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Regulation of Store-Operated Calcium Entry by FK506-Binding Immunophilins

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Abstract

Calcium entry from the extracellular space into cells is an important signaling mechanism in both physiological and pathophysiological functions. In non-excitabile cells, store-operated calcium (SOC) entry represents a principal mode of calcium entry. Activation of SOC entry in pulmonary artery endothelial cells leads to the formation of inter-endothelial cell gaps and subsequent endothelial barrier disruption. Regulation of endothelial SOC entry is poorly understood. In this work, we identify two large molecular weight immunophilins, FKBP51 and FKBP52, as novel regulators of SOC entry in endothelial cells. Using cell fractionation studies and immunocytochemistry we determined that a fraction of these largely cytosolic proteins localize to the plasma membrane where SOC entry channels are found. That FKBP51 and FKBP52 associate with SOC entry channel protein complexes was supported by co-precipitation of the immunophilins with TRPC4, a subunit of the calcium-selective, SOC entry channel I_{SOC} . Dexamethasone-induced upregulation of FKBP51 expression in pulmonary artery endothelial cells reduced global SOC entry as well as I_{SOC} . Similar results were observed when FKBP51 was over-expressed in an inducible HEK293 cell line. On the other hand, when FKBP52 was over-expressed SOC entry was enhanced. When expression of FKBP52 was inhibited, SOC entry was decreased. Collectively, our observations support regulatory roles for these large molecular weight immunophilins in which FKBP51 inhibits, whereas FKBP52 enhances, SOC entry in endothelial cells.

1. Introduction

Store-operated calcium (SOC) entry is a predominant mechanism of calcium entry in non-excitabile cells. SOC entry channels are located on the plasma membrane and are activated upon depletion of calcium from the endoplasmic reticulum (i.e. the calcium store) (reviewed in [1]). These channels are distinct among most other ion channels due to their ability to

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remain open for long periods of time during which calcium enters the cell and participates in a variety of physiological as well as pathophysiological functions (reviewed in [1; 2]). In pulmonary artery endothelial cells (PAECs), activation of SOC entry leads to the formation of inter-endothelial cell gaps and endothelial barrier disruption [3; 4; 5; 6].

Multiple channels can be classified as SOC entry channels, and they may be broadly described as calcium-selective or non-selective channels. The classical SOC entry channel is the calcium-selective Calcium Release-Activated Calcium (CRAC) channel originally identified in mast cells and T-lymphocytes [7; 8]. While the molecular make-up of the CRAC channel has recently been described as a tetrameric configuration of the four transmembrane spanning domain protein orai1 [9], the molecular make-up of other SOC entry channels remains unclear. On the other hand, members of the canonical transient receptor potential (TRPC) subfamily of TRP proteins may contribute to the makeup of some SOC entry channels, and in particular TRPCs 1, 3, 4 and 5 have been shown to exhibit SOC entry characteristics in some systems [10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20]. For example, knock-down of TRPC1 decreased another calcium-selective SOC entry current, I_{SOC} [21]. This current is found in endothelial cells, epithelial cells and other cell types [22; 23; 24; 25]. Similarly, knock-out of TRPC4 virtually abolished I_{SOC} in aortic endothelial cells [26]. Collectively, these data reveal that at least TRPC1 and TRPC4 contribute subunits to the I_{SOC} channel.

All SOC entry channels likely reside in heterocomplexes with other proteins, some of which may be important for regulation of channel function. In general though, regulation of SOC entry is poorly understood. Recent evidence has suggested involvement of members of the family of FK506-binding immunophilins. They are known to bind to and regulate calcium channels such as the ryanodine receptor and inositol-1,4,5-trisphosphate receptor [27; 28; 29; 30]. Of particular interest, the large molecular weight immunophilin FKBP52 has been shown to bind to TRPC1 and TRPC4 in Sf9 insect cells and rat cerebral cortex membrane lysates [31]. In neuronal growth cones, FKBP52 regulates TRPC1 function important for chemotropic guidance [32]. As the endothelial I_{SOC} channel consists, in part, of TRPC1 and TRPC4, we became interested in the immunophilin FKBP52. Recent work by López and colleagues [33] revealed that FKBP52 plays a role in regulation of SOC entry in platelets and MEG 01 (human megakaryoblastic) cells. However, its role in regulation of endothelial SOC entry *per se* is unexplored. We also became interested in closely related immunophilin FKBP51. FKBP52 and FKBP51 are 70% similar and have the same structural organization, yet they exhibit opposing effects on steroid receptor activity [34]. Both *in vitro* and *in vivo*, FKBP52 is stimulatory to steroid receptor function whereas FKBP51 is inhibitory (reviewed in [34; 35]). In this study, we questioned whether FKBP51 and FKBP52 are expressed in pulmonary artery endothelial cells (PAECs) and whether these immunophilins play a role in regulation of SOC entry. Further, as these immunophilins have opposing effects in steroid receptor function, we were interested in determining whether FKBP51 and FKBP52 differentially regulate SOC entry. We observed that in both PAECs and HEK293 cells, upregulation of FKBP51 expression leads to a decrease in SOC entry and I_{SOC} , suggesting that FKBP51 is an inhibitor of SOC entry. Conversely, FKBP52 acts to promote SOC entry, as knock-down of FKBP52 expression led to a decrease in SOC entry and overexpression of FKBP52 enhanced SOC entry. Collectively, our data support regulatory roles of the large molecular weight immunophilins FKBP51 and FKBP52 in SOC entry in endothelial cells.

2. Experimental procedures

2.1. Reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Cell culture medium and penicillin/streptomycin were obtained from Life Technologies

(Grand Island, NY). Heat-inactivated fetal bovine serum (FBS) was purchased from Cell Generation (Fort Collins, CO) or Denville Scientific (Metuchen, NJ). Monoclonal antibodies to FKBP51 and FKBP52 and expression plasmids for human FKBP51 and rabbit FKBP52-Flag have been described previously [36; 37]. Antibodies to β -actin, TRPC4 and N-cadherin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Doxycycline was purchased from Clontech (Mountain View, CA). Superfect Transfection Reagent and HiPerFect siRNA Transfection Reagent were obtained from QIAGEN (Valencia, CA).

2.2. Cell culture

Endothelial cells were isolated as described previously [38]. Cells were grown in DMEM high glucose, supplemented with 10% FBS, 50 U/mL penicillin G, and 0.05 mg/mL streptomycin. T-REx-293-h51 and T-Rex-293-sm51 cells have been previously described [46]. Monolayers of T-REx-293-h51 or T-Rex-293-sm51 cells were grown in DMEM high glucose, supplemented with 10% FBS (Hyclone), 4 mM L-glutamine, 15 μ g/mL blasticidin S, and penicillin/streptomycin as described above. To induce FKBP51 expression in the T-REx-293-H51 or T-Rex-293-sm51 cell lines, cells were treated with 0.1 μ g/mL doxycycline in complete medium, replaced with fresh doxycycline in complete medium every 24 hours prior to cytosolic calcium measurements and patch-clamp experiments as described below.

2.3. Western Blot Analysis

Cell membrane/cytoskeleton extraction was performed as described by Cioffi *et al.* [38], modified from [39]. Briefly, confluent cell monolayers were lysed in buffer [0.1 M Tris chloride (pH 8.0), 1 mM $MgCl_2$, 10% protease inhibitor cocktail (10 $\times$, Sigma)] and pelleted at 3000 $\times$ g (24 min, 4 $^{\circ}$ C). Pellets were resuspended in sucrose buffer [10 mM Tris base (pH 7.4), 250 mM sucrose, 0.9 mM dithiothreitol, 10% protease inhibitor cocktail (10 $\times$, Sigma) and homogenized using a Dounce homogenizer. The homogenized samples were centrifuged (3000 $\times$ g, 17 min, 4 $^{\circ}$ C) following which the supernatant was collected and centrifuged at 50,000 $\times$ g (30 min, 4 $^{\circ}$ C). The pellet was extracted in octylglucoside/potassium iodide extraction buffer [50 mM Tris base (pH 7.5), 150 mM NaCl, 5.2 mM EDTA, 50 mM octylglucoside, 100 mM KI, 10% protease inhibitor cocktail (10 $\times$, Sigma)]. The final centrifugation (145,000 $\times$ g, 50 min, 4 $^{\circ}$ C) resolved a pellet fraction that consists of the cytoskeletal proteins and any protein strongly attached to them, whereas the supernatant fraction contains the dissolved phospholipids, plasma membrane proteins and any protein strongly attached to them. For whole cell extraction, PAECs, T-REx-293-h51 or T-REx-293-sm51 cells were lysed in RIPA buffer (Boston Bioproducts, Ashland, MA) with 1% protease inhibitor cocktail. Membrane/cytoskeleton extracts or whole cell extracts were electrophoresed on 4–15% bis-tris gels (Invitrogen) and then transferred to nitrocellulose membranes at 100 V. Membranes were rocked with primary antibodies for 1 hour at room temperature or overnight at 4 $^{\circ}$ C, and with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. Proteins were visualized via chemiluminescence detection using either Supersignal West Pico or West Femto chemiluminescent substrates (Thermo Scientific, Waltham, MA).

2.4. Ca^{2+} Measurements and Patch-Clamp Electrophysiology

PAECs, T-REx-293-h51, or T-REx-293-sm51 cells were seeded onto 35-mm glass coverslips and grown to confluence. Intracellular calcium concentration was determined using the fluorophore fura 2-acetoxymethyl ester (fura-2 AM; Life Technologies, Grand Island, NY) according to methods described previously [41]. Patch-clamp recordings were performed in whole-cell configuration as previously described [6; 21; 38; 40; 41; 42]. Briefly, transmembrane currents in single, electrically isolated, cells were measured with an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pulse+PulseFit software (Version 8.5, HEKA Elektronik) was used to generate the voltage stimuli and to acquire the

current signals, which were filtered at 2.9 kHz. The currents were evoked by step depolarization (*i.e.*, 200-ms testing pulses ranging from -100 to $+60$ mV with 20-mV increments) from a holding potential of 0 mV; currents were measured as the mean value of the current amplitude during the last 20 ms of each step. The standard pipette solution contained (in mmol/liter) 130 N-methyl-D-glucamine, 10 Hepes, 2 Mg^{2+} -ATP, 1 N-phenylanthranilic acid, 0.1 5-nitro-2-(3-phenylpropylamino)benzoic acid, 2 EGTA, 1 $Ca(OH)_2$ (pH 7.2, adjusted with methane sulfonic acid; the free $[Ca^{2+}]$ was estimated as 100 nmol/L, as calculated by the CaBuf program <G. Droogmans, <ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>>). To examine Ca^{2+} currents, the bath (external) solution contained (in mmol/L) 120 aspartic acid, 5 $Ca(OH)_2$, 5 $CaCl_2$, 10 Hepes, 0.5 3,4-diaminopyridine (pH 7.4 adjusted with tetraethylammonium hydroxide). All solutions were adjusted to 290–300 mOsm with sucrose. Recording pipettes were made of hemo capillaries (Warner Instrument, Hamden, CT), pulled by a two-stage puller (PC-10; Narishige, Tokyo, Japan) and heat-polished with a microforge (MF-200; World Precision Instruments, Sarasota, FL). Pipette resistance was in the range of 3–5 megohms when filled with the standard pipette solution. All of the experiments were performed at room temperature (22–25 °C). Data are expressed as mean $\pm$ SEM for the number of cells (*n*) in which whole-cell patch-clamp recordings were obtained.

2.5. Immunocytochemistry

For FKBP51, FKBP52 and N-cadherin, PAEC monolayers were fixed and permeabilized with 90% ice-cold methanol in PBS. Here, cells were treated with the methanol solution (-20 °C) for three minutes in the -20 °C freezer. For TRPC4, PAECs were fixed and permeabilized with 4% paraformaldehyde / 0.1% Triton X-100. Cells were treated with 4% paraformaldehyde for 15 minutes at room temperature, washed three times with 100 mM glycine in PBS and then permeabilized with 0.1% Triton X-100 in PBS for 2 minutes. Following fixation/permeabilization, cells were incubated in blocking buffer (10% goat serum, 10% donkey serum, 1.5% glycine, 1% bovine serum albumin in Hank's balanced salt solution) for one hour at room temperature. Cells were incubated with primary antibodies diluted in milk (5% non-fat dry milk in PBS supplemented with 0.1% Tween-20) overnight at 4°C. Cells were treated sequentially with secondary antibodies, with a wash in between, for 1 hour at room temperature. Dilutions used for primary antibodies were: FKBP51, 1:100; FKBP52, 1:100; N-cadherin, 1:50; TRPC4, 1:50. Secondary antibodies were used at 1:500 dilution and include goat anti-mouse IgG fluorescein-conjugated (Thermo Scientific; Pittsburg, PA) and AlexaFluor 633 donkey anti-goat IgG (H&L) (Life Technologies; Grand Island, NY). Fluorescent images were obtained using a Leica TCS SP2 confocal laser scanning microscope fitted with a 63 $\times$ water immersion objective

2.6. Immunoprecipitation Experiments

Immunoprecipitation experiments were performed using the Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific, Rockford, IL) following manufacturer's instructions. Briefly, antibody was covalently attached to activated agarose beads using sodium cyanoborohydride. Whole cell lysate was rotated with antibody-coupled beads overnight at 4 °C. After washing, proteins were eluted from beads using a 1% sodium dodecylsulfate treatment for five minutes, repeated twice. Protein eluates were then subjected to SDS-PAGE and immunoblot analysis.

2.7. siRNA Knockdown of FKBP51 and FKBP52

FKBP51 knockdown was achieved via siRNA targeted to the sequence 5'-AAGGCGAGATCTGCCACTTAT-3' (QIAGEN), corresponding to +404 to +424 of the rat FKBP5 gene (NM_001012174). For knockdown of FKBP52, siRNA to the target sequence 5'-CCCAATGATGGCGCTATGGTA-3', corresponding to position +655 to +675 of the rat

FKBP4 gene (XM_001066628.2) was obtained from QIAGEN (Valencia, CA). PAECs were grown to 50–60% confluency, and siRNA was introduced using HiPerFect transfection reagent to a final concentration of 20 nM in serum-free medium. Cells were incubated for three hours following which serum-free medium was replaced with complete medium and then incubated at least an additional 48 hours. Scrambled siRNA was tested to control for off-target effects. Knock-down efficiency was validated by western blot.

2.8. Transient transfection of PAEC or T-REx-293-h51

PAECs or T-REx-293-h51 were grown to 50–60% confluency and transfected with 2 μ g DNA/well in serum-free medium for three hours. Cells were washed and fresh medium was added. Cells were incubated an additional 18–24 hours in complete medium, and $[Ca^{2+}]_i$ was measured as described above. Cellular levels of FKBP51, FKBP52, or FKBP52-Flag were evaluated by western blot.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad version 5.0 software (San Diego, CA). Comparisons between multiple groups were performed using a two-way ANOVA and the Bonferroni posthoc test. Patch-clamp data was analyzed using a student's T-test. Values were considered significantly different when the *P* value was less than 0.05.

3. Results

3.1. FKBP51 and FKBP52 are expressed in pulmonary artery endothelial cells

We first sought to determine whether FKBP51 and FKBP52 are naturally expressed by PAECs. Whole cell lysates were prepared and immunoblot analysis performed revealing that PAECs express both FKBP51 and FKBP52 (Figure 1A). If FKBP51 and/or FKBP52 play a role in regulation of SOC entry, we would expect that these large molecular weight immunophilins reside in the same cellular compartment as SOC entry channels. To date however, FKBP51 and FKBP52 have been generally described as cytosolic proteins. We therefore questioned whether a sub-population of these proteins localizes to the plasma membrane. To address this question we prepared membrane-cytoskeleton fractions of PAECs. These fractions were extracted in the presence of octylglucoside (to solubilize the caveolar membrane compartments [43]) and potassium iodide (KI) to yield a pellet (consisting of the cytoskeleton and proteins strongly attached to it), and a supernatant (comprised of the dissolved phospholipid bilayer, membrane proteins and proteins strongly attached to them). We have previously used this preparation to cleave TRPC components of endothelial SOC entry channels from the cytoskeleton, thus concentrating TRPC proteins in the supernatant. In the current preparation, FKBP51 and FKBP52 also resolved in the salt-dissociated supernatant indicating that at least a fraction of the cellular pool of these immunophilins strongly associates with the membrane or membrane proteins (Figure 1B). We next performed immunocytochemistry to confirm plasma membrane localization of FKBP51 and FKBP52. PAECs were fixed, permeabilized and incubated with antibodies to FKBP51, FKBP52, TRPC4 (integral membrane protein and part of the I_{SOC} channel [38]) and N-cadherin (integral membrane protein prominent at cell-cell junctions (reviewed in [44])). Consistent with our fractionation data, we observed that while most of the endogenous FKBP51 and FKBP52 are cytosolic and nuclear, these proteins are also found at the plasma membrane especially at the cell-cell border regions. Indeed, the staining pattern for FKBP51 and FKBP52 is identical to that observed for TRPC4 and N-cadherin at cell-cell borders (Figure 1C). That FKBP51 and FKBP52 are at the membrane was further supported by colocalization of these proteins with N-cadherin (Figure 1D).

FKBP51 and FKBP52 are part of the TRPC1/4 heterocomplex

As we observed that a portion of the cellular pool of FKBP51 and FKBP52 localizes to the same cellular compartment as SOC entry channels including the TRPC1 and TRPC4 proteins, we next asked whether these immunophilins associate with endothelial SOC entry channel proteins. Using an immunoprecipitation protocol, Sinkins and colleagues [31] showed that FKBP52 interacts with TRPC1 and TRPC4 in rat brain lysates. More recently, López and colleagues [33] demonstrated that in MEG 01 cells there is little to no basal interaction between FKBP52 and TRPC1; however thapsigargin-induced activation of SOC entry promoted interaction between these two proteins. Here we collected whole cell PAEC lysates and immunoprecipitated TRPC4. Following SDS-PAGE, we probed for the co-precipitation of FKBP51 and FKBP52 and observed that both FKBP51 and FKBP52 co-precipitate with TRPC4 (Figure 2A). In the reciprocal experiment, either FKBP51 or FKBP52 was immunoprecipitated and the co-precipitation of TRPC4 probed. As expected, TRPC4 co-precipitated with both FKBP51 and FKBP52 (Figure 2B). Thus, we have shown that FKBP51 and FKBP52 interact with TRPC4 in PAECs. While at this time we do not know whether the interaction is direct or indirect, overall the data suggest that these immunophilins are part of the TRPC1/4 heterocomplex which comprises the I_{SOC} channel.

3.3. FKBP51 decreases SOC entry

In order to determine whether FKBP51 regulates SOC entry, we began by treating PAECs with dexamethasone to increase levels of FKBP51. Cells were treated with 100 nM dexamethasone for 24 and 48 hours, following which FKBP51 expression was determined via immunoblot analysis. At 24 hours, we observed ~11-fold increase in FKBP51 in PAECs (Figure 3A). Cells were loaded with the calcium indicator Fura2/AM and SOC entry initiated by treatment with thapsigargin. While calcium release was not different between treated and untreated cells (low calcium), SOC entry in dexamethasone-treated PAECs was decreased at both the peak (1000 sec) and plateau (1500 sec) levels compared to untreated cells (Figure 3B), although inter-experimental variability was sufficiently large such that differences did not reach statistical significance (Figure 3C). The global, cytosolic calcium response measured by Fura2/AM reflects calcium entry occurring through all open SOC entry channels and does not discriminate between different calcium channels. To determine whether FKBP51 influences that calcium-selective I_{SOC} , whole cell patch clamp was performed. Control cells exhibited a normal I_{SOC} of -43 pA at -100 mV and a reversal potential of ~+40 mV (Figure 3D). In cells treated with dexamethasone for 24 – 40 hours I_{SOC} was reduced by ~ half (-20 pA at -100 mV), suggesting that FKBP51 inhibits channel opening or cation flux through the channel.

We were cognizant of the fact that dexamethasone has numerous cellular effects that are unrelated to an increase in FKBP51, but that may affect calcium entry [45]. Thus, in order to specifically test the effect of FKBP51 on SOC entry we utilized the T-REx-293-h51 cell line, derived from HEK293 cells and engineered to induce FKBP51 expression upon doxycycline treatment [46]. HEK293 cells are frequently used in SOC entry studies and they endogenously express putative components of SOC entry channels including orai1, TRPC1, TRPC3 and TRPC4 [47; 48; 49]. Thus, this cell system should faithfully recapitulate the SOC entry characteristics exhibited by the pulmonary endothelial cells. In doxycycline-treated T-REx-293-h51 cells FKBP51 expression was increased ~3-fold compared to untreated, control cells (Figure 4A). The thapsigargin-induced SOC entry response following doxycycline treatment was decreased compared to control cells in the peak response, but not the plateau (Figures 4B, 4C). We next measured I_{SOC} in the T-REx-293-h51 cell line, in which FKBP51 is upregulated. The I_{SOC} in the T-REx-293-h51 cell line in the absence of doxycycline is a small current (-29.0 pA at -100 mV) that exhibits a reversal potential at +17.6 mV (Figure 5A). Similar to what we observed in PAECs following

dexamethasone treatment, in T-REx-293-h51 cells treated with doxycycline, I_{SOC} was also reduced (-18.3 pA at -100 mV). We then repeated this experiment in the T-REx-293-sm51 cell line which expresses squirrel monkey FKBP51. Not only is the induction of FKBP51 higher in these cells [46], but Denny and colleagues [50; 51] previously determined that squirrel monkey FKBP51 is approximately six times more potent than human FKBP51 in inhibiting glucocorticoid receptor activity. Thus, we reasoned that if FKBP51 indeed inhibits I_{SOC} then an increase in the quantity and/or potency of FKBP51 should elicit an even greater decrease in the current. We observed that I_{SOC} in untreated T-REx-293-sm51 cells is similar to that in T-REx-293-h51 cells, exhibiting a current of -32.4 pA at -100 mV and a reversal potential of $+17.4$ mV (Figure 5B). In the presence of doxycycline, I_{SOC} was reduced to -9.3 pA at -100 mV, a decrease of $> 70\%$. Together, the results from PAECs treated with dexamethasone and T-REx-293-h51 and T-REx-293-sm51 cells treated with doxycycline suggest that FKBP51 is a negative regulator of SOC entry.

If FKBP51 serves to inhibit SOC entry, then we would expect that knock-down of FKBP51 expression should increase SOC entry. To test this idea, we used siRNA to FKBP51 in pulmonary microvascular endothelial cells (PMVECs). PMVECs were chosen over PAECs because we have observed that PMVECs generally express more FKBP51 than PAECs (Figure 6A). Treatment of PMVECs with siRNA to FKBP51 for at least 48 hours resulted in a $\sim 70\%$ decrease in protein expression (Figure 6B). The global cytosolic calcium response to thapsigargin was then measured in Fura2/AM-loaded cells. We observed no difference in SOC entry between FKBP51 siRNA-treated cells and control cells (Figures 6C). To ensure that our results were not due to inefficient knock-down of FKBP51 expression, we repeated these experiments in PAECs. FKBP51 expression was decreased $\sim 90\%$ in siRNA-treated PAECs (Figure 6D). Measurement of the global cytosolic calcium response to thapsigargin again revealed no difference between siRNA-treated cells and control cells (Figure 6E). While at first these observations seemed contrary to what was expected (i.e. an increase in SOC entry following knock-down of FKBP51), they reveal that the important parameter is the “up-regulation” of FKBP51. This idea is supported by the observation that FKBP51 knock-out mice do not exhibit super-sensitivity to glucocorticoids or androgens [52]. Rather it is only when FKBP51 is over-expressed that an effect is observed. Thus, collectively our results support the idea that FKBP51 is a negative regulator of SOC entry.

3.4. FKBP52 potentiates SOC entry

In many systems, FKBP51 and FKBP52 function in opposing manners (reviewed in [34; 35]). As we show here that FKBP51 plays an inhibitory role in SOC entry, we next questioned whether FKBP52 plays an opposite role to FKBP51 that is acting to promote SOC entry. To address this question we over-expressed FKBP52 in T-REx-293-h51 cells. Cells were transfected with an FKBP52-Flag plasmid. At 18–24 hours post-transfection, whole cell lysates were collected and SOC entry measured. Immunoblot analysis for Flag revealed that the FKBP52-Flag was expressed in these cells (Figure 7A). We then measured the global cytosolic calcium response to thapsigargin. Compared to cells transfected with empty vector, the cells over-expressing FKBP52 exhibited an increased response to thapsigargin at its peak entry but not at the plateau (Figures 7B, 7C).

Our second approach to determine whether FKBP52 acts to potentiate SOC entry was to reduce expression of FKBP52 using siRNA. In MEG 01 cells treated with siRNA to FKBP52 López and colleagues [33] observed a decrease in thapsigargin-induced SOC entry. Here, PAECs were treated with siRNA to FKBP52 for 48 hours after which whole cell lysates were collected and SOC entry measured. From immunoblot analysis for FKBP52 it was determined that by 48 hours protein expression was reduced by $\sim 75\%$ (Figure 8A). SOC entry was initiated by thapsigargin and the response compared between siRNA-treated cells and control cells. There was no difference in calcium release but calcium entry was

decreased in the siRNA-treated cells (Figures 8B, 8C). Collectively, we conclude that FKBP52 functions to potentiate SOC entry in endothelial cells and HEK293 cells.

4. Discussion

SOC entry is an important signaling mechanism underlying many physiological and pathophysiological functions (reviewed in [1; 53]). Originally described by Putney in 1986 [54], SOC entry has been intensely studied by many to understand the mechanism linking store depletion to channel activation, to determine the proteins that make up these channels and to decipher factors important for regulation of channel function. In this study, we present data supporting regulatory roles for the large molecular weight immunophilins FKBP51 and FKBP52 in SOC entry in pulmonary endothelial cells. We show that FKBP51 and FKBP52 are endogenously expressed in pulmonary endothelial cells and that a fraction of these proteins localize to the plasma membrane where SOC entry channels are found. Measurement of global changes in cytosolic calcium using Fura2/AM revealed that over-expression of FKBP51 decreased thapsigargin-induced SOC entry, and from whole cell patch clamp analysis, decreased I_{SOC} . On the other hand, when FKBP52 expression was increased the global cytosolic calcium response to thapsigargin also increased, whereas when FKBP52 expression was inhibited the response to thapsigargin was decreased. Collectively, we conclude that FKBP51 functions to inhibit SOC entry, while FKBP52 serves to potentiate SOC entry. Thus, we present the concept that FKBP51 and FKBP52 work in opposing manners in their effects on SOC entry channels. It is notable that changes in the global cytosolic calcium measured by Fura2/AM were only on the order of 20–30% at the peak response, whereas changes in I_{SOC} were typically larger (up to >70%). As the Fura2/AM approach measures increases in cytosolic calcium that occur through all SOC entry channels activated by thapsigargin, patch-clamp electrophysiology measures current through one particular type of SOC entry channel. As such, although I_{SOC} may be completely inhibited, a Fura2/AM response will still be measured. Thus, small changes in the Fura2/AM response can be indicative of large changes in current through one type of channel. Of the many different SOC entry channels which are activated by thapsigargin, we are particularly interested in the I_{SOC} channel because activation of the I_{SOC} channel is an important step leading to formation of inter-endothelial cell gap formation and endothelial barrier disruption [6].

Immunophilins are peptidyl-prolyl isomerases that act as receptors for immunosuppressive drugs but they are also involved in various cellular functions including regulation of some calcium channels [29; 30; 32; 55; 56; 57]. FKBP52 and FKBP51 are large molecular weight immunophilins that consist of three functional domains, the FK1 domain possessing peptidyl-prolyl isomerase (PPIase) activity, the FK2 domain, and the tetratricopeptide repeat (TPR) domain [58; 59]. PPIase activity catalyzes interconversion of proline containing peptide bonds from a *trans* to *cis* or *cis* to *trans* configuration, which can lead to changes in secondary and tertiary protein structure (reviewed in [60]). The TPR domain mediates interaction of the large molecular weight immunophilins with Hsp90 [61; 62]. In the case of the glucocorticoid receptor, the FK1 domain but not PPIase activity of FKBP52 is required for receptor activation [63]. The TPR domain also plays an important role in that FKBP51 inhibits receptor function by competitively binding with FKBP52 to the glucocorticoid receptor through this domain [51]. At this time, we do not know which of these functional units of protein structure are important for SOC entry channel regulation.

In order to understand the mechanisms by which FKBP51 and FKBP52 function to regulate SOC entry, it is important to know the molecular make-up of the channels in question. To this end, the identities of some SOC entry channels are known. For example, the I_{CRAC} channel is composed of an orai1 tetramer [9]. In vascular endothelial cells a heteromeric

TRPV4-TRPC1 channel was recently described to act in a store-dependent manner [64]. Endothelial cells also express a calcium-selective SOC entry channel that is made up in part by TRPC1 [21] and TRPC4 [26]. Additionally, it has been shown that TRPC proteins interact with orai1 in a manner functionally important for SOC entry [23; 65; 66] [67; 68]. We recently demonstrated that orai1 plays an important role in determining the calcium permeation and selectivity of the TRPC1/4 I_{SOC} channel [40]. Further, we showed that orai1 is part of the TRPC1/4 complex isolated by co-precipitation with protein 4.1, collectively suggesting that the functional I_{SOC} channel exists as a heterocomplex of proteins including the TRPC proteins, orai1, protein 4.1 and other regulatory proteins. STIM1 is one such regulatory protein that is an integral member of the SOC entry pathway [69]. STIM1 is principally found in the endoplasmic reticulum and upon store depletion it couples with orai1 on the plasma membrane leading to activation of SOC entry channels (reviewed in [70]). Indeed, protein complexes of STIM1-orai1-TRPCs have been reported [23; 65; 66; 71; 72; 73]. In endothelial cells, Sundivakkam *et al.* [74] recently demonstrated that STIM1 is critical for TRPC1/4-mediated SOC entry. In consideration of a STIM1-orai1-TRPC complex, the TRPC proteins are of particular interest because they have been shown to interact with FK506-binding proteins [31; 32]. All TRPCs express a conserved proline-rich region on their cytosolic carboxy termini (reviewed in [75]). Part of this region, specifically an LP dipeptide sequence, has been implicated in the binding of the FK506-binding immunophilins FKBP52 to TRPC1, 4 and 5 and FKBP12 to TRPC3, 6, and 7 [31]. The idea that some immunophilins are important for regulation of TRPC channel function is supported by the observations that carbachol-stimulated TRPC6 activity was attenuated following disruption of the FKBP12 - TRPC6 interaction [31]. Additionally, co-expression of FKBP52 with TRPC1 enhanced carbachol-induced current, while co-expression of FKBP12 amplified the spontaneous TRPC1 current [32].

While relatively little is known about the interaction of the large molecular weight immunophilins with calcium channels, interaction of the small molecular weight immunophilin FKBP12 with calcium channels has been well studied. FKBP12 most notably interacts with the inositol 1,4,5-trisphosphate (IP₃) and ryanodine receptors [27; 28] regulating channel function [27; 29; 30; 55]. Unlike FKBP51 and FKBP52, FKBP12 does not possess an FK2 or TPR domain [76], thus, functional interaction of FKBP12 with ion channels is likely mediated through the FK1 domain. The FK1 domain possesses PPIase activity, yet the role of this activity in the regulation of ion channels by FKBP12 is still unclear. More specifically, studies by Cameron *et al.* [27] support a role for PPIase activity in channel regulation, while those of Timmerman *et al.* [77] do not. With regards to SOC entry, we do not yet know whether the FK1 domain and/or its PPIase activity are important for channel regulation. Two important studies have addressed actions of FKBP52 on TRP proteins. The first is a 2009 study by Shim *et al.* [32] in which TRPC1 was identified as a PPIase target of FKBP52 (as well as FKBP12). In this study the authors used a nuclear magnetic resonance approach to measure *cis/trans* isomerization of two leucine-proline (L-P) peptide bonds in TRPC1, one on the N terminus and the other on the C terminus. Addition of recombinant FKBP52 (or FKBP12) efficiently catalyzed both L-P bonds. Functional studies revealed that co-expression of wild-type FKBP52 with TRPC1 increased agonist-stimulated current whereas co-expression of PPIase mutant FKBP52 blocked this current. In another study, Gkika *et al.* [56] determined that FKBP52 PPIase activity was important for inhibiting the Ca²⁺ and N⁺ currents through the vanilloid TRP channel, TRPV5.

Given that TRPC1 and TRPC4 are subunits of the I_{SOC} channel, it is tempting to speculate that the PPIase activity of at least FKBP52 is important in regulation of SOC entry. If the PPIase activity of FKBP52 is important for potentiating SOC entry, and FKBP51 also exhibits PPIase activity, then the question arises as to why FKBP51 does not also potentiate

SOC entry. While we currently do not have the answer to this question, we might gain some insight by considering the tertiary structures of FKBP51 and FKBP52. These proteins possess the same three structural domains (FK1, FK2 and TPR) yet the three-dimensional orientation of these domains differs between the two proteins [59]. A hinge region connecting FK1 and FK2 domains differs in conformation between FKBP51 and FKBP52. Further, when using the FK2 domain as a reference, the FK1 domain of FKBP51 is rotated at an angle ($\theta = 24^\circ$, $\omega = 33^\circ$) compared to the FK1 domain of FKBP52. Thus, it is conceivable that the three-dimensional orientations of the FK1 domains of FKBP51 and FKBP52 result in PPIase activity directed towards different proteins in the SOC entry channel heterocomplex. Another possibility is that FKBP51 inhibits SOC entry by simple competition with FKBP52 for binding to the SOC entry heterocomplex, similar to what is observed in glucocorticoid receptor function. While currently speculation, these ideas will be addressed in our future studies.

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Figure 1. Localization of FKBP51 and FKBP52 in PAECs

A. PAECs express FKBP51 and FKBP52. PAEC whole cell lysates were prepared and analyzed via immunoblot for FKBP51, FKBP52 and actin. B. FKBP51 and FKBP52 resolve

in same cellular fraction as TRPC4. Membrane/cytoskeleton fractions of PAECs were prepared and extracted in the presence of octylglucoside and KI to obtain a supernatant fraction (S) containing plasma membrane proteins and other proteins that are strongly attached to the proteins or phospholipids and a pellet fraction (P) consisting of cytoskeletal proteins and other proteins that are strongly attached to them. Immunoblot analysis of S and P revealed that FKBP51 and FKBP52 predominantly reside in the supernatant along with membrane protein TRPC4. C. FKBP52 localizes to the plasma membrane at sites of cell-cell contact. Immunocytochemistry of PAECs reveals that while the majority of FKBP51 and FKBP52 are cytosolic and nuclear, a fraction localizes to the plasma membrane at sites of cell-cell contact. Fluorescent images for TRPC4 and N-cadherin which are integral membrane proteins which localize to sites of cell-cell adhesion are shown to illustrate the staining pattern at cell-cell borders. Arrows indicate sites of cell-cell contact. D. FKBP51 and FKBP52 colocalize with N-cadherin on the membrane at sites of cell-cell contact. Overlay of FKBP51 (*left*) or FKBP52 (*right*) with N-cadherin. Differential interference contrast (DIC) images are shown in top left quadrant. Arrows indicate sites of cell-cell contact.

Figure 2. FKBP51 and FKBP52 interact with TRPC4

A. TRPC4 was immunoprecipitated from PAEC whole cell lysates. Control lane results are from whole cell lysate reacted with antibody that is not covalently attached to agarose beads, whereas sample lane results are from whole cell lysate reacted with antibody which has been covalently attached to agarose beads. Both FKBP51 and FKBP52 co-precipitated with TRPC4. B. The reciprocal immunoprecipitation experiment was performed where either FKBP51 or FKBP52 was immunoprecipitated from PAEC whole cell lysate. TRPC4 co-precipitated with both FKBP51 and FKBP52.

Figure 3. Increased FKBP51 decreases SOC entry and I_{SOC} in PAECs

A. Dexamethasone treatment upregulates FKBP51 expression in PAECs. Cells were treated with 100 nM dexamethasone for 24 hours following which whole cell lysates were prepared. Immunoblot analysis revealed increased expression of FKBP51 in dexamethasone-treated cells with no change in FKBP52 expression. Actin was used as a loading control. B. SOC entry is decreased in PAECs treated with dexamethasone. Thapsigargin was added to cells in low extracellular calcium to reveal release of calcium from the endoplasmic reticulum. Following return to baseline, extracellular buffer was increased to 2 mM extracellular calcium to allow for calcium entry through SOC entry channels. Calcium release was not different between untreated (black curve) and dexamethasone-treated cells (red curve), however calcium entry was reduced in dexamethasone-treated cells. Each tracing represents mean $\pm$ SEM; $n=3$. C. Comparison of peak and plateau 340/380 ratio values from (B) between dexamethasone-treated and control PAECs. Both the peak and plateau calcium entry response was decreased in dexamethasone-treated cells compared to control. D. I_{SOC} activation is attenuated in dexamethasone-treated cells. Whole cell patch-clamp electrophysiology revealed a normal I_{SOC} in untreated PAECs ($-$ Dex) that in dexamethasone-treated cells ($+$ Dex) was reduced. $n=8$ for each. *, significantly different from untreated cells.

Figure 4. Increased FKBP51 decreases SOC entry in T-REx-293-h51 cells

A. T-REx-293-h51 cells were treated for 24 hours with 0.1 $\mu\text{g}/\text{mL}$ doxycycline following which whole cell lysates were prepared. Immunoblot analysis revealed increased expression of FKBP51 with no change in FKBP52 expression in doxycycline-treated cells. Hsp90 was used as a loading control. B. SOC entry was decreased in doxycycline-treated T-REx-293-h51 cells. Doxycycline-mediated increase in FKBP51 (red curve) modestly attenuated thapsigargin-induced SOC entry compared to control (black curve). Each tracing represents the average of at least 5 experiments (mean $\pm$ SEM). C. Comparison of peak and plateau 340/380 ratio values from (B) between doxycycline-treated and control T-REx-293-h51 cells. The peak calcium entry response was decreased in doxycycline-treated cells compared to untreated cells while there was no difference in the plateau entry response. *, significantly different from untreated cells.

Figure 5. Increased FKBP51 decreases I_{SOC} in T-REx-293-h51 and T-REx-293-sm51 cells
 A. (*Top panel*) Representative immunoblot of T-REx-293-h51 cells following treatment with doxycycline for 24 hours reveals an ~3-fold increase in FKBP51 expression. (*Bottom panel*) I_{SOC} in untreated T-REx-293-h51 cells is a small current (-29.0 pA at -100 mV) that exhibits a reversal potential at $+17.6$ mV. Doxycycline treatment reduces I_{SOC} in these cells by 36.9% (-18.3 pA at -100 mV). *Left* – individual current tracings for I_{SOC} at -100 mV in T-REx-293-h51 cells $\pm$ doxycycline. *Center* – I/V plot summarizing currents at each testing potential. *Right* – bar graph showing reversal potential of I_{SOC} in T-REx-293-h51 cells $\pm$ doxycycline. Doxycycline treatment does not affect reversal potential. $n=15$ for each. B. (*Top panel*) Representative immunoblot of T-REx-293-sm51 cells following treatment with doxycycline for 24 hours reveals an ~5-fold increase in FKBP51 expression. (*Bottom panel*) In untreated T-REx-293-sm51 cells, the I_{SOC} is -32.4 pA at -100 mV with a reversal potential of $\sim +17.4$ mV. Following doxycycline treatment, I_{SOC} is reduced by 71.2% exhibiting a current of -9.3 pA at -100 mV. *Left* – individual current tracings for I_{SOC} at -100 mV in T-REx-293-sm51 cells $\pm$ doxycycline. *Center* – I/V plot summarizing

currents at each testing potential. *Right* bar graph showing reversal potential of I_{soc} in T-REx-293-sm51 cells +/- doxycycline. Doxycycline treatment does not affect reversal potential. n=3 for + doxycycline and n=5 for – doxycycline. *, significantly different from untreated cells.

Figure 6. Knock down of FKBP51 expression does not change global SOC entry

A. PMVECs express more FKBP51 as compared to PAECs. Representative immunoblots of PAEC and PMVEC whole cell lysates probed for FKBP51. Actin is shown as loading control. B. Following 48 hour treatment with siRNA to FKBP51, protein expression was decreased ~70% in PMVECs. C. SOC entry was not different in PMVECs following knock down of FKBP51. Thapsigargin-induced SOC entry was unchanged in cells in which FKBP51 was knocked down (red curve) compared to control PMVECs (black curve). There was no difference in calcium release between the groups. Each tracing represents the average of at least 3 experiments (mean $\pm$ SEM). D. Following 48 hour treatment with siRNA to FKBP51, protein expression was decreased ~90% in PAECs. E. SOC entry was not different in PAECs between control cells (black line) and cells in which FKBP51 expression was decreased (red line). Each tracing represents the average of at least 3 experiments (mean $\pm$ SEM).

Figure 7. Over-expression of FKBP52 increases SOC entry in T-REx-293-h51 cells

A. T-REx-293-h51 cells were transfected with FKBP52-Flag plasmid or control plasmid. Immunoblot analysis of whole cell lysates for Flag-tag revealed increased FKBP52 expression in FKBP52-Flag plasmid-treated cells (+ lane) compared to control cells (– lane). Actin was used as the loading control. B. SOC entry was increased in T-REx-293-h51 cells over-expressing FKBP52. Thapsigargin-induced SOC entry was measured. Compared to control cells (black curve), FKBP52 over-expressing cells (red curve) exhibited an increased SOC entry response. There was no difference in calcium release between the two groups. Each tracing represents the average of at least 3 experiments (mean $\pm$ SEM). C. Comparison of peak and plateau 340/380 ratio values from (B) between FKBP52 over-expressing or control T-REx-293-h51 cells. The peak SOC entry response was increased in FKBP52-over-expressing cells. There was no difference in the plateau response. *, significantly different from control cells.

Figure 8. FKBP52 knock-down attenuates SOC in PAECs

A. PAECs were transfected with siRNA to knock down FKBP52. Control cells received scrambled siRNA or HiPerFect alone. Immunoblot analysis revealed ~75% decreased expression of FKBP52 with no change in FKBP51. Actin was used as a loading control. B. SOC entry was decreased in PAECs following knock down of FKBP52. Thapsigargin-induced SOC entry was decreased in cells in which FKBP52 was knocked down (red curve) compared to PAECs receiving HiPerFect reagent (black curve), or scrambled siRNA (data not shown). There was no difference in calcium release between any of the groups. Each tracing represents the average of at least 4 experiments (mean $\pm$ SEM). C. Comparison of peak and plateau 340/380 ratio values from (B) between FKBP52 knock down and control PAECs. *, significantly different than control (-siRNA) cells.