On the role of TRPC1 in control of Ca\(^{2+}\) influx, cell volume, and cell cycle

C. P. Madsen, T. K. Klausen, A. Fabian, B. J. Hansen, S. F. Pedersen, and E. K. Hoffmann

1Department of Biology, University of Copenhagen, Copenhagen, Denmark; 2Institut für Physiologie II, Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany

Submitted 4 August 2011; accepted in final form 21 June 2012

Madsen CP, Klausen TK, Fabian A, Hansen BJ, Pedersen SF, Hoffmann EK. On the role of TRPC1 in control of Ca\(^{2+}\) influx, cell volume, and cell cycle. Am J Physiol Cell Physiol 303: C625–C634, 2012. First published June 27, 2012; doi:10.1152/ajpcell.00287.2011.—Ca\(^{2+}\) signaling plays a crucial role in control of cell cycle progression, but the understanding of the dynamics of Ca\(^{2+}\) influx and release of Ca\(^{2+}\) from intracellular stores during the cell cycle is far from complete. The aim of the present study was to investigate the role of the free extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\text{o}\)]) in cell proliferation, the pattern of changes in the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\text{i}\)]) during cell cycle progression, and the role of the transient receptor potential channel (TRPC1) in these changes as well as in cell cycle progression and cell volume regulation. In Ehrlich Lettré Ascites (ELA) cells, [Ca\(^{2+}\)\(\text{o}\)] decreased significantly, and the thapsigargin-releasable Ca\(^{2+}\) pool in the intracellular stores increased in G1 as compared with G0. Store-depletion-operated Ca\(^{2+}\) entry (SOCE) and TRPC1 protein expression level were both higher in G1 than in G0 and S phase, in parallel with a more effective volume regulation after swelling [regulatory volume decrease (RVD)] in G1 as compared with S phase. Furthermore, reduction of [Ca\(^{2+}\)\(\text{o}\)] as well as two un specific SOCE inhibitors, 2-APB (2-aminoethyldiphenyl borinate) and SKF96365 (1-(β-[3-(4-methoxy-phenyl)propoxy-4-methoxyphenethyl])1H-imidazole-hydrochloride), inhibited ELA cell proliferation. Finally, Madin-Darby canine kidney cells in which TRPC1 was stably silenced [TRPC1 knockdown (TRPC1-KD) MDCK] exhibited reduced SOCE, slower RVD, and reduced cell proliferation compared with mock controls. In conclusion, in ELA cells, SOCE and TRPC1 both seem to be upregulated in G1 as compared with S phase, concomitant with an increased rate of RVD. Furthermore, TRPC1-KD MDCK cells exhibit decreased SOCE, decreased RVD, and decreased proliferation, suggesting that, at least in certain cell types, TRPC1 is regulated during cell cycle progression and is involved in SOCE, RVD, and cell proliferation.

TRPC1: thapsigargin; cell cycle; proliferation; volume regulation; SOCE

IT HAS BEEN SHOWN PREVIOUSLY that the free extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\text{o}\)]) plays an essential role in the regulation of cellular proliferation. Thus, Tani et al. (73) showed that a decrease in [Ca\(^{2+}\)\(\text{o}\)] to 20 μM completely abolished proliferation in rat mast cells (RBL-2H3), and Kahl and Means (22) described how several cell types are arrested in G1 at low [Ca\(^{2+}\)\(\text{o}\)] in general, G1 phase and the transition between G1 and S phase are the most [Ca\(^{2+}\)\(\text{o}\)]-sensitive stages, suggesting that influx of Ca\(^{2+}\) is important in early G1 and in the transition from G1 to S (22).

The resting level of free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(\text{i}\)]) seems to vary through the cell cycle (63), as does the pool of Ca\(^{2+}\) in intracellular stores. Thus, transient changes in [Ca\(^{2+}\)\(\text{i}\)], occur at the exit from quiescence in early G1, at the G1/S phase transition, and at the exit from M phase (35, 37). The sarco-plasmic and endoplasmic reticulum (SR/ER) Ca\(^{2+}\)-ATPase (SERCA) is upregulated in G1/S (33), increasing the amount of Ca\(^{2+}\) in intracellular stores. Furthermore, depletion of intracellular Ca\(^{2+}\) stores with the SERCA pump blocker thapsigargin was found to arrest proliferation in DDTIMF-2 smooth muscle cell (64). Depletion of the intracellular Ca\(^{2+}\) stores results in an increased plasma membrane Ca\(^{2+}\) permeability via store-operated Ca\(^{2+}\) entry (SOCE) (25, 26), which is implicated in regulation of cell proliferation (6, 27, 36, 37, 59, 62). No specific inhibitors of SOCE are available, but two compounds commonly used are 2-aminoethyldiphenyl borinate (2-APB) (5, 31) and SKF96365 (1-(β-[3-(4-methoxy-phenyl)propoxy-4-methoxyphenethyl])1H-imidazole-hydrochloride) (5, 39), 2-APB prolongs S- and G2/M and thus inhibits proliferation in human osteoblast-like cells (27). Inhibition of SOCE by SKF96365 arrested human glioma cells in G2 and M phase and thus inhibited their proliferation (7), and SKF96365 and 2-APB both inhibited proliferation of hippocampal neural progenitor cells (29).

One of the molecular determinants of SOCE is stromal interaction molecule 1 (Stim1), which acts as an intraluminal Ca\(^{2+}\) sensor in the SR (57, 58, 60, 67, 68). The high luminal [Ca\(^{2+}\)\(\text{i}\)] in the SR at rest prevents oligomerization of Stim1, but when luminal [Ca\(^{2+}\)\(\text{i}\)] is lowered, Stim1 oligomerization and clustering of Stim1 in the SR/ER are promoted. While in this conformation, Stim1 interacts with Orai1, an integral plasma membrane protein (13–15, 55, 56). Supporting interaction of Stim1 and Orai1 as a mechanism for SOCE, Orai1 knock-out/ knockdown or the expression of dominant negative forms of Orai1 almost completely prevented SOCE despite normal clustering of Stim1 (55, 56, 75–78). Although it has been proposed that Orai1 by itself could be the Ca\(^{2+}\) channel responsible for the SOCE current, the participation of canonical transient receptor potential channel (TRP) channels alone or in concert with Orai1 in this process has also been suggested (2, 3, 30, 31, 40, 46, 48, 79). Thus siRNA-mediated knockdown of TRPC1 in heart muscle cells reduces SOCE (45), and genetic disruption of TRPC1 in chicken DT40 lymphocytes attenuates the Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (see Ref. 41). The role of TRPC1 in SOCE is highly controversial. Thus some investigations found that TRPC1\(^{−/−}\) mice exhibit decreased SOCE (31), whereas others found no difference in SOCE between TRPC\(^{−/−}\) and TRPC1\(^{+/+}\) mice, neither after stretch nor after addition of thapsigargin (10). Useful reviews on TRPC1 channels in store-operated Ca\(^{2+}\) entry, their functional properties and regulation, and their physiological roles exist (9, 51, 61, 66, 74, 79).

Cell cycle progression depends on an increase in cell volume, and the rate of regulatory volume decrease (RVD) has in certain cell types been shown to change during the cell cycle (see Ref. 19). Accordingly, cell volume was found to be

Address for reprint requests and other correspondence: E. K. Hoffmann, Section of Cell- and Developmental Biology, Dept. of Biology, The August Krogh Bldg., Univ. of Copenhagen, 13 Universitetsparken, DK-2100, Copenhagen, Denmark (e-mail: ekhoffmann@bio.ku.dk).

http://www.ajpcell.org

0363-6143/12 Copyright © 2012 the American Physiological Society

C625

http://ajpcell.physiology.org/ by 10.220.32.247 on July 8, 2017
greatest in the M phase and smallest in the G1 phase in CNE-2Z cells and to increase in parallel to the G1/S transition in fibroblasts (see Ref. 19). In Ehrlich Leukemia Ascites (ELA) cells, we find that there is a significant water uptake and cell swelling in S phase (24). Hence, TRPC1 could in principle impact on cell proliferation through an effect on cell volume. The role of TRPCs in cell volume regulation seems to be to mediate an increase in [Ca\(^{2+}\)], which activates Ca\(^{2+}\)-activated K\(^+\) and Cl\(^-\) channels (19). Several TRPC channels are sensitive to cell swelling or to membrane stretch and might work as volume sensors (see Refs. 52 and 53). For TRPC1 this is, however, a rather controversial subject. TRPC1 was proposed to be involved in the swelling activated cation current in Xenopus oocytes (34), but subsequent studies questioned whether TRPC1 could mediate mechanosensitive currents (10, 17). TRPC1 expression and SOCE are higher in proliferating cells than in cells in G0 (16, 69). Exactly how SOCE is regulated through the cell cycle is not well described, but in rat mast cells it has been shown that SOCE is increased at the G1/S transition, remains high in S phase, and decreases dramatically in M phase (73). To support the conclusion that TRPC1 is implicated in stimulation of proliferation, it is found that TRPC1 antisense oligonucleotides reduce cell proliferation in human pulmonary artery myocytes, and several growth factors activate TRPC1 (16, 21, 45, 70).

Here we investigated the correlation between TRPC1 expression and SOCE through the cell cycle, the role of TRPC1 in Ca\(^{2+}\) influx via SOCE, and the possible roles of TRPC1 in RVD and cell proliferation. We find that [Ca\(^{2+}\)]\(_i\) is decreased and the thapsigargin-releasable Ca\(^{2+}\) stores are increased in G1 as compared with G0 and that SOCE, TRPC1 protein level, and RVD capacity are all slightly increased in G1 and decreased in S. Finally, Madin-Darby canine kidney (MDCK-F) cells in which TRPC1 was stably silenced exhibited reduced SOCE, slower RVD, and reduced cell proliferation. It is suggested that, at least in certain cell types, TRPC1 is involved in SOCE, RVD, and cell cycle progression.

**MATERIALS AND METHODS**

Experimental solutions and reagents. The standard isotonic Ringer IR (310 mOsm) contained (in mM) 143 NaCl, 5 KCl, 1 MgSO\(_4\), 1 Na\(_2\)HPO\(_4\), 1 CaCl\(_2\), 3.3 MOPS, 3.3 TES, and 5 HEPES (pH 7.4); hypertonic Ringer (610 mosM, IR with all concentrations except buffers and CaCl\(_2\) doubled) with 5 mM glucose; and hypertonic Ringer (155 mosM, IR with all concentrations except buffers and CaCl\(_2\) at half concentrations). Ca\(^{2+}\)-free Ringer contained 2 mM EGTA, and NaCl was substituted for CaCl\(_2\); RPMI-1640 incubation medium (Sigma-Aldrich) was supplemented with 10% FBS and 1% penicillin-streptomycin; low Ca\(^{2+}\) RPMI-1640 medium with 43 nM extracellular [Ca\(^{2+}\)] (see above) as indicated, and the cells where incubated for another 24 h. MDCK-F wild-type and transformed cell lines were seeded in black 96 well dishes (Perkin Elmer) with 6.000 cells per well in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FBS and 1% penicillin-streptomycin, at 37°C in a 5% CO\(_2\) atmosphere. After seeding (24 h), the medium was changed to RPMI-1640 with or without inhibitors of TRPC1, or low Ca\(^{2+}\) RPMI-1640 medium with 43 nM extracellular [Ca\(^{2+}\)] was added to the cells. After incubation, the cells were fixed for 30 min and incubated with monoclonal peroxidase-conjugated BrdU antibody for 90 min. After cells were thoroughly washed, 100 µl peroxidase substrate solution was added and chemiluminescence was measured (within 10 min of substrate addition) on a Fluostar-Optima microplate reader (BMGLabtech, Offenburg, Germany).

cDNA synthesis and quantitative real-time PCR. After experiments, cDNA was synthesized using SuperScript II reverse transcriptase, oligo(dt)\(_{12-18}\), and random primers according to the instructions from Invitrogen.
Quantitative real-time PCR (qPCR) was performed using Brilliant QPCR master mix (Stratagene), and the PCR reaction was carried out at 55°C using the MX4000 machine (Stratagene). Primers and Taqman probes targeting mouse TRPC1 (primer forward 5'-CCTCCTTGTCT-GTTTCTCTC-3'; primer reverse 5'-GTCATTGCTTGGCT-GTTC-3'; Taqman probe 5'-CTCAAGCTTACAAAGGGTA-CACCTCCA-3') and β-actin (reference gene) (primer forward 5'-AGACCATAGCTGCTGCCACG-3'; primer reverse 5'-GGATGCT-CACAGGATCTACATCA-3'; Taqman probe 5'-CCCTGAGGCT-CTTTCCAGCTCTTCCTT-3') were purchased from MWG, Bio- tech AG, Germany. The probes were labeled with the reporter fluorescein in the 5'-end and the quencher tetramethylrhodamine in the 3'-end. Primer and probe concentrations were optimized (data not shown). Final probe and primer concentrations in the experiments were 100 and 200 nM, respectively. As internal reference dye, 30 nM 6-carboxyl-x-

Primer and probe concentrations were optimized (data not shown). Final concentration was determined, samples for electrophoresis were prepared, and proteins were separated by SDS-PAGE and Western blot analysis. Data were analyzed by relative quantification according to the method of Pfaff (54) and presented as relative numbers with respect to values in G0 phase.

Measurement of \([Ca^{2+}]_{i}\), \([Ca^{2+}]_{o}\), was estimated using the Ca-sensitive dye Fura-2-AM. ELA cells were seeded on rectangular glass coverslips in 4 ml RPMI medium 24–48 h before the experiment. The cells were ~80–90% confluent at the time of the experiment. Twenty minutes before measurements, cells were loaded in Standard Isotonic Ringer with 10 μM Fura-2-AM and 10 μM of a mix of Pluronic F-127 (Invitrogen) and Cremofer (Sigma), on a vibrating table. The cells were washed twice with fresh Ringer, and the coverslip was placed at an angle of 50° respective to the Xenon lamp light source in a thermostatted and perfused cuvette of a PTI Ratiometer spectrophotometer. The flow rate was 2 ml/min. Fluorescence was measured at 510 nm after excitation at 340 and 380 nm. All experiments were corrected for background fluorescence before the Fura-2 ratio was calculated. The \([Ca^{2+}]_{i}\) was calculated from the Grynkiewicz equation with the following constants: \(R_{min}\), 0.6; \(R_{max}\), 13.61; and KD, 158.77 nM.

Estimation of cell volume changes-light scatter. The cells were grown to 85% confluence on rectangular glass coverslips in 4 well dishes. The cells were placed at a 50° angle, relative to the excitation light, in the stirred, thermostatted cuvette of a PTI Ratiometer spectrophotometer type C-44 in a Ringer solution containing 5 mM glucose, under constant perfusion at 700 μl/min. For estimation of cell volume changes, large angle light scattering was determined by exciting the cells at 560 nm and measuring emission at 562 nm. Light scattering values are presented as the inverse of the intensity of scattered light, normalized to the value obtained under isotonic conditions, i.e., \(I_o/I_i\), for the values to directly reflect relative cell volume.

For the RVD after regulatory volume increase (RVI) protocol, cells were perfused with isotonic Ringer with 5 mM glucose for 10–30 min to obtain a stable baseline signal. The solution was then changed to hypertonic Ringer with 5 mM glucose. To rapidly replace the Ringer in the cuvette, the flow rate was increased to 3.5 ml/min for 2 min and then returned to 0.7 ml/min. After about 20 min, the solution was again replaced with IR, and the signal recorded followed for about 20 min.

SDS-PAGE and Western blot analysis. ELA cells were grown to 80% confluence in 10-cm petri dishes (6 dishes). They were temporarily arrested in the defined G0 stage of the cell cycle by serum starvation for 24 h, and 10% fetal calf serum was then added causing the presynchronized cells to re-enter the cell cycle. At the time points 0, 5, and 15 h, respectively, two petri dishes with cells were washed with ice-cold PBS and harvested by scraping in 100 μl boiling SDS lysis buffer of 1% (vol/vol) SDS, 1 mM Na3VO4, and 10 mM Tris (pH 7.4). Extracts were homogenized by sonication, followed by removal of cell debris by centrifugation at 16,000 g for 5 min. Protein concentration was determined, samples for electrophoresis were prepared, and proteins were separated by SDS-PAGE and electrotransferred essentially as described (47). Primary antibodies (TRPC1 and Histone H3) were applied for 1 h at room temperature. The membrane was then washed, and the secondary alkaline phosphatase conjugated antibodies were applied for 1 h at RT as described previously (47). Immunoreactive bands were detected using BCIP/NBT. The bands were quantified using a HP Scanjet 4600 (Hewlett Packard Palo Alto, CA) and the software UN-SCAN-IT gel version 6.1 for Windows (Silk Scientific). The primary antibodies against TRPC1 and Histone H3 were diluted 1:100 and 1:200, respectively. The secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG were diluted 1:3,000.

Caspase 3/7 assay. ELA cells were seeded in black microtiter plates 1 to 2 days before the experiment and grown to a cell density of ~40,000 cells/well at the start of the experiment. Twenty-four hours before the assay, standard RPMI 1640 cell culture medium was substituted with low Ca2+ (Na+ substituted) RPMI 1640. Caspase 3/7 activity was measured using a fluorometric homogenous caspase assay (Roche), according to the manufacturer’s instructions. Excitation and emission are 499 and 521 nm, respectively. Fluorescence was detected using the microplate FLUOstar OPTIMA (BMG).

Trypan blue staining. ELA cells were grown to subconfluency in 75 cm2 cell culture flasks. Twenty-four hours before the assay, standard RPMI 1640 cell culture medium was substituted with low Ca2+ (Na+ substituted) RPMI 1640 in one of the flasks. After 24 h, all cells were collected. Detached cells were collected from the medium and pooled with the cells obtained from trypsination of the adherent cells. Volume was adjusted to 1 ml, and 0.1 ml 0.4% Trypan blue was added. Blue cells and total cell number were determined by counting under a light microscope. Viable cells exclude Trypan blue, whereas dead cells retain the staining.

Statistical analysis. Data are shown as means of independent experiments, with SE error bars, or as representative individual experiments. Statistical significance was analyzed by Student’s t-test (2-tailed; paired or unpaired as appropriate) for comparisons between two groups and by one-way ANOVA with Tukey-Kramer post-test for multiple comparisons. \(P < 0.05\) was taken as the level of statistical significance.

RESULTS

Role of Ca2+ in cell proliferation, changes in \([Ca^{2+}]_{i}\), and \([Ca^{2+}]_{o}\) release from intracellular stores. To determine whether \([Ca^{2+}]_{i}\) plays a role in the regulation of cellular proliferation, we estimated cell proliferation by measuring BrdU incorporation in cells in medium of normal and low \([Ca^{2+}]_{i}\). Reduction of \([Ca^{2+}]_{i}\) to 43 nM significantly reduced ELA cell proliferation compared with that of cells grown in normal medium (\([Ca^{2+}]_{i}\), 420 μM; Fig. 1A). As ELA cells were maintained in 43 nM external Ca2+ for 24 h, we monitored the possible contribution from cell death to the apparent decrease in proliferation. We found a slight increase in programmed cell death as measured by caspase 3/7 activity (Fig. 1B), whereas there was essentially no increase in Trypan blue staining of the cells (8 ± 1% in controls and 10 ± 4% in low Ca2+ medium after 24 h, \(n = 3\), \(P = 0.7\)).

We next used synchronized cells to measure the resting \([Ca^{2+}]_{i}\), and the amount of Ca2+ in the intracellular stores throughout the cell cycle. Figure 2A shows the resting \([Ca^{2+}]_{i}\), throughout the cell cycle. It is seen that the resting \([Ca^{2+}]_{i}\) is significant higher in the G0 phase compared with G1- and S phase. Accordingly, both the total increase and the rate of increase, in \([Ca^{2+}]_{i}\), after addition of 2 μM thapsigargin (thapsigargin was added to block SERCA, inducing depletion of Ca2+ from intracellular stores), were significantly higher in G1
Fig. 1. The effect of extracellular \([\text{Ca}^{2+}]\) on cell proliferation. Ehrlich Lette Ascites (ELA) cells were grown for 24 h in normal RPMI 1640 medium with 420 \(\mu\)M extracellular \([\text{Ca}^{2+}]\), after which the medium was changed to a low \([\text{Ca}^{2+}]\) medium with 43 nM extracellular \([\text{Ca}^{2+}]\), a value close to the intracellular free \([\text{Ca}^{2+}]\). This was done by substitution of 20% of normal medium with a medium where \(\text{Na}^+\) was substituted for \(\text{Ca}^{2+}\) and 5 mM EGTA was added. A: amount of proliferating cells was measured with the BrdU proliferation assay. B: caspase 3/7 activity was measured using the fluorometric homogenous caspases assay from Roche, according to the manufacturer’s instruction. Excitation and emission are 499 and 521 nm, respectively. Fluorescence was detected using the microplate FLUOstar OPTIMA (BMG). Statistical significance was analyzed by a paired t-test. ***\(P < 0.001\), **\(P < 0.01\). In A, \(n = 8\); in B, \(n = 4\). \([\text{Ca}^{2+}]_{\text{i}}\), free extracellular \([\text{Ca}^{2+}]\) concentration.

SOCE, TRPC1 mRNA, and TRPC1 protein expression during the cell cycle. Figure 4 illustrates the pattern of \([\text{Ca}^{2+}]\) in ELA cells in \(G_0\), \(G_1\), and \(S\) phase upon \([\text{Ca}^{2+}]_{\text{o}}\) removal, depletion of \([\text{Ca}^{2+}]\) from intracellular stores, and reintroduction of extracellular \([\text{Ca}^{2+}]\) as measured using Fura 2-AM. At time 0, the medium was changed to a \([\text{Ca}^{2+}]\)-free Ringer with 2 mM EGTA and thapsigargin (see Fig. 4A). As a result, \([\text{Ca}^{2+}]_{\text{i}}\) increases followed by a return to a low baseline value. When \([\text{Ca}^{2+}]_{\text{i}}\) was stable again, the medium was replaced with standard isotonic Ringer, returning \([\text{Ca}^{2+}]_{\text{i}}\) to 1 mM resulting in influx of \([\text{Ca}^{2+}]\) via SOCE (Fig. 4A). Figure 4B shows that the rise in \([\text{Ca}^{2+}]\), after reintroduction of \([\text{Ca}^{2+}]_{\text{i}}\) is slightly but insignificantly higher in \(G_1\) than in \(G_0\) and significantly lower in \(S\) phase than in \(G_1\). Figure 4C shows the rate of \([\text{Ca}^{2+}]\) influx
TRPC1 in Ca\textsuperscript{2+} influx, cell volume, and cell cycle

Fig. 3. The inhibitory effect of the store-depletion-operated Ca\textsuperscript{2+} entry (SOCE) inhibitor 2-aminoethyldiphenyl borinate (2-APB) and of the general Ca\textsuperscript{2+} channel inhibitor SKF96365 on the cell proliferation. The amount of proliferating cells was measured with the BrdU proliferation assay. ELA cells were grown for 24 h in RPMI 1640 growth medium, after which the medium was changed to a medium with an increasing concentration of either 2-APB (0–500 \mu M) or SKF96365 (0–100 \mu M). Two hours before measurement, BrdU was added to the cells. BrdU incorporation is given relative to the untreated control. A: 2-ABP. The IC\textsubscript{50} value is 84 \mu M, and, respectively, the Hill coefficient (R) is 2.2. B: SKF96365 (for which a Hill coefficient could not be determined). The IC\textsubscript{50} value is estimated at ~5 \mu M. Statistical analysis was performed using repeated-measures ANOVA and Tukey-Kramer post-test (**P < 0.01, and ***P < 0.001). In A, n = 4; in B, n = 6.

\[ \Delta [\text{Ca}^{2+}]_i (\text{nM/s}) \text{ calculated as the difference between } [\text{Ca}^{2+}]_i \text{, before and after addition of extracellular Ca}^{2+} \text{, divided with the time from resting } [\text{Ca}^{2+}]_i \text{, to maximal } [\text{Ca}^{2+}]_i \text{]. \]

The role of TRPC1 in volume regulation in ELA cells. In many cell types, cell proliferation is closely coupled to the capacity of the cell for volume regulation (see Ref. 19). Therefore, next we measured the RVD response after osmotic swelling in the various phases of the cell cycle, using large angle light scattering. As seen, RVD (measured as volume recovery 3 min after max swelling) was significantly greater in G\textsubscript{1} as compared with S phase cells and also tended to be greater compared with G\textsubscript{0} cells, albeit not statistically significant (Fig. 5).

Taken together, these data indicate that TRPC1 is important for cell proliferation and that the protein expression of TRPC1 correlates with SOCE and with RVD in ELA cells.

TRPC1 knockdown and SOCE, volume regulation, and proliferation. To directly investigate whether TRPC1 is involved in SOCE we next monitored SOCE in TRPC1-overexpressing (hTRPC1-HA) and TRPC1 knockdown (TRPC1-KD) MDCK cells, and their respective controls, using the same approach as in Fig. 4. As seen in Fig. 6, SOCE was significantly reduced after TRPC1 knockdown, indicating that TRPC1 contributes to SOCE in MDCK-F cells. In contrast, SOCE was similar in TRPC1 overexpressing cells and their vector controls. We next measured the capacity for volume regulation as RVD after regulatory volume increase in the TRPC1-overexpressing and TRPC1-KD MDCK cells and their respective controls, using large angle light scattering. RVD was measured as either 2 min volume recovery or as initial rate of volume recovery and was significantly inhibited in TRPC1-KD MDCK cells compared with mock cells (Fig. 7). Surprisingly, the TRPC1 overexpressing cells also exhibited a significantly reduced RVD response (19 ± 4% during the 2-min volume recovery period, compared with 76 ± 13% in the vector control cells). Finally, Fig. 8 shows that the proliferation rate was decreased by 20% in the TRPC1-KD MDCK cells compared with their mock controls. Taken together, these data indicate that the cellular level of TRPC1 is important for SOCE, cell proliferation, and RVD.

DISCUSSION

Modification of [Ca\textsuperscript{2+}]\textsubscript{i}, plays an essential role in cell proliferation. TRPC1 is a ubiquitously expressed Ca\textsuperscript{2+} channel that has been proposed to be a component of store-operated channels (SOCE) and has been implicated in the regulation of a wide variety of physiological processes, including volume regulation, cell proliferation, differentiation, and apoptosis. The possible role of TRPC1 in SOCE is, however, controversial, and its involvement in cell proliferation and cell volume regulation-two closely coupled processes—is poorly understood. Here, we sought to obtain further understanding of these questions by investigating the relation between TRPC1, Ca\textsuperscript{2+} influx via SOCE, RVD, and cell proliferation in two different cell types, ELA cells and MDCK-F cells.

Ca\textsuperscript{2+} stores are increased and [Ca\textsuperscript{2+}]\textsubscript{i} reduced in G\textsubscript{1} compared with G\textsubscript{0}. In ELA cells, resting [Ca\textsuperscript{2+}]\textsubscript{i} was significantly higher in G\textsubscript{1} compared with the G\textsubscript{1} and the S phases, suggesting that resting [Ca\textsuperscript{2+}]\textsubscript{i} is increased in quiescent compared with proliferating cells. Previous studies in other cell...
types have shown an increase in \([\text{Ca}^{2+}]_i\) at the exit from quiescence in early G1 and indicated that proliferating cells generally exhibit elevated \([\text{Ca}^{2+}]_i\) compared with quiescent cells (16, 28, 69), possibly related to a role of the \(\text{Ca}^{2+}/\text{calmodulin-regulated protein kinase I}\) as an important regulator of G1 (65). A possible reason for the decrease in \([\text{Ca}^{2+}]_i\) in G1 in ELA cells could be an increased SERCA activity in G1, moving \([\text{Ca}^{2+}]_i\) from the cytoplasm to the ER. That would be consistent with our observation (Fig. 2) that in G1, \([\text{Ca}^{2+}]_i\) n intracellular stores is increased, as is the rate of \([\text{Ca}^{2+}]_i\) release from the stores after blocking SERCA. The higher \([\text{Ca}^{2+}]_i\) in intracellular \(\text{Ca}^{2+}\) stores in the G1 phase would be in agreement with a higher SERCA activity in G1. It is previously found that SERCA is upregulated in the G1/S phase (33), increasing the amount of \(\text{Ca}^{2+}\) in intracellular stores (49, 50), and that the ER \(\text{Ca}^{2+}\) level is vital for the cell cycle (71, 72).

This suggests that SOCE might also vary during the cell cycle, being increased in G1.

Changes in SOCE, TRPC1 expression, and volume regulatory capacity during the cell cycle. To measure SOCE we recorded the increase in the \([\text{Ca}^{2+}]_i\), and the rate of \([\text{Ca}^{2+}]_i\) influx as a result of the addition of extracellular \(\text{Ca}^{2+}\) to cells in which the intracellular \([\text{Ca}^{2+}]_i\) stores had been depleted (see Fig. 4, A–C). The increase in \([\text{Ca}^{2+}]_i\) (the amplitude of the rise) and the rate of \([\text{Ca}^{2+}]_i\) influx both tended to be higher in G1 compared with the G0 and S phases, although the decrease from G1 to S was only significant for the rate of \([\text{Ca}^{2+}]_i\) increase (expected to correspond to SOCE). This has, to our knowledge, not previously been investigated. If the ER \(\text{Ca}^{2+}\) level in G1 is vital for the cell cycle as mentioned above, it seems reasonable that the uptake of \(\text{Ca}^{2+}\) is upregulated in

Fig. 4. SOCE and transient receptor potential (TRP)/C1 expression in the G0, G1, and S phases. A: ELA cells were loaded with 10 \(\mu\text{M Fura} 2\text{-AM as described above. At time 0, the extracellular medium was changed to a Ca}\text{2+}-free Ringer with 2 mM EGTA, and after 300 s 2 \(\mu\text{M TG was added. When [Ca}^{2+}]_i\) was reduced to a stable low value (after about 800 s), the medium was replaced with isotonic Ringer, returning free extracellular Ca\text{2+} concentration ([Ca}^{2+}]_o) to 1 mM. The experiments were performed with synchronized ELA cells as described in MATERIALS AND METHODS. B: increase in [Ca\text{2+}]_i, as a result of store-operated Ca\text{2+}-entry in the different phases in the cell cycle. The increase in [Ca\text{2+}]_i, is significant higher in the G1 compared with the S phase. C: rate of Ca\text{2+} influx, calculated as the difference between [Ca\text{2+}]_i, before and after addition of extracellular Ca\text{2+}, divided by the time from resting to maximum [Ca\text{2+}]_i. The rate of influx is significant higher in the G1 phase compared with the G0 phase. D: TRPC1 mRNA level in ELA cells in G0, G1, and S phases, respectively, determined by quantitative PCR analysis (reference gene β-actin). The TRPC1 mRNA level is significantly higher in S and G1 phases compared with the G0 phase. E: Western blot analysis of TRPC1 protein expression in G0, G1, and S phases as measured by Western blotting using antibodies against TRPC1 (Histone H3 is shown as a loading control) given relative to the value in G0 measured from cells on a parallel petri dish and run on the same gel. The experiments were performed with synchronized ELA cells grown in parallel petri dishes. E, inset, top left: representative Western blot. Statistical analysis was performed using ANOVA (*P < 0.05) with Tukey-Kramer multiple comparisons post test; n = 5 paired experiments in B and C, 12 in D, and 17 in E. E, inset, top right: TRPC1 protein levels in G1 and S phases in paired experiments (paired t-test, ***P < 0.001; n = 17).
the G1 phase to make the refilling of the ER stores more efficient. Whether there is a regulation both of the rate of Ca\(^{2+}\) entry and of the duration of the influx (the peak time for the rise in [Ca\(^{2+}\)]) should be investigated further.

With respect to TRPC1 expression we find that the TRPC1 mRNA level is upregulated in proliferating ELA cells compared with cells in the G0 phase, in agreement with what is found in other cells (16, 69). In contrast, both SOCE and volume regulatory capacity are decreased in S compared with G1 (compare Fig. 4, B and C, and Fig. 5). In agreement with this, TRPC1 protein expression, however, was decreased from G1 to S, correlating with the pattern of SOCE as well as with the volume regulatory capacity of the cells. Furthermore, SOCE was unaffected by TRPC1 overexpression in MDCK-F cells, but was strongly reduced after TRPC1 knockdown. The latter is in agreement with findings in cardiac muscle cells and red blood cells (45), and it is in agreement with the idea that TRPC1 channels, in concert with Orai1, play a role in SOCE, as previously suggested (2, 3, 30, 31, 40, 46, 48, 79). It has also been reported that TRPC\(^{-/-}\) mice have decreased SOCE (31), whereas others did not detect differences in SOCE between TRPC\(^{-/-}\) and TRPC\(^{+/+}\) mice neither after stretch nor after addition of thapsigargin (10). Hence, the role of TRPC1 in SOCE may not be ubiquitous. We speculate that the lack of increase in SOCE after TRPC1 overexpression in MDCK-F cells reflects that the balance between TRPC1 and other players in SOCE is important in determining the specific role of TRPC1.

Role of SOCE in cell proliferation. A role for SOCE in cell proliferation has been proposed previously in various cell types (27, 36, 37, 59). This also appears to be the case in ELA cells, in which we found that two unspecific SOCE inhibitors, 2-APB and SKF96365, inhibited cell proliferation. In agreement with our findings, 2-APB prolonged S and G2/M phase and thus inhibited proliferation in human osteoblast-like cells (27), and inhibition of SOCE with SKF96365 arrested human glioma cells in G2 and M and inhibited proliferation (7). It should be mentioned that 2-APB is found to activate TRPV1, TRPV2, and TRPV3 (20); however, the possible relevance of this to our results is not known.

Stable silencing of TRPC1 inhibited volume regulation and proliferation in MDCK-F cells. In TRPC1-KD MDCK-F cells, proliferation was inhibited by 20%, in agreement with previous findings in human pulmonary artery myocytes. Although cell-type specific differences as well as unspecific effects of the inhibitors may also contribute, the lesser effect of TRPC1 knockdown in MDCK-F cells compared with the effect of pharmacological inhibitors in ELA cells likely, at least in part, reflects the incomplete knockdown. That the 30% remaining TRPC1 in the knockdown cells is partially sufficient for the contribution of TRPC1 to SOCE in MDCK-F cells would also account for the fact that overexpression of TRPC1 in these cells did not increase SOCE. Supporting the idea of TRPC1 playing a role in proliferation is the observation that growth factors angiotensin II and endothelin-1 activate TRPC1 (17, 39, 56). Other TRP channels play roles in cell proliferation. Thus, in HeLa cells, downregulation of TRMP4 reduced growth significantly, apparently reflecting delayed progression from G1 to S phase of the cell cycle (4).

Fig. 6. Effect of TRPC1 knockdown in Madin-Darby canine kidney (MDCK-F) cells on SOCE. The experimental protocol was as described in Fig. 4. A: increase in [Ca\(^{2+}\)], as a result of Ca\(^{2+}\) influx through SOCE is significant higher in mock transfected cells compared with TRPC1 knockdown (TRPC1-KD) MDCK cells. B: rate of SOCE calculated as the difference between [Ca\(^{2+}\)], before and after addition of extracellular Ca\(^{2+}\), divided by the time from resting to maximum [Ca\(^{2+}\)]. The rate of influx is significantly higher in the mock control cells (Ctrl.) compared with the TRPC1-KD cells. Unpaired Student’s t-test (***(P < 0.001; n = 7 paired experiments). Max, maximum.
Volume regulatory mechanisms play an essential role in cell cycle progression (14, 25). In ELA cells, the RVD process was significantly inhibited in the S phase compared with the G1 phase, in parallel with the decreases in TRPC1 protein level and SOCE. In addition, RVD was significantly inhibited in TRPC1-KD MDCK cells compared with mock transfected cells, suggesting that in these cells, TRPC1 is involved in a Ca\textsuperscript{2+} influx during RVD, which is important for the RVD response. An involvement of Ca\textsuperscript{2+} in the RVD response following cell swelling has been found in many cell systems, particularly those of epithelial origin (15) such as the MDCK cells investigated here. Swelling of such cells appears to induce either a single increase in [Ca\textsuperscript{2+}]\textsubscript{i}, or a biphasic increase, in which both phases depend on Ca\textsuperscript{2+} influx from the extracellular compartment, whereas the second phase additionally represents Ca\textsuperscript{2+}-induced release of Ca\textsuperscript{2+} from internal stores (18). Several channels have been suggested to be responsible for swelling-induced Ca\textsuperscript{2+} entry, including nonselective stretch-activated channels, TRP channels, and voltage-dependent L-type Ca\textsuperscript{2+} channels (see Refs. 15 and 64). It has been shown that Ca\textsuperscript{2+} entry through TRPM7 channels stimulates the volume regulated anion channel activation during RVD in human epithelial cells (42). Furthermore, in mouse astrocytes, activation of volume regulated anion channel after addition of bradykinin can be mediated by “nanodomains” of high [Ca\textsuperscript{2+}] generated by both Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores and Ca\textsuperscript{2+} entry at the plasma membrane involving Orai1, TRPC3, and especially TRPC1 (1). The role of TRPC1 in RVD and in cell proliferation seen in ELA and MDCK cells in the present investigation might reflect a similar mechanism, with TRPC1 being involved in localized Ca\textsuperscript{2+} influx creating a nanodomain of high [Ca\textsuperscript{2+}]. It is noteworthy, however, that

Fig. 7. Regulatory volume decrease after regulatory volume increase (RVD after RVI) in TRPC1-KD cells and mock controls (Ctrl.). Relative cell volume during a RVD after RVI protocol was measured using large angle light scattering, in mock control MDCK-F cells (A) and TRPC1-KD MDCK cells (B). Relative cell volume is shown as the inverse of the intensity of scattered light, normalized to the value obtained under isotonic conditions, i.e., I/I\textsubscript{0}. After signal stabilization, the solution is changed to hypertonic Ringer for 20 min, followed by return to isotonic Ringer (now hypertonic to the cells), and the scatter followed over time. Quantification of the initial rate of recovery of the RVD response (C) and percent volume recovery after 2 min (D) are based on the experiments shown in A and B. Statistical analysis was performed using an unpaired Student’s t-test (*P < 0.05; n = 3). The number of independent experiments is 3 in all cases.

Fig. 8. Proliferation rate in TRPC1-KD MDCK-F cells and mock controls (Ctrl.). Proliferation was measured by BrdU incorporation. MDCK-F wild-type and TRPC1-KD cells were seeded in the black 96 well dishes with 6,000 cells per well and grown in parallel in MEM for 48 h. Two hours before measurements, BrdU was added. After incubation measurements were performed as described in MATERIALS AND METHODS. BrdU incorporation is given relative to the mock control at the same plate. Statistical analysis was performed using a paired Student’s t-test (*P < 0.05; n = 16). It should be noted that 2 of the 16 experiments are very uncommon (mock control is lower than TRPC1-KD) and responsible for the large SD and the relative small effect.
the specific roles of TRPC1 in Ca\(^{2+}\) influx, and in SOCE in particular, differ between cell types, as does the role of Ca\(^{2+}\) in RVD; hence, future studies should address whether the relation of TRPC1 to these processes can be extended to other cell types.

In conclusion, the present investigation shows an upregulation of SOCE and TRPC1 protein level in the G1 compared with the G0 and the S phases, in parallel with an increased capacity of volume regulation. In addition, TRPC1-KD MDCK cells exhibit decreased SOCE, decreased RVD response, and decreased proliferation rate. Based on these results, it is suggested that TRPC1 plays an essential role in SOCE, in cell volume regulation, and in cell cycle progression in at least certain cell types. The precise mechanisms through which TRPC1 may integrate cell volume regulation, Ca\(^{2+}\) influx, and cell cycle progression require further investigation.

ACKNOWLEDGMENTS

We thank Morten Møller Christensen and David Gram Naym for technical assistance.

GRANTS

This work was supported by The Danish Council for Independent Research/ Natural Sciences and by the Lundbeck Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.P.M., T.K.K., A.F., and B.J.H. performed the experiments; C.P.M., T.K.K., B.J.H., S.F.P., and E.K.H. analyzed the data; C.P.M., T.K.K., S.F.P., and E.K.H. interpreted the results of the experiments; S.F.P. prepared the manuscript; T.K.K., B.J.H., S.F.P., and E.K.H. analyzed the data; C.P.M., T.K.K., S.F.P., and E.K.H. designed the research; E.K.H. approved the final version of the manuscript.

REFERENCES


AJP-Cell Physiol • doi:10.1152/ajpcell.00287.2011 • www.ajpcell.org
TRPC1 IN Ca\(^{2+}\) INFLUX, CELL VOLUME, AND CELL CYCLE


