [14–16].

The immunophilin family comprises immunophilins (FKBPs), cyclophilins (Cyps), and junglone. They are chaperone proteins with peptidyl-prolyl isomerase activity [17]. Regarding FKBP subfamily structure, FKBP12 shows the most basic structure among the members of the FKBP subfamily, containing just one FK506-binding domain (FKBD). Other FKBPs, like FKBP52 and FKBP64, have two or three FKBD domains [17]. The affinity of the FKBD region for FK506 is different among the members of the family and some FKBPs have affinity for other macrolide

Abbreviations: Cyps, cyclophilins; FKBPs, immunophilins; FKBD, FK506-binding domain; ER, rough endoplasmic reticulum; SERCA, sarco-endoplasmic Ca^{2+}-ATPase; IP_3Rs, inositol 1,4,5-trisphosphate receptors; IP_3, inositol 1,4,5-trisphosphate; TRPC, transient receptor potential channel; CsA, cyclosporin A; FK506, tacrolimus; CaN, calcineurin; CaM, calmodulin; Cyp, cypermethrin; $[Ca^{2+}]c$, cytosolic free-calcium concentration; Thr, thrombin; siRNA FKBP, small interfering RNA of FKBP; SOCE, store-operated calcium entry; NCCE, non-capacitative calcium entry; STIM1, stromal interaction molecule 1; Orai1, calcium release-activated calcium modulator; OAG, 1-oleoyl-2-acetyl-glycerol; DAG, diacyl glycerol; DTT, dithiothreitol; BSA, bovine serum albumin; PRP, plasma rich platelet; HBS, HEPES buffered saline; ACD, acid citrate dextrose buffer; TBST, tris-buffered saline supplemented with 0.1% Tween 20 detergent; WT, wild type; CAP, control antigen peptide; ADP, adenosine 5′-diphosphate sodium salt; WCL, whole cell lysate

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lactones like rapamycin; for instance, it was reported that FKBP25 has more affinity for rapamycin than for FK506 [18,19]. In the presence of FK506, FKBPs interfere with calcineurin activity; meanwhile, in cells incubated with rapamycin, FKBPs interact with and inhibit mTOR activity, which participates in the cell cycle by downregulating the PI3K/AKTPKB/mTOR complex. Moreover, the FKBD region might be mutated such as in the case of FKBP38 [20], which was not considered to have neither peptidyl-prolil isomerase activity nor FK506 affinity. In fact, FKBP38 has a very anomalous structure as compared to other immunophilin members, since it presents three TPR domains and a leucine-zipper repeat that facilitate its interaction with other proteins [20]. The FKBP38 activity and its association with FK506 were later demonstrated, although it was subordinated to previous complexing with CaM [21,22]. The intracellular location of the FKBP members might differ. For instance, FKBP25 and FKBP133 are mostly located in the nucleus; meanwhile FKBP12, FKBP25, FKBP38, FKBP51, and FKBP52, are located in the ER and mitochondrial membranes, or in the proximity of the ribosomes, like FKBP38 and FKBP25. FKBP38 has a particular C-terminal domain and three TPR domains that facilitate the associations to cellular membranes, such as the mitochondrial membrane, and the interaction with other proteins, respectively [17]. FKBP51 might change their intracellular localization, migrating from the mitochondria to the nucleus [17,23].

FKBPs regulate intracellular Ca^{2+} homeostasis by interacting with different Ca^{2+} channels, like ryanodine or IP_3 receptors [24–28]. FKBPs also interact with plasma membrane Ca^{2+} channels. For instance, FKBP12 and FKBP52 interact with TRPCs [29–32] and in human platelets, Orai1 and TRPC1 interact with FKBP12 and FKBP52 in response to thrombin (Thr) or the SERCA inhibitor, thapsigargin [30]. Studies in cells from the rat cerebral cortex and HEK cells have revealed that FKB12 interacts with TRPC3, TRPC6, and TRPC7; while FKBP52 interacts with TRPC1, TRPC4, and TRPC5 [29]. Furthermore, TRPC6 overexpressed in HEK293 interacts with the native FKBP13 [29]. Hence, in the present study we have investigated the role of FKBPs in the activation of NCCE in non-excitable cells.

2. Experimental

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM) and fluo-4 acetoxymethyl ester (fluo-4/AM) were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), adenosine 5′-diphosphate sodium salt (ADP), aspirin, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), ionic detergent tween 20 (Tween-20), Na_3VO_4, cypermethrin, bovine serum albumin (BSA), geneticin G418 disulfate salt solution, and anti-actin antibody were from Sigma® (Madrid, Spain). FK506 was from Selleck Chemical® (Huston, Texas, U.S.A.). OAG was from Calbiochem® (Merck© Millipore, Darmstadt, Germany, UK). Protein A-agarose was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Amaxa Kit-C for MEG-01 transfection and culture media were from Lonza® (Cultek, Madrid, Spain). Goat IgG antibody, anti-FKBP38 antibody, siRNA control, and siRNAs against FKBP25 and FKBP38 were purchased from Santa Cruz biotechnology Inc. (Texas, U.S.A.). FKBP38 overexpression was achieved using the vector of expression pEGFP-C1 containing the fused protein eGFP-FKBP38 that was kindly provided by Dr. Gunter Fischer (Max Planck Research Unit for Enzymology of Protein Folding, Halle/Saale, Germany). Transfecting Lipid Reagent was from Bio-Rad (Madrid, Spain). The anti-PMCA, anti-calmodulin, anti-TRPC6, anti-TRPC3, and anti-TRPC1 antibodies were from Alomone (Jerusalem, Israel). Horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibodies, hyperfilm ECL and molecular weight markers were from GE Healthcare Ltd (Chalfont St. Giles, UK). All other reagents were of analytical grade.

2.2. Animals

TRPC6-deficient (TRPC6 KO) and wild type (WT) mice were purchased from Deltagen, Inc. (San Mateo, CA) through the European Mouse Mutant Archive (EMMA) network (B6;129P2-Trpc6tm1Dgen/H; http://www.emmanet.org/deltagen/DELTAGEN_T881/). Briefly, murine TRPC6 allele expression was disrupted by substitution of 13 nucleotides located into the exon four (E4) with a selection cassette containing an internal ribosome entry site, β-galactosidase and the neomycin resistant gene. Chimeric mice were backcrossed with C56BL/6 mice to obtain the first generation of heterozygous mice, which were subsequently intercrossed to obtain homozygous mice for the Trpc6-null allele. TRPC6 silencing using this crossing has been previously reported by our research group [33,34]. All experiments were conducted in mice between 8 and 12 weeks of age in accordance with the regulations of the Local Ethical Committee.

2.3. Human and mouse platelet preparations

Human platelets were prepared as described previously [30,35]. 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 then aspirin (100 μM) and apyrase (40 μg/mL) were added. Platelets were then centrifuged at 350 ×*g* for 20 min, and resuspended in HEPES-buffered saline (HBS) containing (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.

Mice were bled through the retro orbital plexus upon being anesthetized with isoflurane [33,34] and blood was collected into a test tube containing 300 μL of ACD. Mouse blood was centrifuged in a Galaxy 7D centrifuge (VWR International, PA, U.S.A.) at 300 ×*g* for 5 min. The supernatant was centrifuged again at 100 ×*g* for 5 min to obtain the PRP. PRP was centrifuged at 600 ×*g* for 5 min in the presence of aspirin (0.1 μg/mL) and the pellet was resuspended in Ca^{2+}-free Tyrode's buffer (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 12 mM, NaH_2PO_4 0.43 mM, glucose 0.1%, HEPES 5 mM, BSA 0.35%, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, pH 7.13) containing apyrase (40 μg/mL).

2.4. Culture cell lines and protein silencing

HEK293 cells, stably expressing TRPC6, were grown using DMEN supplemented with antibiotics and 10% FBS at 37 °C with 5% CO_2. HEK 293 cells were detached from the culture dish using trypsin solution (0.25% trypsin + 0.1% EDTA) and stored in the culture medium at room temperature for patch-clamp experiments within 5 h [36,37]. Additionally, for Ca^{2+} experiments, cells were passed to a 96 hole plate 24 h before the beginning of the experiments.

The human megakaryoblastic cell line MEG-01 was obtained from ATCC (Manassas, VA, U.S.A.) and cultured at 37 °C and 5% CO_2 in RPMI media, supplemented with 10% FCS, 2 mM L-glutamine and antibiotics.

MEG-01 cells were transiently transfected, in parallel experiments, with siRNA control, siRNA FKBP25 and siRNA FKBP38, using the kit-C and following manufacturer's instructions [31,38]. Two micrograms of siRNA control, siRNA FKBP25, and siRNA FKBP38 were added to the cuvette. Upon transfection, cells were transferred to fresh medium and kept for 72 h. Protein silencing was verified by Western blotting using specific antibodies as described below. Additionally, HEK293 cells at 60–90% confluence were transfected with 10 μg siRNA FKBP25 and siRNA FKBP38 using Transfecting Lipid Reagent. Upon 5 h of incubation at 37 °C with the mix, cells were supplemented with fresh medium and they were analyzed 72 h after transfection.

Finally, HEK293 cells were transfected with a FKBP38 overexpression plasmid using the vector of expression pEGFP-C1 containing the fused protein eGFP-FKBP38 (2 μg/mL) and the Transfecting Lipid Reagent following the manufacturer's instructions. 24 h after transfection, cells positively transfected were identified using an epifluorescence microscope equipped with a 60× objective.

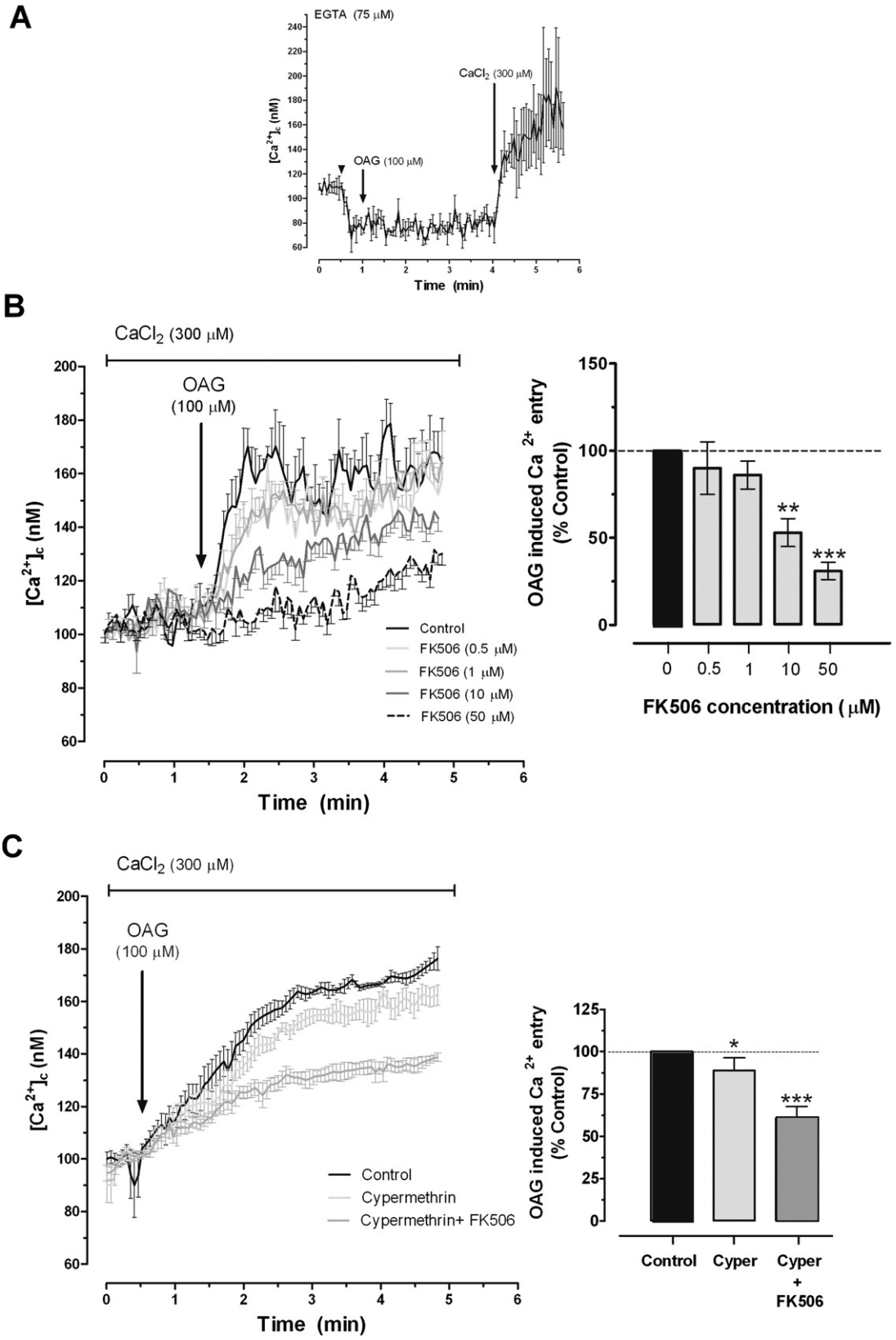

Fig. 1. FK506 reduces NCCE independently of its inhibitory effect in CaN activity. (A) Fura-2-loaded human platelets resuspended in Ca^{2+} free HBS medium (75 μM EGTA was added as indicated with the arrowhead) and they were subsequently stimulated with OAG (100 μM). (B) Fura-2-loaded human platelets were suspended in HBS medium containing 300 μM Ca^{2+} and preincubated for 5 min with increasing concentration of FK506 (0.5–50 μM), after which OAG (100 μM) was added to initiate NCCE. (C) NCCE was stimulated by adding OAG (100 μM) to platelets suspended in HBS containing 300 μM Ca^{2+} and previously incubated for 30 min in the absence or presence of the CaN inhibitor cypermethrin (100 nM, cyper) alone or in combination with FK506 (50 μM) that was added to the cuvette 5 min before starting the Ca^{2+} records. Graphs represent the mean ± standard deviation of six independent experiments using different blood donors. Histograms represent OAG-evoked Ca^{2+} entry expressed as percentage of control (cells not treated with FK506). Data are presented as mean ± S.E.M. *, **, ***: represent $P < 0.05$, <0.01, and <0.001, as compared to control, respectively.

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

PRP obtained from both human and mouse blood was incubated at 37 °C with 2 μM of fura-2/AM for 40 min in order to evaluate the changes in the $[Ca^{2+}]_c$ evoked during platelets activation. Fluorescence was recorded using a spectrofluorophotometer (Cary Eclipse, Varian, Madrid) in 2 mL aliquots of magnetically stirred human platelet suspensions (10^8 cells/mL in HBS) at 37 °C. Mouse platelets were suspended in 600 μL of tyrode's buffer containing 50 μM of $CaCl_2$, and the $[Ca^{2+}]_c$ was monitored using a RF-5301PC spectrofluorophotometer (Shimadzu®, Japan). Samples were alternatively excited at the wavelengths of 340 and 380 nm and emission was recorded at 510 nm. Changes in $[Ca^{2+}]_c$ were determined using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz [39]. Alternatively, MEG-01 cells were incubated for 30 min with 2 μM of fura-2/AM at room temperature, centrifuged for 2 min at 100 ×*g*, and then resuspended in fresh HBS medium supplemented with 50 μM of $CaCl_2$. MEG-01 and HEK293 cells were transferred to a perfusion chamber that was placed under an inverted microscope. Emission of fura-2 fluorescence as result of changes in the $[Ca^{2+}]_c$ in MEG-01 and HEK293 cells were monitored using a single-cell configuration equipment and was processed using Aquacosmos software (Hamamatsu ®, Japan). Data are expressed as the change in fluorescence after the addition of different stimulus (Fn) divided by the fluorescence emitted by the cells under resting conditions (Fo). Finally, HEK293 cells overexpressing TRPC6 were loaded with fluo-4 by incubating the cells for 60 min at 37 °C. $[Ca^{2+}]_c$ in HEK293 cells were monitored using a Flexstation 3 system (Molecular Devices®, U.S.A.), in which cells were stimulated by 100 μM of OAG, 300 μM of $CaCl_2$, using a robotic arm, and following the setting established by the main handling control unit.

2.6. Patch-clamp experiments

Whole-cell patch-clamp recordings were performed as previously described elsewhere [36,37]. Patch pipette solution contained (in mM): 40 EGTA, 10 HEPES, 48 L-glutamic acid, 8 NaCl, 2 $MgCl_2$, 10 Na_2 ATP, 10 Hepes, 17 $CaCl_2$ (osmolarity was adjusted to ~300 mOsm with mannitol and pH was titrated to 7.2 using CsOH). The extracellular (bath) solution contained (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 $BaCl_2$, 10 HEPES, 10 glucose (osmolarity ~290 mOsm and pH 7). A salt-agar bridge was used to connect the Ag–AgCl ground wire to the bath. Signals were amplified with an Axopatch 200B patch-clamp amplifier and controlled with Signal software 3.05 (CED). A 200 ms ramp protocol from −100 mV to +100 mV was applied at a frequency of 0.1 Hz from a holding potential of −60 mV. Current signals were analogue filtered (Bessel) at 1 kHz and digitally sampled at 3 kHz. TRPC6 current was measured at -80 and +80 mV, and the differences in the current between control and FK506-treated cells were estimated and represented as % respect to control.

2.7. Platelet aggregation

Platelets were suspended in 400 μL of HBS and incubated for 1 h at 37 °C with 1 μg/mL of rabbit anti-TRPC6 antibody, 1 μg/mL of rabbit IgG or 1 μg/mL anti-TRPC6 antibody neutralized by preincubation for 1 h with 1 μg/mL CAP. Platelet aggregation stimulated by 10 μM ADP was monitored in the presence of 1 mM $CaCl_2$ using a Chronolog aggregometer (Havertown, Pa, U.S.A.). Platelets were kept during the experiments at 37 °C under stirring conditions (1200 rpm) [65].

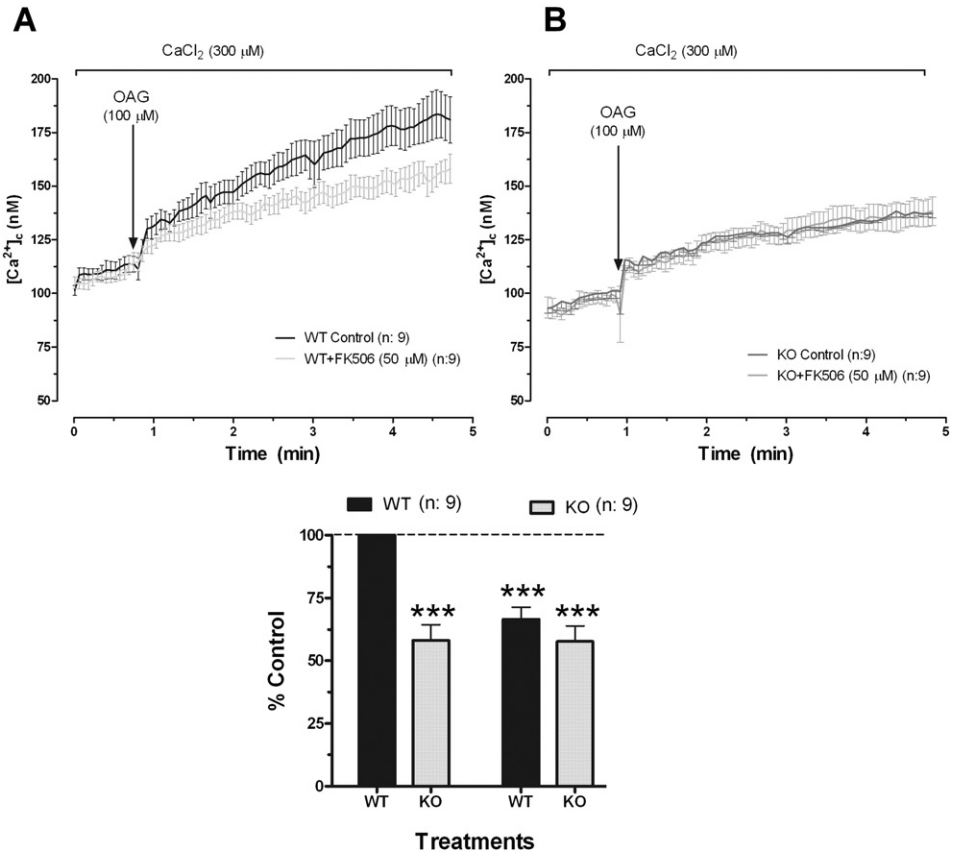

Fig. 2. NCCE entry is not affected by FK506 in murine TRPC6$^{-/-}$ platelets. Fura-2-loaded platelets were drawn from WT (A) and TRPC6$^{-/-}$ mice (B), and were suspended in tyrode's buffer-Ca^{2+} rich medium, respectively. Platelet suspensions were then incubated for 5 min with FK506 (50 μM), upon which NCCE was stimulated with 100 μM of OAG. Graphs represents the mean ± standard deviation of six independent experiments using 9 different mice of 8 to 10 weeks old, while histograms show the average of the percentage and S.E.M. respect to the controls. ***: $P < 0.001$.

2.8. Biotinylation protocol

Aliquots of 500 μL of platelets (1×10^8 cells/mL) were suspended in HBS and were centrifuged and resuspended in PBS/EDTA (500 μM), and the cells were then centrifuged (600 ×*g* for 5 min). Pellet was then suspended in biotynilation buffer containing: 50 mM $NaHCO_3$ + 0.9% NaCl. Finally, 150 μL of biotin-containing buffer was added and then were incubated for 1 h at ice-cold temperature. The biotinylation reaction was terminated by addition of 1% NH_4Cl and then we supplemented the buffer with 100 μL of PBS/EDTA (500 μM). Following biotinylation, cells were washed four times in PBS/EDTA, and resuspended in IP buffer and subsequently lysed with IGEPAL (2% solution was mixed). Samples were incubated with 25 μL streptavidin beads overnight at 4 °C and resuspended in Laemmli's buffer for subsequent analysis by Western blotting.

2.9. Immunoprecipitation and Western blotting

Aliquots (500 μL) of human platelet suspensions (10^9 cells/mL) were stimulated with OAG in the absence or presence of FK506, and then, cells were lysed by mixing with an equal volume of lysis buffer, RIPA (2×) (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 Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 100 μg/mL leupeptin and 10 mM benzamidine. MEG-01 and HEK293 cells were lysed in buffer NP-40 (2×) containing: 40 Tris-HCl, 277 NaCl, 20% Glycerol, 2% Nonidet, 4 EDTA. Aliquots of platelets, MEG-01 and HEK293 cell lysates (1 mL) were immunoprecipitated by incubating cells with 1 μg/mL of anti-TRPC6, anti-FKBP25, anti-FKBP38 antibodies, rabbit IgG, and 25 μL of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were solved by 10% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight in tris-buffered saline with 0.1% Tween 20 containing 10% (w/v) BSA (TBST) to block residual protein binding sites. Immunodetection was achieved using the anti-TRPC6 antibody diluted 1:1000 in TBST, anti-TRPC3 antibody diluted 1:1000 in TBST, anti-TRPC1 antibody diluted 1:200 in TBST, anti-CaM diluted 1:500 in TBST, anti-FKBP12 diluted 1:500 in TBST, anti-FKBP25 diluted 1:500 in TBST, anti-FKBP38 diluted 1:500 in TBST, anti-FKBP52 diluted 1:500 in TBST, and incubated for 2 h, or using an anti-actin antibody incubated for 1 h at 4 °C and diluted 1:1000 in TBST. 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 1:10,000 in TBST, depending on the primary antibody used, and then exposed to enhanced chemiluminescence reagents for 5 min. Non-specific bands due to the secondary antibodies were assessed by reprobing the membranes in the absence of primary antibodies.

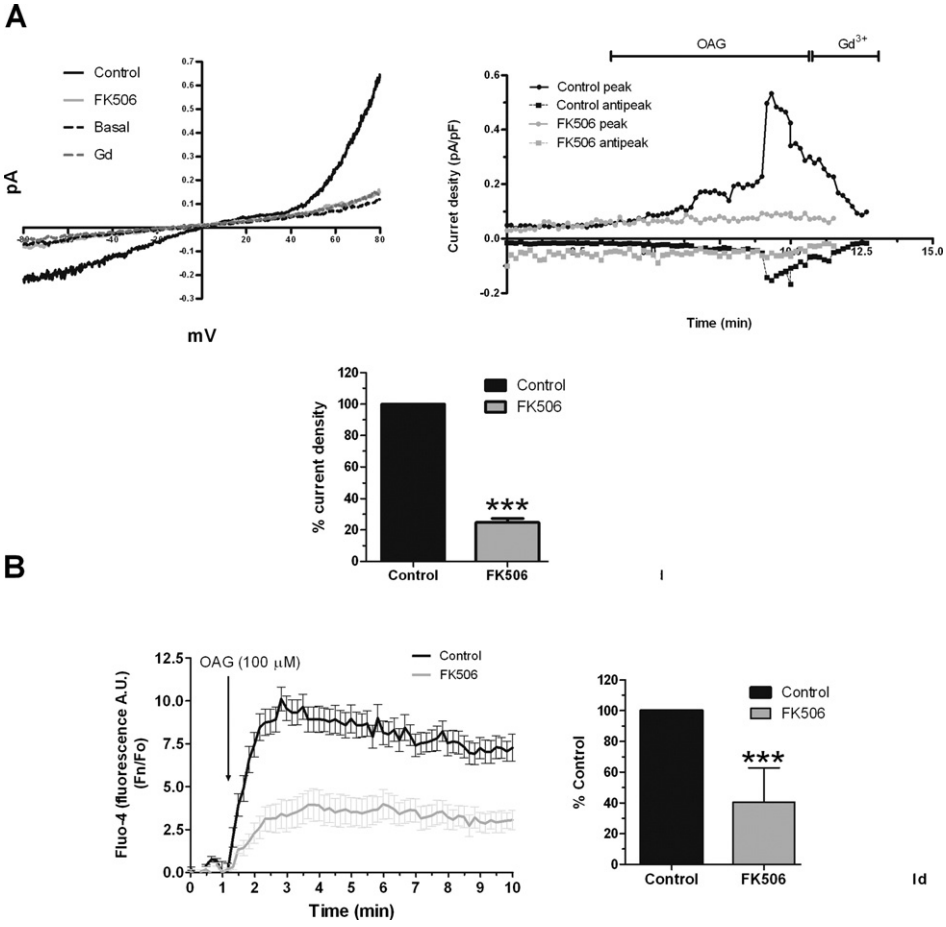

Fig. 3. FK506 almost completely abolishes the TRPC6 current and Ca^{2+} permeability in HEK293 cells overexpressing TRPC6. HEK293 constitutively expressing TRPC6 cell line were culture in D-MEN over coverslips. (A) Patch-clamp experiments were performed to monitor TRPC6-dependent current in presence or absence of FK506 (50 μM). Time course of FK506-evoked inhibition of the hTRPC6 currents activated by OAG (100 μM) is represented on the left hand side; current amplitudes were measured at -80 mV and +80 mV. Inward and outward current densities of OAG-activated hTRPC6 are represented in the right hand side. Gd^{3+} was added to the chamber at the end of each cuvette to corroborate that the current monitored relay on TRPC6 permeability. (B) HEK293 cells constitutively expressing TRPC6, were culture in DMEN over coverslips and they were detached 24 h before the beginning of the experiments and added to the 96-hole plate. Fluo-4 loaded cells were incubated in the presence or absence of FK506 (50 μM) for 5 min, and they where automatically stimulated with OAG (100 μM). $[Ca^{2+}]_c$ changes were analyzed using a Flexstation system®. Patch-clamp results are representative of nine independent experiments, while Flexstation experiments were reproduced six times and expressed as mean ± standard deviation of changes in the arbitrary unit of fluo-4 fluorescence (F_n/F_0) of 4 independent experiments, F_0: fluo-4 fluorescence unit before adding the stimulus. ***: $P < 0.001$, as compared to control.

The bands resulting of the Western blotting were acquired using the C-digit device (Licor®). The density of bands on the film was measured using the Image J free software from NIH. Biotinylated samples were additionally developed using anti-PMCA antibody (1 h; 1:1000) in order to demonstrate that similar amount of proteins have been biotinylated independently of the stimulation procedure apply to platelets. Stripping of the membranes and reprobing using antibody against the immunoprecipitated proteins or with anti-actin antibody was done to corroborate that a similar amount of protein was loaded in each lane.

2.10. Statistical analysis

Analysis of statistical significance was performed with Student's unpaired *t* test. For multiple comparisons, a one-way analysis of variance combined with the Dunnett tests was used.

3. Results

3.1. Immunophilins are involved in NCCE in human platelets

Association to and regulation of SOCE channels by FKBPs have been previously demonstrated [29,31,32], but their role in NCCE remains poorly investigated. As shown in Fig. 1, OAG (100 μM) was unable to evoke changes in the basal $[Ca^{2+}]_c$ in human platelets suspended in a Ca^{2+}-free medium; meanwhile an increase in $[Ca^{2+}]_c$ was observed when 300 μM of $CaCl_2$ was added to the extracellular medium (Fig. 1A), thus indicating that OAG activates NCCE, as previously reported in platelets and other cell types [4,40–43]. Preincubation of human platelets for 5 min at 37 °C with increasing concentrations of FK506 resulted in a concentration-dependent inhibition of OAG-evoked NCCE, which might be indicative of a potential role of FKBPs in NCCE (Fig. 1B). A significant reduction of 47.0 ± 8.0% ($P < 0.01$; n = 6) was observed using 10 μM of FK506, as previously shown in neurons [32,44], but the higher effect was observed at the concentration of 50 μM (69.0 ± 5.0% of control; $P < 0.001$; n = 6), therefore, we have used this concentration throughout the study, as previously reported [45]. FK506 (50 μM) was still able to reduce OAG-evoked NCCE in platelets previously incubated for 30 min with 100 nM of cypermethrin, a specific calcineurin (CaN) antagonist (Fig. 1C); thus suggesting that although part of the effect observed using FK506 might relay on its indirect regulatory role over CaN, a bigger inhibition is found in the presence of FK506; hence, we cannot consider that the FK506-dependent NCCE impairment is based on the inhibition CaN activity exclusively, as previously reported by others [46,47].

3.2. Immunophilins regulate NCCE through TRPC6

NCCE in human platelets has been reported to be mainly conducted through TRPC6 and TRPC3 channels [11,13]. In order to investigate whether TRPC6 is regulated by immunophilins, we perform a series of

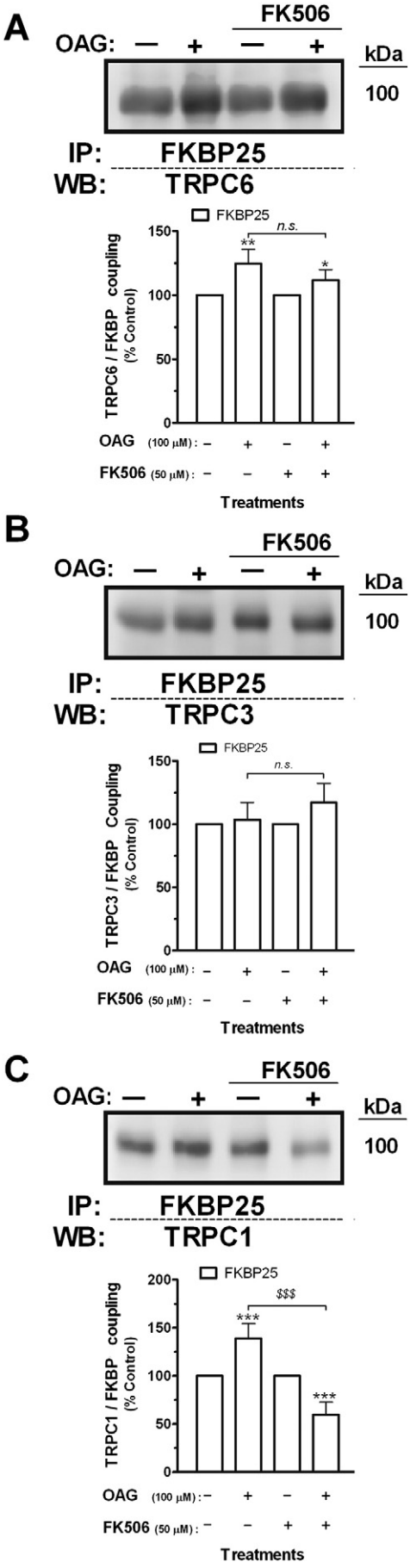

Fig. 4. FKBP25 interacts with TRPCs in human platelets. Human platelets were incubated for 5 min in the absence or presence of FK506 (50 μM), and they were subsequently stimulated with OAG for 1 min (100 μM) in HBS-rich Ca^{2+} medium or kept under resting conditions. Upon stimulation time was over, platelets were fixed by mixing with RIPA. FKBP25 was immunoprecipitated from the cell fragments solution by incubating overnight with 1 μg/mL of anti-FKBP25 and agarose bead conjugated with protein A. Upon washing, immunoprecipitated complexes were analyzed by performing Western blotting using anti-TRPC3, anti-TRPC6 and anti-TRPC1 antibodies. Reprobing of the membranes was done with either anti-FKBP25 to corroborate the homogeneity of the samples loaded in the gel (data not shown). Images in the panels are representative of four independent experiments, while histograms represent the changes in the protein complexes expressed as the percentage and the S.E.M respect to control resting platelets. *, **, ***: represent $P < 0.05$, <0.01, and <0.001 with respezct to control resting platelets; $^{\$\$\$}$: $P < 0.001$ with respect to OAG stimulated platelets but non-treated with FK506.

experiments in platelets from TRPC6 deficient ($TRPC6^{-/-}$) and WT mice [33,34]. As depicted in Fig. 2, $TRPC6^{-/-}$ exhibited a significantly reduced OAG response, which strongly indicates that TRPC6 plays a relevant role in NCCE in these cells, as previously reported [48]. In WT mice, FK506 reduced OAG-induced NCCE by 33.6 ± 4.9% (Fig. 2A; $P < 0.001$; n = 9), while it was without effect in platelets from $TRPC6^{-/-}$ mice (Fig. 2B), thus suggesting that the effect of FK506 was mediated through TRPC6. These findings were further supported by patch-clamp analysis in HEK293 cells constitutively overexpressing TRPC6. As shown in Fig. 3A, inward and outward TRPC6 current resulted modified by preincubating cells with FK506 for 5 min. Ga^{3+} was added to the cells at the end of the experiment to confirm OAG-induced TRPC6 activation. Our results indicate a reduction of 75.2 ± 4.4% ($P < 0.001$; n = 8, Fig. 3A) in the current density through TRPC6 channel in the presence of FK506 as compared with control untreated cells. Similarly, we have found that NCCE induced by stimulating TRPC6-overexpressing HEK293 with 100 μM OAG was reduced in the presence of FK506 by 59.5 ± 22.4% (Fig. 3B; $P < 0.001$; n = 4).

3.3. FKBP25 interacts with TRPCs

FKBPs interact with TRPCs in a number of cell types. In non-excitable cells, we and others have recently identified FKBP52 and FKBP12 as possible regulators of the SOC channels Orai1 and TRPC1 [29,31,32,49]. Here, we have looked for alternative immunophilins such as FKBP25 and FKBP38 that are receiving more attention from the scientific community due to their role in cell survival and subsequently cancer progression. As shown in Fig. 4, in human platelets, FKBP25 was found to associate with TRPC6 and TRPC3 under resting conditions. FKBP25 and TRPC6 coupling was enhanced by 24.7 ± 10.9% ($P < 0.01$; n = 4; Fig. 4A) upon platelet stimulation with OAG (100 μM) for 1 min, which was unaffected by treatment with FK506 (50 μM) for 5 min. Stimulation with OAG had no effect on the association between FKBP25 and TRPC3, which was detectable under resting conditions ($P > 0.05$; n = 4). Interestingly, FKBP25 was also found to interact with TRPC1 under resting conditions and a significant increase of 39.1 ± 15.3% ($P < 0.001$; n = 4; Fig. 4C) was induced by OAG. Treatment for 5 min with FK506 significantly reduced by 79.5 ± 13.2% OAG-evoked coupling between FKBP25 and TRPC1 (Fig. 4C; $P < 0.001$; n = 4). Concerning FKBP38, we did not found detectable interaction between this immunophilin and TRPC channels (data not shown).

By evaluating these patterns of interaction between the immunophilins and the different TRPCs, it seems that TRPC6 plays a more relevant role than TRPC3 during the coupling process evoked by OAG. Hence, we reproduced the same experimental protocols in platelets from $TRPC6^{-/-}$ mice. As shown in Fig. 5, in platelets from WT mice, FKBP25 and FKBP38 were found to associate with TRPC6 (Fig. 5A; n = 3) and this interaction was impaired in the murine $TPRC6^{-/-}$ mice (Fig. 5B), which suggests that FKBP25 and FKBP38 might regulate NCCE by interaction with TRPC6, at least in mice platelets. Interestingly, we have not observed compensation of TRPC6 with TRPC3 in $TPRC6^{-/-}$ mice (see Fig. 5B middle panel where TRPC3 expression in WT and $TRPC6^{-/-}$ is analyzed by Western blotting) (WCL: whole cell lysate).

Functional TRPCs are supposed to be located in the plasma membrane of platelets; however current evidence indicates that TRPC1, 3 and 6 might be also found in intracellular locations, such as the expression of TRPC6 in the dense tubular system of murine platelets and TRPC3 in the mitochondrial membrane in brain and liver cells [50,51]. Thus, we explored whether the coupling between FKBPs and TRPC6 takes place in the plasma membrane by biotinylation. As shown in Fig. 6A, FKBP25 and FKBP38 was found in the biotinylated samples, and while FKBP25 location was not altered upon platelet stimulation with OAG (100 μM), FKBP38 presence in the membrane was significantly reduced upon platelet stimulation (Fig. 6A, middle panel).

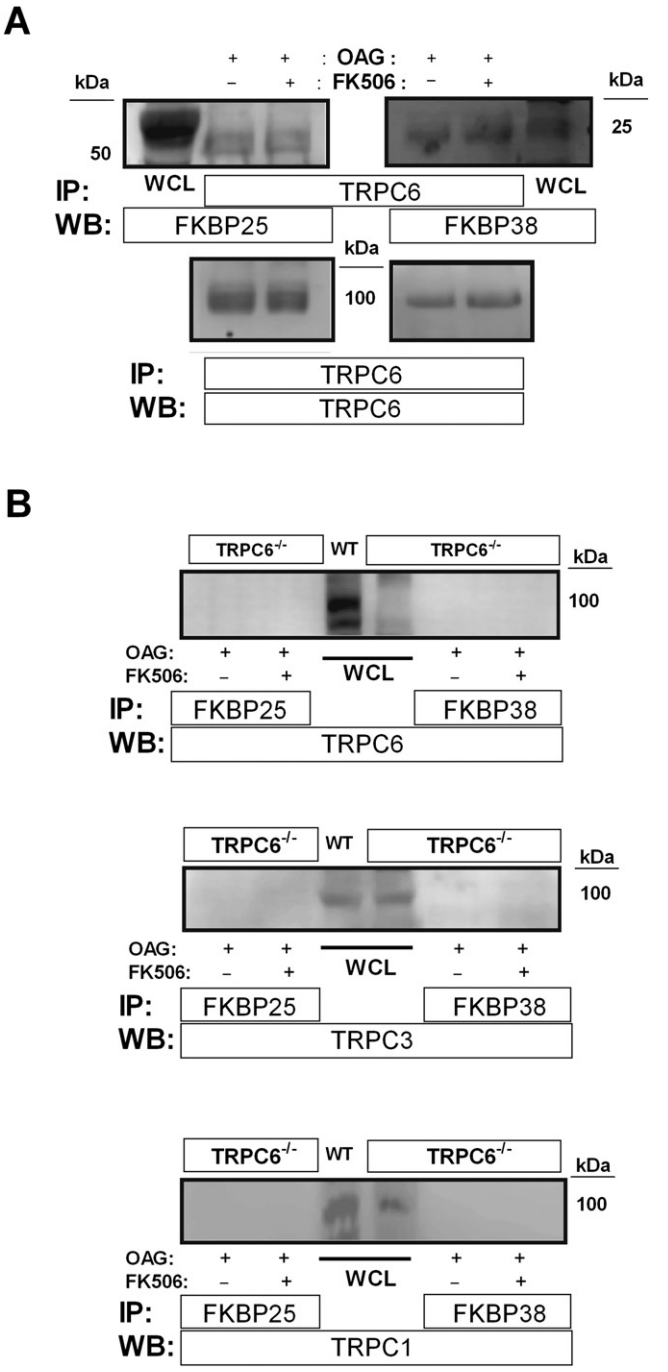

Fig. 5. FKBP25 and FKBP38 interact with TRPC6 but not with other TRPCs in murine platelets. Platelets drawn from WT (A) or $TRPC6^{-/-}$ (B) mice were suspended in tyrode's Ca^{2+}-rich medium (300 μM was added), and then were incubated for 5 min in the absence or presence of FK506 (50 μM), after which they were stimulated for 1 min with OAG (100 μM) or kept under resting conditions, and finally fixed using RIPA. Immunoprecipitation of the proteins of interest was achieved by incubating the cell sample solutions with either anti-TRPC6 (A) or anti-FKBP25 (B) or FKBP38 (B) and beads of agarose conjugated with protein A. Immunoprecipitated protein complexes were analyzed by Western blotting using anti-FKBP25 (A) and anti-FKBP38 (A), or using anti-TRPC1 (B), anti-TRPC3 (B) and anti-TRPC6 (B). Reprobing of the membranes with the appropriate antibody confirmed that same amount of proteins were loaded in each gel lane. WCL: whole cell lysate. Images are representative of four independent experiments.

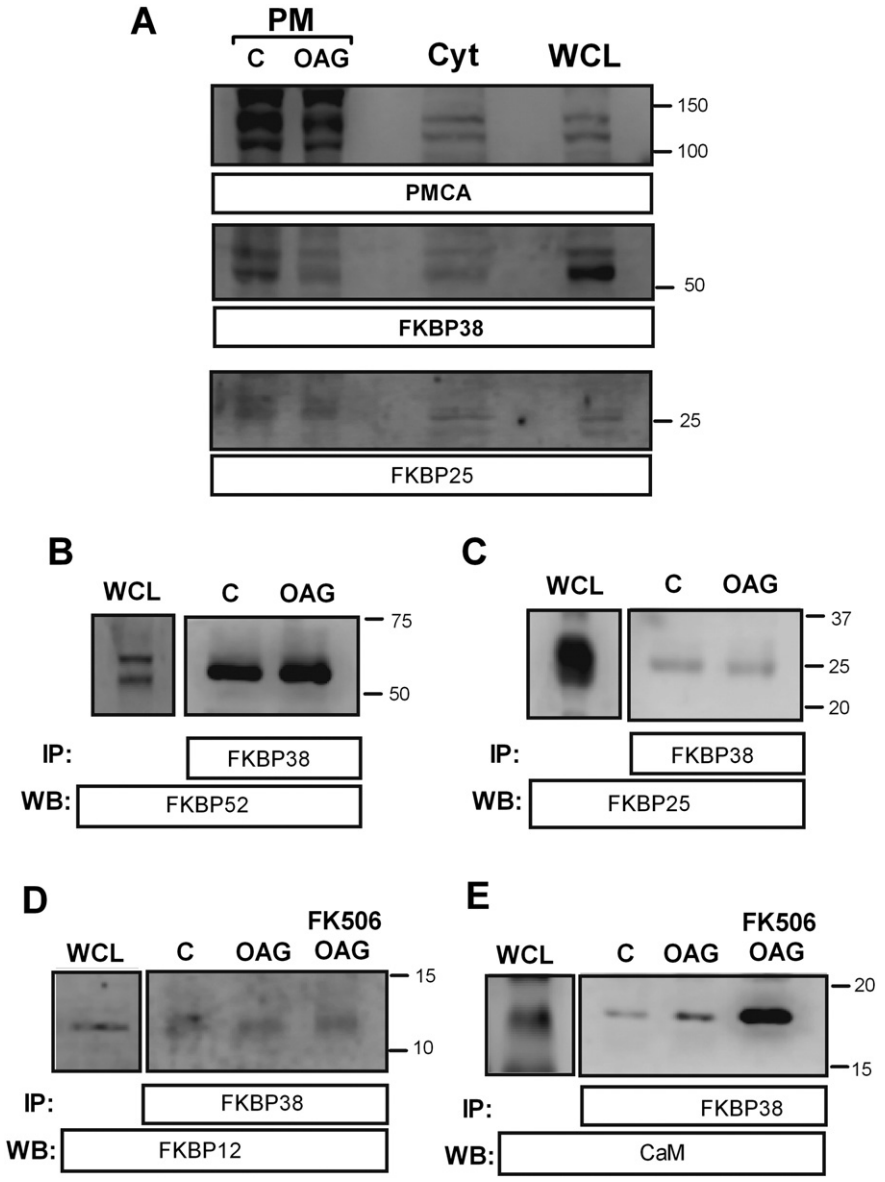

Fig. 6. FKBP25 and FKBP38 are located at the plasma membrane and would interact with other FKBPs and CaM. Human platelets suspended in HBS-rich Ca^{2+} medium (300 μM of $CaCl_2$ was added) were preincubated in the absence (A–E) or presence of FK506 (50 μM for 5 min, D–E) and were subsequently stimulated with OAG for 1 min (100 μM) or kept under resting conditions. Upon which platelets were subjected to biotinylation (A) or immunoprecipitation protocols (B–E), which are performed as described in Materials section. Anti-PMCA antibody was used to verify that similar amounts of membrane protein were loaded in the gel, while reproving with anti-FKBP38 was done as loading control (data not shown). Images are representative of three independent experiments.

3.4. FKBPs modify the structural composition of NCCE channels

We have further evaluated the association between TRPC6 and TRPC3 in both murine and human platelets during NCCE activation. As shown in Fig. 7A, during OAG-evoked NCCE (100 μM) a significant increase in the association between TRPC3 and TRPC6 was observed (109.9 ± 3.0%; $P < 0.05$; n = 4). This interaction was significantly reduced by incubating platelets for 5 min with 50 μM FK506 (85.5 ± 4.8%; $P < 0.05$; n = 4). OAG-evoked association between TRPC6 and TRPC3 resulted also impaired in platelets from WT mice incubated with 50 μM FK506 (Fig. 7B; n = 4), which further support a role of FKBPs in the assembly of the TRPC6/TRPC3 heteromultimeric complex.

Orai1 has been reported as the main SOC channel, with a role for TRPC channels in receptor-operated Ca^{2+} influx. In order to ascertain whether TRPC6 plays a role in human platelet physiology, we performed experiments where platelet aliquots of 400 μL were incubated with either 1 μg/mL anti-TRPC6 antibody, or anti-TRPC6 previously preincubated with CAP, or rabbit IgG of the same nature as the anti-TRPC6 antibody. As observed in the Fig. Supplementary 1, a significant reduction in the aggregation evoked by ADP (10 μM) was found in platelets preincubated with anti-TRPC6 antibody, while incubation with anti-TRPC6 + CAP as well as with rabbit IgG was without effect. In the absence of specific TRPC6 inhibitors and taking into account that RNA-based techniques are scarce in human platelets, this approach provides evidence for a role of TRPC6 in human platelet physiology, in agreement with a recent manuscript supporting a role for TRPC6 in platelet activation [52].

3.5. FKBP25 and FKBP38 silencing inhibits the activation of NCCE

As shown in Fig. 8, we have further explored the relevance of FKBP25 in NCCE in two different culture cell types, MEG-01 and HEK293 cells.

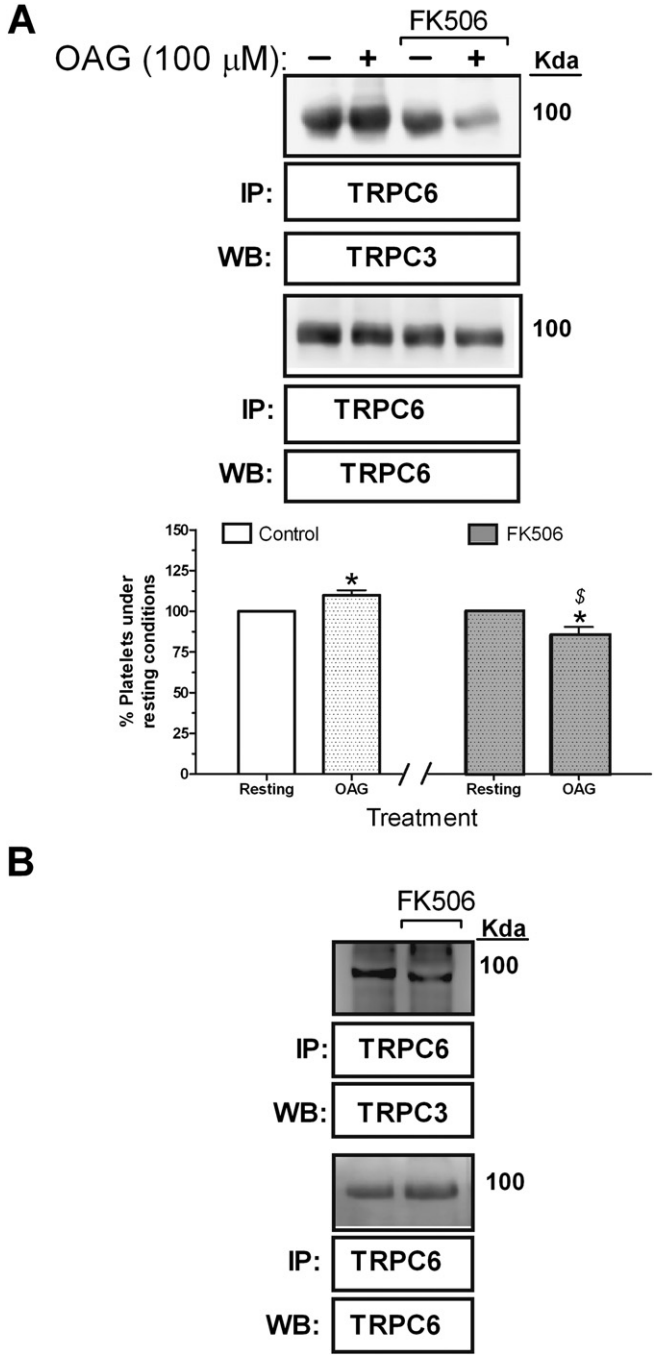

Fig. 7. Immunophilins participate in the formation of the NCCE heteromultimeric complexes. Human (A) or WT mice (B) platelets were suspended in a HBS-Ca^{2+} rich medium or tyrode's Ca^{2+}-rich medium, respectively, and then were incubated for 5 min in the presence or absence of FK506 (50 μM) and were subsequently stimulated with OAG (100 μM) for 1 min or left non-stimulated. Upon cell fixation by mixing with equal volume of RIPA, protein of interest were immunoprecipitated using anti-TRPC6 and beads of agarose conjugated with protein A. Immunoprecipitated proteins were solved by Western blotting using anti-TRPC3 antibody. Images in the panel are representative of four independent experiments. As shown, reprobing of the membranes was done with anti-TRPC6 antibody to corroborate the homogeneity of the sample loaded in each gel lane. *: $P < 0.05$ respect control platelet under resting conditions, and $: $P < 0.05$ respect to control platelets stimulated with OAG.

The reduction in the expression of FKBP25 significantly attenuated the OAG-evoked NCCE by 52.1 ± 14.4% ($P < 0.001$; n = 4) in MEG-01 cells (Fig. 8A), and by 38.9 ± 11.9% ($P < 0.001$; n = 4) in HEK293 (Fig. 8B), respectively. Cell transfection with the empty plasmid did not alter NCCE evoked by OAG (100 μM) in HEK293 cells; hence demonstrating that neither transfection nor introduction of a exogenous genetic material affect to this mechanism, and supporting the fact that the NCCE inhibition registered in cells transfected with siRNA was likely due to the reduction in the amount of the targeted protein.

Similarly, treatment of MEG-01 and HEK293 cells with siRNA FKBP38 significantly attenuated OAG-evoked NCCE by 37.59 ± 15.5% ($P < 0.001$; n = 4) and 48.5 ± 14.4% ($P < 0.001$; n = 4). Overexpression of GFP-FKBP38 in HEK293 did not alter OAG-evoked Ca^{2+} entry compared to WT cells (cells transfected but non-expressing the FKBP38 fused protein) (see Fig. 8B left hand side graph at the bottom of the figure).

Genetic manipulation of FKBP25 and FKBP38 in MEG-01 and HEK293 cells did not alter the expression of TRPC3 or TRPC6 (data not show), but slightly, although non-significantly, attenuated the TRPC6/TRPC3 coupling, while transfecting with siRNA control was without effect in the TRPC6/TRPC3 coupling (data not shown).

4. Discussion

Human platelets have different mechanisms for Ca^{2+} entry. SOCE, a mechanism regulated by the filling state of the Ca^{2+} stores, has been shown to be conducted through Orai1 channels [53], although other channels like TRPC1 or TRPC6 are also involved in Ca^{2+} entry, as demonstrated in different cell types, including the megakaryoblastic cell line, MEG01 [45,54,55]. Additionally, TRPC6 also acts as a non-capacitative channel in platelets [48,51], and, as for other TRPC channels, it might be subjected to different postraductional modifications that affect their permeability [4,35,56–58]. TRPC6 is one of the two classic TRPCs involved in NCCE operated by DAG. The relevance of NCCE in human platelets is evidenced in patients suffering type 2 diabetes mellitus, where an enhanced NCCE through TRPC6 seems to underlie the hyperaggregability state observed in these patients [59–61]. Here, we have explored the possible role of immunophilins in the regulation of NCCE stimulated by OAG, a cell-permeable DAG synthetic derivative.

Our results provide clear evidence for a role of immunophilins in NCCE that can be attributed to the activity of the FKBP members, and not just due to the indirect inhibitory effect of FK506 on CaN, as previously suggested by others [44,62]. Inhibition of immunophilins did not alter NCCE in platelets from mice lacking TRPC6, which provides a strong evidence in favor of that TRPC6 might be the target channel of the immunophilin activity [63].

Previous studies have reported direct interaction of immunophilins with TRPC channels. For instance, in neurons, FKBP52 presents two possible binding regions in both extremes of the TRPC1 structure (^{19}LPSSP^{23} and ^{644}LPPPF^{648}), being the latest region overlapped with the homer binding region, and it is close to the STIM1 interaction region (two aspartate residues immediately after TRP box 2) [32]. Interaction between immunophilins and TRPC6 might occur through a unique residue, such as occurs with FKBP12 that recognizes the Ser768 and Ser714 of TRPC6 upon their phosphorylation by PKC [63].

We describe here that the interaction between TRPC6 and FKBP25 occurs under resting conditions and is modulated upon stimulation with OAG. FKBP25 has been described to act as a nuclear factor that has the ability of interacting with pre-rRNA, thus affecting to ribosomal protein synthesis [64], and subsequently, to the general protein expression in the cells. FKBP25 was shown to be downregulated by rapamycin and FK506, having more affinity for rapamycin than FK506, hence it represents a good candidate for the alterations observed in patients suffering cancer or neurodegenerative disorders, and are medicated with these macrolides [65–67].

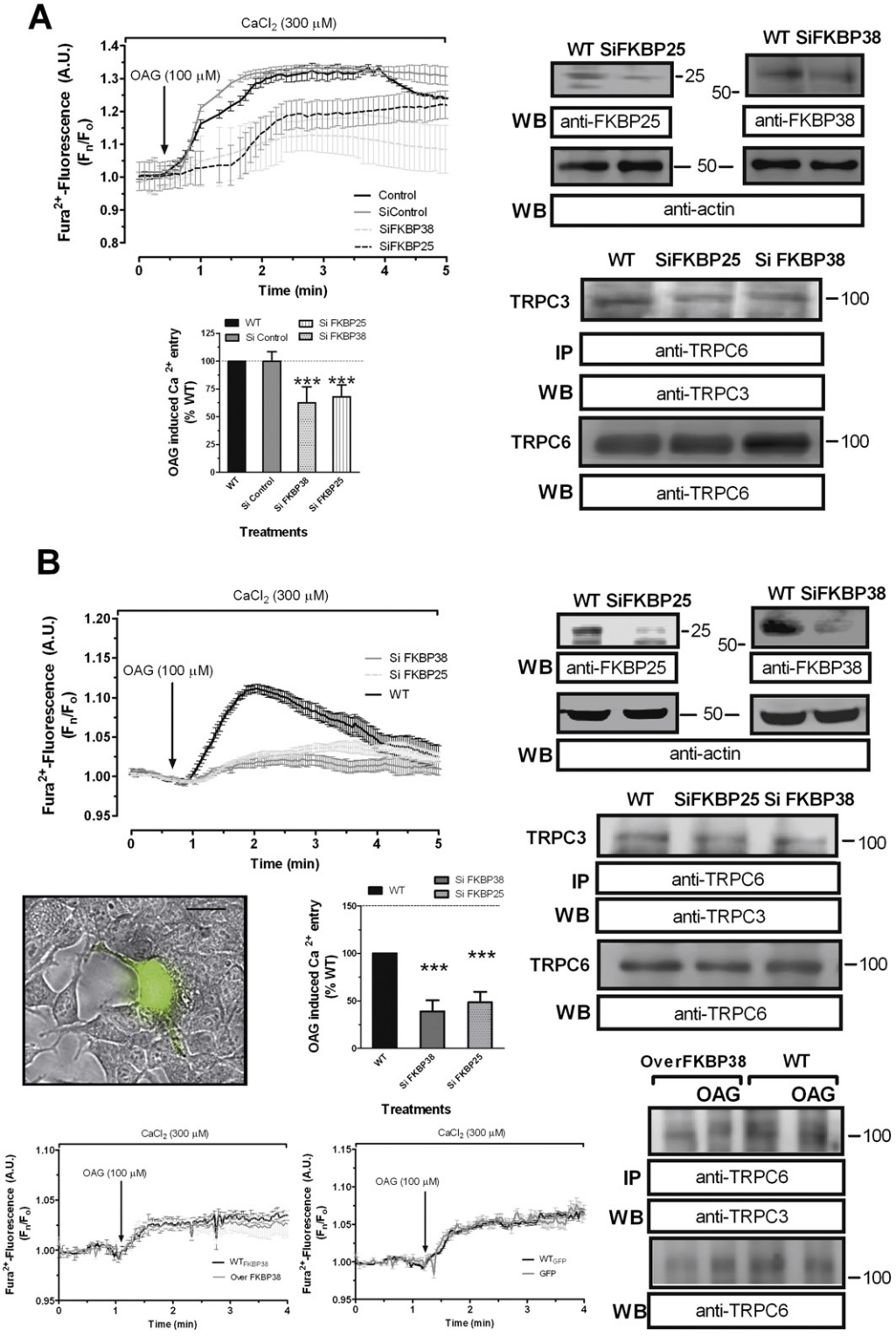

Fig. 8. Silencing of FKBP25 and FKBP38 reduces NCCE in MEG-01 and HEK293 cells. MEG-01 (A) and HEK293 (B) cells were transfected with 2 μg/mL of siRNA against FKBP25 and FKBP38 or were left non-transfected (WT). Internal experimental controls consisted in transfecting MEG-01 cells with 2 μg/mL of siRNA control. Additionally, HEK293 was transfected with 2 μg/mL of either an empty overexpressing plasmid (GFP-plasmid) or with the plasmid containing the GFP-FKBP38 fused protein. The reduction in the expression of the FKBPs was confirmed either by Western blotting (right hand side images) or by epifluorescent microscopy (in case of the HEK293 transfected with the GFP-FKBP38 protein; Scale bar is 15 μm), which occurred 72 h or 24 h after transfection, respectively. After confirming that cells were genetically modified they were loaded with fura-2 and mounted in a perfusion chamber of a single cells imaging system. Cells were then perfused with HBS-rich Ca^{2+} (50 μM) containing, and were then stimulated with OAG solution (containing 300 μM of $CaCl_2$). Additionally, OAG-evoked coupling between TRPC6 and TRPC3 was explored in both cell types and compared to control non-transfected cells (WT) (Right hand side images). Reprobing of the membranes with anti-TRPC6 antibodies or anti-actin antibodies was done following the appropriate protocols in order to corroborate that similar amount of proteins was loaded in each gel lane. Graphs represent the mean ± standard deviation of 3–4 independent cell transfections; meanwhile, histograms represent the mean of the percentage respect to control ± S.E.M. Images in the panels are representative of 3 to 4 independent experiments. ***: $P < 0.001$.

We have described in the present study that FKBP25 is acting as positive regulator of OAG-dependent calcium entry, as well as contrary to previous publication that limit its intracellular distribution to the ER and nucleus, we have detected FKBP25 at the plasma membrane of platelets by performing biotinylation experiments, but the amount seems to be scarce compared with the cytosolic location. Furthermore, we found that it associates with other FKBPs like FKBP38, so perhaps favoring a FK506-dependent inhibition of FKBP38 non-described yet. The interaction of FKBP25 with TRPC6 and the effect of FKBP25 downregulation on NCCE in MEG01 and HEK293, indicate that FKBP25 might be a positive regulator of TRPC6. Although non-significant, a reduction in TRPC6/TRPC3 interaction could be observed in these cells transfected with the siRNA FKBP25.

FKBP38 is an immunophilin activated by CaM/Ca^{2+} after intracellular Ca^{2+} bursts [68]. FKBP38 N-terminal domain presents a charge-sensitive functional loop with affinity for Ca^{2+} that regulates the interaction with their substrates [70]. We have not detected direct interaction of FKBP38 with TRPC6; however, RNAi-based analysis revealed downregulation of OAG-evoked NCCE in both, MEG-01 and HEK293 cells. Moreover, the biotinylation experiments together with the epifluorescence microscopy observations done in HEK293 overexpressing GFP-FKBP38, which evidenced a random cytosolic distribution of this protein, lead us to conclude that FKBP38 might be found in intracellular locations different from the ER and mitochondrial associated membranes described by others [73]. In contrast, the overexpression of FKBP38 did not enhance NCCE activated by OAG which was unexpected and might indicate that it could be required somehow during the activation of this mechanism, so this part will deserve future research to find out the precise modulating role of FKBP38 over TRPC6 during NCCE.

Regarding the role of FKBP38, we would suggest that it would have a regulatory role on TRPC6. Nevertheless, according to the IP control experiments performed (data not shown), FKBP38/TRPC6 interaction would not occur directly, but it would happen through other intermitted proteins like FKBPs (perhaps FKBP25) or CaM instead, which were found here to associate with this channels as well as it has been previously reported by other research groups [74,75]. Nowadays, there are contradictory results in the literature regarding the effect of FK506 in FKBP38 PPIase activity, like FK506 binding domain is not altered in presence of FK506, like it does in the FKBP12 structure [76]. On the contrary, other research groups have reported inhibition of FKBP38 activity in the presence of FK506; this inhibition might result of its interaction either with other immunophilins or with CaM, all of them are shown in the present study [69,71,72]. In fact, incubation of platelets with FK506 and subsequent stimulation with OAG evoked enhanced CaM/FKBP38 interaction. Other nonimmunosuppresive and neurotrophic compound, GPI1046, was also able to inhibit FKBP38 when combined in a functional complex with CaM/Ca^{2+} [22]. Hence, FKBP25 and other immunophilins might be favoring the generation of the full active NCCE channels resulting of the complex between both channels with the immunophilins, and FKBP38 might modulate this macrocomplex during NCCE in non-excitable cells, although the mechanisms underlying this regulation deserve further studies.

Summarizing, here we describe for the first time the interaction between FKBP25 and TRPC6, which plays a relevant role in the activation of NCCE. FKBP25 might interact with the NCCE channels through TRPC6, as demonstrated by using platelets from TRPC6-deficient mice. FKBP38 also play a role in NCCE although the mechanism underlying this process seems different from that reported to FKBP25.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.07.023.

Author contribution

Esther Lopez performed most of the experiments. Alejandro Berna Erro was the one responsible for developing $TRPC6^{-/-}$ mice. Pedro C. Redondo provided conceptual input and designed the experiments as well as participated in analysis, discussion, and interpretation of data together with Gines M. Salido and Juan A. Rosado. Pedro C. Redondo wrote the final form of the manuscript prior to submission and all the authors revised the final submitted material and gave final approval.

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Conflict of interest

We report no conflict of interests.

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