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TRPC6 silencing in primary airway smooth muscle cells inhibits protein expression without affecting OAG-induced calcium entry

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Abstract

TRPC proteins have been described as non-selective cation channels and are thought to be involved in the regulation of Ca^{2+} movement in various cells, including airway smooth muscle (ASM) cells. In order to study the role of these channels in ASM cells, transfection of a small interfering RNA (siRNA) designed against the TRPC6 channel was performed in guinea pig primary ASM cells. This specific siRNA was complexed with the new X-TremeGene (X-TG) chemical transfection reagent, whose efficiency and low cytotoxicity were determined by the use of a non-silencing rhodamine-tagged siRNA. It was found that more than 95% of cells were transfected by an optimized protocol. Verification of TRPC6 transcript down-regulation was determined by RT-PCR while Western blot analysis attested to lower protein content in the microsomal fraction. Micro-spectrofluorimetry measurements of control and siRNA-treated cells revealed that lower TRPC6 expression did not affect OAG-induced intracellular Ca^{2+} movement. Thus, TRPC6 channels cannot be defined as simple Ca^{2+} transporters but more likely as protein complexes supporting monovalent cation conductance in ASM cells. These conductances would in turn facilitate membrane depolarization of high input resistance cells, Ca^{2+} channel activation and tone increase. In conclusion, this study defines a valuable model of RNA interference study in primary cultures of ASM cells, eventually allowing for silencing of other target proteins for which no pharmacological modulators are currently available. (Mol Cell Biochem **296:** 193–201, 2007)

Key words: airway smooth muscle, calcium regulation, cell culture, gene expression, siRNA, TRPC

Introduction

In smooth muscles (SM), intracellular calcium concentration controls cell contraction through formation of Ca^{2+}-calmodulin complexes and phosphorylation of 20 kDa myosin light chain (MLC20) by myosin light chain kinase (10S kDa MLCK) [1]. The regulation of intracellular [Ca^{2+}] is achieved through two main pathways, most notably by Ca^{2+} release from internal stores and Ca^{2+} entry through channels located on the surface membrane [2]. In airway smooth muscles (ASM), the activation of G-protein coupled receptors (GPCR) activates the PLC pathway, which in turn hydrolyzes PIP_2 to produce DAG and inositol triphosphate ($InsP_3$) [3]. $InsP_3$ binds and activates its receptor, a ligand-gated Ca^{2+} release channel located on the ER membrane, while DAG and its analogs, arachidonic acid (AA) metabolites, likely

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activate TRPC (Transient Receptor Potential, Canonical) channels, as reported previously [4].

TRPC channels have often been considered to be involved in two complementary processes: receptor operated channels (ROC) and store operated channels (SOC), which serve in regulating [Ca^{2+}] [5]. This sub-family of the TRP channels has been linked to both of these pathways, [6–8], although their precise role and regulation remain a mater of debate [9]. It is known that guinea pig ASM tissues express many isoforms of the TRPC sub-family [10], and previous studies have reported that exogenous OAG and 20-HETE are able to activate non-selective cationic currents in cell cultures transfected with rat TRPC6 channels [11]. A complementary report by our group, through indirect pharmacological and electrophysiological experimentation, provided evidence for the activation of a non-selective cationic current by 20-HETE in guinea pig ASM cells, undoubtedly linked to TRPC and most likely to TRPC6 isoforms [12].

While recent publications [13, 14] have demonstrated the putative manner in which TRPC6 are involved in intracellular [Ca^{2+}] regulation, the majority of the described studies were performed using cell lines, which may not be representative of primary ASM cell responses. This is an important issue, since investigation of TRPC channels has been hampered by many contradictory findings [15], hence justifying the use of primary cultures of ASM cells, as well as fresh and organ tissue cultures [16] to study the role and regulation of TRPC channels. Other difficulties in studying TRPC channels reside in the absence of specific pharmacological inhibitors and the ability for these channels to form homo- and hetero-tetramers with other TRPC isoforms [17]. This is the major reason why an efficient strategy for the study of TRPC channels with primary ASM cells requires a rapid and efficient down-regulation of this protein.

Several putative biochemical activators have been described for members of the TRP sub-family, including DAG, 1-oleoyl-2-acetyl-sn-glycerol (OAG) for TRPC 3, 6, and 7, and capsaicine for Transient Receptor Potential of the vanilloid receptor (TRPV1) [18]. However, since there are no known specific pharmacological inhibitors against TRPC channels, the ability to quickly activate or efficiently silence a specific TRPC isoform has become particularly relevant. Such TRPC silencing has recently been achieved by the use of specific antisense [19] and small interfering RNA (siRNA) in vascular cells lines [13] and in organ tissue culture [20]. In cultured ASM cells, siRNA technology is rapidly becoming a very useful tool for elucidating the role of many gene products [21], including TRPC channels [13].

The aim of this study was hence to prevent the translation of a specific TRPC6 channel protein over a relatively short period of time (a few days). Immuno-cytofluorimetry, RT-PCR as well as Western Blot analysis were used to demonstrate TRPC6 protein down-regulation while intracellular calcium measurements were performed to determine the precise role, if any, of TRPC6 channels in the regulation of calcium entry in primary cultures of ASM cells.

Material and methods

ASM cell culture

Male or female albino guinea pigs (weighting 350–450 g; Hartley) were anesthetized with a lethal dose of pentobarbital sodium (50 mg/kg ip) and sacrificed by abdominal exsanguination. All procedures involving animal tissues were performed according to current Canadian Council for Animal Care (CCAC) guidelines. The trachea was excised aseptically and placed immediately on ice in sterile Hank's Buffered Saline Solution (HBSS): 138 mM NaCl, 5 mM KCl, 1 mM Glucose, 4 mM $NaHCO_3$, and 0.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4. Under sterile conditions, the trachea was dissected free of excess tissue and cut longitudinally on the opposite side of the SM. The epithelial cells were removed mechanically with a sterile cotton swab. The SM tissue was minced and incubated 45 min in HBSS solution containing 2-mg/ml collagenase (type IV) and 0.5-mg/ml elastase (type IV). After filtration with a 100-μm filter into 16 ml of HBSS solution, the suspension was centrifuged for 6 min at 500 g, after which the pellet was resuspended in 1 ml of DMEM/F12 solution. The resulting cell suspension was aliquoted onto coverslips placed in Petri dishes or directly into 25 cm^2 plastic flasks, containing 5 ml of DMEM/F12 solution supplemented with 1% penicillin/streptomycin and 10% FBS. Isolated ASM cells were allowed to recover for at least 24 h prior to experimentation, and cultures were tested positive with an anti-SM α-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Primers and siRNA design

Specific sense and antisense primers were designed from guinea pig mRNA TRPC6 sequences, with construction of primers against the guinea pig GAPDH mRNA transcript serving as internal controls (see Table 1). All primers were complementary to their guinea pig target. To assess for potential genomic DNA contamination, the sense and antisense strand targets were located on different exons of the gene they targeted. To down-regulate the expression of TRPC6 mRNA, a siRNA was designed with a complementary sequence to guinea pig TRPC6 mRNA. This siRNA was also complementary to the human (ensembl ENST00000344327), rat (ensembl ENSRNOT00000008905), and mouse (ensembl ENSMUST00000050433) sequences, making it a potential tool for directly regulating the expression of TRPC6 in other species.

Table 1. RT-PCR primers and siRNA sequences used

Guinea Pig TRPC6 sequence Genbank accession number		AB090950
A		
Guinea Pig TRPC6 primers:	Sense	GTCCGGCTTACCTCTCACTG
	Antisense	ATGGCTACACCAAGGACCAC
Guinea Pig GAPDH sequence Genbank accession number		AB060340
Guinea Pig GAPDH primers:	Sense	ACCCAGAAGACTGTGGATGG
	Antisense	TGCTGTAGCCGAACTCATTG
B		
Guinea Pig TRPC6 siRNA	Sense	UCGAGGACCAGCAUACAUGdTdT
Target: AATCGAGGACCAGCATACATG	Antisense	CAUGUAUGCUGGUCCUCGAdTdT
Rhodamine-tagged non-silencing siRNA	Sense	UUCUCCGAACGUGUCACGUdTdT
Modification: sense, 3′-Rhodamine	Antisense	ACGUGACACGUUCGGAGAAdTdT

As a control, a non-silencing siRNA tagged with rhodamine at the 3′ end was used for micro-spectrofluorimeter experiments in order to determine transfection efficiency. All primers and siRNA were obtained from Invitrogen Inc. (Carlsbad, CA).

siRNA transfection

Comparison of various transfection reagents was conducted to determine optimal transfection conditions for transfection of ASM primary cultures. Superfect (Qiagen Canada, Mississauga, ON) and Lipofectamine (Invitrogen Canada, Burlington, ON) were eventually discarded due to the high level of cytotoxicity induced by these reagents in primary ASM cultures. Ultimately, the X-TremeGene (X-TG) (Roche Diagnostics, Laval, QC) transfection reagent was chosen for its ability to successfully transfect primary ASM cells while maintaining a low cytotoxicity level.

A total of 10 μl of X-TG stock solution were mixed with 12 μl of 20 μM siRNA solution, a ratio within the dilutions recommended by the manufacturer. A stock solution of X-TG (10 μl) was diluted in 90 μl of DMEM/F12 medium. The chosen siRNA was used by diluting the calculated siRNA stock solution (12 μl) in 88 μl DMEM/F12 medium. This solution was added to the X-TG solution and mixed by up and down pipetting. After 15 min at room temperature to ensure complete formation of the siRNA/X-TG complex, DMEM/F12 medium was added to a total volume of 1 ml. Cells were then incubated and transfected in the above solution for 4 h at 37 °C, after which the solution was replaced with DMEM/F12 + 1% Penicilin/Streptomycin and 10% FBS and cells subsequently cultured for 72 h.

RT-PCR

Guinea pig primary ASM cells were isolated and cultured in 25 cm^2 plastic flasks. A total of 72 h following transfection (or mock transfection / control cells), total RNA was extracted from cultured cells using the Qiagen RNeasy Mini Kit (Qiagen Canada), according to the manufacturer's recommended instructions. A spectrophotometer (Pharmacia Biotech Ultrospec 2000) was used to quantify the concentration and quality of isolated total RNA by calculating the ratio of absorbance of the samples at λ = 260 nm versus λ = 280 nm. RNA used for subsequent experiments consistently exhibited a ratio of 2.1–2.2, thus ensuring minimal contamination by other genomic material. A total of 400 ng total RNA was reverse-transcribed into cDNA with the aid of a Qiagen Omniscript RT kit (Qiagen Canada) according to the manufacturer's recommended instructions. Aliquots of this reverse-transcribed cDNA were then amplified by PCR reactions (30 cycles). Following PCR, the samples were migrated for 75 min on a 1% agarose gel + ethydium bromide, and images acquired under ultraviolet light. Quantitative analysis of the gel was performed using the LabImage software v.2.72 (Kapelan GmbH, Germany).

SDS-PAGE and Western Blot analysis

Protein samples (20 μg protein/well), from specified fractions were dissolved in 2% SDS and separated on 10% SDS-PAGE, using a 3% stacking gel. Gels were cast into a mini-protean III dual cell (Bio-Rad). Antibodies against TRPC6 -goat Ab sc-19196- (Santa Cruz) and anti-SM α-actin were used to perform Western blot analysis. The separated proteins from SDS-PAGE were electrophoretically transferred at 70 V onto nitrocellulose membranes (Bio-Rad) for 2 h at 4°C. The membranes were washed in Tris Buffered Solution + 0.1% Tween 20 (TBST) for 15 min, blocked with TBST + 5% non-fat diet milk overnight and then washed 3 times for 5 min in TBST, before overnight incubation with selected specific antibodies in TBST. After three washes in TBST, the membranes were incubated for 1 h at 23°C with peroxidase-conjugated goat

anti-mouse IgG antiserum for α-actin detection and bovine anti-goat sc-2384 for TRPC6 detection (SantaCruz). The membranes were washed three times for 5 min in TBST and revealed using an Enhanced Chemiluminescence kit (Roche (Roche Diagnostics, Laval, QC).

Spectrofluorimetry

Primary guinea pig ASM cells were cultured for 24 h prior to transfection with an X-TG + non-silencing rhodamine-tagged siRNA complex (see protocol described above). Control experiments, without complex or fluorescent siRNA, were performed in parallel. The Petri dishes containing cultured cells were examined with an Nikon Eclipse TE300 microscope equipped with 20×, 40×, and 100× objectives using a CoolSnaps FX (Photometrics Inc., Tucson AZ) camera for imaging under visible and fluorescent light. To assess transfection efficiency, fluorescent siRNA localization was determined by counting cells exhibiting rhodamine fluorescence at 500 nm vs. non-fluorescent cells. The Metamorph software v4.6 r10 (Molecular Devices, Sunnyvale, CA) was used for data acquisition and processing. All measurements consisted of data acquired from at least 15 cell-filled fields, in 3 separate Petri dishes.

Intracellular calcium measurement and data analysis

A Ca^{2+}-free external solution for cell culture was prepared as follows (mM): 107 NaCl, 7.2 KCl, 1.2 $MgCl_2$, 11.5 glucose, 20 HEPES, and pH set at 7.2 with NaOH. Control or X-TG/siRNA complex-transfected primary guinea pig ASM cells were cultured on glass coverslips in DMEM/F12 solution for 72 h, following the transfection procedure. Cell cultures were thereafter incubated for 30 min in 1 ml of Ca^{2+}-free external solution containing 2 μl Fura-2 AM (Invitrogen). After loading, intracellular Fura-2 was allowed to hydrolyze, following three washes with 1 ml of external solution + 10% BSA, for 30 min in the same solution. Coverslips were then mounted on the Nikon Eclipse TE300 microscope and changes in intracellular calcium were measured using the Metafluor software (Molecular Devices) at λ = 340 and 380 nm excitation wavelengths, and 505 nm emission wavelength, respectively. Intracellular calcium measurements were performed following sequential addition of 3 mM of $CaCl_2$ and 100 μM OAG, or in reverse order. Recordings of emission at 505 nm were acquired every 5 s to determine OAG-induced calcium entry in presence of extracellular Ca^{2+}. All intracellular calcium measurements consisted of acquired data from four experiments with an average of 10 cells per field. In order to compare and average the results, data was expressed as percentage values: 100% corresponding to the difference between 1 and the fluorescence ratio in the absence of $CaCl_2$ (resting fluorescence ratio). The percentage of change induced by the extracellular $CaCl_2$ and OAG compound was calculated within this range. Values are presented as means ± SEM.

Results

Transfection efficiency

Following isolation from airway tissues, primary cultured ASM cells displayed mainly long-spindle shape morphology, when plated in either plastic flasks or on glass coverslips in Petri dishes. After 24 h, isolated ASM cells exhibited typical polygonal multiplicative phenotype as described previously. Expression of SM α-actin was also detected in these cells by immuno-cytofluorimetry as reported previously [22]. In order to optimize transfections, isolated cells were grown 48–72 h to allow reaching 60% confluency and thereafter transfected with either X-TG or X-TG + non-silencing fluorescent siRNA. Optical and spectro-fluoro microscopy were performed to compare fluorescence levels in the above cultured cells (Fig. 1 A and B). Following sequential washes with HBSS solution, the fluorescence was only detected in cultures transfected with the rhodamine-tagged siRNA complexes. Fluorescence was localized in the cytoplasm and surrounding the nucleus (Fig. 1B). By simple counting of non-fluorescent versus total cells, it was determined that a very low percentage of the cells were untransfected. The calculated transfection rate revealed a mean efficiency rate of 96.3 ± 1.5% in these isolated ASM cells.

RT-PCR and Western Blots analysis

To assess the effects of a silencing siRNA designed against a specific guinea pig TPRC6 sequence (see Table 1), RT-PCR experiments were performed using specific sense and antisense primers, on several ASM cell cultures. Parallel experiments were performed to prepare cytosolic and microsomal fractions, followed by Western blot analysis. Total RNA was extracted following 4-h transfections and a 72-h culture period. There were no observed differences in the average concentration of total extracted RNA between control (547 μg / ml) and X-TG/siRNA-treated cells (544 μg / ml). However, the RNA concentration from X-TG-treated only samples was lower (408 μg/ml). After separation of RT-PCR reaction products on agarose gels, image analysis revealed an 88% reduction in TRPC6 transcript levels in cells transfected with X-TG siRNA against TRPC6 complexes in comparison to corresponding control cultures or X-TG treated cells (Fig. 2A). Controls performed with GAPDH primers demonstrated a

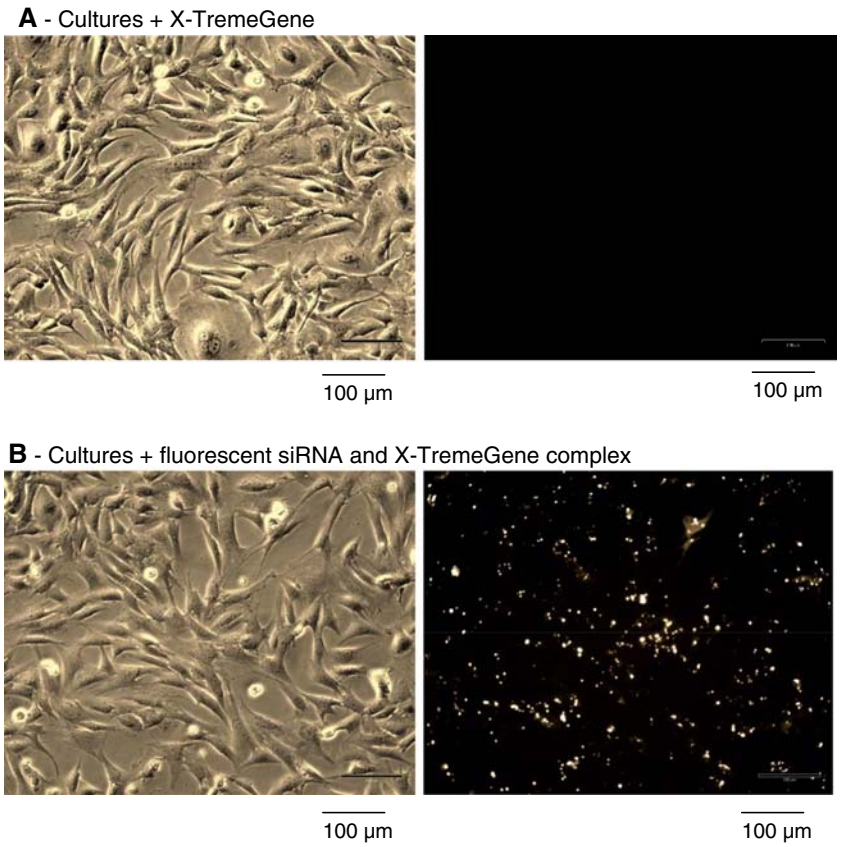

Fig. 1. Comparison of guinea pig ASM cell cultures transfected with X-TG-siRNA complexes. (A) Cells were transfected with X-TG alone or (B) with a complex formed by rhodamine-tagged non-silencing siRNA and X-TG. Image acquisition was used to determine siRNA transfection efficiency in primary cell cultures. Visible (left panels) and fluorescent (right panel) imaging was performed 24 h after transfection. All red fluorescence vas observed in the cytoplasm especially in the perinuclear region, surrounding the nucleus.

steady-state expression level of this housekeeping gene transcript between control, X-TG treated and X-TG / siRNA against TRPC6 transfected cells (Fig. 2A right panel). There were no observable bands in lanes corresponding to wells loaded either with "No Template" or RNA only, indicating the absence of any external or genomic DNA contamination.

Western blot analysis confirmed a significant decrease in TRPC6 protein expression in cells transfected with the X-TG-TRPC6 specific siRNA complex, comparatively to cells from control or X-TG treated cultures. Anti-SM α-actin antibodies did not exhibit any significant variation in protein content between the various cell samples. These data thus corroborate and strengthen the results obtained from RT-PCR experiments

Intracellular calcium measurements

OAG, a known PKC agonist and a TRPC 3, 6, and 7 activator [23] was used to test whether siRNA-dependent inhibition of TRPC6 protein expression actually affects calcium signaling. Intracellular Ca^{2+} measurements were performed using two distinct protocols. The first set of experiments was performed in zero nominal Ca^{2+} in the extracellular medium. Measurement of background fluorescence, corresponding to resting intracellular Ca^{2+} levels, yielded 340/380 ratios between 0.65 and 0.72. Addition of 3 mM $CaCl_2$ in the external medium slightly increased the fluorescence ratio to a steady-state level (Fig. 3A and B), attesting that surface membrane integrity of control and X-TG + siRNA transfected cells was maintained. Exogenous addition of 100 μM OAG, in the extracellular medium, consistently triggered a rapid increase in fluorescence ratio – corresponding to an increase in cytosolic free $[Ca^{2+}]$ – with a slow inactivation rate, which eventually triggered repetitive fluorescent signals (Fig. 3A and B). Figure 3C illustrates results obtained after OAG addition on both control and X-TG + siRNA transfected cells. On average, in guinea pig ASM cells, inhibition of TRPC6 protein expression had no effect on the amplitude of OAG-activated Ca^{2+} signals derived from the first peaks.

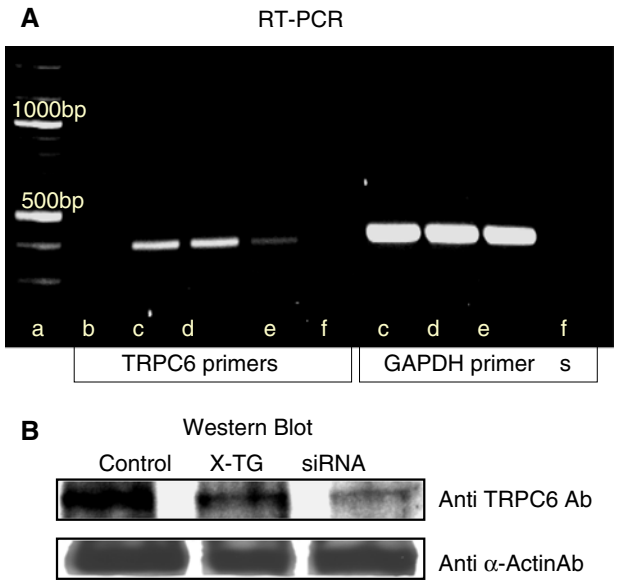

Fig. 2. Determination of TRPC6 transcript expression and Western blot analysis in ASM cells following treatment with anti-TRPC6 siRNA. (A) RT-PCR analysis performed on total RNA samples from guinea pig ASM cells in primary culture. Cultures were either not transfected (c), transfected with X-TG alone (d) or with the X-TG-siRNA complex, against TRPC6 (e). Controls consisted in using a "No Template" lane (b) or "total RNA" only (f) as a means of assessing possible contamination. (a) DNA ladder. Primers against guinea pig TRPC6 and GAPDH sequences were used as defined in Table 1 (B) Western blot of guinea pig ASM cell microsomal fractions. Experiments were performed in parallel on fractions derived from cell cultures, either not transfected (Control), transfected with X-TG alone (X-TG) or with an X-TG siRNA complex (siRNA). Anti-TRPC6 and anti-SM α-actin antibodies were used to determine relative protein content in the various samples applied to the same gel. The illustrated results are representative of three identical experiments.

Complementary experiments in which OAG and $CaCl_2$ were added in reverse order revealed that OAG alone had no effect in the absence of extracellular Ca^{2+}. However, in the presence of OAG, addition of $CaCl_2$ triggered similar transient signals in both preparation types (Fig. 3D, E). Under these conditions and despite identical mean signal amplitudes of the first peak (Fig. 3F), rapid inactivations were observed on raw recordings (Fig. 3D, E), suggesting that the Ca^{2+} entry was followed by a Ca^{2+} inactivation process.

Discussion

The present study demonstrates the validity of RNA interference technology as a valuable tool for studying TRPC channels in primary cultures of ASM cells. It also provides an ideal model for quickly and efficiently evaluating the effect of a specific gene transcript for which pharmacological antagonists are not yet available. The data reported herein also demonstrates that a reduction in specific protein isoform expression can be achieved by the use of siRNA complexes against TRPC6 in primary ASM cultures.

Transfection efficiency and TRPC6 silencing

Due to the sensitivity of primary cell cultures to various transfection reagents (Lipofectamine, Surpefect) and the limited physiological relevance of cell lines, an optimization of the transfection protocol was hence necessary to circumvent cytotoxicity in ASM cells. Selection of the chemical transfection reagent (X-TG) enabled the use of siRNA technology with minimal interference on cell growth, while demonstrating high transfection efficiency. Using the current protocol, it was determined that more than 95% of cells were transfected with the X-TG siRNA complex. Moreover, results show that it is possible to efficiently silence a specific gene transcript from primary ASM cells. This data provides further confirmation that the results obtained on isolated cells were indeed valid, since the presence of siRNA was evenly distributed (Fig. 1). Furthermore, the transfection protocol induced a significant decrease in both expression of TRPC6 transcripts and protein expression levels, confirming the ability of this chemical transfection reagent approach to quickly silence the expression of a target protein in primary ASM cells, and thus allowing to study its putative role in regulating electrophysiological properties of SM cells. The decrease in TRPC6 protein was found to be both specific and significant (Fig. 2). A similar strategy involving an electroporation system was recently used on A7r5 vascular SM cells to knock down the TRPC6 channels [13]. Our attempts at this latter approach revealed that while the electroporation system can be very successfully applied to cell lines and cells in suspension, the majority of primary SM cells however did not survive the electroporation transfection step (data not shown).

OAG-induced Ca^{2+} entry

Spectrofluorimetric measurements revealed that in the presence of extracellular calcium, addition of OAG to cell cultures did increase intracellular calcium levels, although this effect was not significantly different between control and siRNA-transfected ASM cells. Addition of OAG and extracellular calcium in reverse order did not reveal any difference in Ca^{2+} signaling thus confirming OAG-induced Ca^{2+} entry in primary ASM cells, in accordance with results reported previously in SM cell lines [13, 24]. However, in pulmonary arterial SM cells, it was reported that application

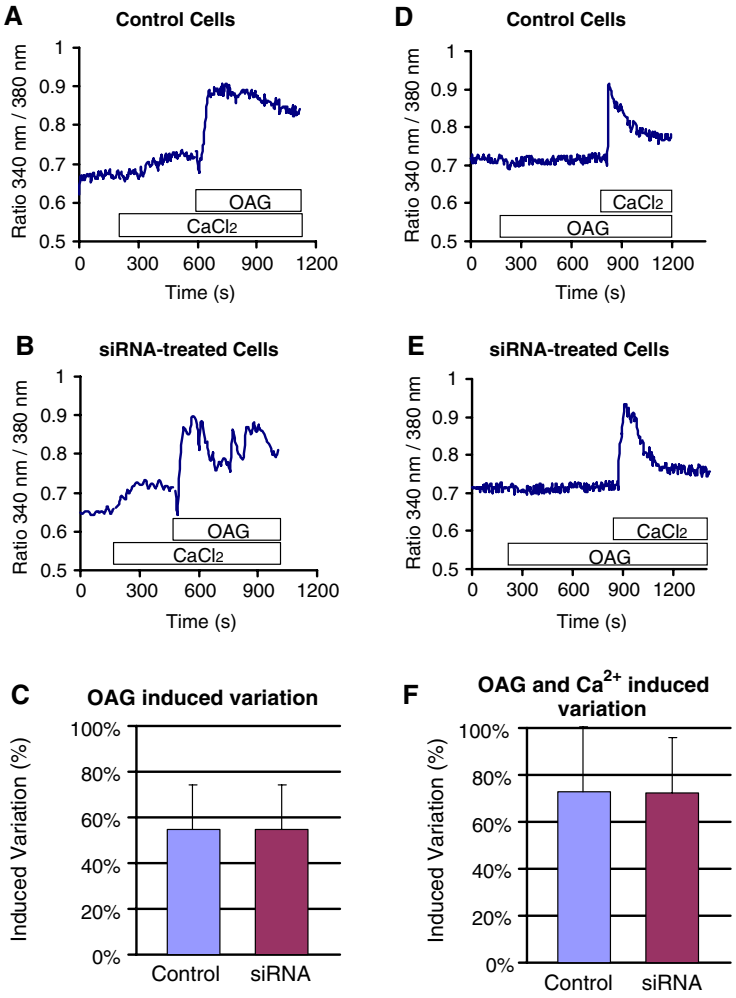

Fig. 3. OAG-induced intracellular calcium mobilization in control and siRNA-treated cells. Following Fura2-AM incubation and hydrolysis, cells were incubated and initially tested in modified Ca^{2+}-free physiological solution. Left panels: addition of 3 mM $CaCl_2$ and 100 μM OAG induced an increase in intracellular free calcium in (A) Control, (B) X-TG-siRNA (against TRPC6) treated ASM cells and **C**: average responses; n = 41 for both conditions. Right panels: treatment with 100 μM OAG and addition of 3 mM $CaCl_2$ induced calcium changes in (D) Control, (E) X-TG-siRNA treated ASM cells and (F) average responses; n = 39 and 48, respectively.

of OAG to directly activate ROC, or thapsigargin to deplete Ca^{2+} stores, caused a large cations entry measured by Mn^{2+} quenching of fura-2 and Ca^{2+} transients [25]. While siRNA knockdown of TRPC1 and TRPC6 specifically attenuated OAG-induced cation entry [25], Zhang and Saffen reported that expression of the rat TRPC6 longest isoform (rTRPC6A) in COS cells, causes Ba^{2+} entry upon OAG application [26]. A recent publication, using HEK cells over-expressing the TRPC6 channels, reported that Na^+ is likely the main charge carrier while Ca^{2+} likely acts as a blocking ion [14]. This analysis is consistent with our observations in ASM cells in which reduction of TRPC6 did not correlate with changes in OAG-induced Ca^{2+} movement. Several reports have concluded that Na^+ transport through TRPC channels results in cell depolarization [12], which indirectly activate Ca^{2+} transport either through Ca^{2+} channels or activation of Na^+/Ca^{2+} exchangers [11, 13, 27].

TRPC channels are known to form hetero-tetramers with other TRPC subunits as previously reported [23, 28]. This observation is of particular interest since various TRPC channels are expressed in guinea pig ASM cells, including TRPC3 and TRPC6 [10, 12]. Moreover, TRPC3/6 hetero-multimers were also found in epithelial cells [29]. In our transfected cell preparations, the stoichiometric ratio of TRPC expression was not maintained due to TRPC6 silencing. Thus, formation of TRPC3 homo-tetramers could mediate OAG-induced calcium entry [26] despite reduction in TRPC6 expression.

Use of the protocol on new targets

While this study demonstrates the possibility of using a siRNA transfection protocol on primary ASM cells, this approach could also be used to elucidate the role of other proteins involved in various pathways of airway pathophysiology, including its use in organoid cultured tissues [16]. The concomittent use of multiple siRNA in the same culture could also be envisioned as a method of combining the effects of several siRNA on protein expression. Multiple, although partial, ''knockout'' studies would hence be possible. For example, such an approach could be used in the analysis of siRNA transfection against TRPC6 and TRPC3 channels, reported to form hetero-multimers and whose activation is mediated through PLC-dependent DAG production and putative DAG metabolites.

In conclusion, through the use of siRNA technology, we were able to down-regulate TRPC6 expression in primary ASM cells. Intracellular calcium measurements allowed to demonstrate the absence of change in OAG-stimulated Ca^{2+} signaling following TRPC6 silencing. Furthermore, TRPC6 channel cannot be defined as a simple Ca^{2+} channel, but more likely as a non-selective cation channel for Na^{+} and Ca^{2+} currents. Depolarization induced by these ionic currents may be responsible for the activation of voltage-activated Ca^{2+} channels.

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