Role of Trpc channels, Stim1 and Orai1 in $PGF_{2\alpha}$-induced calcium signaling in NRK fibroblasts.

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Abstract

Normal rat kidney (NRK) fibroblasts exhibit growth-dependent changes in electrophysiological properties and intracellular calcium dynamics. The transition from a quiescent state to a density-arrested state results in altered calcium entry characteristics. This coincides with modulation of the expression of the genes encoding the calcium channels Trpc1, Trpc6 and Orai1, and of the intracellular calcium sensor Stim1. In the present study we have used gene selective short hairpin (sh) RNAs against these various genes to investigate their role in a) capacitative store-operated calcium entry (SOCE); b) non-capacitative OAG-induced receptor-operated calcium entry (ROCE); and c) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced Ca^{2+}-oscillations in NRK fibroblasts. Intracellular calcium measurements revealed that knockdown of the genes encoding Trpc1, Orai1 and Stim1 each caused a significant reduction of SOCE in NRK cells, whereas knockdown of the gene encoding Trpc6 reduced only the OAG-induced ROCE. Furthermore, our data show that knockdown of the genes encoding Trpc1, Orai1 and Stim1, but not Trpc6, substantially reduced the frequency (up to 60%) of $PGF_{2\alpha}$-induced Ca^{2+} oscillations in NRK cells. These results indicate that in NRK cells distinct calcium channels control the processes of SOCE, ROCE and $PGF_{2\alpha}$-induced Ca^{2+} oscillations.

Key words: NRK; $PGF_{2\alpha}$; Trpc; SOCE; ROCE; Stim1; Orai1; Ca^{2+} oscillation.

1. Introduction

In many cell types activation of phospholipase C (PLC)-coupled receptors does not only result in an IP_3-mediated increase in intracellular calcium concentration, but also in enhanced calcium entry across the plasma membrane. In general, two distinct mechanisms for calcium uptake can be discriminated; one is referred to as receptor-operated calcium entry (ROCE), which involves non-selective calcium channels that are activated by phosphatidylinositol 4,5-bisphosphate (PIP_2) metabolites. The other mechanism is referred to as store-operated calcium entry (SOCE) and involves calcium channels that are activated by IP_3-induced depletion of intracellular calcium stores [1-4]. Despite intensive studies the calcium channels specifically involved in ROCE and SOCE have not been identified yet in a conclusive manner.

During the last decade, the mammalian homologues of *Drosophila* canonical Transient Receptor Potential Channels (TRPCs) have been proposed to be involved in both SOCE and ROCE [5]. There are seven related members of the TRPC family, designated in humans as TRPC1-7. Based on biochemical and functional similarities, the TRPC family of ion channels can be divided into a number of subfamilies, one consisting of TRPC1, TRPC4 and TRPC5, and another one consisting of TRPC3, TRPC6 and TRPC7[6]. Based on overexpression and knockdown studies, as well as on pharmacological approaches, it has been proposed that TRPC1 is particularly involved in SOCE and becomes activated upon depletion of intracellular calcium stores. Moreover, there is general agreement that members of the TPRC3/TRPC6/TRPC7 subfamily are involved in ROCE, since they can be activated by the diacylglycerol analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) [7-9].

The recent identification of two new protein families has helped greatly to better understand the nature and regulation of ion channels involved in SOCE. It has been shown that STIM1 (Stromal Interaction Molecule 1) acts as a Ca^{2+} sensor inside the cell, which links the filling state of the intracellular Ca^{2+} stores to the regulation of plasma membrane Ca^{2+} channels [10-13]. Knockdown of STIM1 gene expression by siRNA reduced SOCE in HEK293, SH-SY5Y, Jurkat T, and HeLa cells significantly, indicating that STIM1 plays an essential role in the regulation of this process. In contrast, over-expression of the STIM1 gene only modestly enhanced SOCE in HEK293 cells, indicating that physiological levels of STIM1 are already optimal for SOCE control [14].

Secondly, a new calcium release-activated calcium channel (CRACM1 or ORAI1) has been identified, which acts as a STIM-1 activated calcium channel in lymphocytes [15]. Experimental evidence indicates that knockdown of the ORAI1 gene decreases SOCE.[16, 17]. A mutation in the ORAI1 gene, resulting in an R91W substitution, has been shown to be responsible for a familial form of Severe Combined Immunodeficiency (SCID) in humans [18]. Interestingly, human cells transfected with the R91W-ORAI1 mutant gene did not only show a reduction in SOCE, but also in ROCE [19]. Furthermore, there is increasing evidence that STIM1 and ORAI1 can form dynamic complexes with both TRPC1 and TRPC6 [20, 21]. Several recent studies have revealed that STIM1 regulates the opening of ORAI and TRPC channels. Whereas STIM1 is obligatory for the functioning of ORAI channels [22, 23], TRPC channels appear to be able to function both as STIM1-dependent and STIM1-independent channels. Structural studies have indicated that binding of the so-called SOAR domain of STIM1 is sufficient to mediate activation of ORAI1, but that in the case of TRPC channels this requires the additional binding of the STIM1 polylysine domain [24]. However, the exact mechanism by which STIM1 and ORAI1 regulate SOCE and ROCE, both in the presence and absence of TRPC proteins, is still largely unknown.

Normal rat kidney fibroblasts (NRK) represent an excellent in vitro model system for studying the control mechanisms of cellular growth and phenotypic alterations upon cellular transformation [25]. Normal rat kidney (NRK) fibroblasts have intracellular calcium dynamics that strongly depend on their growth stage NRK cells can be grown to confluence in monolayer culture and made quiescent by serum deprivation. The exposure of these quiescent cells to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) causes individual cells in the monolayer to exhibit calcium oscillations at variable frequency, in spite of their metabolic and electrical, gap junction-mediated coupling When grown to density-arrest following incubation with EGF and insulin, these fibroblasts start to exhibit spontaneous firing of repetitive calcium action potentials, which are associated with near-synchronous Ca^{2+} transients [26, 27]. We have previously shown that $PGF_{2\alpha}$ plays an important role in both processes, since knockdown of the FP receptor gene *(Ptgfr)*, which is specifically activated by $PGF_{2\alpha}$, completely abolishes both the Ca^{2+} transients and the action potentials [28].

Previous studies have shown that in NRK cells SOCE is essential for maintaining intracellular calcium homeostasis, including membrane excitability and the refilling of calcium stores [29]. We have recently presented pharmacological evidence that in NRK fibroblasts SOCE and ROCE are differentially regulated in quiescent and density-arrested cultures [30]. The aim of the present study was to investigate the role of TRPCs, STIM1 and ORAI1 in these calcium entry mechanisms and in $PGF_{2\alpha}$-induced calcium oscillation in NRK cells, by using a knockdown approach against their individual genes.

Abbreviations:

SOCE, store-operated calcium entry; ROCE, receptor-operated calcium entry; TRPC, Transient Receptor Potential Channels; STIM1, Stromal Interaction Molecule 1; NRK, normal rat kidney; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha;}$ OAG, 1-oleoyl-2-acetyl-sn-glycerol; BHQ, 2,5-di-t-butyl-1,4-benzohydroquinone; ER, endoplasmic reticulum; $IP_{3,}$ inositol 1,4,5-trisphosphate; Q-cells, quiescent NRK cells; DA-cells, density-arrested NRK cells.

2. *Materials &Methods*

2.1 Cell culturing

Normal rat kidney fibroblasts (NRK clone 49F, American Type Culture Collection, Manassas, VA) were cultured in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% newborn calf serum (NCS; HyClone Laboratories, Logan, UT). Confluent cultures were made quiescent by subsequent incubation for 2-3 days in serum-free DF medium (1:1 mixture of DMEM-Ham's F-12 medium; Invitrogen) supplemented with 30 nM Na_2SeO_3 and 10 μg/ml human transferrin. Density-arrested monolayers were obtained by a subsequent 2-days incubation in the presence of 5 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA) in combination with 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO).

2.2 Total RNA extraction and reverse transcription Polymerase Chain Reaction analysis

Total RNA was isolated and purified by applying Trizol reagent (Invitrogen) to NRK monolayer cells according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis, 2 μg of total RNA were reverse transcribed from random hexamer primers using the SUPER SCRIPT II Ribonuclease H⁻ reverse transcriptase kit (Invitrogen). Reverse transcriptase PCR was performed in order to determine mRNA levels of *Stim1* and *Orai1* in NRK cells. Specific primers were designed using Oligo Perfect designed tool (Invitrogen). PCR amplification was performed on a PERKIN ELMER Gene Amp PCR System 2400 (Norwalk, CT, USA) using 2 μl of first-stranded cDNA reaction, 150 pmol of each degenerate primer, 50 μM dNTPs, 2 U Bio Therm Taq polymerase and 2.5 mM $MgCl_2$ in total volume of 50 μl. Following a hot start (3 min at 94 ^{0}C), the sample was subjected to 30 cycles of 30 sec at 94 ^{0}C, followed by 30 sec at 60 ^{0}C, 30 sec at 72 ^{0}C, and a final extension for 7 min at 72 ^{0}C. Reaction products were separated by standard agarose gel electrophoresis and cloned into the pCR-2.1 TOPO vector (Invitrogen) using the protocol supplied by the manufacturer. The identity of the inserts was subsequently confirmed by ABI PRISM sequencing. Table 1 shows the designed primers used for *Stim1* and *Orai1* (rat sequences) with their amplified product size, whereby the identity of the products was confirmed by direct sequencing.

2.3 Real-Time quantitative PCR analysis

For real-time quantitative RT-PCR analysis, the ABI Prism Sequence Detection System 5700 and Primer Expressk software (Applied Biosystems, Carlsbad, CA) were used. For each gene, a set of primers was designed using sequences obtained from Genbank (Table 2). Prior to complementary DNA (cDNA) synthesis, 2 μg of total RNA were treated with DNase for 15 min at 37 ^{0}C, after which RNA was reverse transcribed from random hexamer primers using the SUPERSCRIPTk II reverse transcriptase kit (Invitrogen). Subsequently, 0.2 μg of total cDNA was amplified using SYBR Green PCR Mastermix (Applied Biosystems) under the following conditions: initial denaturation for 10 min at 95 ^{0}C, followed by 40 cycles consisting of 15 sec at 94 ^{0}C and 1 min at 60 ^{0}C. Expression values were calculated from threshold cycles at which an increase in reporter fluorescence above baseline signal was first detected (Ct).

2.4 shRNA constructs, virus production and cell infections

In order to knockdown the expression of rat *Trpc1, Trpc6, Stim1 and Orai1*, at least two different sets of siRNAs sequences were chosen by entering the nucleotide sequence of each gene into the web-based design tool from Dharmacon (http://design.dharmacon.com). Verification of these siRNA sequences for their specificity by BLAST database search did not show significant homology to any other known gene sequence in the human, mouse and rat genome. Specific and control short hairpin (shRNA) template oligonucleoties were designed by entering the siRNA target sequences into the siRNA Wizard web-based design tool (http://www.sirnawizard.com/construct.php) (Table 3). The obtained complementary oligonucleotides were synthesized by Sigma-Aldrich, annealed, and ligated into the linearized pSUPER.retro puro vector (Oligo Engine, Seattle, WA) according to pSUPER RNAi system protocol.

The resulting constructs, shRNA-Trpc1, shRNA-Trpc6, shRNA-Stim1, shRNA-Orai1 and control shRNA, were transfected into the Phoenix packaging cell line (Nolan Lab, Stanford, CA) in order to produce ecotropic retroviral supernatants. A negative control vector, expressing a hairpin shRNA with limited homology to any known sequences in rat genome, was used as a control. Phoenix cells were seeded in

tissue culture dishes in DMEM supplemented with 10% NCS and pre-treated with chloroquine at a final concentration of 25 μM. One day before transfection, Phoenix cells were seeded in culture dishes at a density of $4.0x10^4$ cells/cm^2 in order to reach 60% confluence at the time of transfection. Cells were then transfected with 20 μg of viral vector DNA using the calcium–phosphate precipitation method [31, 32]. After 48 hours of transfection, the culture medium was filtered through a 0.45 μm filter and the viral supernatant was used for infection of NRK cells pre-treated with 4 μg/ml of polybrene (Sigma-Aldrich). After infection, NRK cells were incubated for 24 hours at 37 ^{0}C. Subsequently, the medium was replaced by fresh virus-free medium and NRK cells were allowed to recover for 48 hours at 37 ^{0}C. Infected cells were selected by culturing them in the presence of puromycin (6 μg/ml) for 5 days. Target gene expression in NRK wild-type cells, empty vector cells and shRNA producing cells was analyzed by real-time quantitative RT-PCR and Western blot analysis, as described in detail below.

2.5 Western blot analysis

NRK cells were plated in dishes at a density of $1.9x10^4$ cells/cm^2 under the conditions described above. Cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitor mixture), supplemented with 50 mM NaF, 1 mM Na$_3$VO$_4$, and 10 mM β-glycerol phosphate. Cell lysates were incubated for 1 hour on ice and centrifuged at 12,000xg to collect supernatants. Protein concentration in the supernatants was measured by the Bradford method [33, 34]. After addition of sample buffer and boiling of this suspension, 75 μg of the denatured proteins were separated on 10% SDS–PAGE gels and subsequently transferred to nitrocellulose papers. After a 1-hour blocking period, nitrocellulose papers were incubated with specific antibodies. The primary antibodies used were: monoclonal anti-Stim1 and polyclonal anti-Trpc1, both form Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-Orai1 antibodies from BioCat GmbH (Heidelberg, Germany) and polyclonal anti-Trpc6 and anti-Actin antibodies from Sigma-Aldrich. HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Immunolabelling was visualized using the ECL procedure (Amersham Biosciences, Uppsala, Sweden). Bands were quantified by densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden).

2.6 Intracellular Ca^{2+} measurements

Glass coverslips grown with quiescent monolayers of NRK fibroblasts were placed in a Leiden cell chamber and loaded for 30 min at room temperature with 4 µM Fura-2/AM (Invitrogen) in serum-free DF medium. Medium was replaced by Ca^{2+}-free HEPES-buffered saline (Ca^{2+}-free HBS, containing 143 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES-KOH, pH 7.4). Ca^{2+}-containing HEPES-buffered saline (Ca^{2+}-containing HBS, containing 128 mM NaCl, 10 mM $CaCl_2$) was added to an equal amount of Ca^{2+}-free medium to obtain a 5 mM Ca^{2+}-containing medium in the Leiden chamber. Dynamic calcium video imaging was performed as described [34]. Excitation wavelengths of 340 nm and 380 nm (bandwidth 8-15 nm) were provided by a 150 W Xenon lamp (Ushio UXL S150 MO, Ushio, Tokio, Japan), while fluorescence emission was monitored above 440 nm, using a 440 nm DCLP dichroic mirror and a 510 nm emission filter (40 nm bandwidth) in front of the camera. Image acquisition, using a camera pixel binning of 4 and computation of ratio images (F340/F380), was every 4 sec and operated through Metafluor software v.6.2 (Universal Imaging Corporation, Downingtown, PA, US). Camera acquisition time was 100 msec per excitation wavelength.

2.7 Data analysis

Each experiment was performed at least 5 times; per experiment 50 to 60 cells were recorded. The traces of these cells were averaged per experiment and further analyzed. Mean values of cytosolic calcium increase via release and influx was determined by dividing the mean value of 5 data points before increase (R0 and R2 for respectively Ca^{2+}-release and –influx) by the mean value at maximum ratio levels after release and influx (R1 and R3 respectively). Student's *t*-test was used for statistical comparisons. Numeric data are represented as mean ± SEM throughout this article, whereby *n* represents the number of replicates of each experiment. Frequency of calcium oscillations was determined by counting the peaks using 'pick peak' algorithm of Origin 6.0 (Microcal, Northampton, MA), using a minimal peak height of 10% of the total signal as selection criteria.

3. Results

3.1 Effect of knockdown of Trpc1 and Trpc6 on SOCE in NRK cells

We recently reported on the differential role for SOCE and ROCE in the calcium dynamics of quiescent and density-arrested NRK fibroblasts. Furthermore, we have shown that NRK fibroblasts express Trpc1 and Trpc6, and that expression of these proteins depends on the growth stage of the NRK fibroblasts [30]. In the present study we have investigated the role of these various calcium channel proteins in mediating Ca^{2+} entry during the different NRK growth stages. For this we employed an shRNA approach to generate stable NRK cell lines in which the expression of specific *Trpc* genes was targeted and the resulting mRNA and protein levels were subsequently monitored by quantitative RT-PCR and Western blotting. Fig.1A shows that in NRK cells transfected with shRNA against *Trpc1* gene (dTRPC1 cells) the mRNA level of *Trpc1* was reduced to 37.6 ± 5.8 % (n=4), when compared to control cells that were transfected with control shRNA, while no effect on *Trpc1* expression was observed in cells transfected with shRNA against *Trpc6* (dTRPC6 cells). On the other hand, *Trpc6* expression was reduced to 39.8 ± 2.6 % (n=4) in dTRPC6 cells, while no reduction was observed in dTRPC1 cells (Fig. 1B). Western blot analysis showed a significant reduction in expression of Trpc1 protein in dTRPC1 cells, without affecting the protein level of Trpc6 (Fig.1C,D). Similarly, the protein level of Trpc6, but not of Trpc1, was reduced in dTRPC6 cells (Fig. 1E,F), when compared to the expression level of smooth muscle α-actin. These results indicate that expression of Trpc channels can be selectively repressed by an shRNA-mediated knockdown approach.

3.2 Effect of knockdown of Trpc1 and Trpc6 on store-operated Ca^{2+} entry (SOCE)

In order to study the role of specific Trpc channels on SOCE in quiescent NRK cells, single-cell Ca^{2+} imaging experiments were performed, in which BHQ-treated cells were changed from a nominal calcium-free medium to a medium containing 5 mM Ca^{2+}, and the resulting increase in Fura-2 fluorescence was quantified. Fig. 2A shows the obtained traces for control cells, dTRPC1 cells and dTRPC6 cells. Upon averaging (Fig. 2B) our data show that in quiescent NRK cells the increase in calcium uptake was significantly reduced in dTRPC1 cells (to 44.7 ± 9.2%; n=5) when compared to control cells, while no significant effect was observed in dTRPC6 cells. Also in density-arrested cells, in which expression of Trpc6 is upregulated six-fold [30], we observed no difference in SOCE between BHQ-treated control and dTRPC6

cells, which was assayed in the presence of nifedipine to prevent spontaneous calcium action potentials (Fig. 2C,D). This observation argues against the possibility that expression levels of Trpc6 are too low to contribute significantly to SOCE. On the other hand, similarly as in quiescent cells the uptake of calcium in density-arrested dTRPC1 cells was significantly reduced (to 38.3 $\pm$ 2.2%; n=4) when compared to control cells. These results are againindicative for the involvement of Trpc1, but not Trpc6, in SOCE of NRK cells.

3.3 Effect of knockdown of Trpc1 and Trpc6 on receptor-operated Ca^{2+} entry (ROCE)

We have recently shown that both quiescent and density-arrested NRK cells exhibit SOCE, while only density-arrested cells exhibit ROCE. In order to determine the role of Trpc1 and Trpc6 in ROCE of density-arrested NRK cells, the increase in intracellular calcium was measured following addition of OAG, a membrane permeable DAG analogue, to nifedipine-treated dTRPC1, dTRPC6 and control cells. Fig. 3A (traces) and 3B (statistics) show that in dTRPC6 cells OAG-induced calcium entry was significantly reduced (to 54.1% $\pm$ 8.9%; N=4) when compared to control cells, while no reduction was observed in dTRPC1 cells. These result show that Trpc6, but not Trpc1, is involved in ROCE in density-arrested NRK cells, which is in line with the observation that *Trpc6* expression is upregulated six-fold when quiescent NRK cells are grown to density-arrest.

3.4 Knockdown of Stim1 and Orai1in NRK fibroblasts

In order to determine the potential involvement of Stim1 and Orai1 in Ca^{2+} entry in NRK cells, we stably transfected these cells with an shRNA-expressing vector to target either of these genes. Figure 4A shows that in cells expressing *Stim1* shRNA (dSTIM1 cells) the *Stim1* mRNA level was reduced to 41.3 $\pm$ 5.3% (n=3) compared to control transfected cells, while Fig. 4B shows that in cells expressing *Orai1* shRNA (dORAI1 cells) the *Orai1* mRNA level was reduced to 49.2 $\pm$ 2.4% (n=3) compared to control transfected cells. Western blot analysis confirmed that a reduction in mRNA level resulted in a specific reduction of Stim1 (Fig. 4C,D) and Orai1 (Fig. 4E,F) protein levels in dSTIM1 and dORAI1 cells, respectively.

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3.5 Effect of knockdown of Stim1 and Orai1 on SOCE in NRK cells

Figures 5A,B show that addition of 5 mM Ca^{2+} to BHQ-treated dSTIM1 and dORAI1 cells in the quiescent state resulted in a significantly reduced Ca^{2+} uptake, when compared to control NRK cells. When averaging over 225 cells in 5 independent experiments, a reduction in calcium influx to $65.1 \pm 6.4\%$ was observed for dSTIM1 cells, and to $56.9 \pm 4.1\%$ for dORAI1 cells. These results show that both Orai1 and Stim1 are directly involved in store-operated Ca^{2+} entry in NRK cells.

3.6 Effect of knockdown of Stim1, Orai1, Trpc1 and Trpc6 on $PGF_{2\alpha}$ -induced Ca^{2+} oscillations

We have previously shown that addition of $PGF_{2\alpha}$ to quiescent NRK cells induces calcium oscillations in more than 90% of the cells in the monolayer, with a frequency that is highly variable between individual cells. The initial calcium peak results from intracellular calcium release from IP_3-sensitive stores, whereas the subsequent calcium transients are mediated by an interplay between IP_3-sensitive calcium stores and an influx of extracellular calcium [26]. Since constitutive Ca^{2+} entry is needed for sustained Ca^{2+} oscillations and our above data show that Trpc1, Orai1 and Stim1 are all three involved in capacitative Ca^{2+} entry of NRK cells, we hypothesized that these proteins may play an important role in the maintenance of Ca^{2+} oscillations induced by $PGF_{2\alpha}$.

To investigate this hypothesis, we examined the effects of knockdown of *Stim1*, *Orai1*, *Trpc1* and Trpc6 on the Ca^{2+} oscillations induced by 100 nM $PGF_{2\alpha}$ in quiescent NRK monolayers. Figure 6A shows a typical example of $PGF_{2\alpha}$-induced Ca^{2+} oscillations in NRK control monolayers, whereby the oscillations in individual cells differ in their frequency, but show a sustained character as long as 1 mM Ca^{2+} is present in the extracellular medium. In dSTIM1, dORAI1 and dTRPC1 cells (Fig. 6B, 6C and 6D, respectively) the frequency of these $PGF_{2\alpha}$-induced oscillations was significantly reduced, whereas no reduction was observed in dTRPC6 cells (Fig. 6E).

Because of the high variability of these oscillations, we analyzed 253 individual cells in 5 independent monolayer cultures. The $PGF_{2\alpha}$-induced calcium responses were divided into three categories, characterized as either: (i) no secondary transients after an initial calcium transient, (ii) less than five secondary transients after an initial calcium transient within a time frame of 15 min, (iii) five or more secondary calcium transients within a time frame of 15 min (Fig. 6F). The data show that in

control populations the majority of cells ($89.0 \pm 9.2\%$) have a dominant response of 5 or more secondary transients within 15 min after stimulation with PGF$_{2\alpha}$. In contrast, in dSTIM1, dORAI1 and dTRPC1 cultures the cells predominantly showed less than 5 transients within 15 min: 82.5 % of the dSTIM1 cells, 75% of the dORAI1 cells and 55% of the dTRPC1 cells. On the other hand, no significant difference in oscillation frequency was observed between dTRPC6 and control cells, which both showed more than 90% of the cells with more than 5 transients within 15 min. The observation that knockdown of either *Stim1*, *Orai1* or *Trpc1* results in a reduced frequency of PGF$_{2\alpha}$-induced Ca^{2+} oscillations supports our previous conclusion that constitutive calcium entry across the plasma membrane is required for maintaining calcium oscillations [29]. Moreover, these data underline that Stim1, Orai1 and Trpc1 are all three involved in store-operated calcium entry in NRK cells.

*4. **Discussion***

The aim of the present study was to investigate the molecular components that play a role in the influx of extracellular Ca^{2+} in NRK fibroblasts. Here we show that NRK cells express various members of the Trpc family of calcium transporters, as well as Stim1 and Orai1, which have both been involved in capacitive calcium entry. Using an shRNA approach against these various transporters, we have shown that Trpc1, Stim1 and Orai1 are particularly involved in store-operated calcium entry, while Trpc6 is mainly involved in receptor-operated calcium entry in these cells. Our data furthermore indicated that knockdown of either *Trpc1*, *Stim1* or *Orai1* results in a reduced frequency of PGF$_{2\alpha}$-induced Ca^{2+} oscillations in NRK cells, indicating that uptake of extracellular calcium is important for continuation of these oscillations. In combination, our data indicate that Trpc1, Stim1 and Orai1 interplay in regulating calcium uptake in NRK cells under conditions that intracellular calcium stores have become depleted.

Initially, we tested at least three different shRNA constructs for each gene to downregulate its expression, and chose the one which induced the highest reduction in mRNA level, as measured by quantitative PCR, and protein level, as measured by Western blotting. In none of the cases did stable transfection with an shRNA against one of these genes affect expression of any of the other transport genes. This was also true for the various *Trpc* genes, in spite of their sequence homology (see Fig. 1).

Our previous studies have indicated that density-arrested NRK cells produce and secrete low amounts of PGF$_{2\alpha}$ [27]. This prostaglandin is able to activate a Gq-protein-coupled FP receptor, which results in PLC-dependent hydrolysis of PIP$_2$ into IP$_3$ and DAG. As a result density-arrested NRK cells can show an increased level of intracellular DAG, which is sufficient to activate ROCE. Our observation that density-arrested cells but not quiescent NRK cells exhibit ROCE, may be related to the six-fold increase in *Trpc6* expression which is observed when quiescent cells are grown to density-arrest [30]. Our observation that Trpc6 is involved in ROCE is in agreement with the general hypothesis that members of the TRPC3/TRPC6/TRPC7 subfamily form receptor-operated and not store-operated calcium channels [8, 9]. However, several other studies have recently reported that TRPC6 may also be involved in SOCE [35, 36]. The other OAG-inducible channels, Trpc3 and Trpc7, are not detectable in NRK cells, but we have shown that in addition to Trpc1 and Trpc6, these cells also express Trpc5 [30]. We were able to generate knockdown of *Trpc5* by

a specific shRNA that resulted in 85% reduction of the *Trpc5* transcript (data not shown). However, we did not observe any effect on either the BHQ-induced calcium entry (SOCE) or on the OAG-induced calcium entry (ROCE) in NRK cells. The latter result may be explained by the observation that TRPC5 may become desensitized by PKC upon treatment of cells with the DAG analogue OAG [37]. Moreover, *TRPC5* overexpression studies in HEK293 and DT40 cells have shown that this channel is not activated upon depletion of intracellular calcium stores following addition of thapsigargin [9].

We have previously shown that the *Trpc6* mRNA level increases six-fold upon growing NRK cells from a quiescent to the density-arrested state, while that of *Trpc1*does not change significantly [30]. Still, the present knockdown studies show that Trpc1, and not Trpc6 controls the frequency of calcium oscillations in both quiescent and density-arrested cells. Although mRNA levels determined by quantitative PCR cannot readily be compared for different genes, it appears that mRNA level for *Trpc1* is higher than that of *Trpc6*, under all growth conditions tested. Therefore, it can be safely assumed that either the contribution of the Trpc6 channels is additive to Trpc1 or that Trpc1 exerts the major contribution to the Ca^{2+} entry and consequently to the modulation of the frequency of the Ca^{2+} oscillations.

The present observation that a reduction of Trpc6 levels by shRNA has no effect on the $PGF_{2\alpha}$-induced Ca^{2+} oscillations in quiescent NRK cells, contrasts a previous report [38] in which it has been shown that siRNA-mediated knockdown of *Trpc6* in rat A7r5 vascular smooth muscle cells results in a strong suppression of vasopressin-induced Ca^{2+} oscillations. Again, this discrepancy might be explained by the fact that in NRK cells Trpc1 provides a major route for Ca^{2+} entry in the presence of the $PGF_{2\alpha}$. On the other hand, recent studies have shown that in native vascular myocytes stimulation by angiotensin II resulted in inhibition of Trpc6 channels by Trpc1/c5 channel activity through a Ca^{2+}- and PKC-dependent mechanism [39]. In conclusion, these findings indicate that the function of specific Trpc channels may be strongly cell-type dependent.

Finally, our results show that individual knockdown of *Trpc1*, *Orai1* or *Stim1* reduced SOCE in NRK cells to a similar extent. This may indicate that a complex of these three proteins mediates SOCE. Although TRPC6 and ORAI1 can clearly function independently of each other and have different channel properties when

activated by STIM1 [40], abundant evidence supports the formation of heteromeric complexes of these proteins. First, overexpression of *ORAI1* into TRPC1 expressing cells induced enhanced SOCE, which suggests a functional association between ORAI1 and TRPC channels [20]. Biochemically it has been shown that ORAI1 interacts with both the C- and N-terminal region of TRPC channels [41]. Furthermore, it has been shown in human salivary gland cells that STIM1 assembles with the ORAI1/TRPC1 complex and that all three proteins are essential for generation of SOCE in these cells [21]. This important finding could indicate that in NRK cells Trpc6-mediated ROCE functions independently of Stim1 activity, while SOCE may require the assembly of a Trpc1-Stim1-Orai1 ternary complex. Verification of these hypotheses will require further research, as well as of the mechanism whereby DAG and IP$_3$ can activate the two modes of calcium entry upon FP receptor activation.

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Legends to figures

Fig.1. Assessment of *Trpc1* and *Trpc6* expression in NRK cells stably expressing shRNA targeting specific Trpc genes. (A and B) NRK cells stably expressing shRNA constructs specific for *Trpc1* (dTRPC1 cells, dark grey), *Trpc6* (dTRPC6 cells, white) and control shRNA (control cells, black) were established. Quantitative PCR analysis of mRNA levels for *Trpc1* (A) and *Trpc6* (B) is shown for each of the three cell lines and expressed relative to control cells. Data represent mean and SEM of at least four independent experiments. Asterisk denotes a significant difference ($p < 0.05$) compared to control cells. (C and D) Western blot analysis of Trpc1 levels in whole cell lysates of three cell lines, as visualized by specific antibodies against rat Trpc1. A typical example is shown, as well as the statistics (mean $\pm$ SEM) for triplicate experiments. Asterisk denotes a significant difference ($p < 0.05$) compared to control cells. (E and F) Similar for Trpc6 using specific antibodies against Trpc6. Staining with antibodies against rat α-actin was used as a loading control.

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Fig. 2. Effect of knockdown of *Trpc1* and *Trpc6* on store-operated calcium entry in quiescent NRK cells.

BHQ-induced store-operated calcium entry in quiescent cells (A) and density-arrested cells (C) of control (black), dTRPC1 (dark gray) and dTRPC6 (light gray) cultures. Cells were loaded with Fura 2-AM for 30 min and treated with BHQ (50 µM) in the absence of Ca^{2+}. After depletion of the calcium stores (first phase of calcium increase), 5 mM of extracellular Ca^{2+} were added and ion uptake was measured as a function of time (second phase of calcium increase). The traces are averages of 45 cells from a single experiment. All recordings in density-arrested cells were performed in presence of nifedipine to prevent entry of calcium through L-type calcium channels. The gray band around the traces represents the SEM-error bars for every data point. (B and D) Data summarized for the amplitude of the calcium influx (second phase), expressed as mean $\pm$ SEM for at least 4 independent experiments. Asterisk denotes a significant difference ($p < 0.05$) compared to control cells.

Fig.3. Effect of knockdown of *Trpc1* and *Trpc6* on receptor-operated calcium entry in density-arrested NRK cells.

(A) Density-arrested control (black), dTRPC1 (dark gray) and dTRPC6 (light gray) cells were loaded with Fura-2AM and treated with OAG (100 μM) and nifedipine (1 μM), after which the amplitude of the influx of Ca^{2+} was measured. The traces are averages of 45 cells from a single experiment, as representative for four independent experiments. (B) Data summarized for the amplitude of OAG-induced Ca^{2+}-entry upon addition of 5 mM-extracellular Ca^{2+} (peak-entry). Data represent mean ± SEM for at least 3 independent experiments, whereby the asterisk denotes a significant difference ($p<0.05$) compared to control cells.

Fig. 4. Effect of knockdown of *Stim1* and *Orai1* on expression levels in NRK fibroblasts.

NRK cells were stably transfected with either shRNA against *Stim1* (dSTIM1 cells) or against *Orai1* (dORAI1 cells). (A) Quantitative PCR analysis of *Stim1* RNA levels in quiescent NRK control cells and dSTIM1 cells. (B) Quantitative PCR analysis of *Orai1* RNA levels in quiescent NRK control cells and dORAI1 cells. Single PCR products were identified in NRK cells for *Stim1* (A) and *Orai1* (B) of the expected size. Data are representative of three independent experiments, carried out in duplicate. Asterisk denotes a significant difference ($p<0.05$) compared to control cells. (C and D) Western blot analysis of Stim1 levels in control cells and dSTIM cells, using antibodies against rat Stim1. A typical example is shown , as well as statistics (mean ± SEM) for triplicate experiments. (E and F) Western blot analysis of Orai1 in control cells and dORAI1 cells, using antibodies against rat Orai1. A typical example is shown, as well as statistics (mean ± SEM) for triplicate experiments. Asterisk denotes a significant difference ($p<0.05$) compared to control cells. Antibodies against rat α-actin were used as a loading control.

Fig. 5. Effect of knockdown of *Stim1* and *Orai1* on store-operated Ca^{2+} entry in NRK fibroblasts.

(A) Comparison of 5 mM Ca^{2+}-induced calcium entry in BHQ-treated NRK control, dSTIM1 and dORAI1 cells. Each trace is the average of 45 cells from a single experiment, which is representative for five distinct experiments. (B) Data summarized for control cells, dSTIM1 and dORAI1 cells, whereby the peak values of

Ca^{2+} entry were averaged over all 5 experiments (mean $\pm$ SEM). Asterisk denotes a significant difference ($p<0.05$) compared to control cells.

Fig. 6. Effect of knockdown of *Stim1*, *Orai1*, *Trpc1 and Trpc6* on PGF$_{2\alpha}$- induced calcium oscillations in NRK fibroblasts.

Single cell dynamic calcium video imaging measurements were carried out on Fura-2 AM loaded quiescent NRK control cells (A), dSTIM1 cells (B), dORAI1 cells (C), dTRPC1 cells (D) and dTRPC6 cells (E), after stimulation with 100 nM PGF$_{2\alpha}$ in the presence of 1 mM of extracellular Ca^{2+}. (F) For each individual cell, the oscillation frequency was determined by the number of Ca^{2+} spikes during a 15 min interval after the initial peak induced by agonist treatment. Based on these data cells were divided into three categories: no oscillations; 1-5 oscillations; 6 or more oscillations. At least 40 to 50 traces were analyzed in each preparation of 5 independent experiments for each cell line and data are presented as the mean $\pm$ SEM.

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Table.1. Oligonucleotide sequences of primers used for Quantitative RT-PCR

Gene	Accession No.	Size bp	Primers	Location
rTrpc1	AF061266	105	For: 5-GGCAGAACAGCTTGAAGGAG-3 Rev:5-GTCGCATGGAGGTCAGGTAT-3	2057-2161
rTrpc6	NM_053559	114	For: 5-GCATCATCGATGCAAATGAC-3 Rev:5-TGATCTGAGGATCGGTAGGG-3	1664-1777
rOrai1	NM_001013982	117	For: 5-TCACTTCTACCGCTCACTGG-3 Rev:5-AGAGAATGGTCCCCTCTGTG-3	859-975
rStim1	XM_341896	100	For: 5-AGCTCCTGGTATGCTCCTGA-3 Rev:5-GCCTCTCTGCATTTTGCTTC-3	1583-1682
Rat 18s rRNA	XM_341896	109	For: 5-CGGCTACCACATCCAAGGAA-3 Rev:5-GCTGGAATTACCGCGGCT-3	462-571

Table.2. Target sequences for constructing shRNA specific for TRPC homologs

Gene	Accession No.	Hairpin oligonucleotide sequences (5'-3') target sequence printed in bold	Location
rTrpc1	AF061266	For:GATCCCC**GGGTGACTATTATATGGTT**TTCAAGAGAAACCATATAATAGTCACCCTTTTTA Rev:AGCTTAAAAAGGGTGACTATTATATGGTTTCTCTTGAAAACCATATAATAGTCACCCGGG	246-264
rTrpc6	NM_053559	For:GATCCCC**TCGAGGACCAGCATACATGTT**CAAGAGACATGTATGCTGGTCCTCGATTTTTA Rev:AGCTTAAAAATCGAGGACCAGCATACATGTCTCTTGAACATGTATGCTGGTCCTCGAGGG	670-678
rOrai1	NM_001013982	For:GATCCCC**GCAACGTCCACAACCTCAACT**TTCAAGAGAAGTTGAGGTTGTGGACGTTGCTTTTTA Rev:AGCTTAAAAAGCAACGTCCACAACCTCAACTTCTCTTGAAAGTTGAGGTTGTGGACGTTGCGGG3	543-563
rStim1	XM_341896	For:GATCCCC**GCATGGAAGGCATCAGAAGTGTATA**TTCAAGAGATATACACTTCTGATGCCTTCCATGCTTTTTA Rev:AGCTTAAAAAGCATGGAAGGCATCAGAAGTGTATATCTCTTGAATATACACTTCTGATGCCTTCCATGCGGG	635-655
control		For:GTACCCC**AATTCTCCGAACGTGTCAG**TTCAAGAGACTGACACGTTCGGAGAATTTTTTTA Rev:AGCTTAAAAAAATTCTCCGAACGTGTCAGTCTCTTGAACTGACACGTTCGGAGAATTGGG	

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