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Thapsigargin activates Ca\(^{2+}\) entry both by store-dependent, STIM1/Orai1-mediated, and store-independent, TRPC3/PLC/PKC-mediated pathways in human endothelial cells

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**Abstract**

The ER Ca\(^{2+}\) sensor STIM1 and the Ca\(^{2+}\) channel Orai1 are key players in store-operated Ca\(^{2+}\) entry (SOCE). In addition, channels from the TRPC family were also shown to be engaged during SOCE, while their precise implication remains controversial. In this study, we investigated the molecular players involved in SOCE triggered by the SERCA pump inhibitor thapsigargin in an endothelial cell line, the EA.hy926. siRNA directed against STIM1 or Orai1 reduced Ca\(^{2+}\) entry by about 50–60%, showing that a large part of the entry is independent from these proteins. Blocking the PLC or the PKC pathway completely abolished thapsigargin-induced Ca\(^{2+}\) entry in cells depleted from STIM1 and/or Orai1. The phorbol ester PMA or the DAG analog OAG restored the Ca\(^{2+}\) entry inhibited by PLC blockers, showing an involvement of PLC/PKC pathway in SOCE. Using pharmacological inhibitors or siRNA revealed that the PKC\(\eta\) is required for Ca\(^{2+}\) entry, and pharmacological inhibition of the tyrosine kinase Src also reduced Ca\(^{2+}\) entry. TRPC3 silencing diminished the entry by 45%, while the double STIM1/TRPC3 invalidation reduced Ca\(^{2+}\) entry by more than 85%. Hence, in EA.hy926 cells, TG-induced Ca\(^{2+}\) entry results from the activation of the STIM1/Orai1 machinery, and from the activation of TRPC3.

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**1. Introduction**

Store-operated Ca\(^{2+}\) entry (SOCE) is a ubiquitous mechanism of regulated Ca\(^{2+}\) influx, which is getting activated upon Ca\(^{2+}\) store depletion [1]. By contributing to cytosolic Ca\(^{2+}\) elevation and to the replenishment of the ER Ca\(^{2+}\) compartment [2,3], SOCE is a key regulator of many Ca\(^{2+}\) dependent physiological processes [4]. Under physiological conditions, SOCE is induced by the activation of cell surface receptors coupled to e.g. phospholipase C (PLC) that leads to the generation of inositol 1,4,5-trisphosphate (IP\(_3\)), and the release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) [5]. The extent to which SOCE is engaged during agonist stimulation might greatly vary depending on the level of ER Ca\(^{2+}\) depletion [6,7]. Experimentally, SOCE is frequently stimulated by blocking the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA), with drugs like thapsigargin (TG) that passively deplete the ER. During the last 20 years, the family of TRPC (canonical transient receptor potential) channels has been proposed as candidates for SOCE. Number of reports has shown a reduced SOCE activity when TRPC expression was knocked down or knocked out [8–11]. When exogenously expressed some TRPC channels displayed SOCE activity [12–14]. However, numerous studies also demonstrated that TRPC channels did not operate as SOCE channels, but rather are gated by second messengers like Ca\(^{2+}\) or diacylglycerol (DAG; [15–17]). Hence, the involvement of TRPC channels in the SOCE mechanism is a controversial issue, and appears to dependent on the cell types investigated, and importantly on the expression level of the TRPC [14,18,19].

More recently, two major SOCE components have been identified: the Stromal Interacting Molecule 1 (STIM1) [20–22] predominantly located in the ER membrane and that senses the luminal Ca\(^{2+}\) concentration, and the Ca\(^{2+}\) channel Orai1 (also known as CRACM; [23–25]). Upon Ca\(^{2+}\) store depletion, STIM1 oligomerizes, translocates close to the plasma membrane and aggregates into punctate structures [21,26]. The interaction between STIM1 and Orai1 leads to channel opening and Ca\(^{2+}\) entry [23,25,27]. An additional level of complexity emerged as several recent reports showed that STIM1 binds to TRPC1, TRPC4 and TRPC5 [28] and indirectly regulates TRPC3 and TRPC6 [29]. Furthermore, it was...
reported that Orai1 and TRPC proteins form complexes together with STIM1 that participate to Ca$^{2+}$ entry [30–35].

In endothelial cell lines, several studies have demonstrated that TRPC1 [36] and TRPC4 [37,38] participate to SOCE. On the contrary, a recent study showed in primary human umbilical vein endothelial cells (HUVECs) that SOCE is mediated almost exclusively by the STIM1/Orai1 machinery [39].

In the current study, we investigated the molecular identity of SOCE in a human umbilical vein endothelial cell line, the EA.hy926. To this aim, we evaluated the involvement of STIM1, Orai1 and TRPCs proteins in TG-induced Ca$^{2+}$ entry. We showed that STIM1, Orai1 or TRPC3 invalidation reduced Ca$^{2+}$ entry by about 50%, while the combination of STIM1 and TRPC3 knockdown reduced the entry by 85%. Moreover, indirect evidence is provided that TRPC3 is getting activated following a PLC/PKC signaling pathway initiated by the addition of TG.

### 2. Material and methods

#### 2.1. Materials

DMEM, penicillin and streptomycin were obtained from Invitrogen. Fetal calf serum (FCS) was from PPA Laboratories (Linz, Austria). Thapsigargin, U73122, U73343, PP1, Src inhibitor-1, staurosporine, chelerythrine, rottlerin, PMA and OAG were obtained from Sigma. Acetoxyethyl ester form of Fura-2 (Fura-2/AM) was from Molecular Probes Europe (Leiden, the Netherlands). The myristoylated peptide PKCta was from Calbiochem and GF103209X from Tocris.

#### 2.2. Cell culture and transfection

Experiments were performed on the human umbilical vein endothelial cells derived cell line EA.hy926 at passages > 45. Cells were grown in DMEM containing 15% FCS, 0.5% fungizone, 1% penicillin and streptomycin, and were maintained at 37 °C in 5% CO$_2$ atmosphere.

#### 2.3. siRNA knockdown

EA.hy926 cells were transfected in suspension by incubating 3 × 10$^5$ cells in a solution containing 6 μl of lipofectamine RNAiMax (Invitrogen) and 100 nM of a specific siRNA (Ambion, Invitrogen or Qiagen) according to manufacturer protocols (Invitrogen). Experiments using one gene invalidation (Ca$^{2+}$ imaging, QRT-PCR, Western blot analysis) were performed 48 h post-transfection, while for the double STIM1/TRPC3 knockdown experiments, the siSTIM1 was transfected 24 h before siTRPC3 (72 h and 48 h post-transfection for STIM1 and TRPC3, respectively). Sequences of the different siRNA are summarized in Table 1. The siRNA scramble from Ambion was used as negative control. The siRNA transfection efficiency was approximately 90%, estimated by Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen).

#### 2.4. Quantitative real-time PCR

Real-time experiments were performed at the genomics platform of the NCCR Frontiers in Genetics (Geneva). For each PCR reaction, 1/20th of the cDNA template was PCR amplified in a 7900HT SDS System using Power SYBR Green PCR master mix (both from Applied Biosystems, Foster City, CA, USA). We obtained raw threshold-cycle (Ct) values using SDS 2.0 software (Applied Biosystems). A mean quantity was calculated from triplicate PCR reactions for each sample, and this quantity was normalized to the average of four endogenous control genes (glucuronidase B, GAPDH, TBP and EE-EF1) as described by [40]. Primers used for the real-time PCR are described in Table 1.

#### 2.5. Cytosolic calcium measurements

For Ca$^{2+}$ imaging, cells were plated on 30 mm glass cover slips. The changes in cytosolic Ca$^{2+}$ concentration were measured with Fura-2. Cells were loaded with 2 μM Fura-2/AM plus 1 μM pluronic de-esterification. Ratiometric images of Ca$^{2+}$ signals were obtained using a microscope (Axio Observer, Zeiss) equipped with a Lambda GD4 illumination system (Sutter Instrument Company, Novato, CA, USA), which rapidly changed the excitation wavelengths between 340 nm (340AF15; Omega Optical) and 380 nm (380AF15; Omega Optical). Emission was collected through a 415DCLP dichroic mir-

### Table 1

Sequences of siRNA and primers used for the real-time RT-PCR (all sequences are oriented 5′–3′).

<table>
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<th>Sense</th>
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Primers used for the real-time PCR are described in Table 1.
Fig. 1. Effect of STIM1 and Orai1 knockdown on TG-induced Ca\(^{2+}\) entry. (A) Cytoplasmic Ca\(^{2+}\) was assessed on EA.hy926 cells loaded with 2 µM Fura-2. SOCE was triggered by depletion of the stores with 1 µM thapsigargin (TG) in the absence of external Ca\(^{2+}\) (1 mM EGTA). 2 mM Ca\(^{2+}\) was then re-added to the extracellular medium as indicated. The control experiment without stimulation, showed a minimal Ca\(^{2+}\) entry. (B) Cells were transiently transfected with control siRNA (scramble, siControl), or siRNA against STIM1 or Orai1. Each trace represents the Ca\(^{2+}\) entry phase following store depletion, and is the mean of a representative coverslip. (C) Quantification of the effects of siSTIM1, siOrai1 and siSTIM1/Orai1 on the initial slope of ratio increases, after Ca\(^{2+}\) re-addition. Bars are mean ± SEM. The number of cells is written on the bar graph.

Experiments were performed at room temperature in Hepes-buffered solution containing (in mM): 135 NaCl, 5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 Hepes, 10 glucose, pH adjusted at 7.45 with NaOH. The Ca\(^{2+}\)-free solution contained 1 mM EGTA instead of 2 mM CaCl\(_2\). Due to the high trans-
Fig. 2. Effect of STIM1 and Orai1 knockdown on mRNA and protein levels. (A) C mRNA levels were assessed by quantitative RT-PCR 48 h after transfection with siSTIM1 (A) or siOrai1 (C) (mean ± SEM, n = 3 or 4 independent experiments). (B and D, left panels) Western blots showing the decrease of the STIM1 or Orai1 bands 48 h after the transfection with siSTIM1 (B) or siOrai1 (D). β-Tubulin was used as a loading control. Right panels: quantification of Western blots from 4 to 5 different experiments.

2.6. Western blots

Western blots were performed as previously described [41]. Briefly, the endothelial cells were lysed using modified NP40 cell lysis buffer (Invitrogen), 50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4 and 1% Nonidet P40.

Total proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in T-TBS (0.1% Tween 20, 20 mM Tris–HCl pH 7.5, 137 mM NaCl) and 5% non-fat milk. Blots were incubated with the primary antibodies diluted in T-TBS and nonfat milk as follows: rabbit polyclonal anti-STIM1 antibody (1:1000; Sigma), rabbit polyclonal anti-Orai1 antibody (1:1000; Sigma), rabbit polyclonal anti-PKCε antibody (1:700; Santa Cruz Biotechnology), rabbit polyclonal anti-TRPC3 antibody (1:1000; Sigma) and mouse monoclonal antibody against β-tubulin (clone DM1A, Sigma) 1:10,000. Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted 1:10,000 (BioRad) or with HRP-conjugated goat anti-rabbit diluted 1:10,000 (BioRad), respectively. Antibodies were revealed using ECL reagents and hyperfilm ECL (Amersham Biosciences). Image-J Software was used to quantify the level of protein expression.

3. Results

3.1. STIM1 and Orai1 are involved in store-operated Ca2+ influx

To measure the store-operated Ca2+ entry (SOCE), the cells were stimulated with 1 μM thapsigargin (TG) in the absence of external Ca2+, and 2 mM Ca2+ was re-added to the extracellular medium to elicit Ca2+ entry (Fig. 1A). Only a very small response was detected in the absence of TG stimulation (Fig. 1A). To compare Ca2+ influx between different conditions, we measured the initial slope of fluorescence increase (Fig. 1C).

First, we investigated the role of STIM1 and Orai1 proteins on SOCE, by transiently transfecting the EA.hy926 cells with the respective siRNA designed against STIM1 and Orai1. The basal Ca2+ entry, in the absence of TG stimulation, was not different between...
control cells, and cells invalidated for STIM1 or Orai1 (data not shown). The addition of TG in Ca\(^{2+}\)-free medium elicited similar responses in control and STIM1 or Orai1 knockdown cells (data not shown), whereas the response elicited by Ca\(^{2+}\) re-addition was inhibited by 60% in STIM1 knockdown cells (Fig. 1B and C). In Orai1 silenced cells, the SOCE was reduced by 43% (Fig. 1B and C). These results confirmed that STIM1 and Orai1 proteins contribute to SOCE in this endothelial cell line.

### 3.2. Effect of STIM1 and Orai1 knockdown on mRNA level and protein expression

We performed a quantitative RT-PCR to evaluate the extend of gene silencing upon siRNAs transfection. As shown in Fig. 2A, STIM1 mRNA level was decreased by 86% in cells transfected with siRNA against STIM1 \((n = 4)\) when compared to cells exposed to scramble siRNA (noted siControl). The amount of STIM1 protein measured by Western blot 48 h after transfection was reduced by 67% in cells treated with siSTIM1 \((n = 4)\); Fig. 2B) confirming the efficiency of STIM1 silencing. Orai1 mRNA levels were decreased by 99% in Orai1 silenced cells \((n = 3)\) (Fig. 2C) when compared with control cells. The amount of Orai1 protein 48 h after transfection was reduced by 81% in cells treated with siOrai1 \((n = 5)\); Fig. 2D) confirming that Orai1 silencing was highly efficient. In double (STIM1/Orai1) knockdown cells, the level of STIM1 mRNA was decreased by about 67% and 99% for Orai1 mRNA \((n = 2);\) Fig. S1). The double knockdown (Orai1/STIM1) did not reduce the SOCE more than siRNA against STIM1 alone (Fig. 1C), suggesting that STIM1 is the limiting player in SOCE mechanism. Altogether, these data confirmed the role played by STIM1/Orai1 machinery in TG-induced Ca\(^{2+}\) entry, but suggest also the involvement of other proteins in this process.

### 3.3. The phospholipase C inhibitor (U73122) blocks the residual Ca\(^{2+}\) influx in STIM1 or Orai1 knockdown cells

Several studies have shown that PLC is involved in TG-induced Ca\(^{2+}\) entry \([42-44]\). We thus used the membrane-permeable phospholipase C (PLC) inhibitor U73122 to evaluate the implication of PLC in TG-induced Ca\(^{2+}\) entry. In control cells, a 5 min preincubation (before TG stimulation) with 2 \(\mu\)M U73122 resulted in a reduction of the Ca\(^{2+}\) influx by 20% (Fig. 3B). In STIM1 silenced
Fig. 4. Role of protein kinase C (PKC) in TG-induced Ca\(^{2+}\) entry. (A) Cells were transiently transfected with siSTIM1 or control siRNA. The PKC inhibitor staurosporine was added 2 min before TG stimulation. The Ca\(^{2+}\) entry phase is depicted and each trace is the mean of a representative coverslip. (B) Statistic evaluation of the effect of staurosporine and chelerythrine on TG-induced Ca\(^{2+}\) influx in control and STIM1 silenced cells (\(n\) ranges from 36 to 335 cells). (C) The addition of 1 \(\mu\)M PMA or 100 \(\mu\)M OAG, 5 min before TG, restored the Ca\(^{2+}\) entry in cells treated with the PLC inhibitor U73122. Bar graphs are the quantification of the effect of PMA and OAG on the initial slope of ratio increase, after Ca\(^{2+}\) re-addition (\(n\) ranges from 41 to 335 cells). The bars of panel B and C, representing the control experiments (for control, siSTIM1, siOrai1 and siSTIM1/Orai1) are the same as presented in Fig. 1.

Cells, the U73122 pretreatment completely prevented the residual Ca\(^{2+}\) influx (Fig. 3A and B). The remaining Ca\(^{2+}\) influx in Orai1 and Orai1/STIM1 knockdown cells was also completely abolished after PLC inhibition (Fig. 3B). On the contrary, the inactive analog U73343 (5 \(\mu\)M) did not affect the Ca\(^{2+}\) influx activated by TG (Fig. 3). Neither U73122 nor U73343 had an effect on the ability of TG to release calcium from the stores (data not shown). Interestingly, the addition of 2 \(\mu\)M U73122 just prior the Ca\(^{2+}\) re-addition phase or during the Ca\(^{2+}\) re-addition phase did not prevent Ca\(^{2+}\) influx (Fig. S2). This suggests that this compound does not act as a direct channel
Fig. 5. Inhibition or invalidation of PKC\(\eta\) reduced TG-induced Ca\(^{2+}\) entry. (A) The myristoylated peptide MPI-PKC\(\eta\) was preincubated 30 min before TG stimulation. The bar graphs represent the slope of ratio increase following Ca\(^{2+}\) re-addition (n ranges from 65 to 130 cells). (B) Statistical evaluation of the effect of siRNA against PKC\(\eta\) on TG-induced Ca\(^{2+}\) entry. The bars represent the slope of the ratio increase after Ca\(^{2+}\) re-addition (n ranges from 72 to 130 cells). (C, left panel) Western blot showing the decrease expression level of STIM1 and PKC\(\eta\), 48 h after transfection, either alone or in combination. \(\alpha\)-Tubulin was used as a loading control. Right panel: quantification of the Western blot, showing also the combination of siOrai1 with siPKC\(\eta\) from 2 to 4 different experiments.
3.4. The protein kinase C (PKC) pathway is involved in TG-induced Ca\textsuperscript{2+} entry

The activation of PLC induces the cleavage of phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) \[5\]. In turn, DAG is an activator of the "classical" and "novel" PKC isoforms \[45\]. To investigate the potential involvement of PKC in endothelial SOCE, we first used two types of PKC inhibitors: chelerythrine (a widely generic inhibitor of PKC) \[46,47\] and staurosporine \[48,49\]. Each inhibitor was added 2 min before TG stimulation, in control and STIM1 knockdown cells. In control cells chelerythrine and staurosporine reduced the Ca\textsuperscript{2+} influx by about 45\% (Fig. 4B), while in STIM1 knockdown cells, the Ca\textsuperscript{2+} influx was inhibited by 70\% for staurosporine (Fig. 4A and B) and by 90\% for chelerythrine (Fig. 4B). The same inhibitory effect of PKC blockers was observed in Orai1 and Orai1/STIM1 silenced cells (data not shown). Like for the PLC inhibitor U73122, the PKC blockers had no effect on Ca\textsuperscript{2+} influx when they were added before or during the Ca\textsuperscript{2+} re-addition phase (Fig. S3), suggesting that these compounds did not act as direct channel blockers. In order to further support our hypothesis that the PLC/PKC pathways is involved in TG-induced Ca\textsuperscript{2+} entry, we performed rescue experiments on cells treated with the PLC inhibitor U73122. In this condition, the addition of either the PKC activator PMA (phorbol myristate acetate 1 \mu M), or the membrane-permeable DAG analog OA\textsubscript{G} (1-oleoyl-2-acetyl-sn-glycerol 100 \mu M) restored the Ca\textsuperscript{2+} entry in control cells and STIM1 or Orai1 knockdown cells (Fig. 4C). These data suggest that the PLC metabolite DAG activates the PKC, limiting the possible PKC isoforms engaged in the Ca\textsuperscript{2+} entry as those that belong to the "classical" (activated by Ca\textsuperscript{2+} and DAG) or "novel" family (activated by DAG but nor by Ca\textsuperscript{2+}). On EA.hy926 cells, the PKC\textalpha and PKC\beta, from the "classical" family, as well as the PKC\delta, PKC\epsilon and PKC\eta from the "novel" family are present \[50\]. Thus we applied inhibitors directed against different isoforms of PKC. GF103209X that preferentially blocks the PKC\alpha/\beta did not reduce Ca\textsuperscript{2+} entry (Fig. S4A). On the contrary, rottlerin (Fig. S4B) and the myristoylated peptide MPI-PKC\beta (Fig. 5A), which inhibit PKC\delta and PKC\eta, respectively, reduced by more than 50\% TG-induced Ca\textsuperscript{2+} entry, both in control cells, and in cells invalidated for STIM1 or Orai1. To confirm these data, we transiently transfected the cells with siRNA directed against these two PKCs isoforms. The invalidation of the PKC\delta reduced by about 15\% the Ca\textsuperscript{2+} entry in control cells, but did not affect Ca\textsuperscript{2+} entry in STIM1 or Orai1 invalidated cells (Fig. S5A). On the contrary, the knockdown of PKC\eta reduced TG-induced Ca\textsuperscript{2+} entry by 20\% in control cells, and by about 40\% in STIM1 and Orai1 invalidated cells (Fig. 5B). To measure the efficiency of gene silencing, we performed Western blots 48 h after transfection. The siRNA
Fig. 7. Effect of STIM1 and TRPC3 knockdown on TG-induced Ca\(^{2+}\) entry. (A) EA.hy926 cells were loaded with 2 \(\mu\)M Fura-2 to monitor cytosolic Ca\(^{2+}\) changes. Traces are the mean of a representative coverslip showing the Ca\(^{2+}\) entry phase. Cells were transiently transfected with control siRNA, siRNA against STIM1, or siRNA against STIM1 together with siRNA against TRPC3; siSTIM1 was transfected 24 h before siTRPC3 (72 h and 48 h after transfection for STIM1 and TRPC3, respectively). (B) The bars represent the slope of the ratio increase after Ca\(^{2+}\) re-addition in siControl, siSTIM1 and siSTIM1/siTRPC3 silenced cells. Bars are mean ± SEM (\(n\) ranges from 46 to 190 cells). (C) Western blots showing the disappearance of the STIM1 protein and the TRPC3 protein expression after the transfection with siSTIM1/siTRPC3. \(\alpha\)-Tubulin was used as a loading control. (D) Quantification of Western blots from 2 different experiments.

against PKC\(\eta\) was efficient in reducing protein levels, both on single transfection, and in combination with STIM1 and Orai1 silencing. The same efficiency on protein levels was obtained with the siRNA against PKC\(\delta\) (Fig. S5B), suggesting that PKC\(\delta\) is not essential for TG-induced Ca\(^{2+}\) entry.

3.5. Involvement of canonical transient receptor potential in TG-induced Ca\(^{2+}\) entry

We next tested the involvement of TRPC in TG-induced Ca\(^{2+}\) entry. To this aim, we designed siRNA against each of the five isoforms expressed in this endothelial cells line (TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6). Interestingly, only TRPC3 appear to be involved in TG-induced Ca\(^{2+}\) entry, as shown by the reduction of the initial slope of Ca\(^{2+}\) influx by 45% (Fig. 6A and B). The efficiency of each siRNA in silencing its messenger RNA was verified by quantitative RT-PCR, and the mRNA levels were reduced between 50% and 90% (Fig. 6C). At the protein level, TRPC3 was decreased by around 70% (Fig. 6D). We confirmed this result by using two other siRNA against TRPC3, which gave similar reduction of the Ca\(^{2+}\) entry (Fig. S6A). The combination of siRNA against STIM1 and TRPC3 reduced Ca\(^{2+}\) entry by 85% (Fig. 7A and B). In these conditions, the efficiency of TRPC3 and STIM1 invalidation was between 70 and 85% at the protein level (Fig. 7C and D). These data confirmed the involvement of STIM1 and TRPC3 protein in TG-induced Ca\(^{2+}\) influx in endothelial cells.

3.6. Effect of PLC, PKC or Src inhibitors on TRPC3 knockdown cells

To explore the putative target of the PKC pathway, we tested the PLC and PKC inhibitors on TRPC3 knockdown cells. U73122 reduced by about 12%, and chelerythrine by 25% TG-induced entry (Fig. S6B). Staurosporine on the other hand did not inhibit Ca\(^{2+}\) entry in TRPC3 knockdown cells (Fig. S6B), and neither OAG nor PMA rescued the Ca\(^{2+}\) entry after U73122 treatment in siTRPC3 treated cells (Fig. S6C). The MPI-PKC\(\eta\) reduced by about 25% the slope of Ca\(^{2+}\) influx, while the siRNA against PKC\(\eta\) did not reduce, and even paradoxically increased the entry (Fig. 8A). The absence (staurosporine, siPKC\(\eta\)) or small inhibitory effect (U73122, chelerythrine, MPI-PKC\(\eta\)) of the PLC/PKC pathway inhibitors on TRPC3 knockdown cells, suggested that TRPC3 is likely the target of the pathway engaged upon TG stimulation. The non-receptor tyrosine
Fig. 8. Effect of PKC\(\eta\) and Src inhibition on TG-induced Ca\(^{2+}\) entry. (A) Statistical evaluation of the effect on TG-induced Ca\(^{2+}\) entry, of MPI-PKC/H9257 and siPKC\(\eta\). The bar graphs represent the initial slope of the ratio increase following Ca\(^{2+}\) re-addition \((n\) ranges from 37 to 130 cells). (B) Effect of two Src inhibitors, PP1 and Src inhibitor-1 (Src1) on TG-induced Ca\(^{2+}\) entry. The Src inhibitors were incubated 30 min before TG stimulation. Experiments were performed in STIM1, Orai1 or TRPC3 invalidated cells. Bars are mean \(\pm\) SEM of the initial slope following Ca\(^{2+}\) re-addition \((n\) ranges from 24 to 141 cells). The bars representing the control experiments (for control, siSTIM1, siOrai1) are the same as presented in Fig. 5.

Kinase Src was shown to be essential for TRPC3 activation [51]. We thus tried two different Src inhibitors, PP1 and Src inhibitor-1 on TG-induced Ca\(^{2+}\) entry. As shown in Fig. 8B, Src inhibition reduced Ca\(^{2+}\) entry in STIM1 and Orai1 silenced cells, but not in TRPC3 invalidated cells, confirming again TRPC3 as a probable target of the PLC/PKC signaling pathway.

4. Discussion

In the present work, we studied the molecular identity and the mechanism of TG-induced Ca\(^{2+}\) entry in human umbilical vein endothelial cell line, the EA.hy926. By siRNA approach, we assessed the involvement of STIM1, Orai1 and TRPCs proteins in this process. We showed that TG-induced Ca\(^{2+}\) entry is partially mediated by STIM1, Orai1 and TRPCs proteins in this process. We showed that TG-induced Ca\(^{2+}\) entry is partially mediated by STIM1 and Orai1, and partially by TRPC3, and the double STIM1/TRPC3 invalidation reduced the entry by 85%. Interestingly, the residual STIM1/Orai1-independent Ca\(^{2+}\) entry is fully abolished following inhibition of PLC and/or PKC, while the residual TRPC3-independent entry is almost not affected by PLC or PKC inhibition. These data strongly suggested that PLC is getting activated upon TG stimulation, and that the downstream signaling pathway, involving PKC and TRPC3, account for a large part in TG-induced Ca\(^{2+}\) entry in endothelial cells.

Since 2005 and the discovery of the major role played by STIM1 and Orai1 proteins in SOCE, the function of TRPCs isoforms in this mechanism remained to be firmly established. In this study, we confirm that STIM1 and Orai1 are important players of SOCE, as invalidation of these proteins reduced the Ca\(^{2+}\) entry by 45% (Orai1) to 65% (STIM1). Interestingly, even though the silencing efficiency was of between 65 and 80% at the protein level, an important residual Ca\(^{2+}\) entry persisted upon store depletion. Such a “partial” SOCE reduction was already observed, for instance in smooth muscle cells [52–54] or HEK cells [20], but in most cell types, the inhibition of SOCE upon STIM1 invalidation is very pronounced [3,39,55,56]. This finding prompted us to investigate the mechanism of the residual TG-induced Ca\(^{2+}\) entry.

Several studies have reported a reduction of SOCE following inhibition of the PLC by U73122 [42], or after PLC\(\gamma\) knockdown [43,44]. In our hands, U73122 reduced by only 20% the SOCE in control cells, while in STIM1 and/or Orai1 knockdown cells, the Ca\(^{2+}\) entry was completely abolished. To investigate whether the downstream products of PLC activation might affect Ca\(^{2+}\) entry after TG
stabilization, we used two PKC inhibitors, staurosporine and chelerythrine. Both compounds reduced by about 45% TG-induced Ca^{2+} entry in control cells, and completely inhibited Ca^{2+} entry in STIM1 and/or Orai1 knockdown cells. The involvement of PKC was further confirmed by using PMA to directly activate PKC on cells treated with the PLC blocker. This treatment restored the Ca^{2+} influx to levels achieved without blocking the PLC, both in control cells, and in cells invalidated for STIM1 or Orai1. Additionally, the DAG analog OAG also restored Ca^{2+} entry upon PLC inhibition. It thus appears that the lipase activity of PLC is required for TG-induced Ca^{2+} entry, in particular through the production of DAG that activates the PKC.

In previous studies reporting the importance of PLC for SOCE, it was shown that either the lipase activity of the enzyme was not required [44], or the production of DAG seems to be essential for TG-induced Ca^{2+} entry in control cells, and completely inhibited Ca^{2+} entry in STIM1/H9259 and PKC inhibition was shown to be essential for TG-induced Ca^{2+} entry. Next, we wanted to determine which isoform of the PKC was involved in the Ca^{2+} entry. The PKC family consists of ten isozymes [57]. Based on their mechanism of activation, the PKC are divided into three subfamilies, the "conventional" (or "classical"), the "novel," and the "atypical" PKC. "Conventional" PKCs contain the isoforms α, β, δ, and γ. They require Ca^{2+}, DAG, and phospholipids such as phosphatidylserine for their activation. "Novel" PKCs include the δ, ε, η, and θ isoforms that only require DAG, but not Ca^{2+} to be activated. Thus, "conventional" and "novel" PKCs are activated downstream of the PLC signal transduction pathway. The "atypical" PKCs (including protein kinase Mζ and s/δ isoforms) neither require Ca^{2+} nor diacylglycerol for their activation.

GF103209X, an inhibitor of the classical PKC had no inhibitory effect on Ca^{2+} entry. On the contrary, the rottlerin and MPI-PKC that inhibit PKCδ and PKCγ, respectively, markedly prevented TG-induced Ca^{2+} entry. Caution had to be taken by using PKC inhibitors, in particular rottlerin that was shown to have serious side effects [58]. We thus used siRNA against PKCδ and PKCγ to confirm our data. While the knockdown of PKCδ did not inhibit Ca^{2+} entry, the invalidation of PKCγ negatively impacted on TG-induced Ca^{2+} entry, both in control and STIM1 and Orai1 silenced cells. Altogether, these results strongly point to a role played by the "novel" PKC family (likely PKCγ), in TG-induced Ca^{2+} entry.

The inhibitory effect of PLC and PKC blockers was observed in control cells, but also in cells invalidated for STIM1 or Orai1. Hence, it is unlikely that STIM1/Orai1 proteins are the target(s) of PKC, even though we could not completely rule this out, as the silencing of these two proteins was not complete. We thus investigated what other proteins could be implicated in TG-induced Ca^{2+} entry, focusing on the TRPC channel family and found out that only TRPC3 invalidation reduced the Ca^{2+} entry. The double STIM1/TRPC3 invalidation almost completely prevented Ca^{2+} entry, strongly suggesting that TG-induced Ca^{2+} entry in endothelial cells was constituted by the STIM1/Orai1 machinery for one part, and for the other part by TRPC3. Interestingly however, when TRPC3 was knocked-down, the efficiency of PLC of PKC inhibition was much less pronounced, with an absence of significant inhibition using staurosporine or siPKCγ, and a modest reduction of the Ca^{2+} entry of about 15–20%, upon U73122, chelerythrine and MPI-PKC application. In addition, when TRPC3 was down-regulated, neither OAG nor PMA potentiate the Ca^{2+} entry. We thus propose that TRPC3 channel is likely a target of the PLC/PKC pathway activation. PKC was shown to phosphorylate TRPC3 on Ser 712, resulting in channel inactivation [59,60], whereas in our study PKC inhibition decreased Ca^{2+} entry. Hence, in our cellular system, it is unlikely that PKC directly phosphorylates TRPC3. PKC might phosphorylate an intermediate protein that in turn positively regulates the activity of TRPC3. It was shown that the non-receptor tyrosine kinase Src is essential for TRPC3 activation [51], by phosphorylating the channel on a tyrosine at position 444 [61]. Using two different Src inhibitors, we showed that both reduced Ca^{2+} entry in STIM1 and Orai1 knockdown cells, while not in TRPC3 invalidated cells. This result suggests that TRPC3 is the target of phosphorylation by Src. We can reasonably postulate that Src is getting activated indirectly by PKC, as it was reported in smooth muscle cells [62]. The precise mechanism between PKC activation and TRPC3 opening remains however to be firmly established, but our data suggested that Src is, at least partially, involved in this process. Considering that the inhibition of the Ca^{2+} entry varied between about 40 and 60% upon src inhibition or siRNA treatment against PKCγ, it is possible that for a part, TRPC3 is also getting directly activated by DAG, like it was already reported [15,63,64]. This point, however, remains to be further investigated.

Moreover, recent papers showed that STIM1 directly or indirectly binds to all TRPC (except TRPC7) and activated them [28,29]. Orai1 was also shown to be associated with the complex formed by STIM1 and TRPC. Hence, the idea emerged that the function of TRPC channels might well depend on their association or not with the STIM/Orai molecules [30–35]. Our experimental evidence suggested that TRPC3 function independently from STIM/Orai and that its activity is not linked to the Ca^{2+} content of the store.

In summary, we showed that in EA.hy926 cells, the influx elicited upon TG application has two components, one being the "classical" STIM1 and Orai1 machinery, and the other one that comprises TRPC3 being activated by a rather unusual mechanism: TG stimulation leads to PLC activation and the production of DAG that in turns activates a "novel" PKC (likely PKCγ). This presumably results in an activation of Src that phosphorylates TRPC3 leading to Ca^{2+} entry. This chain of event is unexpected following TG stimulation, even though PLC was already reported to be involved in SOCE. While we can certainly call the STIM1/Orai1-dependent Ca^{2+} entry SOCE, the machinery involving PLC/PKC/Src/TRPC3 is not necessarily store-operated. Indeed, we do not know whether the depletion of the ER per se leads to PLC activation, or whether the consequence of the depletion, for instance the cytosolic Ca^{2+} elevation, is responsible for this activation. In any instance, in EA.hy926 cells, TG stimulation engaged more molecules than STIM1 and Orai1, and surprisingly activated a Ca^{2+} signaling pathway usually triggered by agonist stimulation.

Conflict of interest

There is no conflict of interest

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Appendix A. Supplementary data


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