Biochimica et Biophysica Acta 1843 (2014) 464–472

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Are Orai1 and Orai3 channels more important than calcium influx for cell proliferation?☆

Anne-Sophie Borowiec [a], Gabriel Bidaux [a,1], Rachida Tacine [c], Pauline Dubar [b], Natascha Pigat [d], Philippe Delcourt [a], Olivier Mignen [b], Thierry Capiod [a,c,d,*]

[a] INSERM, U1003, IFR147, Univ Lille 1, Villeneuve d'Ascq F-59655, France
[b] INSERM U613, Université Bretagne Occidentale, 46 rue Felix Le Dantec, Brest F-29218, France
[c] INSERM U807, Hôpital Necker Enfants Malades, Université Paris 5, 156 rue de Vaugirard, Paris F-75730, France
[d] INSERM U845, Growth and Signalling Research Center, Université Paris 5, Bâtiment Leriche, 96 rue Didot, Paris F-75993, France

ARTICLE INFO

Article history:
Received 12 June 2013
Received in revised form 9 November 2013
Accepted 27 November 2013
Available online 7 December 2013

Keywords:
Store-independent calcium entry
Store-operated calcium entry
Mitosis
Cell cycle

ABSTRACT

Transformed and tumoral cells share the characteristic of being able to proliferate even when external calcium concentration is very low. We have investigated whether Human Embryonic Kidney 293 cells, human hepatoma cell Huh-7 and HeLa cells were able to proliferate when kept 72 h in complete culture medium without external calcium. Our data showed that cell proliferation rate was similar over a range of external calcium concentration (2 μM to 1.8 mM). Incubation in the absence of external calcium for 72 h had no significant effect on endoplasmic reticulum (ER) Ca^{2+} contents but resulted in a significant decrease in cytosolic free calcium concentration in all 3 cell types. Cell proliferation rates were dependent on Orai1 and Orai3 expression levels in HEK293 and HeLa cells. Silencing Orai1 or Orai3 resulted in a 50% reduction in cell proliferation rate. Flow cytometry analysis showed that Orai3 induced a small but significant increase in cell number in G_2/M phase. RO-3306, a cdk-1 inhibitor, induced a 90% arrest in G_2/M reversible in less than 15 min. Our data showed that progression through G_2/M phase after release from RO-3306-induced cell cycle arrest was slower in both Orai1 and Orai3 knock-downs. Overexpressing Orai1, Orai3 and the dominant negative non-permeant mutants E106Q-Orai1 and E81Q-Orai3 induced a 50% increase in cell proliferation rate in HEK293 cells. Our data clearly demonstrated that Orai1 and Orai3 proteins are more important than calcium influx to control cell proliferation in some cell lines and that this process is probably independent of I_{CRAC} and I_{arc}.

1. Introduction

The relation between cell proliferation and calcium channel expression is now well established and recent reviews have summarized the nature of calcium channels involved and emphasized their role in pathophysiological proliferation, thus revealing their oncogenic potential [1–4]. Normal and tumoral tissues often display different calcium channel expression and we recently showed that stronger levels of the TRPC6 cation channel are observed specifically in area of tumoral human livers [5]. Store-operated calcium entry (SOCE), activated following intracellular calcium store depletion [6], is clearly the major calcium entry pathway involved in cancer [7]. Therefore, calcium channels are nowadays considered as potential targets for anticancer therapies with only a few drugs undergoing clinical tests. Carboxyamidotriazole (CAI), a calcium channel blocker originally described as a tumor repressor in several cancers [8,9] and now used in lung cancer phase III clinical trials [10], was also recently shown to inhibit mitochondrial activity in the same range of concentrations [11]. Other blockers include verapamil, diltiazem, nifedipine, amlodipine, mibefradil, TH-1177, 2-APB, and SK&F 96365 but none of these drugs has clear selectivity for one type of calcium channels [12,13].

If the role of calcium channels seems to be rather clear, the need for external calcium for cell proliferation can be questioned [12,13]. During the seventies, several scientists have studied the dependence on external calcium of normal and transformed cells [14–16] and they established that, while normal cells need external calcium to proliferate, transformed cells are less sensitive [14,17,18]. In fact, transformed cells proliferate even in the presence of 20 μM external calcium with maximum proliferation rates achieved in the range of 50 to 100 μM while non-tumorigenic cells had higher requirement (500 to 1000 μM).

The need for calcium influx during cell cycle is not definitively settled in mammalian cells. Indeed, it is known that, first, blockade of cell cycle was associated to a decrease in SOCE amplitude [19,20], and second, calcium channel blockers inhibited cell proliferation [21]. SOCE is

☆ Supported by a grant from the Comité du Septentrion, Ligue Contre le Cancer (TC).
* Correspondence to: T. Capiod, INSERM U845, Growth and Signalling Research Center, Université Paris 5, Bâtiment Leriche, 96 rue Didot, Paris F-75993, France. Tel.: +33 172607371; fax: +33 172606401.
E-mail address: thierry.capiod@inserm.fr (T. Capiod).
[1] Present address: Laboratoire Biophotonique Cellulaire Fonctionnelle, Institut de Recherche Interdisciplinaire, USR3078 CNRS, Parc scientifique de la Haute Borne, 50 Avenue du Halley, Villeneuve d'Ascq, France.

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.11.023

usually measured after ER Ca^{2+} depletion following thapsigargin (TG) treatment in calcium imaging experiments or in whole-cell voltage-clamp cells using Ca^{2+}-buffered solution and stable InsP3 receptor agonists [6]. However, there is no clear evidence that SOCE is activated in cells continuously stimulated with growth factors. Indeed, calcium transients, and probably transient ER Ca^{2+} store depletion, were recorded during fertilization or meiosis in oocytes but this is far from clear during mammalian cell cycle.

The relation between calcium channel expression and cell cycle is better documented. First, cell cycle arrest, whatever the cell cycle phase and the mechanism used to induce this blockade, induced a clear decrease in SOCE amplitude [13]. Second, knocking down Orai, TRPC or TRPM channels resulted in cell cycle arrest [13]. Third, amplitude of SOCE and the archetype current I_{CRAC} is modulated during cell cycle [22] and is totally inhibited during mitosis due to several potential mechanisms including internalization [23,24], phosphorylation [25] or decreased expression [20].

Here, we have investigated whether external calcium was needed for cell proliferation, mitosis and cell cycle progression. HEK293 cells, human hepatoma cell line Huh-7 and HeLa cells were used to establish the differential roles of calcium channels and calcium influx in cell division.

2. Materials and methods

2.1. Cell culture

HEK293 stably transfected with the human m3 muscarinic receptor (HEKm3), human hepatoma cell line Huh-7 and HeLa cells were cultured as described before [11] in DMEM with Glutamax-I supplemented with fetal calf serum (FCS, 10%), penicillin (200,000 U/ml) and streptomycin (100 μg/ml) (Life Technologies, Cergy-Pontoise, France).

2.2. Cell proliferation

Cell proliferation rates were determined using two separate methods. Cells were seeded in 96-well plates for 3 days before medium substitution. Medium was renewed every day and proliferation monitored for 3 days in DMEM without Ca^{2+} supplemented with EGTA 150 μM, fetal calf serum (FCS, 10%), penicillin (200,000 U/ml) and streptomycin (100 μg/ml) and Glutamax-I (Life Technologies, Cergy-Pontoise, France). Proliferation rates were determined as described before [5] or by measuring cell number in cells kept 3 days in different external Ca^{2+} concentrations. Cell viability was assessed using Trypan Blue staining of dead cells.

2.3. Mitotic index

Cells were collected by trypsinization, resuspended in 300 μl PBS–EDTA (5 mM) and fixed with 700 μl absolute ethanol at −20 °C overnight. Cells were washed twice with wash solution (PBS/0.05% Triton X-100). Cells were then incubated in staining solution (PBS/0.2% Triton X-100/2% FCS) containing 1 μg/ml anti-phospho-Histone H3 (Ser10; Millipore 05-806) for 2 h at 4 °C. Cells were washed twice with wash solution and secondary antibodies (anti-mouse IgG coupled to Alexa Fluor-488) diluted at 1/2000 were incubated with cells for 2 h at 4 °C in the dark. Afterwards, cells were washed out 3 times at RT for a total of 30 min. Finally, cells were resuspended in PBS and treated with ribonuclease at a final concentration of 2 μg/ml for 30 min and stained with propidium iodide at a final concentration of 25 μg/ml. The stained samples were analyzed using a Becton Dickinson FACScan cytofluorometer.

2.4. Calcium measurements

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) and thapsigargin-induced responses were monitored in a multiplate reader Berthold TriStar (Berthold France SAS, Thoiry, France). Cells were plated in 24-well plates and incubated for 72 h in complete DMEM in the presence or absence of external Ca^{2+}. Cells were loaded with 4 μM fluo4-AM for 45 min then washed with recording medium containing (mM) NaCl 116, KCl 5.6, $MgCl_2$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 5, Hepes 20 (pH 7.3) supplemented with 1.8 mM Ca^{2+} or 150 μM EGTA. Thapsigargin (1 μM) was added in the absence of external Ca^{2+} and areas of the responses calculated using Origin. Recordings were performed at 37 °C and fluorescence signals calibrated by successive additions of 5 mM digitonin containing 5 mM Ca^{2+}, and 50 mM EGTA (pH 7.3 NaOH). $[Ca^{2+}]_i$ was determined using the equation Kd*((F-Fmin)/(Fmax-F)), with Kd of fluo4 for Ca^{2+} taken as 345 nM, Fmax and Fmin, the maximum and minimum fluorescence signals after digitonin and EGTA addition respectively.

2.5. Cell cycle analysis

Cells were seeded for at least 24 h before transfection with siRNA. Cells were treated for 24 h with 10 μM RO-3306 on the next day. Cells were then washed in complete medium without RO-3306 for the period of time indicated. The same protocol was used for non-transfected cells with two washes in Ca^{2+}-free solutions when needed. Cells were harvested and washed with phosphate-buffered saline and fixed overnight with 70% ice-cold ethanol at −20 °C. The fixed cells were stained with a solution containing Propidium Iodide (25 μg/ml), RNase A (2 μg/ml) and Triton X-100, and then analyzed with a Becton Dickinson FACScan cytofluorometer. G_0/G_1, S and G_2/M phases were assessed by fitting the cell distribution using ModFit software (Verity Software House, ME, USA). 10% FCS contains about 100 μM (measured in fura2 solution, data not shown) close to values reported in the literature [26] and we therefore estimated the Ca^{2+} concentration of the Ca-free medium to approximately 140 μM as Ca^{2+}-free DMEM medium contains about 40 μM free Ca^{2+}. After the addition of 150 μM EGTA, free calcium was therefore calculated to 2 μM using MaxChelator (Ca–Mg–ATP–EGTA Calculator v1.0 http://maxchelator.stanford.edu). Data acquisition and data analysis were performed on the IFR94 Cytometry Facility (Growth and Signalling Research Center, Université Paris 5, Paris, France).

2.6. Transfection

SiRNA was transfected with HiPerfect lipofectant (Qiagen). Transfection protocols were performed following the manufacturer's instructions. SiRNA sequences (Table 1) against Orai1 and Orai3 were previously validated [20,27]. Control experiments were performed by transfecting a specific siRNA against Luciferase (noted siLuc).

2.7. Reverse-transcription and real-time PCR analysis

Total RNA was extracted using the Trizol method (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. After a DNase I (Invitrogen) treatment, 2 μg of total RNA was reverse transcribed into cDNA at 42 °C using random hexamer primers (Applera)

Table 1
siRNA and primers sequences respectively used for silencing protein expression and detection in qPCR experiments.

	siRNA sequence	
Si Luc	5′-CUUACGCUGAGUACUUCGA(dTdT)-3′	
Si Orai1	5′-UGAGCAACGUGCACAAUCU(dTdT)-3′	
Si Orai3	5′-UUGAAGCUGUGAGCAACAU(dTdT)-3′	
	Primers sequence	
Orai1	5′-ATGGTGGCAATGGTGGAG-3′	5′-CTGATCATGAGCGCAAACAG-3′
Orai3	5′-GGCCAAGCTCAAAGCTTCC-3′	5′-CCTGGTGGGTACTCGTGGT-3′
GAPDH	5′-ATCTCTGCCCCCTCTGCT-3′	5′-GCAGGAGGCATTGCTGAT-3′
HPRT	5′-GGCGTCGTGATTAGTGATGAT-3′	5′-CGAGCAAGACGTTCAGTCCT-3′

and MuLV reverse transcriptase (Applera) in a 20 µl final volume. Expression levels of PCR products were quantified by real-time quantitative PCR on a Cfx C1000 system (Biorad). For each reaction, 5 µl of a 20 times diluted cDNA was placed in a final reaction mixture of 15 µl containing 7.5 µl of 2× Evagreen Master Mix (Applied Biosystems) and 250 nM primer pairs. The housekeeping gene glyceraldehyde-3phosphate dehydrogenase (GAPDH) and hypoxanthine–guanine phosphoribosyltransferase (HPRT) (see sequence in Table 1) were used as an endogenous control to normalize variations in RNA extractions, the degree of RNA degradation and variability in RT efficiency. Dissociation curve was done to assess specificity of amplification. The comparative Ct method was used to quantify gene expression.

2.8. Western-blots

An ice-cold buffer (pH 7.2) containing 10 mM PO4Na2/K buffer, 150 mM NaCl, 1 g/100 ml sodium deoxycholate, 1% Triton X-100, 1% NP40, a mixture of protease inhibitors (Sigma-Aldrich), and a phosphatase inhibitor (sodium orthovanadate; Sigma-Aldrich) was applied to previously PBS washed cells in dishes. After 30 min incubation on ice, the protein extract was transferred to 1.5 ml tubes and subjected to sonication. After 10 min of centrifugation at 15,000 *g*, the pellet was transferred into a clean tube prior to a determination of the protein concentration using a BCA Protein Assay (Pierce). 50 µg of total protein was loaded onto a 12% polyacrylamide gel before an SDS-page was performed. After electrophoresis, proteins were transferred to a PVDF membrane using a semi-dry electroblotter (Bio-Rad). The membrane was blocked in a TNT +3% (W/V) milk (15 mM Tris buffer, pH 8, 140 mM NaCl, 0.05% Tween 20, and 3% non-fat dried milk) for 30 min at room temperature, then incubated with primary Orai1 antibody (O8264; Sigma-Aldrich) diluted (1/1000) in TNT +1% milk for overnight at +4 °C. After three washes in TNT, the membrane was soaked in secondary antibody diluted in TNT +1% milk for 1 h at room temperature. The membrane was processed for chemiluminescence detection using Luminata Forte Western HRP Substrate (Millipore) according to the manufacturer's instructions.

2.9. Evaluation of half-time decay of cell cycle release

Quantification of speed release from cell cycle arrest was evaluated as the time at which half of the maximal proportion of cells was excluded from G_2/M (cell cycle experiment) or M phase (Mitotic index experiment), and was defined as the half-time decay, $\tau_{(1/2)}$. Values representing the proportion of cells in G_2/M or M phase over time were plotted as the function %cell = f(t). $\tau_{(1/2)}$ was estimated graphically on interpolated curves as $f(t) = (\%_{max} - \%_{min}) / 2$; $\%_{max}$ is the proportion of cells in G_2/M or M phase 30 min after the release of cell cycle arrest; and $\%_{min}$ is the minimal proportion of cells in G_2/M or M phase after the release of cell cycle arrest.

2.10. Statistics

An unpaired Student *t* test was used and results are expressed as means ± s.e.m., with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results

The influence of calcium channels on cell cycle progression and mitosis was investigated by using the cyclin-dependent kinase 1 inhibitor RO-3306 [28]. We previously showed that 10 µM RO-3306 for 24 h resulted in a massive blockade in G_2/M and a large decrease in SOCE amplitude in HEK293 cells [20]. SOCE was fully recovered within 4 h after RO-3306 wash off and we have investigated here the kinetics of G_2/M exit, mitosis and G_0/G_1 entry using cell cycle analysis with flow cytometry in HEK293 and HeLa cells. 24 h in the presence of 10 µM RO-3306 induced a cell cycle arrest with 89.8 ± 0.9% (n = 12) of HEK293 cells (Fig. 1) and 88.3 ± 0.9% (n = 8) of HeLa cells in G_2/M phase confirming previous work [28]. We then monitored the release from blockade over

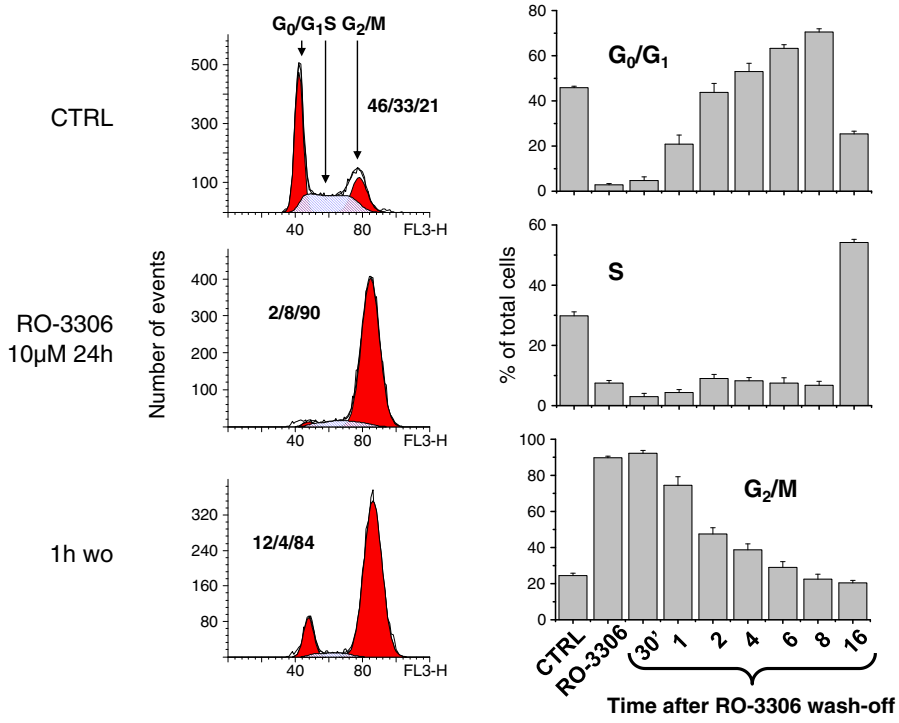

Fig. 1. Cell cycle analysis by DNA content estimation with flow cytometry in HEK293 cells. Left: Representative cell cycle distribution in control cells (CTRL), in cells treated for 24 h with 10 µM RO-3306, and 1 h after RO-3306 washout (1 h wo). Numbers indicate the percentage of cells in G_0/G_1, S and G_2/M respectively. Right: Histograms showing the distribution in the different phases of the cell cycle in control cells (CTRL), in cells treated for 24 h with 10 µM RO-3306, and at different times (from 30 min to 16 h) after release from cell cycle arrest.

a period of time of 16 h. The distribution of the cell cycle phases was not changed 30 min after release but the cell number in G_0/G_1 started to increase after 1 h, with a parallel decrease in the proportion of cells in G_2/M. The simultaneous increase in G_0/G_1 and decrease in cell number in G_2/M were observed for 8 h with no change in S phase. G_0/G_1 increased from 2.8 ± 0.6% in the presence of RO-3306 to 70.5 ± 1.4 (n = 12) 8 h after release from cell cycle arrest while cell number in G_2/M decreased from 89.8 ± 0.9% to 22.5 ± 2.7 (n = 12) in the same conditions. One issue of cell cycle analysis with flow cytometer is the inability to discriminate between G2 and M phases. It is thus almost impossible to determine whether the delay of cell exit from G_2/M phase is dependent on G2 transition time, M transition time or both. We therefore calculated the decay of mitotic index after RO-3306 removal. It has been previously demonstrated that R0-3306-induced G_2/M-phase-arrested cells are actually arrested in G2 phase as they did not showed Ser10 phosphorylation of histone H3 [28]. This latter has been demonstrated to occur from prophase to telophase of mitosis. As seen in Supplemental Fig. 1, we showed that the half-time decay ($\tau_{(1/2)}$) of the relative mitotic index is similar to the half-time decay of G_2/M-phase-arrested HEK293 cells since both are reached 1 h after R0-3306 removal. This suggests that, although R0-3306 induced cell cycle arrest in G2 phase, cells enter mitosis several minutes after R0-3306 removal. Consequently, G_2/M cell population detected by DNA content analysis with flow cytometry in cells treated with RO-3306 could be considered as M cell population from 30 min to 4 h after R0-3306 removal.

Cell cycle analysis by DNA content estimation with flow cytometry was also used to study the role of external Ca^{2+} on mitosis and cell cycle progression in HEK293 and HeLa cells. Cells were blocked in G_2/M phase using RO-3306 and released either in Ca^{2+}-containing or Ca^{2+}-free medium in the presence of 10% FCS and 150 μM EGTA. Cell cycle progression was monitored over a time period of 24 h in HEK293 and HeLa cells. Exit from G_2/M arrest and mitosis was insensitive to external Ca^{2+} in both HEK293 and HeLa cells (Fig. 2A and B). HEK293 and HeLa cells were able to progress through all the phases at the same speed in the absence or presence of external Ca^{2+} with a cell cycle length of about 20–22 h (Fig. 2A and B).

We also checked whether cell proliferation was blocked in the absence of external Ca^{2+}. HEK293, HeLa and Huh-7 cells were seeded in complete culture medium then incubated for another 3 days in the presence or absence of external Ca^{2+}. Our data showed (Fig. 3A) that incubating all 3 cell types in the absence of external Ca^{2+} had no effect on cell proliferation. The dependence of external Ca^{2+} concentration was also investigated and no significant difference was observed over a range from 2 μM up to 1800 μM (Fig. 3B). Cell viability was similar in all conditions and ranged from 95 to 99% in all cell types.

Incubating the cells for 72 h in the absence of external Ca^{2+} could result in a decrease in ER Ca^{2+} content and $[Ca^{2+}]_i$. ER Ca^{2+} content was assessed by measuring the area under the thapsigargin-evoked rise in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} as described before [29]. Our data showed no significant change in ER Ca^{2+} content in Huh-7, HeLa and HEK293 cells in the presence or absence of external Ca^{2+} after 72 h (Fig. 4A) while $[Ca^{2+}]_i$ was decreased from 113 ± 13 nM (n = 15) to 20 ± 3 nM (n = 30), 108 ± 9 nM (n = 20) to 90 ± 4 nM (n = 54), and 82 ± 6 nM (n = 25) to 7 ± 1 nM (n = 9) in Huh-7, HeLa and HEK293 cells respectively (Fig. 4B).

Several studies have clearly demonstrated that cell proliferation is related to calcium channel expression [13]. The apparent contradiction between these observations and our data led us to investigate the role of Orai1 and Orai3 on cell cycle progression and cell proliferation in HEK293 cells. Cell cycle analysis by DNA content estimation with flow cytometry was performed after transfection with siOrai1 (50 nM), siOrai3 (75 nM) and both siOrai1 and siOrai3 (25 and 75 nM respectively). Knock-down efficiency of these siRNAs was performed with quantitative PCR (Supplemental Fig. 2). Our results showed that siOrai3 and siOrai1 + 3 induced a significant increase in cell number in G_2/M

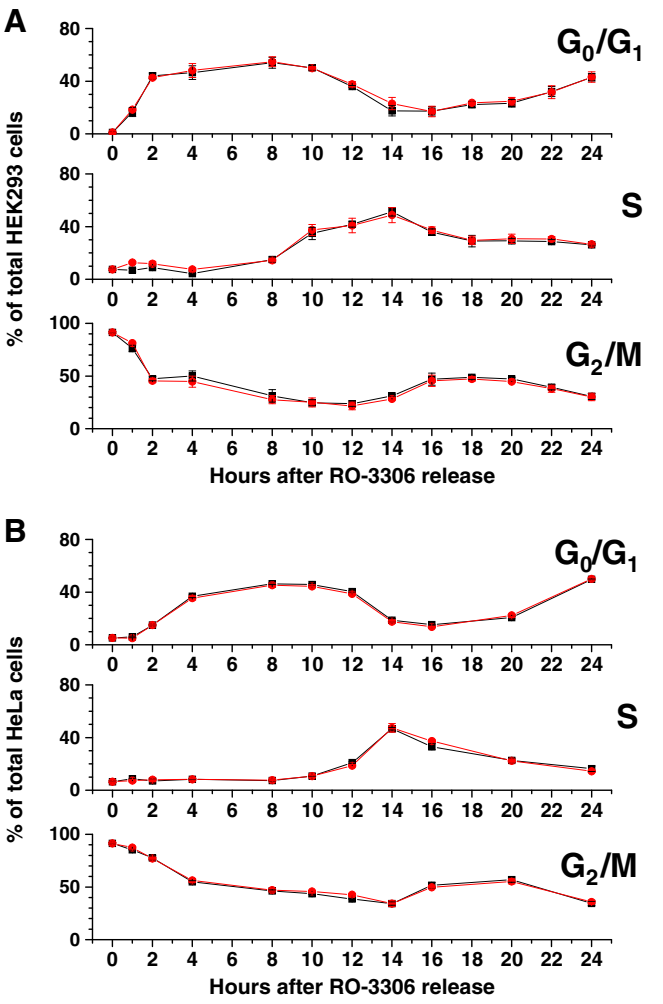

Fig. 2. Cell cycle analysis by DNA content estimation with flow cytometry in HEK293 (A) and HeLa cells (B) in Ca-free medium. Cell cycle progression was monitored after release from RO-3306 induced cell cycle arrest. Cells were treated for 24 h with 10 μM RO-3306 (t = 0) and released in complete DMEM containing 1.8 mM Ca^{2+} (black trace) or Ca-free complete DMEM supplemented with Glutamax and 150 μM EGTA (red trace) with G_0/G_1 (top), S (middle) and G_2/M (bottom). HEK293 and HeLa cells displayed a similar pattern in both conditions.

phase and that siOrai1 + 3 induced a significant decrease in S phase (Fig. 5). G_2/M was increased from 26.6 ± 1.3 (n = 15) in control cells to 31.5 ± 1.3 (n = 13) and 35.1 ± 1.6 (n = 10) in siOrai3 and siOrai1 + 3 cells respectively. S phase was decreased from 25.7 ± 1.4 (n = 15) in control cells to 20.3 ± 1.6 (n = 10) in siOrai1 + 3 cells.

Exit from mitosis and cell cycle progression was monitored in siRNA transfected HEK293 cells. Cells were blocked in G_2/M after 24 h in the presence of 10 μM RO-3306 then released in complete DMEM. Transfection with siRNA against Orai1, Orai3 and both Orai1 and Orai3 had no effect on RO-3306 induced cell cycle arrest in G_2/M (Fig. 5). Re-entry in cell cycle was checked 4 h and 16 h after release from blockade. A decrease expression in Orai1, Orai3 or both, resulted in a slower increase in G_0/G_1 and a decrease in G_2/M phases respectively 4 h after release from blockade. The number of cells in G_0/G_1 was reduced from 43.8 ± 1.1 (n = 12) in siLuc transfected control cells to 28.9 ± 3.1 (n = 14), 25.4 ± 2.6 (n = 11) and 29.3 ± 4.1 (n = 7) in siOrai1, siOrai3 and both siOrai and siOrai3 respectively. In contrast, the number of cells in G_2/M remained higher in siOrai1 (64.6 ± 2.8, n = 16), siOrai3 (66.7 ± 3.0, n = 11) and both siOrai and siOrai3 (65.4 ± 4.4, n = 7) than in siLuc cells (46.4 ± 1.4, n = 12). A very different pattern was observed 16 h after release from blockade. Indeed, siLuc transfected cells progressed along the cell cycle and entered S phase with less cells

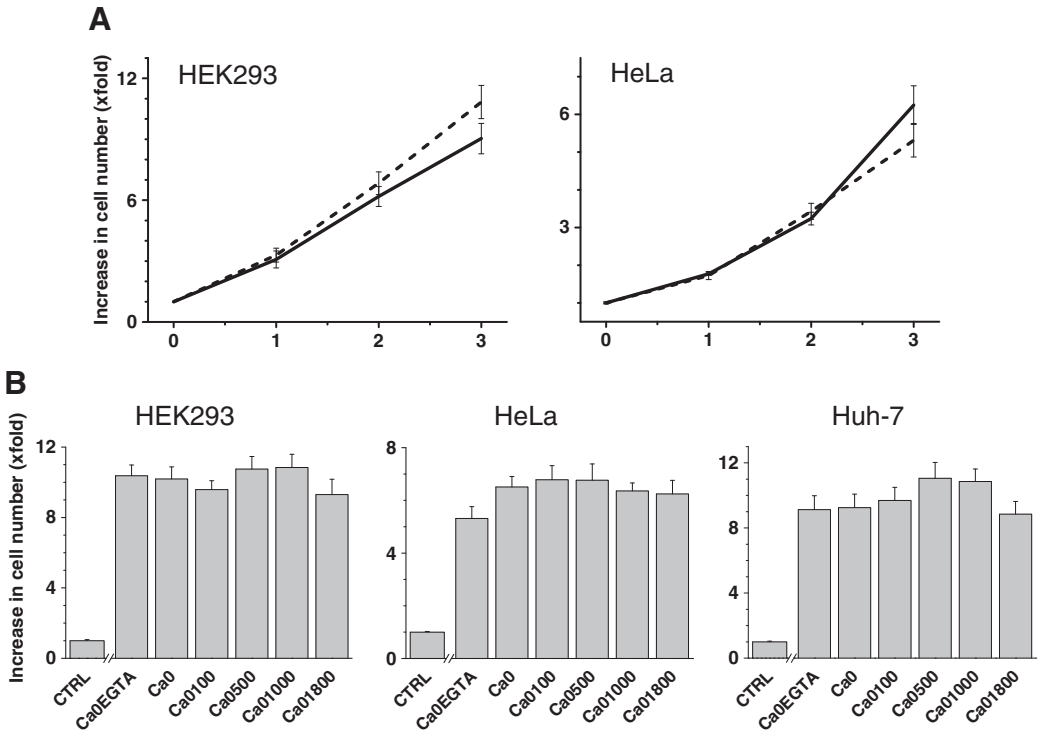

Fig. 3. Cell proliferation in HEK293, Huh-7 and HeLa cells. (A) HeK293 and HeLa cells were incubated for 3 days in the presence (solid) or absence (dash) of external Ca^{2+} as in Fig. 2. Cells were counted every day and data are summary of 3 separate experiments done in triplicate. (B) HEK293, Huh-7 and HeLa cells were incubated for 3 days in Ca-free DMEM medium supplemented with 10% FCS, Glutamax and 150 μM EGTA (Ca0EGTA), no EGTA (Ca0), 100 μM $CaCl_2$ (Ca0100), 500 μM $CaCl_2$ (Ca0500), 1000 μM $CaCl_2$ (Ca01000) and 1800 μM $CaCl_2$ (Ca01800). Cells were counted just before incubation (CTRL) in these different culture media and results are expressed as fold increase from this initial value. Histograms summarized cell numeration in 3 separate experiments done in triplicate. No significant difference was observed in all conditions.

in G_0/G_1 and G_2/M. Cell number in S phase was greatly reduced in all Orai1 and Orai3 knock-down conditions, probably due to the delayed re-entry in G_0/G_1 phase. A marked difference was observed in G_2/M phase as cells remained for a longer time in this phase in the absence of Orai3 (54.9 ± 5.3, n = 11) or both Orai1 and Orai3 (48.3 ± 4.8, n = 8) than in siOrai1 alone (32.1 ± 2.0, n = 16). This strongly suggested that Orai proteins are needed for mitosis progression with Orai3 playing the main role in this process. Mitotic index was measured in the absence of Orai1 or Orai3 using the same method as described above. While $\tau_{(1/2)}$ of the relative mitotic index after RO-3306 removal was about 1 h in siLuc transfected HEK293 cells, $\tau_{(1/2)}$ in Orai1 or Orai3 knock-downs conditions were 1 h50 and 1 h29, respectively

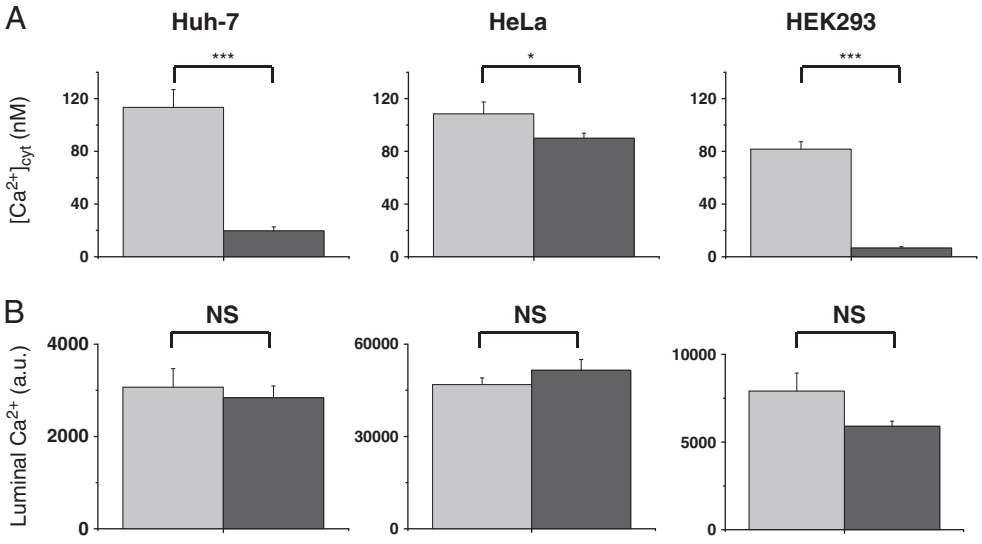

Fig. 4. Intracellular calcium concentrations in HEK293, Huh-7 and HeLa cells. Cells were incubated for 3 days in the presence (light gray) or absence (dark gray) of external Ca^{2+} as described in the previous figures. Cells were loaded for 45 min in the presence of 4 μM Fluo4-AM and washed 3 times in recording medium with or without Ca^{2+}. (A) Cytosolic calcium concentration was determined after calibration using successive applications of 10 μg/ml digitonine in the presence of 5 mM Ca^{2+} and 50 mM EGTA buffered with NaOH (pH 7.3). *P < 0.05; ***P < 0.001. (B) Aera of the fluorescence signal under the thapsigargin-induced response in the absence of external Ca^{2+}. The values in the presence or absence of external Ca^{2+} were not statistically different in all cell types. Histograms summarized data obtained in 4 separate experiments.

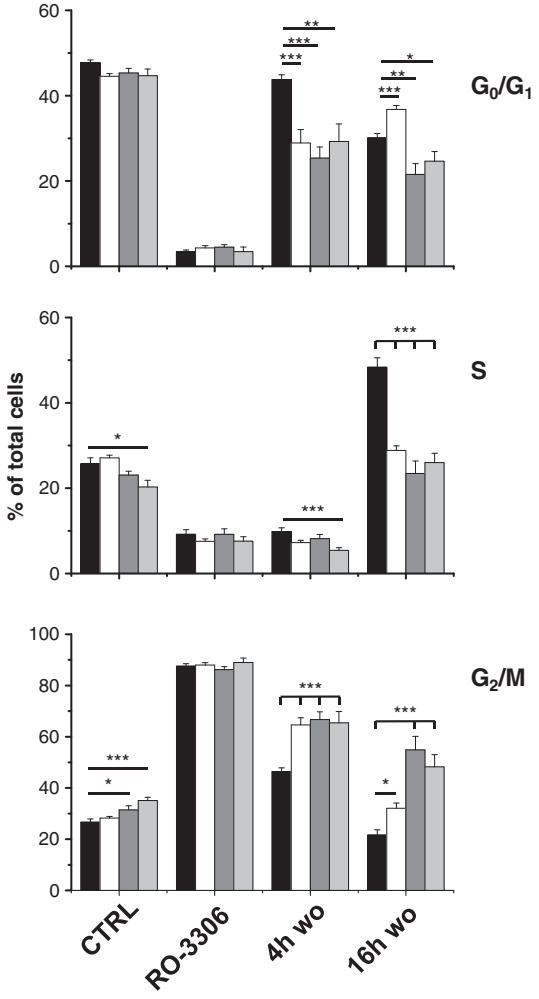

Fig. 5. Flow cytometry analysis of cell cycle after down-regulation of Orai1 and Orai3 calcium channels in HEK293 cells. Cells were transfected with siRNA against luciferase (black column), Orai1 (white), siOrai3 (dark gray) and both Orai1 and Orai3 (light gray) and cell cycle analyzed 3 days after transfection. Cell cycle was determined in control conditions (CTRL), after 24 h in the presence of 10 μM RO-3306, 4 (4 h wo) and 16 (16 h wo) after release from cell cycle arrest. The 3 sets of histograms represent the G_0/G_1 (top), S (middle) and G_2/M (bottom) phases and summarized data in 3–4 separate experiments with n ranging from 11 to 12 (CTRL), 14 to 16 (siOrai1), 9 to 11 (siOrai3) and 7 to 8 (siOrai1 + 3). *P < 0.05; **P < 0.01, ***P < 0.001.

(Supplemental Fig. 3) Similar decay times were observed for G2/M phase release (cell cycle analysis), strongly suggesting that Orai3 and in a lesser extent Orai1, are essential in mitosis transition.

The relation between Orai1 and Orai3 expression levels and cell proliferation was also investigated. Knock-down and overexpression experiments were performed to assess the role of these two calcium channels. We already showed that siRNA against Orai1 resulted in a dramatic reduction in cell proliferation rate [20]. Here, we obtained a 50% decrease of HEK293 cell number after a 48–72 h transfection with siRNA targeting either Orai3 or both Orai1 and Orai3 (Fig. 6A). Similar results were obtained in HeLa cells with siRNA against Orai1, Orai3 or both Orai1 and Orai3 (Fig. 6A). Opposite results were observed when Orai1 or Orai3 channels were overexpressed in HEK293 cells. Our results showed first that cell number was increased by 30% 72 h after transfection as compared to controls and second, that cell number increased by 8 fold within 72 h indicating that cell cycle duration was reduced to less than 16 h in overexpressing conditions as compared to 20–24 h in control conditions (Figs. 2A, 3A and 6B). Cell proliferation was also monitored in the presence of the dominant negative non-permeant mutants E106Q (Orai1) and E81Q (Orai3). It has previously showed that overexpressing E106Q and E81Q mutants resulted in the inhibition of I_{crac} and I_{arc} respectively [30,31]. Our data showed that cell proliferation rates in the presence of these mutants were increased to the same levels as for Orai1 and Orai3 channels strongly suggesting that the observed effects were independent of any increase in calcium currents. Cell cycle analysis showed a small but significant decrease in G_2/M phase in Orai3 overexpressing HEK293 cells (Fig. 6C). Overexpression of both mutants was confirmed by qPCR (Supplemental Fig. 4) and western-blots (Supplemental Fig. 5) for Orai1.

4. Discussion

The intimate relation between calcium channels expression and cell proliferation is well documented [2,3,13,32]. However, our present results showed that it is the Orai proteins themselves more than its intrinsic primary function that are required for cell proliferation in some cell types. Indeed, both Orai1 and Orai3 channels are clearly involved in I_{CRAC} and I_{arc} activity as already shown in HEK293 cells [30,33] but we assume that this may be incidental in the control of cell proliferation. In one hand, knocking down Orai channels as well as other channels involved in SOCE such as TRPC channels resulted in a reduced cell proliferation and cell cycle arrest [13]. On another hand, blocking cell proliferation resulted in a decrease calcium channel expression as previously shown for Orai1 in HEK293 [20]. Thus, blocking cell proliferation induces a decrease in SOCE as calcium channel expression is decreased but it is still unclear whether SOCE is really needed for cell proliferation. There is also evidence that knocking down STIM1 has virtually no effect on cell proliferation in an increasing number of cell types while it almost totally inhibits SOCE activity [20,34–37]. Knocking-down Orai3 was much more effective than reducing external calcium concentration in reducing cell proliferation in MCF-7 cells [38]. It was also shown that knocking down TRPC1 or TRPC4 in human endothelial cells reduced cell proliferation rate but had no effect on SOCE amplitude [34]. Finally, on the basis of our results demonstrating the lack of effect of external calcium in Huh-7 cell proliferation, our own previous work on the role of TRPC6 in Huh-7 proliferation could be interpreted as a result of channel expression rather than calcium influx amplitude [5]. Thus, the two processes, cell proliferation and calcium influx, could be unconnected, questioning the source of calcium triggering the Ca^{2+} transients observed at different stages of the cell cycle [39,40]. The other evidence in favor of an effect of calcium influx on cell proliferation comes from the use of calcium channel blockers. We, and other laboratories, have shown that calcium channel blockers, such carboxyamidotriazole or 2-APB, totally blocked cell proliferation [21,41] but it is also clear that at the concentrations used in these studies that both blockers may affect ER Ca^{2+} release mechanisms.

Cell proliferation and cell cycle arrest depend on Orai1 and Orai3 expression levels. The data here showed that Orai3 or both Orai1 and Orai3 silencing significantly increased cell number in G_2/M and blocked cell proliferation. We previously described a small increase in cell number in G_2/M in the presence of siOrai1 [20] but in this new set of experiments, this increase was no longer statistically different. Other studies have described a G_0/G_1 decrease and G_2/M increase in Orai1 silencing experiments [34] and cell proliferation inhibition in several cell types [13] and an increase in cell number in G_0/G_1 and cell proliferation inhibition in overexpressing conditions [42]. Orai3 silencing has no effect on vascular smooth muscle cell proliferation [43] but induced an increase in cell number in G_0/G_1 and cell proliferation inhibition in MCF-7 breast cells while it has no effect in another breast cell line MCF-10A [38]. Our data showed that cell proliferation was significantly increased in Orai1 and Orai3 overexpressing experiments. Overexpression of non permeant mutants E106Q and E81Q for Orai1 and Orai3 respectively suggested that this was independent of calcium influx as both mutants have been shown to block Icrac (E106Q) [31] or Iarc (E81Q) [30]. This increase in cell proliferation rates seen when overexpressing Orai channels and their respective mutants, has no significant effects on cell cycle

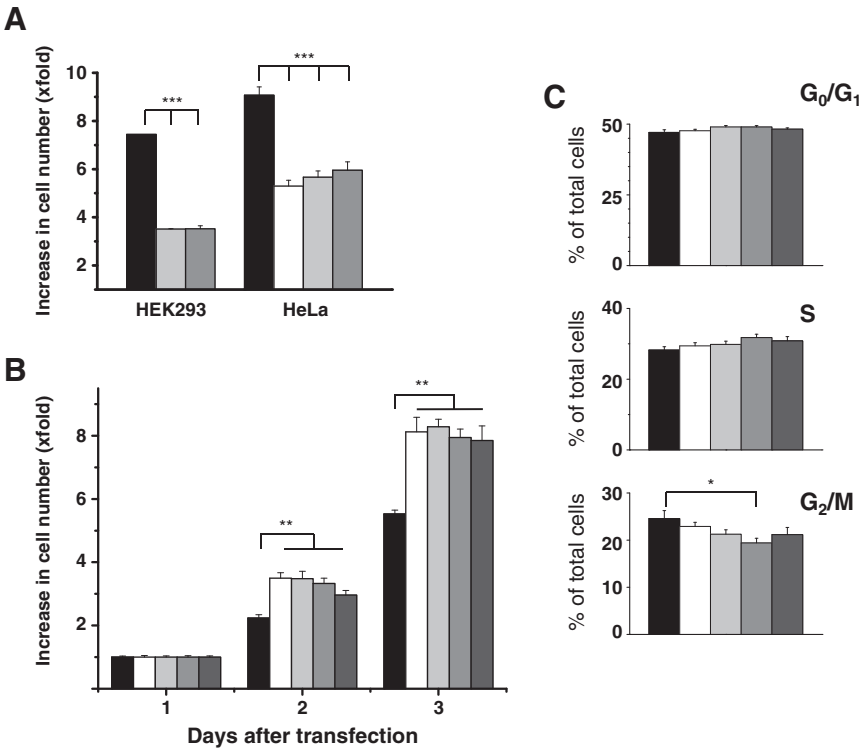

Fig. 6. Cell proliferation, cell cycle analysis and Orai channel expression A) Cell proliferation in SiRNA Orai3 and both Orai1 and Orai3-transfected HEK293 cells and in siRNA Orai1, Orai3 and both Orai1 and Orai3-transfected HeLa cells. Cells were plated and transfected the following day with siRNA against luciferase (100 nM, black), Orai1 (25 nM, white), Orai3 (75 nM, gray) or both 25 nM Orai1 and 75 nM Orai3 (dark gray). Cell number was determined 72 h after transfection and graphs are summarizing the results obtained in 3 separate experiments. $P < 0.001$ for all conditions compared to siLuc. B) HEK293 cells were transfected then trypsinized 12 h later and seeded in 60 mm dishes. Cells were transfected with empty plasmid MO70 (black), Orai1 (white), E106Q Orai1 (light gray), Orai3 (gray) and E81Q Orai3 (dark gray). Cells were counted 30, 54 and 78 h after transfection (days 1, 2 and 3 respectively) and results were normalized to cell numeration at day 1. Cell number was determined every day in triplicates for 3 days, and graphs are summarizing the results obtained in 4 separate experiments. $P < 0.001$ for all conditions compared to MO70 at day 2 and day 3. C) Flow cytometry analysis of cell cycle in the presence of Orai1, Orai3 and their respective mutants E106Q and E81Q in HEK293 cells. Cells were transfected with empty plasmid MO70 (black), Orai1 (white), E106Q Orai1 (light gray), Orai3 (gray) and E81Q Orai3 (dark gray) and cell cycle analyzed 3 days after transfection. The 3 sets of histograms represent the G_0/G_1 (top), S (middle) and G_2/M (bottom) phases and summarized data in 4 separate experiments with $n = 12$ in all conditions. No significant difference was observed compared to MO70 except in G_2/M phase in Orai3 overexpressing cells ($P < 0.05$).

distribution except for Orai3 where a small but significant decrease in G_2/M was observed in HEK203 cells strengthening the putative role for Orai3 in the transition from mitosis. We therefore suggest that the channel structure is more important than its function to control cell proliferation in the cell types used in this study. Supporting experiments using truncated Orai1 or Orai3 proteins are now needed to establish which parts of the channels are important in triggering cell proliferation. Orai1 or Orai3 channels may interact with proteins or kinases involved in cell cycle independently of calcium fluxes. TRPV1 can be directly phosphorylated by CdK5 and inhibition of CdK5 activity by roscovitine decreases TRPV1 function and Ca^{2+} influx [44] or L-type $Ca_v1.2$ activity [45]. InsP3 receptors also are likely to be regulated by cyclin-dependent kinases [46,47]. However, to date, there is no evidence for Orai1 or Orai3 interactions with any member of the cyclin/cyclin-dependent kinase complexes.

Another tempting hypothesis is based on STIM1 and STIM2 potential to bind several other intracellular proteins than Orai channels [48,49]. An increase or a decrease in Orai channels expression therefore may affect the amount of STIM proteins available for alternative signaling pathways. A domino like effect would even complicate data analysis when the outputs are not direct as they are in SOCE activation for instances but delayed in time when cell cycle is involved. Studies aimed at identification of associating partners of Orai and STIM proteins in non-CRAC channel-related functions should greatly enhance our understanding of their physiological roles.

Frequency of $[Ca^{2+}]_i$ transients is crucial in determining the specificity of downstream cellular effectors [50,51]. The role of calcium influx in the control of this frequency is controversial. If it is recognized that calcium influx plays a major role in controlling this frequency in agonist-evoked Ca^{2+} signals [52,53], there is evidence showing that these Ca^{2+} transients are observed in the presence of channel blockers [54,55] or in Orai1 [56] or Orai3 [57] knock-down HEK293 cells. In our experiments, cells are kept in the continuous presence of 10% fetal calf serum and we have no evidence for spontaneous calcium transients in these conditions, hence for SOCE activation. All together, these data strongly suggest that Ca^{2+} influx, whatever its nature, is not essential for $[Ca^{2+}]_i$ transients and for cell proliferation and that another source of Ca^{2+} may be of major importance in these physiological responses.

Cell proliferation is also highly sensitive to ER Ca^{2+} content [58–60]. In our experimental conditions, Huh-7, HeLa and HEK293 cells, incubated for 72 h in a complete culture medium containing approximately 2 μM free Ca^{2+}, had the same amount of intraluminal Ca^{2+} as in control cells. Maintaining ER Ca^{2+} content could be obtained through an inhibition of ER Ca^{2+} leakage. The nature of the channel involved is not established yet and includes a variety of ER proteins including the translocon complex, polycystin-2, presenilins, members of anti-apoptotic proteins of the Bcl2 and Bcl-2-associated X protein (Bax)-inhibitor-1 (BI-1) families, and pannexins. They have all been reported to produce an ER Ca^{2+} leak as part of their cellular mechanism [61]. In contrast, $[Ca^{2+}]_i$ was significantly reduced in all 3 cell types. Lower $[Ca^{2+}]_i$ may participate in ER channel regulation. InsP3 and ryanodine receptors are less likely to open at low $[Ca^{2+}]_i$ [62,63] and it is possible that leak ER channels follow a similar behavior. Indeed, this is yet to be proven but this would represent an interesting mean to maintain ER Ca^{2+} content in cells kept in the absence of external Ca^{2+} for a long period of time. In the same extent, calcium extrusion should be reduced at

low $[Ca^{2+}]_i$ as PMCA activity is decreased [64]. Altogether, it is likely that the combination of all these effects led to cell survival at least over the 3-day period of our study.

Knocking down Orai1 and Orai3 resulted in a clearer effect on cell proliferation than on cell cycle arrest. Cell proliferation was slower but not totally inhibited when both calcium channels were down-regulated. The small but significant rise in cell number in G_2/M observed in the absence of Orai3 and both Orai1 and Orai3, is probably underlying a bottle-neck effect rather than a complete cell cycle arrest. Our results showed that cells are still able to progress through mitosis but at a reduced rate. This is also probably due to the fact that both channels are still present. We previously observed a 70% reduction in Orai1 expression in our experimental conditions [20] and our data showed that Orai3 mRNA was also reduced by the same amount. Knocking-down Orai1 had no effect on mRNA Orai3 levels and conversely (Supplemental Fig. 2). However, as cell proliferation is greatly reduced in siOrai3 condition, it is likely that Orai3 protein expression may also interfere in the other cell cycle phases as the significant increase in cell number in G_2/M phase is about 10%. The question now is to understand how these two channels can control mitosis and cell cycle progression independently of external calcium and therefore calcium influx.

Recent work has demonstrated that many ion channels can directly influence biochemical events in ways that do not directly depend on their function as ion channels [65]. Although many signaling molecules are known to associate with ion channels, the finding that an integral element of the channel also catalyzes an enzymatic reaction or participates in cell–cell interactions strongly suggests that changes in channel activity could influence these processes [65]. New functions for voltage-gated Ca^{2+} channel auxiliary beta subunits have been recently identified. These functions appear to be regulated by the beta subunit alone, independently of any effects on the Ca^{2+} influx; hence, the beta subunit may not be truly "auxiliary" and may play more fundamental roles in Ca^{2+} homeostasis or gene regulation [66,67]. More recently, a proteolytic fragment of the C-terminus of the pore-forming subunit of L-type channels (Cav1.2) was shown to act as a transcription factor [68]. For instances, it is likely that the deletion of the N-term or C-term of Orai1 and Orai3 channels should provide valuable clues on the putative regions linking channel expression and cell proliferation. It is therefore possible that new functions for ORAI channels could be uncovered in near future and that a direct link between the channel structure and transcription factors would explain why some cells need the channel more than the function to proliferate.

5. Conclusion

Cell proliferation is regulated by calcium channel expression although it is insensitive to external calcium in several cell lines. This original observation may imply additional functions for these proteins and further experiments are now needed to reveal how they could interfere with cell cycle progression and mitosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.11.023.

References

[1] A. Panner, R.D. Wurster, T-type calcium channels and tumor proliferation, Cell Calcium 40 (2006) 253–259.
[2] A.B. Parekh, Store-operated CRAC channels: function in health and disease, Nat. Rev. 9 (2010) 399–410.
[3] J.T. Taylor, X.B. Zeng, J.E. Pottle, K. Lee, A.R. Wang, S.G. Yi, J.A. Scruggs, S.S. Sikka, M. Li, Calcium signaling and T-type calcium channels in cancer cell cycling, World J. Gastroenterol. 14 (2008) 4984–4991.
[4] C. Van Haute, D. De Ridder, B. Nilius, TRP channels in human prostate, ScientificWorldJournal 10 (2010) 1597–1611.
[5] C. El Boustany, G. Bidaux, A. Enfissi, P. Delcourt, N. Prevarskaya, T. Capiod, Capacitative calcium entry and transient receptor potential canonical 6 expression control human hepatoma cell proliferation, Hepatology 47 (2008) 2068–2077.
[6] A.B. Parekh, J.W. Putney Jr., Store-operated calcium channels, Physiol. Rev. 85 (2005) 757–810.
[7] S.J. Roberts-Thomson, A.A. Peters, D.M. Grice, G.R. Monteith, ORAI-mediated calcium entry: mechanism and roles, diseases and pharmacology, Pharmacol. Ther. 127 (2010) 121–130.
[8] N. Azad, A. Perroy, E. Gardner, C.K. Imamura, C. Graves, G.A. Sarosy, L. Minasian, H. Kotz, M. Raggio, W.D. Figg, E.C. Kohn, A phase I study of paclitaxel and continuous daily CAI in patients with refractory solid tumors, Cancer Biol. Ther. 8 (2009) 1800–1805.
[9] M.M. Hussain, H. Kotz, L. Minasian, A. Premkumar, G. Sarosy, E. Reed, S. Zhai, S.M. Steinberg, M. Raggio, V.K. Oliver, W.D. Figg, E.C. Kohn, Phase II trial of carboxyamidotriazole in patients with relapsed epithelial ovarian cancer, J. Clin. Oncol. 21 (2003) 4356–4363.
[10] E.A. Johnson, R.S. Marks, S.J. Mandrekar, S.L. Hillman, M.D. Hauge, M.D. Bauman, E.J. Wos, D.F. Moore, J.W. Kugler, H.E. Windschitl, D.L. Graham, A.M. Bernath Jr., T.R. Fitch, G.S. Soori, J.R. Jett, A.A. Adjei, E.A. Perez, Phase III randomized, double-blind study of maintenance CAI or placebo in patients with advanced non-small cell lung cancer (NSCLC) after completion of initial therapy (NCCTG 97-24-51), Lung Cancer 60 (2008) 200–207.
[11] O. Mignen, C. Brink, A. Enfissi, A. Nadkarni, T.J. Shuttleworth, D.R. Giovannucci, T. Capiod, Carboxyamidotriazole-induced inhibition of mitochondrial calcium import blocks capacitative calcium entry and cell proliferation in HEK-293 cells, J. Cell Sci. 118 (2005) 5615–5623.
[12] T. Capiod, Cell proliferation, calcium influx and calcium channels, Biochimie 93 (2011) 2075–2079.
[13] T. Capiod, The need for calcium channels in cell proliferation, Recent Pat. Anticancer Drug Discov. 8 (2013) 4–17.
[14] S.D. Balk, Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium, Proc. Natl. Acad. Sci. U. S. A. 68 (1971) 271–275.
[15] A.L. Boynton, J.F. Whitfield, R.J. Isaacs, H.J. Morton, Control of 3T3 cell proliferation by calcium, In Vitro 10 (1974) 12–17.
[16] S.H. Swierenga, J.F. Whitfield, S. Karasaki, Loss of proliferative calcium dependence: simple in vitro indicator of tumorigenicity, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 6069–6072.
[17] A.L. Boynton, J.F. Whitfield, Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells, Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 1651–1654.
[18] S.H. Swierenga, J.F. Whitfield, H.P. Morris, The reduced extracellular calcium requirement for proliferation by neoplastic hepatocytes, In Vitro 14 (1978) 527–535.
[19] M. Bodding, Reduced store-operated Ca(2+) currents in rat basophilic leukaemia cells cultured under serum-free conditions, Cell Calcium 30 (2001) 141–150.
[20] C. El Boustany, M. Katsogiannou, P. Delcourt, E. Dewailly, N. Prevarskaya, A.S. Borowiec, T. Capiod, Differential roles of STIM1, STIM2 and Orai1 in the control of cell proliferation and SOCE amplitude in HEK293 cells, Cell Calcium 47 (2010) 350–359.
[21] A. Enfissi, S. Prigent, P. Colosetti, T. Capiod, The blocking of capacitative calcium entry by 2-aminoethyl diphenylborate (2-APB) and carboxyamidotriazole (CAI) inhibits proliferation in Hep G2 and Huh-7 human hepatoma cells, Cell Calcium 36 (2004) 459–467.
[22] D. Tani, M.K. Monteilh-Zoller, A. Fleig, R. Penner, Cell cycle-dependent regulation of store-operated I(CRAC) and Mg2+-nucleotide-regulated MagNuM (TRPM7) currents, Cell Calcium 41 (2007) 249–260.
[23] F. Yu, L. Sun, K. Machaca, Orai1 internalization and STIM1 clustering inhibition modulate SOCE inactivation during meiosis, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17401–17406.
[24] F. Yu, L. Sun, K. Machaca, Constitutive recycling of the store-operated Ca2+ channel Orai1 and its internalization during meiosis, J. Cell Biol. 191 (2010) 523–535.
[25] J.T. Smyth, J.G. Petranka, R.R. Boyles, W.I. DeHaven, M. Fukushima, K.L. Johnson, J.G. Williams, J.W. Putney Jr., Phosphorylation of STIM1 underlies suppression of store-operated calcium entry during mitosis, Nat. Cell Biol. 11 (2009) 1465–1472.
[26] E.W. Moore, Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes, J. Clin. Invest. 49 (1970) 318–334.
[27] Y. Gwack, S. Srikanth, S. Feske, F. Cruz-Guilloty, M. Oh-hora, D.S. Neems, P.G. Hogan, A. Rao, Biochemical and functional characterization of Orai proteins, J. Biol. Chem. 282 (2007) 16232–16243.
[28] L.T. Vassilev, C. Tovar, S. Chen, D. Knezevic, X. Zhao, H. Sun, D.C. Heimbrook, L. Chen, Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 10660–10665.
[29] S. Bergling, R. Dolmetsch, R.S. Lewis, J. Keizer, A fluorometric method for estimating the calcium content of internal stores, Cell Calcium 23 (1998) 251–259.
[30] O. Mignen, J.L. Thompson, T.J. Shuttleworth, Both Orai1 and Orai3 are essential components of the arachidonate-regulated Ca2+-selective (ARC) channels, J. Physiol. 586 (2008) 185–195.
[31] M. Vig, A. Beck, J.M. Billingsley, A. Lis, S. Parvez, C. Peinelt, D.L. Koomoa, J. Soboloff, D.L. Gill, A. Fleig, J.P. Kinet, R. Penner, CRACM1 multimers form the ion-selective pore of the CRAC channel, Curr. Biol. 16 (2006) 2073–2079.
[32] N. Prevarskaya, L. Zhang, G. Barritt, TRP channels in cancer, Biochim. Biophys. Acta 1772 (2007) 937–946.
[33] S. Feske, Y. Gwack, M. Prakriya, S. Srikanth, S.H. Puppel, B. Tanasa, P.G. Hogan, R.S. Lewis, M. Daly, A. Rao, A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function, Nature 441 (2006) 179–185.
[34] I.F. Abdullaev, J.M. Bisaillon, M. Potier, J.C. Gonzalez, R.K. Motiani, M. Trebak, Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation, Circ. Res. 103 (2008) 1289–1299.
[35] F. Antigny, N. Girardin, M. Frieden, Transient receptor potential canonical channels are required for in vitro endothelial tube formation, J. Biol. Chem. 287 (2012) 5917–5927.

[36] B. Darbellay, S. Arnaudeau, S. Konig, H. Jousset, C. Bader, N. Demaurex, L. Bernheim, STIM1- and Orai1-dependent store-operated calcium entry regulates human myoblast differentiation, J. Biol. Chem. 284 (2009) 5370–5380.
[37] J. Li, P. Sukumar, C.J. Milligan, B. Kumar, Z.Y. Ma, C.M. Munsch, L.H. Jiang, K.E. Porter, D.J. Beech, Interactions, functions, and independence of plasma membrane STIM1 and TRPC1 in vascular smooth muscle cells, Circ. Res. 103 (2008) e97–e104.
[38] M. Faouzi, F. Hague, M. Potier, A. Ahidouch, H. Sevestre, H. Ouadid-Ahidouch, Down-regulation of Orai3 arrests cell-cycle progression and induces apoptosis in breast cancer cells but not in normal breast epithelial cells, J. Cell. Physiol. 226 (2011) 542–551.
[39] L. Santella, The role of calcium in the cell cycle: facts and hypotheses, Biochem. Biophys. Res. Commun. 244 (1998) 317–324.
[40] R.A. Steinhardt, J. Alderton, Intracellular free calcium rise triggers nuclear envelope breakdown in the sea urchin embryo, Nature 332 (1988) 364–366.
[41] H. Liu, J.D. Hughes, S. Rollins, B. Chen, E. Perkins, Calcium entry via ORAI1 regulates glioblastoma cell proliferation and apoptosis, Exp. Mol. Pathol. 91 (2011) 753–760.
[42] M.F. Hou, H.C. Kuo, J.H. Li, Y.S. Wang, C.C. Chang, K.C. Chen, W.C. Chen, C.C. Chiu, S. Yang, W.C. Chang, Orai1/CRACM1 overexpression suppresses cell proliferation via attenuation of the store-operated calcium influx-mediated signalling pathway in A549 lung cancer cells, Biochim. Biophys. Acta 1810 (2011) 1278–1284.
[43] M. Potier, J.C. Gonzalez, R.K. Motiani, I.F. Abdullaev, J.M. Bisaillon, H.A. Singer, M. Trebak, Evidence for STIM1- and Orai1-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration, FASEB J. 23 (2009) 2425–2437.
[44] T.K. Pareek, J. Keller, S. Kesavapany, N. Agarwal, R. Kuner, H.C. Pant, M.J. Iadarola, R.O. Brady, A.B. Kulkarni, Cyclin-dependent kinase 5 modulates nociceptive signaling through direct phosphorylation of transient receptor potential vanilloid 1, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 660–665.
[45] V. Yarotskyy, K.S. Elmslie, Roscovitine, a cyclin-dependent kinase inhibitor, affects several gating mechanisms to inhibit cardiac L-type (Ca(V)1.2) calcium channels, Br. J. Pharmacol. 152 (2007) 386–395.
[46] K. Malathi, X. Li, O. Krizanova, K. Ondrias, K. Sperber, V. Ablamunits, T. Jayaraman, Cdc2/cyclin B1 interacts with and modulates inositol 1,4,5-trisphosphate receptor (type 1) functions, J. Immunol. 175 (2005) 6205–6210.
[47] V. Vanderheyden, B. Devogelaere, L. Missiaen, H. De Smedt, G. Bultynck, J.B. Parys, Regulation of inositol 1,4,5-trisphosphate-induced Ca2 + release by reversible phosphorylation and dephosphorylation, Biochim. Biophys. Acta 1793 (2009) 959–970.
[48] S. Srikanth, Y. Gwack, Orai1, STIM1, and their associating partners, J. Physiol. 590 (2012) 4169–4177.
[49] S. Srikanth, B. Ribalet, Y. Gwack, Regulation of CRAC channels by protein interactions and post-translational modification, Channels (Austin) 7 (2013).
[50] P. De Koninck, H. Schulman, Sensitivity of CaM kinase II to the frequency of Ca2 + oscillations, Science 279 (1998) 227–230.
[51] R.E. Dolmetsch, K. Xu, R.S. Lewis, Calcium oscillations increase the efficiency and specificity of gene expression, Nature 392 (1998) 933–936.
[52] T. Kawanishi, L.M. Blank, A.T. Harootunian, M.T. Smith, R.Y. Tsien, Ca2 + oscillations induced by hormonal stimulation of individual fura-2-loaded hepatocytes, J. Biol. Chem. 264 (1989) 12859–12866.
[53] M.D. Bootman, K.W. Young, J.M. Young, R.B. Moreton, M.J. Berridge, Extracellular calcium concentration controls the frequency of intracellular calcium spiking independently of inositol 1,4,5-trisphosphate production in HeLa cells, Biochem. J. 314 (Pt 1) (1996) 347–354.
[54] G.S. Bird, J.W. Putney Jr., Capacitative calcium entry supports calcium oscillations in human embryonic kidney cells, J. Physiol. 562 (2005) 697–706.
[55] J. Sneyd, K. Tsaneva-Atanasova, D.I. Yule, J.L. Thompson, T.J. Shuttleworth, Control of calcium oscillations by membrane fluxes, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 1392–1396.
[56] B. Wedel, R.R. Boyles, J.W. Putney Jr., G.S. Bird, Role of the store-operated calcium entry proteins Stim1 and Orai1 in muscarinic cholinergic receptor-stimulated calcium oscillations in human embryonic kidney cells, J. Physiol. 579 (2007) 679–689.
[57] J.L. Thompson, T.J. Shuttleworth, Orai channel-dependent activation of phospholipase C-delta: a novel mechanism for the effects of calcium entry on calcium oscillations, J. Physiol. 589 (2011) 5057–5069.
[58] D.L. Gill, R.T. Waldron, K.E. Rys-Sikora, C.A. Ufret-Vincenty, M.N. Graber, C.J. Favre, A. Alfonso, Calcium pools, calcium entry, and cell growth, Biosci. Rep. 16 (1996) 139–157.
[59] L. Lipskaia, J.S. Hulot, A.M. Lompre, Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation, Pflugers Arch. 457 (2009) 673–685.
[60] A.D. Short, J. Bian, T.K. Ghosh, R.T. Waldron, S.L. Rybak, D.L. Gill, Intracellular Ca2 + pool content is linked to control of cell growth, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 4986–4990.
[61] E. Sammels, J.B. Parys, L. Missiaen, H. De Smedt, G. Bultynck, Intracellular Ca2 + storage in health and disease: a dynamic equilibrium, Cell Calcium 47 (2010) 297–314.
[62] I. Bezprozvanny, The inositol 1,4,5-trisphosphate receptors, Cell Calcium 38 (2005) 261–272.
[63] G. Meissner, Ryanodine receptor/Ca2 + release channels and their regulation by endogenous effectors, Annu. Rev. Physiol. 56 (1994) 485–508.
[64] F. Di Leva, T. Domi, L. Fedrizzi, D. Lim, E. Carafoli, The plasma membrane Ca2 + ATPase of animal cells: structure, function and regulation, Arch. Biochem. Biophys. 476 (2008) 65–74.
[65] L.K. Kaczmarek, Non-conducting functions of voltage-gated ion channels, Nat. Rev. Neurosci. 7 (2006) 761–771.
[66] M. Barbado, K. Fablet, M. Ronjat, M. De Waard, Gene regulation by voltage-dependent calcium channels, Biochim. Biophys. Acta 1793 (2009) 1096–1104.
[67] M. Rousset, T. Cens, P. Charnet, Alone at last! New functions for Ca2 + channel beta subunits? Sci. STKE 2005 (2005) pe11.
[68] N. Gomez-Ospina, F. Tsuruta, O. Barreto-Chang, L. Hu, R. Dolmetsch, The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor, Cell 127 (2006) 591–606.