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A Key Role for Mg²⁺ in TRPM7's Control of ROS Levels During Cell Stress

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SYNOPSIS

The TRPM7 channel has been shown to play a pivotal role in cell survival during brain ischemia as well as in the survival of other cell types challenged with apoptotic stimuli. Ca²⁺ is thought to be central to the channel's ability to regulate reactive oxygen species (ROS) production. However, channel-mediated entry of Mg²⁺ and Zn²⁺ have also been implicated in cell death. Here we show that depletion of TRPM7 by RNA interference in fibroblasts increases cell resistance to apoptotic stimuli by decreasing ROS levels in a Mg²⁺-dependent manner. Depletion of TRPM7 lowered cellular Mg²⁺, decreased the concentration of ROS and lessened p38 MAP kinase and JNK activation as well as decreased caspase-3 activation and PARP cleavage in response to apoptotic stimuli. Re-expression of TRPM7 or of a kinase-inactive mutant of TRPM7 in TRPM7-knockdown cells increased cellular Mg²⁺ and ROS levels, as did expression of the Mg²⁺ transporter SLC41A2. In addition, expression of SLC41A2 increased TRPM7-knockdown cells' sensitivity to apoptotic stimuli as well as boosted ROS generation in response to cell stress. Together these data uncover an essential role for Mg²⁺ in TRPM7's control of cell survival and in the regulation of cellular ROS levels.

Keywords

magnesium; transient receptor potential melastatin 7 (TRPM7); reactive oxygen species (ROS)

INTRODUCTION

TRPM7 is one of only two vertebrate ion channels to contain both ion channel and kinase domains [1, 2]. The other bifunctional channel is TRPM6, mutations in which cause familial hypomagnesemia with secondary hypocalcemia (HSH), an autosomal-recessive disease characterized by low serum Mg²⁺ accompanied by hypocalcemia [3, 4]. Whereas TRPM6 is expressed mainly in the kidney and the intestine [3, 5], TRPM7 is expressed in nearly all cell types [1, 2]. Evidence points to a pivotal role for TRPM7 in cellular Mg²⁺ homeostasis. Scharenberg and colleagues revealed that TRPM7-deficient DT40 B-cells exhibited Mg²⁺ deficiency and growth arrest [6, 7]. Supplementation of growth medium with extracellular Mg²⁺ or expression of the Mg²⁺-transporter SLC41A2 (solute carrier family 41 member 2) in TRPM7-deficient cells restored the ability of TRPM7-deficient DT40 cells to proliferate

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AUTHOR CONTRIBUTION

Hsiang-Chin Chen, Li-Ting Su, Omayra González-Pagán, and Jeffrey D. Overton performed the experiments. Loren W. Runnels, and Hsiang-Chin Chen planned the experiments, analyzed the experimental data and wrote the manuscript.

[8]. In mice, homozygous deletion of *TRPM7* resulted in early embryonic lethality [9]. Early developmental arrest caused by loss of the channel-kinase in mice appears to be related to the channel's ability to permeate Mg^{2+} , as depletion of TRPM7 in *Xenopus laevis* embryos produced a disruption in convergent-extension cell movements during gastrulation that could be prevented by Mg^{2+} supplementation as well as by expression of the Mg^{2+} -transporter SLC41A2 [10]. Later in development, other physiological functions have been ascribed to the channel-kinase, including skeletogenesis and melanophore maturation, kidney and pancreatic development, synaptic vesicle fusion, and thymopoiesis [9, 11–15]. The pleiotropic phenotypes caused by loss of the channel-kinase is likely due to TRPM7's bifunctional nature as well as to the channel's ability to permeate multiple species of divalent cations [16]. The best illustration of this comes from studies of the channel's role in cell death.

TRPM7 appears to be playing a major role in a cell's response to cell stress. The first and perhaps most striking example of the channel's influence on this process comes from the collective work by Tymianski, MacDonald and colleagues [17–19]. Their studies revealed that TRPM7 constitutes a Ca^{2+} -permeable nonselective cation conductance (I_{OGD}) that becomes activated by reactive oxygen/nitrogen species to promote Ca^{2+} overload and anoxic death in cultured cortical neurons subjected to oxygen glucose deprivation (OGD) [17]. Suppressing TRPM7 expression using small interfering RNA (siRNA) reduced the ischemia-induced current, decreased Ca^{2+} uptake and increased cell viability [17]. Using intrahippocampal injections of adeno-associated viral vectors packaged with short hairpin RNA specific for TRPM7, a subsequent study by Sun and colleagues provided *in vivo* evidence that regional TRPM7 suppression provides a comparable level of protection against brain ischemia [18]. Importantly, depletion of the channel had no negative effect on animal survival, dendritic morphology, neuronal excitability or synaptic plasticity [18]. In addition to its contribution to Ca^{2+} overload during OGD, TRPM7 is also required for Zn^{2+} -induced neuronal cell death, indicating that permeation of Ca^{2+} and Zn^{2+} both contribute to the TRPM7 channel's ability to mediate cell death in neurons [20]. More recently, knockdown of TRPM7 in hippocampal neurons has been shown to reduce the increase in intracellular Mg^{2+} levels detected following OGD, suggesting that conduction of Mg^{2+} by the channel during ischemia may also be contributing to neuronal cell death [21].

Consistent with the notion that conduction of multiple ions are involved in TRPM7's ability to mediate cell death, overexpression of TRPM7 in human embryonic kidney (HEK-293) cells increased Mg^{2+} and Ca^{2+} influx, which led to increased production of reactive oxygen species (ROS) and nitric oxide (NO) production [22]. The resultant oxidative stress caused by overexpression of the channel in turn activated the stress-activated protein kinases p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), which caused loss of cell adhesion and increased cell death [22, 23]. Conversely, depletion of TRPM7 in HEK-293 cells was protective against many forms of cell stress, including the apoptosis inducer doxorubicin, translation inhibitor cycloheximide, and broad kinase inhibitor staurosporine [23]. To further uncover how TRPM7 affects the cellular response to stress we have employed a stable TRPM7-knockdown Swiss 3T3 fibroblast line (M7shRNA6 cells), which we previously used to investigate the mechanisms by which TRPM7 controls cell motility [24]. M7shRNA6 cells exhibit defects in the ability to form lamellipodia and migrate directionally, which can be rescued by re-expression of TRPM7 as well as by expression of the Mg^{2+} transporter SLC41A2 [24]. In the present study, we show that depletion of TRPM7 from fibroblasts lowered intracellular Mg^{2+} , rendered cells more resistant to apoptotic stimuli and lowered the concentration of cellular ROS, which can all be reversed by re-expression of TRPM7, as well as by expression of the Mg^{2+} transporter SLC41A2. These results indicate that Mg^{2+} is not only playing a key role in TRPM7's

ability to control cell survival, but is also critical to the regulation of cellular ROS levels, both in the absence and presence of cell stress.

EXPERIMENTAL

Reagents

All chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). Staurosporine (STS), a broad inhibitor of protein kinases, was obtained from Calbiochem/EMD Biosciences (San Diego, CA). Mag-Indo-1, Fluo-4-AM, and CM-H₂DCFDA were obtained from Invitrogen (Carlsbad, CA).

Cell Lines

The TRPM7-knockdown fibroblast cell line (3T3-M7shRNA6) and the non-silencing control line (3T3-shRNA-C) originated from Swiss 3T3 cells (American Type Culture Collection number: CCL-92) and were made by standard approaches using a previously characterized shRNA (shRNA6) that specifically target mouse TRPM7 and using a non-silencing control shRNA (shRNA-C) [24]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum (FBS), unless otherwise indicated. Cells from frozen stocks were used 1–2 weeks after thawing to allow them to recover their normal morphology. The methods used to create the cell lines and a description of their full characterization have been previously described [24].

Cell Viability Measurements

Cell viability in response to treatment of cells with doxorubicin (DOX), staurosporine (STS) and cycloheximide (CHX) was determined by MTT assay or by manual cell counting using trypan blue exclusion. For the MTT assay, cells were incubated with 1 µg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours and then dissolved in 150 µl dimethyl sulfoxide. The absorbance at 570 nm was measured and normalized to untreated cells.

Detection of PARP, caspase-3 and MAPKs

Cells were lysed in ice-cold RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 % IGEPAL CA-630, 0.5 % Deoxycholate and 0.1 % SDS) with protease inhibitors (Roche, Basel, Switzerland). Samples of the lysates were analyzed by SDS-PAGE and Western blotting using standard procedures. PARP and caspase-3 were detected with rabbit polyclonal antibodies obtained from Cell Signaling Technology (Danvers, MA). Extracellular-signal related kinase 1/2 (ERK1/2) and JNK were detected with polyclonal antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Level of p38 MAPK and phospho-ERK1/2 (Thr202/Tyr204), were detected with monoclonal antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Activated p38 MAPK (phospho-p38 (Thr180/Thr182)) and JNK (phospho-JNK (Thr183/Thy185)) were detected with antibodies from Cell Signaling Technology (Danvers, MA). For immunochemiluminometric detection, SuperSignal West Maximum Sensitive Substrates (Pierce, Rockford, IL) Pico and Dura were used, depending the strength of the signal.

Measurement of ROS levels

Swiss 3T3 fibroblasts were trypsinized, washed with phosphate-buffered saline (PBS) and resuspended in phenol red-free culture medium. Cells were then labeled with 5 µM CM-H₂DCFDA for 20 min at 37 °C under 5 % CO₂. After being washed with PBS, the cells were resuspended in phenol red-free DMEM and analyzed using a Beckman Coulter

Cytomics FC500 Flow Cytometer equipped with a 488-nm argon ion laser and a 525-nm bandpass emission filter.

Measurement of intracellular Mg^{2+} and ATP

For measurement of intracellular Mg^{2+} , Swiss 3T3 fibroblasts were trypsinized, washed twice with PBS, and resuspended in phenol red-free DMEM. Cells were then labeled with 5 μM Mag-Indo-1 and incubated for 35 minutes at 37 °C under 5 % CO_2 . After being washed twice with PBS, cells were resuspended in phenol red-free DMEM, and blue and violet fluorescence emissions were collected using a Beckman Coulter MoFlo XDP cell sorter equipped with a 355-nm laser at room temperature. For measurement of intracellular ATP, cells were plated at a cell density of 2.5×10^3 and intracellular ATP levels were measured after 12 hr using EnzyLight™ ATP assay (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Luminescence was measured on a TECAN GENios Pro Microplate Reader (TECAN Group, Ltd, Switzerland) and cellular ATP levels were quantified using ATP standards.

Cell Cycle Analysis

Cells were cultured in DMEM with 10 % FBS and harvested at 70 % confluence for cell cycle analysis. To enrich cells in the G_0/G_1 phase of the cell cycle, cells were serum starved in media containing 0.1 % fetal bovine serum and kept at 100 % confluence for 48 hours. Cells were harvested and washed with PBS and then fixed in 70 % ethanol for 30 minutes. Cells were then washed again with PBS and then resuspended in PBS containing propidium iodide (10 $\mu g/ml$) and RNase (200 $\mu g/ml$) at room temperature for 30 minutes. Samples were analyzed using a Beckman Coulter Cytomics FC500 Flow Cytometer.

Adenoviral transduction

To confirm the role of TRPM7 in cell survival and its control of ROS levels we conducted gain-of-function studies by re-expressing TRPM7 in M7shRNA6 cells using a recombinant adenovirus that expresses silencing-resistant mouse TRPM7 (SR-TRPM7). To assess the contribution of TRPM7's kinase domain and its ability to permeate Mg^{2+} to these processes we also conducted gain-of-function studies using recombinant adenoviruses expressing a silencing-resistant kinase-inactive mutant of TRPM7 (SR-TRPM7-G1618D) and the FLAG-tagged magnesium-specific transporter SLC41A2 (FLAG-SLC41A2). Expression of LacZ was used as the negative control for adenovirus transduction. Detailed information regarding the creation of these reagents has been previously described [24]. Viral titers were determined by a plaque-forming assay using HEK-293A cells (Invitrogen). For gain of function studies, M7shRNA6 cells were transduced with recombinant adenoviruses at a MOI (multiplicity of infection) ranging from 100 to 200 and analyzed 5 days post-transduction.

RESULTS

Several investigations have indicated that the TRPM7 channel contributes to the demise of neurons in response to oxygen glucose deprivation and other forms of cellular stress. Depletion of the channel by RNA interference has been shown to decrease Ca^{2+} overload, Zn^{2+} influx, and lower cellular Mg^{2+} levels, all of which may be acting in concert to influence cell survival [17, 20, 21]. In a previous study we showed that HEK-293 cells could be rendered more resistant to several forms of apoptotic stimuli by reducing TRPM7 protein expression by RNA interference as well as by direct pharmacological blockade of the channel, indicating that the channel is influencing the cell death process in other cell types besides neurons [23]. Despite this progress, the molecular mechanism by which the channel affects cell survival has remained poorly understood.

Depletion of TRPM7 increases cell viability in response to apoptotic stimuli

To further understand the process by which TRPM7 influences cell survival we employed a TRPM7-knockdown fibroblast cell line (M7shRNA6) derived from Swiss 3T3 cells, which we recently used to demonstrate a critical role for the channel in polarized cell movements [24]. M7shRNA6 cells express a small hairpin RNA (shRNA) that specifically targets the channel-kinase, reducing TRPM7 expression by approximately 80 percent compared to a control fibroblast cell line expressing a non-silencing shRNA (shRNA-C) [24, 25]. To determine whether M7shRNA6 cells are a good model system for investigating TRPM7's role in cell death, we evaluated their resistance to apoptotic stimuli. TRPM7-knockdown cells were more resistant than wild-type Swiss 3T3 cells (WT) or control cells (shRNA-C) to cell death following treatment with the apoptotic inducers doxorubicin, cycloheximide and staurosporine, as assessed by MTT assay (Figure 1A). Similar results were obtained using the Trypan Blue exclusion assay (Supplementary Figure 1). These results establish M7shRNA6 cells as a suitable system for investigating the mechanism by which TRPM7 affects cell survival.

As TRPM7 has also been shown to be critical for cell growth and proliferation, we next investigated whether dysregulation of the cell cycle was affecting the resistance of M7shRNA6 cells to cell death. Consistent with previous reports, TRPM7-knockdown fibroblasts (M7shRNA6 cells) proliferated more slowly, with a higher percentage of cells in the G₀/G₁ phase of the cell cycle, compared to shRNA-C control cells (Supplementary Figures S2A and S2B) [6–8]. However, M7shRNA6 cells grown to confluence and serum starved, to enrich cells in the G₀/G₁ phase of the cell cycle, showed a similar decreased sensitivity to apoptotic stimuli compared to arrested control shRNA-C cells (Supplementary Figure 3). This result indicates that the small variance in cell cycle distribution between M7shRNA6 and shRNA-C cells was not responsible for the difference in cell viability between the two cell lines. To determine whether M7shRNA6 cells' decreased sensitivity to apoptotic stimuli was due to a disruption in apoptotic signaling, we next examined the cleavage of poly ADP-ribose polymerase (PARP) and of caspase-3 following treatment of cells with the apoptosis inducer doxorubicin. Caspase-3 is a proenzyme activated by proteolytic cleavage during apoptosis by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways [26, 27]. PARP is a substrate of caspase-3 that is inactivated by caspase-mediated proteolysis [28]. M7shRNA6 cells exhibited reduced PARP and caspase-3 cleavage following doxorubicin treatment (Figure 1B). Conversely, levels of pro-survival extracellular-signal-regulated kinases 1 and 2 (ERK1/2) and its activated forms were elevated in TRPM7-knockdown cells compared to control shRNA-C cells (Figure 1B). As apoptosis is an ATP-dependent process, we investigated whether TRPM7-knockdown cells have lower levels of ATP compared to control cells, which could possibly account for the difference in apoptotic signaling we observed [29, 30]. However, the concentration of ATP in the two cell lines was very similar (Supplementary Figure 4). So while these results indicate that depletion of TRPM7 is interfering with apoptotic signaling, the mechanism by which TRPM7 is affecting cell survival remained unclear.

Depletion of TRPM7 interferes with p38 MAPK and JNK activation and lowers ROS levels

In a previous study we showed that overexpression of TRPM7 in HEK-293 cells increased levels of reactive oxygen species (ROS) and concomitantly stimulated the activation of p38 MAPK and JNK [22]. These results prompted us to investigate whether depletion of TRPM7 has the opposite effect and diminishes p38 MAPK and JNK activation in response to oxidative stress. Application of H₂O₂, from which hydroxyl radicals are produced by the Fenton reaction, induced robust activation of p38 MAPK and JNK in shRNA-C cells compared to M7shRNA6 cells (Figure 2A). We next investigated whether depletion of TRPM7 lowers ROS levels by measuring the concentration of ROS by flow cytometry using

the ROS indicator CM-H₂DCFDA. TRPM7-knockdown cells (M7shRNA6) had a significantly lower concentration of ROS compared to shRNA-C cells, both in the absence of apoptotic stimuli and following treatment with apoptotic inducers (Figure 2B). This data indicates that TRPM7 is affecting ROS levels both in the absence and presence of cellular stress.

To confirm TRPM7's impact on cellular ROS levels, we next investigated whether re-expression of TRPM7 can restore the concentration of ROS in M7shRNA6 cells to the level found in control cells. In a previous study we created a recombinant adenovirus that expresses a "silencing-resistant" version of TRPM7 (SR-TRPM7), which was designed to evade RNA interference from expression of a TRPM7-specific shRNA (shRNA6) [24]. We recently employed this reagent in gain-of-function studies with M7shRNA6 cells to demonstrate a role for TRPM7 in polarized cell movements [24]. Transduction of M7shRNA6 cells with the recombinant SR-TRPM7 adenovirus increased the concentration of ROS to a similar level as detected in control shRNA-C cells (Figure 3). M7shRNA6 cells transduced with a recombinant LacZ-expressing adenovirus as a negative control had a similar concentration of ROS compared to non-transduced M7shRNA6 cells. We next investigated whether TRPM7's kinase was affecting cellular ROS levels by transducing a recombinant kinase-inactive TRPM7 (SR-TRPM7-G1618D) adenovirus into M7shRNA6 cells and determining whether expression of the kinase-dead mutant could also increase the concentration of ROS [24]. Re-expression of SR-TRPM7-G1618D in M7shRNA6 cells was as effective in boosting ROS levels as expression of the wild-type channel (Figure 3). These results indicate that loss of TRPM7's channel activity is responsible for the decrease in ROS in TRPM7 knockdown cells. However, the mechanism by which the channel is affecting the concentration of ROS remained unclear.

Expression of the Mg²⁺ transporter SLC41A2 increases ROS levels and restores sensitivity to apoptotic stimuli

Deletion of TRPM7 in the DT40 B cell line causes proliferation arrest in G₀/G₁ and G₂ phases of the cell cycle that can be reversed by supplementation of the growth media with 10–15 mM Mg²⁺ or by expression of the Mg²⁺ transporter SLC41A2 [6, 7, 31]. We have recently shown that depletion of TRPM7 during early embryonic development can interfere with convergent extension cell movements during gastrulation [10]. The gastrulation defect can be prevented by Mg²⁺ supplementation as well as by co-expression of Mg²⁺ transporter SLC41A2 with antisense TRPM7 morpholinos that target *Xenopus Laevis* TRPM7 [10]. As mentioned above, reducing TRPM7 expression in Swiss 3T3 fibroblasts impairs the ability of cells to execute polarized cell movements [24]. The defect in cell polarity in TRPM7-knockdown cells can also be rescued by expression of the Mg²⁺ transporter SLC41A2 [24]. We therefore investigated whether expression of SLC41A2 could increase the concentration of ROS in M7shRNA6 cells. Viral transduction of the recombinant Mg²⁺ transporter SLC41A2 adenovirus into M7shRNA6 cells increased ROS levels in the TRPM7-knockdown cells and elevated them to a level similar to that measured in shRNA-C cells. As expected, expression of LacZ in M7shRNA6 cells did not change the concentration of ROS (Figure 4). Interestingly, supplementation of the growth media with 10 mM Mg²⁺ increased ROS levels in shRNA-C cells, but not in the TRPM7-knockdown cell line (Figure 4). Moreover, expression of SLC41A2 in M7shRNA6 cells restored the ability of Mg²⁺ supplementation to increase ROS levels. This data strongly suggest that changes in intracellular Mg²⁺ affect the concentration of ROS in cells.

To confirm that depletion of TRPM7 is affecting intracellular Mg²⁺ we measured the concentration of free Mg²⁺ in the wild-type (WT) Swiss 3T3 cells, TRPM7-knockdown M7shRNA6 cells, and control shRNA-C cells by flow cytometry using the Mg²⁺ indicator mag-indo-1. The concentration of free Mg²⁺ in M7shRNA6 cells was significantly lower

than WT and control cells (shRNA-C) (Figure 5). Similar results were obtained using the Mg^{2+} indicator mag-fluo-4 (Supplementary Figure 5). To evaluate whether re-expression of TRPM7 was able to restore intracellular Mg^{2+} in M7shRNA6 cells to a level similar to that in control cells, we transduced the recombinant adenovirus expressing silencing-resistant TRPM7 (SR-TRPM7) into TRPM7-knockdown cells. Re-expression of SR-TRPM7 in M7shRNA6 cells brought the concentration of intracellular Mg^{2+} concentration to a similar level as that found in shRNA-C cells. In addition, the kinase activity of TRPM7 was not required to elevate free cellular Mg^{2+} levels, as M7shRNA6 cells virally transduced with the kinase-inactive SR-TRPM7-G1618D was equally effective in increasing intracellular Mg^{2+} as expression of the wild-type TRPM7 channel (Figure 5). Finally, we investigated whether expression of the Mg^{2+} transporter SLC41A2 would increase the concentration of free Mg^{2+} in M7shRNA6 cells. As expected, viral transduction of SLC41A2 increased levels of free Mg^{2+} in TRPM7-knockdown cells. However, supplementation of the growth media with 10 mM Mg^{2+} did not increase free Mg^{2+} in M7shRNA6 cells, suggesting that TRPM7 constitutes a major Mg^{2+} transport pathway in Swiss 3T3 fibroblasts. Together, these results support the hypothesis that changes in intracellular Mg^{2+} concentration affect ROS levels.

To further examine the effect of Mg^{2+} on the cellular response to apoptotic stimuli, we next investigated whether expression of the Mg^{2+} transporter SLC41A2 in TRPM7-knockdown M7shRNA6 cells decreases cell viability in response to apoptotic stimuli. M7shRNA6 cells transduced with the recombinant SLC41A2 adenovirus rendered TRPM7 knockdown cells more sensitive to cell death induced by the apoptotic inducers doxorubicin, cycloheximide and staurosporine (Figure 6A). In addition, following treatment with apoptotic inducers, levels of ROS were significantly more elevated in M7shRNA6 cells expressing SLC41A2 compared to M7shRNA6 cells expressing LacZ (Figure 6B). Collectively, these results indicate that intracellular Mg^{2+} potentially regulates ROS levels as well as the cellular response to apoptotic stimuli, revealing a heretofore unrecognized connection between TRPM7's control of Mg^{2+} homeostasis and the channel's impact on cell survival.

DISCUSSION

Ca^{2+} has a clearly established role in apoptotic signaling pathway, however there is very little evidence linking Mg^{2+} to this important cellular process [27]. Here, we have uncovered a role for TRPM7's control of cellular Mg^{2+} homeostasis in regulating ROS production during cell stress. In the present study, we showed that knockdown of TRPM7 in Swiss 3T3 fibroblasts increased cellular viability in response to a range of apoptotic stimuli. This result parallels the increased resistance to apoptotic stimuli detected in HEK-293 cells depleted of the channel-kinase [23]. Reduction of TRPM7 expression in fibroblasts decreased apoptotic signaling as revealed by reduced proteolytic cleavage of caspase-3 and PARP. Moreover, knockdown of TRPM7 diminished the activation of p38 MAPK and of JNK in response to cell stress. TRPM7-knockdown fibroblasts had lower amounts of ROS than control cells. The decrease in ROS caused by depletion of the channel was reversed by re-expression of TRPM7. Surprisingly, expression of the Mg^{2+} -specific transporter SLC41A2 in TRPM7-knockdown cells was equally effective in restoring levels of ROS as well as in re-sensitizing cells to apoptotic stimuli. Another important finding of this study was that supplementation of the growth media with 10 mM Mg^{2+} increased ROS levels for shRNA-C cells and for TRPM7-knockdown M7shRNA6 cells expressing SLC41A2. In contrast, Mg^{2+} supplementation of the growth media did not affect the concentration of intracellular free Mg^{2+} in M7shRNA6 cells nor did it elevate ROS levels, indicating TRPM7 likely constitutes the major pathway for Mg^{2+} entry in Swiss 3T3 fibroblasts. This finding explains why in our previous study we found that the defect in polarized cell movements by TRPM7-knockdown fibroblasts could be rescued by expression of the Mg^{2+} -transporter SLC41A2, but not by Mg^{2+} supplementation [24]. Mg^{2+} supplementation is, however, able to rescue

the proliferation arrest caused by deletion of *TRPM7* in DT40 B cells [6, 7]. The most parsimonious explanation for this difference is that Swiss 3T3 fibroblasts rely primarily on TRPM7 for Mg^{2+} entry, whereas DT40 cells employ TRPM7 as well as another Mg^{2+} transporter, such as SLC41A2, for Mg^{2+} transport, and are thus able to compensate for loss of the channel when cells are cultured in high Mg^{2+} . Thus, the failure to rescue a phenotype caused by loss of TRPM7 using Mg^{2+} supplementation should be interpreted cautiously, as the success of this approach is likely dependent upon the presence of an alternate pathway for cellular Mg^{2+} entry.

Our observation that Mg^{2+} influences the concentration of ROS is consistent with the findings of Wolf and colleagues, who reported that HC11 mouse mammary epithelial cells cultured in medium containing low extracellular Mg^{2+} had decreased ROS levels [32]. Adding Mg^{2+} back to the growth medium was able to return the concentration of ROS in HC11 cells to a normal level. The mechanism by which Mg^{2+} controls cellular ROS levels remains unclear. Lower intracellular Mg^{2+} may be slowing ROS production, increasing ROS turnover, or some combination of both. For example, HC11 cells cultured in low Mg^{2+} had increased glutathione transferase activity [32]. Glutathione transferases are a family of scavenger enzymes involved in the detoxification of free radical species [33]. In addition, other studies have reported that Mg^{2+} can stimulate the activity of NADPH oxidase, a major source of ROS within cells [34, 35]. Mg^{2+} may also be affecting ROS production by mitochondria [36]. At this point, the role of Mg^{2+} in controlling ROS levels still remains undefined, and additional work is needed to give a clearer picture of how Mg^{2+} regulates oxidative stress. Nevertheless, our study has shown that changes in Mg^{2+} homeostasis can affect ROS levels, which in turn can potentially influence the cellular response to apoptotic stimuli. Thus, the mechanism by which TRPM7 controls cell survival is more complex than originally envisioned.

Tymianski, MacDonald, and colleagues were the first to identify a critical role for TRPM7 in neuronal cell death during ischemia, showing that knockdown of TRPM7 expression by RNA interference decreased neuronal death and Ca^{2+} overload in response to oxygen glucose deprivation [17]. The connection between TRPM7, Ca^{2+} and cell stress was also revealed by another study, which found that suppression of TRPM7 expression in PC12 cells reduced the increase in intracellular Ca^{2+} caused by lipopolysaccharide (LPS) treatment [37]. Undoubtedly, intracellular Ca^{2+} plays a key role in apoptotic pathway and is important to TRPM7's control over cell survival. However, evidence suggests that other cations contribute to neuronal cell death via TRPM7. For example, Zn^{2+} -induced neurotoxicity was revealed to be dependent on TRPM7's ability to transport Zn^{2+} [20]. In addition, TRPM7 has been shown to contribute to increases in intracellular Mg^{2+} in rat hippocampal neurons following hypoxia [21]. Our results suggest that during ischemia, changes in intracellular Mg^{2+} levels could be affecting ROS levels and susceptibility to neuronal cell death. Additional studies are required to evaluate this hypothesis as well as to determine whether Mg^{2+} homeostasis affects ROS levels in other systems, especially as different cell types have their own distinctive response to cell stress in addition to their own unique ion transport mechanisms. It is becoming increasingly clear that different ions act in concert to regulate cell death, and that additional studies are required to understand how various divalent cations function together. TRPM7, with its ability to permeate multiple species of cations, is uniquely adapted to orchestrate their collective actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TRPM7	transient receptor potential melastatin 7
SLC41A2	solute carrier family 41 member 2
ROS	reactive oxygen species
MOI	multiplicity of infection
HEK-293	human embryonic kidney 293 cells
shRNA	short hairpin RNA

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Figure 1. Depletion of TRPM7 increases cell resistance to apoptotic stimuli and reduces apoptotic signaling

(A) MTT assay demonstrating increased survivability of TRPM7-knockdown Swiss 3T3 fibroblasts (M7shRNA6) compared to wild-type (WT) or control cells expressing a non-silencing shRNA (shRNA-C) in response to the apoptotic stimuli doxorubicin (DOX), cycloheximide (CHX) and staurosporine (STS). Cells were treated for 48 hours with 2 μ M DOX, 10 μ g/ml CHX, and 2 μ M STS. Values are presented as the mean $\pm$ standard deviation from three independent experiments. The asterisk indicates a significant difference in cell viability between TRPM7-knockdown cells and WT and control cells using Student's *t* test ($P < 0.05$). (B) TRPM7-knockdown cells exhibited reduced cleavage of PARP and caspase-3 compared to control shRNA-C cells, but showed increased amounts of ERK1/2 and ERK1/2 phosphorylation following 24 and 36 hour (hr) treatment with 2 μ M DOX. β -actin is shown as a loading control for the samples.

Figure 2. Depletion of TRPM7 reduces activation of p38 MAPK and JNK and lowers ROS production in response to cell stress

(A) Western blot analyses of levels of activated p38 and JNK, and the total amount of each protein in cells lysates after the 15 and 30 min treatment of with varying amounts of H₂O₂. TRPM7-knockdown fibroblasts (M7shRNA6) exhibited decreased activation of p38 MAPK and JNK in response to H₂O₂ compared to control cells (shRNA-C). β-actin was shown as the loading control for the samples. (B) Measurement of ROS production in cells labeled using fluorescent ROS indicator CM-H₂DCFDA by FACS analysis. TRPM7-knockdown cells (M7shRNA6) have lower level of ROS than controls cells (shRNA-C) in the absence apoptotic stimuli and following treatment with 0.5 mM H₂O₂ (30 min), 2 μM DOX (24 hr) and 10 μg/ml CHX (24 hr). Values are presented as the mean ± standard deviation from three independent experiments. The asterisk indicates a significant difference in ROS levels between TRPM7-knockdown cells and control cells using Student's *t* test (*P* < 0.05).

Figure 3. Re-expression of TRPM7 restores ROS production in TRPM7-knockdown cells
 ROS production in fibroblasts was measured by FACS analysis labeled with the fluorescent ROS indicator CM-H₂DCFDA. Adenovirus-mediated re-expression of silencing-resistant TRPM7 (SR-TRPM7) or of the kinase-inactive SR-TRPM7 (SR-TRPM7-G1618D) in TRPM7-knockdown cells (M7shRNA6) increased ROS production to a level similar to that of control fibroblasts (shRNA-C), whereas expression of LacZ had no effect. Values are presented as the mean $\pm$ standard deviation from two independent experiments. The asterisk indicates a significant difference in ROS production in TRPM7-knockdown cells and TRPM7-knockdown cells expressing LacZ compared to control cells (shRNA-C) using Student's *t* test ($P < 0.05$).

Figure 4. Expression of Mg²⁺ transporter SLC41A2 restores ROS production in M7shRNA6 cells

Expression of the Mg²⁺ transporter SLC41A2 in TRPM7-knockdown cells (M7shRNA6) increased ROS production to a similar level as control cells (shRNA-C) expressing LacZ. In addition, supplementation of the growth medium with excess 10 mM Mg²⁺ augmented ROS production in shRNA-C cells and SLC41A2 expressed-M7shRNA6 cells, but not in TRPM7-knockdown cells (M7shRNA6) expressing LacZ. Values are presented as the mean $\pm$ standard deviation from two independent experiments. “N” indicates cells grown in normal Mg²⁺ (0.95 mM) and “H” signifies cells grown in high Mg²⁺ (10 mM). The hash symbol indicates a significant difference in the ROS production between M7shRNA6 cells and M7shRNA cells expressing SLC41A2. The asterisk indicates a significant difference in the ROS production between samples grown in normal growth medium and samples grown in the medium supplemented with 10 mM Mg²⁺ using Student’s *t* test ($P < 0.05$).

Figure 5. Knockdown of TRPM7 decreases cellular free Mg²⁺

Measurement of cellular free Mg²⁺ with the Mg²⁺ indicator Mag-indo-1 revealed that knockdown of TRPM7 (M7shRNA6) lowered free Mg²⁺ compared to wildtype (WT) and control cells expressing a non-silencing shRNA (shRNA-C). Re-expression of SR-TRPM7 and the kinase-inactive SR-TRPM7-G1618D, and expression of the Mg²⁺ transporter SLC41A2 restored Mg²⁺ concentrations to a level similar to controls, whereas expression of lacZ or supplementation of the growth medium with excess Mg²⁺ was ineffective in increasing cellular free Mg²⁺. To verify the specificity of the Mag-indo-1 indicator we treated WT fibroblasts with thapsigargin (TG) (0.2 ng/μl, 10 min), an inhibitor of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) which can increase cytoplasmic Ca²⁺, and did not detect an increase in the fluorescence intensity of mag-indo-1. Whereas treatment of WT cells with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 μM), a mitochondrial uncoupler known to increase cellular free Mg²⁺, produced a robust increase in Mag-indo-1 fluorescence intensity. Values are presented as the mean ± standard deviation from at least three experiments. The asterisk indicates significant difference in cellular free Mg²⁺ compared to the control cells using Student's *t* test (*P*<0.05).

Figure 6. Expression of Mg^{2+} transporter SLC41A2 increases the sensitivity of M7shRNA6 cells to apoptotic stimuli and increases ROS levels

(A) Expression of Mg^{2+} transporter SLC41A2 in TRPM7-knockdown cells (M7shRNA6) reduced the cellular viability of M7shRNA6 cells in response to apoptotic stimuli as assessed using the MTT assay. Cells were transduced with adenovirus expressing LacZ, as a negative control, and SLC41A2 for 5 days, and then were treated with 2 μ M doxorubicin (DOX), 10 μ g/ml cycloheximide (CHX) and 2 μ M staurosporine (STS) for 48 hours. Values are presented as the mean $\pm$ standard deviation from three independent experiments. The asterisk indicates a significant difference in ROS levels between TRPM7-knockdown cells expressing LacZ and cells expressing SLC41A2 using Student's *t* test ($P < 0.05$). (B) Expression of the Mg^{2+} transporter SLC41A2 in TRPM7-knockdown cells enhanced the ability of apoptotic stimuli to increase ROS levels. Cells were transduced with adenovirus expressing LacZ, as a negative control, and SLC41A2 for 5 days, and then were treated with 2 μ M doxorubicin (DOX) and 10 μ g/ml cycloheximide (CHX) for 18 hours. Values are presented as the mean $\pm$ standard deviation from three independent experiments. The asterisk indicates a statistical difference in ROS level between TRPM7-knockdown cells expressing LacZ and cells expressing SLC41A2 using Student's *t* test ($P < 0.1$).