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TRPM7 channel activity in Jurkat T lymphocytes during magnesium depletion and loading: implications for divalent metal entry and cytotoxicity

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Abstract

TRPM7 is a cation channel-protein kinase highly expressed in T lymphocytes and other immune cells. It has been proposed to constitute a cellular entry pathway for Mg^{2+} and divalent metal cations such as Ca^{2+} , Zn^{2+} , Cd^{2+} , Mn^{2+} and Ni^{2+} . TRPM7 channels are inhibited by cytosolic Mg^{2+} , rendering them largely inactive in intact cells. Dependence of channel activity on extracellular Mg^{2+} is less well studied. Here, we measured native TRPM7 channel activity in Jurkat T cells maintained in external Mg^{2+} concentrations varying between 400 nM and 1.4 mM for 1–2 days, obtaining an IC_{50} value of 54 μM . Maintaining the cells in 400 nM or 8 μM $[Mg^{2+}]_o$ resulted in almost complete activation of TRPM7 in intact cells, due to cytosolic Mg^{2+} depletion. 1.4 mM $[Mg^{2+}]_o$ was sufficient to fully eliminate the basal current. Submillimolar concentrations of amiloride prevented cellular Mg^{2+} depletion, but not loading. We investigated whether the cytotoxicity of TRPM7 permeant metal ions Ni^{2+} , Zn^{2+} , Cd^{2+} , Co^{2+} , Mn^{2+} , Sr^{2+} and Ba^{2+} requires TRPM7 channel activity. Mg^{2+} loading modestly reduced cytotoxicity of Zn^{2+} , Co^{2+} , Ni^{2+} and Mn^{2+} but not of Cd^{2+} . Channel blocker NS8593 reduced Co^{2+} and Mn^{2+} but not Cd^{2+} or Zn^{2+} cytotoxicity and interfered with Mg^{2+} loading as evaluated by TRPM7 channel basal activity. Ba^{2+} and Sr^{2+} were neither detectably toxic, nor permeant through the plasma membrane. These results indicate that in Jurkat T cells entry of toxic divalent metal cations primarily occurs through pathways distinct from TRPM7. By contrast, we found evidence that Mg^{2+} entry requires TRPM7 channels.

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Keywords

metal toxicity; immunotoxicity; cadmium; lanthanum; amiloride; NS8593; HAP1 cells

Introduction

TRPM7 is a dual ion channel-protein kinase, ubiquitously expressed in mammalian tissues. TRPM7 channels belong to the transient receptor potential (TRP) channel family and are inhibited by cytoplasmic Mg^{2+} , polyamines and protons [24]. Like other TRP family members TRPM7 is a tetrameric nonselective cation channel [9,21]. Numerous studies have attempted to elucidate the functions of this protein using a variety of systems: primary cell culture, immortalized cell lines, heterologous expression and transgenic mice. TRPM7 kinase activity supports murine T-cell proliferation [3] and gut-homing of intraepithelial lymphocytes [67]. TRPM7 channels, on the other hand, are thought to mediate Mg^{2+} influx into cells, as well as governing blood magnesium homeostasis [76,75]. Sensitivity to physiological levels of intracellular Mg^{2+} , however, renders the majority of channels in intact cells silent, due to tonic inhibition by Mg^{2+} [13]. TRPM7 kinase is functional in the absence of channel activity [58,48] and its activity is regulated by divalent metal cations, such as Mg^{2+} , Mn^{2+} , Zn^{2+} and Co^{2+} [73,58]. These characteristics raise interesting questions about the possible mode(s) of activation of TRPM7 channels in intact cells in the body, questions that remain open.

Traditionally, TRPM7 channel currents are recorded in whole-cell patch clamp by infusing the cell with low micromolar $[Mg^{2+}]$ in conjunction with metal chelators (e.g. EGTA, HEDTA) to deplete cellular Mg^{2+} [64,47,60,38,13,90]. During Mg^{2+} washout from cytosol, TRPM7 channels open sequentially over several minutes [14]. Adding higher $[Mg^{2+}]$ internally results in current inhibition which is reversible and mainly affects the number of conducting channels with a small reduction (~20%) of unitary conductance [14]. Of interest, TRPM7 channels over-expressed in mammalian cells show a high degree of basal activity, even before Mg^{2+} depletion (e.g. [60,58,90]). Like other TRPM (melastatin) subfamily channels, TRPM7 requires the plasma membrane phospholipid PI(4,5) P_2 for ion channel function [72,48]. We recently demonstrated that the characteristic inhibition of TRPM7 by cytoplasmic Mg^{2+} , spermine and protons is indirect and represents electrostatic screening of PI(4,5) P_2 negative charges, preventing channel activation [90].

Despite the obvious importance of Mg^{2+} in lymphocyte function, the cellular uptake pathways of this cation are poorly understood [8,92]. Congenital mutations in MagT1 (magnesium transporter 1) were shown to result in an immunodeficiency in patients, and it was proposed that MagT1 is the long-sought Mg^{2+} transporter in lymphocytes [26,51,85,52,19,91]. More recent studies, however, have cast doubt on the idea that MagT1 is a Mg^{2+} transporter *per se*, and suggested that it is a protein involved in glycosylation [6]. TRPM7 has been the focus of studies showing that it can provide a pathway for Mg^{2+} entry in the gut and other tissues [45,77,59]. In human colon cell lines, on the other hand, knockdown of TRPM7 resulted in a paradoxical increase in cytoplasmic Mg^{2+} [54]. Additionally, in *lck-Cre* mice, where *Trpm7* was selectively deleted, no change in body

Mg²⁺ homeostasis was observed due to TRPM7 channel disruption [39]. Thus, the involvement of TRPM7 in Mg²⁺ transport requires further investigation.

TRPM7 channels have been reported to conduct Ca²⁺, Mg²⁺ and other divalent metal cations such as Zn²⁺, Cd²⁺, Mn²⁺, Co²⁺, Ba²⁺, Sr²⁺ in the inward direction when these ions are added acutely [60,33,53,17,79]. Zn²⁺, Co²⁺ and Cd²⁺ influx through TRPM7 was suggested to be responsible for cytotoxicity [36,50,1,57,69]. Alternative influx pathways for Cd²⁺, such as divalent metal transporter 1 (DMT1), have also been proposed [7]. Interestingly, many of these permeant cations can inhibit TRPM7 channels from inside in a voltage-independent manner, similar to Mg²⁺ [46,58]. It is therefore plausible that significant entry of divalent metal cations into the cell will result in TRPM7 channel inhibition. The consequences of divalent metal loading and feedback inhibition on basal TRPM7 channel activity have not been explored.

In the present study, we aimed to elucidate the role of TRPM7 channels as an entry pathway for Mg²⁺ and other divalent metal cations in Jurkat T lymphocytes, which express high levels of TRPM7 [13]. We investigated the consequences of varying the extracellular Mg²⁺ concentration over several days on TRPM7 channel basal activity. We determined the dependence of native TRPM7 channel currents on *external* [Mg²⁺] and found that it resembles the dose-response relationship obtained from directly changing *internal* Mg²⁺ [13]. Using cytotoxicity as an assay of entry of external divalent metal cations in the cytosol, we defined the cytotoxicity sequence of metal cations as Cd²⁺>Zn²⁺>Co²⁺>Ni²⁺>Mn²⁺ (or Mn²⁺>Ni²⁺ in other experiments) which differed from the known permeability sequence of TRPM7 to these ions. Thus, Cd²⁺ was the most toxic cation yet is only modestly permeant through TRPM7 [60,53,55]. Incubation of cells in elevated [Mg²⁺]_o (1.4 mM) resulted in complete elimination of basal TRPM7 channel activity and modestly reduced killing by Zn²⁺, Co²⁺, Ni²⁺ and Mn²⁺ without affecting Cd²⁺ toxicity. Maintaining Jurkat T cells in the presence of La³⁺, which at high concentrations blocks TRPM7 currents at negative membrane potentials, moderately reduced Cd²⁺, Zn²⁺, Co²⁺ and Mn²⁺ cytotoxicity. NS8593, which blocks TRPM7 channels in a voltage-independent manner did not reduce killing by Cd²⁺ and Zn²⁺. Interestingly, incubation with NS8593 but not La³⁺ prevented Mg²⁺ loading. We conclude that TRPM7 significantly contributes to the uptake of Mg²⁺ and, to a lesser degree Zn²⁺, Co²⁺, Mn²⁺ and Ni²⁺ in Jurkat T cells. In TRPM7 CRISPR knockout HAP1 cells, Co²⁺, Mn²⁺ and Ni²⁺ cytotoxicity was reduced compared to WT cells, whereas Cd²⁺ and Zn²⁺ cytotoxicity was unaffected. Based on our findings, we propose that divalent metal ion entry pathways must exist that preferentially transport Cd²⁺, Co²⁺ and Zn²⁺ and are not Mg²⁺-sensitive. Neither Ba²⁺ nor Sr²⁺ accumulated in Jurkat T cells and were not toxic, despite being highly permeant through TRPM7 channels. Long-term activation of TRPM7 channels under conditions when external Mg²⁺ is pathologically low, which occurs during hypomagnesemia, may present TRPM7 as a previously unknown player in the progression of this disease state.

Materials and methods

Cell culture

Jurkat human leukemic T lymphocytes (ATCC, Manassas, VA) were grown in 5% CO₂ humidified atmosphere at 37°C in RPMI 1640 (GE Healthcare, HyClone Laboratories, Logan, UT) or Advanced RPMI 1640 (Gibco/ThermoFisher, Waltham, MA) medium supplemented with 10% or 5% heat-inactivated fetal bovine serum (FBS) (Corning, Manassas, VA), respectively, and penicillin/streptomycin (Life Technologies, Grand Island, NY). Both RPMI formulations contain 0.42 mM Ca²⁺ and 0.4 mM Mg²⁺. In experiments with elevated external Mg²⁺, 1 mM MgCl₂ was added to the medium bringing the total Mg²⁺ concentration to 1.4 mM. In experiments with reduced Ca²⁺ (see Fig. 6), 1.4 mM MgCl₂ was added to RPMI treated with chelating resin (Chelex 100 ion exchange resin, 50–100 mesh, Sigma-Aldrich, St. Louis, MO). The complete RPMI containing serum was mixed with 5% (5 grams per 100 mls) Chelex 100 sodium form, stirred for 1 hour at room temperature and filtered through a 0.22 µm polyethersulfonate (PES) vacuum filter (CELLTREAT Scientific Products, Pepperell, MA) to remove the Chelex particles and sterilization, as described previously [3]. pH of the Chelex-treated RPMI was adjusted to 7.3. To prepare low Mg²⁺-containing RPMI, the complete medium was first treated with Chelex to remove Ca²⁺ and Mg²⁺, followed by addition of 0.42 mM CaCl₂, 10 or 40 µM HEDTA and 10 µM MgCl₂ as specified in figure legends. The free Mg²⁺ concentrations of 10 µM MgCl₂/10 µM HEDTA and 10 µM MgCl₂/40 µM HEDTA-containing RPMI were estimated at 8 µM and 400 nM, respectively, at 37°C (Maxchelator software <https://web.stanford.edu/~cpatton/webmaxcS.htm>). Chelex 100 binds Ca²⁺, Mg²⁺ and other divalent metal cations and has been used to treat FBS-supplemented RPMI, resulting in Ca²⁺ and Mg²⁺ concentrations lower than 25 µM and 40 µM, respectively [66,11,12]. One drawback of using Chelex is that it can deplete proteins and amino acids in culture media, another is its preference for heavy metals over Ca²⁺ and Mg²⁺ [11].

In experiments with Zn²⁺, Ba²⁺, Ni²⁺, Cd²⁺, Co²⁺, Mn²⁺ and Sr²⁺ supplementation of growth medium, Zn₂SO₄, BaCl₂, NiCl₂, CdCl₂, CoCl₂, MnCl₂, SrCl₂ were added to Chelex-treated complete RPMI with or without CaCl₂.

HAP1, a near haploid human cell line derived from chronic myelogenous leukemia (CML) cell line KBM-1 (Horizon Discovery, Waterbeach, Cambridge, UK) was grown at 37°C, 5% CO₂ humidified atmosphere in IMDM (Gibco/ThermoFisher) supplemented with 10% FBS and penicillin/streptomycin. Cells were passaged 1–2 times a week. For HAP1 cell viability measurements, Chelex-treated complete RPMI was supplemented with 1.4 mM CaCl₂ (equal to the Ca²⁺ concentration in IMDM) and other metal cations as specified in Fig. 7. HEK293 cells were grown in RPMI supplemented with 10% FBS and antibiotics and passaged twice weekly. Cells were transfected with GFP-tagged murine TRPM7 cDNA as described in [90].

Patch-clamp electrophysiology

Electrophysiological experiments were performed in whole-cell or perforated patch clamp recording configurations using EPC10 (HEKA, Holliston, MA) patch clamp amplifier as

previously described [13,90]. Instantaneous current-voltage (I-V) relations were acquired by applying command voltage ramps spanning -100 to $+85$ mV every 1.5 or 2.5 sec. For whole-cell recordings of native TRPM7 Jurkat T-cell currents, the basic internal (pipette) solution contained (in mM): 112 glutamic acid, 8 NaCl, 5 CsF, 12 EGTA, 10 HEPES, pH 7.3 adjusted with CsOH. The external (bath) recording solution contained 2 mM CaCl_2 , 3 mM CsCl, 140 mM Na aspartate, 10 mM HEPES- Na^+ , pH 7.3 with added 30 μM MgCl_2 , unless otherwise specified. Perforated-patch recordings were performed as previously described [48,90] with a pipette solution containing 55 mM CsCl, 50 mM Cs_2SO_4 , 7 mM MgCl_2 , 1 mM CaCl_2 , pH 7.3. On the day of experiment, amphotericin B (Sigma) 60 mg/ml stock solution prepared in DMSO was diluted in the pipette solution yielding 0.24 mg/ml final concentration [90]. At the end of each experiment, brief suction was applied to achieve whole-cell configuration upon which TRPM7 currents were rapidly inhibited with 7 mM Mg^{2+} and 1 mM Ca^{2+} present in the pipette solution (see Fig. 3). Deionized water was used for preparing all recording solutions. Osmolalities were measured with a freezing point osmometer (Osmette, Norwood, MA) and adjusted with D-mannitol (Sigma). Experiments were performed at room temperature. Jurkat T cells were spun down and transferred to RPMI containing specified Mg^{2+} and other metal cation concentrations and grown in 37°C CO_2 incubator as described above. Recordings were made the next day, the day after or on the third day (referred to in the text as 1, 2, 3 days, respectively). Cells were kept in the recording solution containing 30 μM MgCl_2 for not longer than approximately 1 hour. The durations of Mg^{2+} depletion and loading (e.g. Fig. 2) represent the time period between placing cells in modified Mg^{2+} medium and the time the recordings were made, and therefore include 1 hour when the cells were kept at room temperature in the recording chamber in presence of 30 μM Mg^{2+} . Electrophysiological data were analyzed with Patchmaster (HEKA) and Origin (OriginLab, Northampton, MA) software. Two-sample Student's tests were performed using Origin to determine significant differences. In some cases t test results were confirmed with non-parametric Wilcoxon rank sum test as specified in figure legends.

Cell viability measurements

The viability of Jurkat T lymphocytes and HAP1 cells grown in the presence of various metal cations was measured by trypan blue dye exclusion using Vi-CELL automated viability analyzer (Beckman Coulter, Brea, CA) [25,3]. In the majority of experiments the cells were grown at 37 °C, 5% CO_2 in Chelex-treated or normal RPMI supplemented with 0.42 mM CaCl_2 and 0.4 mM chloride (for Mn^{2+} , Ni^{2+} , Co^{2+} , Ba^{2+} , Cd^{2+} , Sr^{2+}) or sulfate salt (for Zn^{2+}) of the metal ion. 0.4 mM Na_2SO_4 was tested as a control in separate experiments and did not have a noticeable effect on cell viability (data not shown). In experiments described in Fig. 6, CaCl_2 was added at 40 μM or entirely omitted. In experiments with elevated Mg^{2+} , complete RPMI (without Chelex treatment) was supplemented with 1 mM MgCl_2 and various metal cations at 0.4 mM, except in Figs. 5c, d and 6 where MgCl_2 was added to Chelex treated complete RPMI. Cell viability was assessed approximately 24 hours after adding the metal cation. In experiments described in Fig. 5d, cells were incubated at low and normal magnesium concentrations for 24 hours, counted and then exposed to divalent metal cations for 24 hours. Aliquots of cell culture were spun down to test cell viability after treatment with divalent metal cations. Briefly, 1 ml of cell

suspension was added to a sample cup at room temperature and mixed with trypan blue by the automated cell counter. 100 cell images were acquired and analyzed by the Vi-CELL software. The software detects cells that are viable and those that are dead, based on trypan blue exclusion or accumulation in the cells. Vi-CELL data were graphed using Origin. Salts were purchased from Sigma, Acros Organics (Geel, Belgium) and Fisher. Stock solutions of NS8593 hydrochloride (Sigma), amiloride hydrochloride (Sigma), FTY720 hydrochloride (Cayman Chemical Company, Ann Arbor, MI), FTY720-phosphate (Cayman) and imipramine hydrochloride (Sigma) were prepared in DMSO and diluted to the final concentration on the day of experiment. EDTA was from Research Products International (Mt Prospect, IL), HEDTA was from Acros Organics (Geel, Belgium).

Western blot analysis

Detection for the levels of endogenous TRPM7 protein was performed as previously described [3]. HAP1 cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 mM DTT, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100 supplemented with protease inhibitors) on ice for 20 min and insoluble materials were removed by centrifugation at 20,000 g for 10 min. To concentrate the TRPM7 protein by immunoprecipitation, cell lysates were incubated with rabbit polyclonal anti-TRPM7 antibody (AB15562; Millipore, Billerica, MA) or control IgG overnight at 4°C, followed by incubation with protein A sepharose beads for 1 hour. The beads were washed three times in lysis buffer and proteins bound to the beads were eluted using Laemmli sample buffer. The eluted proteins and whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked using Blocking One (Nacalai Tesque, Kyoto, Japan) and then probed with anti-TRPM7 or mouse monoclonal anti-β-actin antibodies (Medical and Biological Laboratories, Nagoya, Japan) overnight at 4°C before being washed and incubated for 1 hour with the secondary HRP-conjugated anti-rabbit IgG or anti-mouse IgG antibodies (Dako, Carpinteria, CA). Before development, the membranes were washed and incubated with Amersham ECL Prime (GE Healthcare, Piscataway, NJ) and the immunoreactive proteins were visualized using ImageQuant400 (GE Healthcare).

RT-PCR

RT-PCR was performed on RNA isolated from Jurkat T cells essentially as previously described [13,3]. RNA was isolated from cells grown for 24 hours in Chelex-treated RPMI with 10 μM MgCl₂/10 μM HEDTA or 0.4 mM MgCl₂, using Tri Reagent RT (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. OneStep RT-PCR Kit (Qiagen, Hilden, Germany) was used with primer sets specific for TRPM7, SLC41A1, SLC41A2 and GAPDH (Integrated DNA Technologies, Coralville, IA). Primer sequences were: hTRPM7 Fwd 5'-CTGAAGAGGAATGATTATACGCC-3'/Rev 5'-GCCTAACCCTGATTCATAAA-3' [13], hSLC41A1 Fwd 5'-GTGATTGAGTCTCGGGCCAA/Rev GTTCCAGGTAGAGTCCCCCA, hSLC41A2 Fwd 5'-CTTGCCATATTGGCTTGGAT/Rev CAACCTTTGGGTTTCATCAGG [74]. 25 cycle amplification was performed for TRPM7 and SLC41A1, A2 and 35 cycles for GAPDH. Amplicons were separated by agarose gel electrophoresis run together with GenerRuler1kb Plus DNA ladder (Thermo Scientific).

Results

Effects of Mg^{2+} depletion and loading on TRPM7 channel activity

Based on RT-PCR results, Jurkat T lymphocytes express high levels of TRPM7 but not TRPM6, a closely related channel-kinase [13]. This presents an advantage for studying Mg^{2+} regulation and TRPM7 in this cell line since TRPM6 has also been implicated in Mg^{2+} transport in other cell types and can combine with TRPM7 to form heterotetramers [83,18,15,5,54]. Given that most TRPM7 channels are normally inhibited in Jurkat T cells, presumably due to inhibition by cytoplasmic Mg^{2+} [13], we investigated if prolonged removal of external Mg^{2+} would activate TRPM7 channels by depletion of Mg^{2+} from the cell. Growing Jurkat T cells in 8 μM Mg^{2+} -containing medium, in the presence of normal 0.42 mM Ca^{2+} for 1–2 days resulted in an almost full activation of TRPM7 channels estimated as preactivation index I_0/I_{max} (Fig. 1a, b). We chose this parameter because it accurately quantifies the number of open channels in an intact cell at break-in (I_0) compared to the total number of channels present in the plasma membrane (I_{max}) (see also Fig. 2 below). I_{max} for each cell was estimated after cell dialysis with Mg^{2+} -free internal solution for 5–6 minutes (Fig. 1a, c). Elevating external Mg^{2+} to 1.4 mM in the growth medium for 1–2 days resulted in full inhibition of basal channel activity (Fig. 1b, d).

We next measured TRPM7 preactivation at low and intermediate $[Mg^{2+}]_o$ (Fig. 1e). In agreement with our previous study [13], basal channel activity was minimal in most cells at 400 μM $[Mg^{2+}]_o$ present in normal RPMI (Fig. 1e). We constructed a dose-response curve for external Mg^{2+} , which yielded IC_{50} of 54 μM (Fig. 1f). This value is close to that determined in whole-cell recordings where pipette $[Mg^{2+}]$ was systematically varied [13].

We investigated in detail the dependence of TRPM7 current preactivation on the duration of exposure to 8 μM Mg^{2+} (i.e. depletion step). Cells grown in normal RPMI were transferred to 8 mM Mg^{2+} -containing Chelex-RPMI for various time periods up to 60 hours and TRPM7 current amplitudes were compared (Fig. 2). We found that the preactivation index rose steeply in the majority of cells after incubating for ~12 hours and remained high for at least up to 55 hours (Fig. 2a). The maximum TRPM7 current rose gradually during that time period, however this rise was substantially slower than that for preactivation index (Fig. 2b). We compared the maximum TRPM7 current amplitudes in cells grown in 8 μM Mg^{2+} vs. elevated 1.4 mM Mg^{2+} for one day. On average, there was a modest increase of maximum current amplitude in cells grown in low Mg^{2+} (Fig. 2c). We investigated further if increase maximum current was due to increased expression of TRPM7 in low Mg^{2+} . RT-PCR experiments using TRPM7 specific primers were performed on RNA isolated from cells grown in 8 μM and 0.4 mM Mg^{2+} for 1 day. However, there was a slight decrease, rather than an increase of TRPM7 expression in low Mg^{2+} (Fig. 2d).

Unlike whole cell, the perforated-patch configuration prevents changes in concentrations of divalent cations such as Mg^{2+} and Ca^{2+} inside the cell. In Mg^{2+} -depleted cells we recorded TRPM7 currents in this configuration. Fig. 3a shows TRPM7 current-voltage relation obtained in a cell grown in 8 μM Mg^{2+} for two days. As expected, TRPM7 amplitude did not change during the entire recording, reflecting the fact that the majority of TRPM7 channels are active in an intact cell under these growth conditions (Fig. 3b). At the end of

experiment, whole-cell configuration was obtained causing the current to be inhibited fully by influx of 7 mM Mg^{2+} and 1 mM Ca^{2+} present in the internal solution (Fig. 3a, b).

Experiments presented in Figs. 1 and 2 and Suppl. Fig. 1 demonstrate that removal of external Mg^{2+} and resulting cellular depletion of this cation is sufficient to activate TRPM7 currents. Conversely, loading of cells in presence of moderately elevated Mg^{2+} (1.4 mM) is sufficient to inhibit the great majority of channels. We previously showed that TRPM7 channels can be inhibited by cytoplasmic polyamines and protons in addition to Mg^{2+} [48,90]. The observation that in perforated-patch recordings TRPM7 currents were maximally activated under low Mg^{2+} conditions (Fig. 3), similar to whole-cell (Fig. 1a) suggest that the primary inhibitory cation in Jurkat T cells is Mg^{2+} , whereas polyamines, such as spermine, and protons may play a minor role. Thus, TRPM7 channels can be used to estimate cellular Mg^{2+} levels in intact cells determined by extracellular $[\text{Mg}^{2+}]$. TRPM7 channels under low Mg^{2+}_o conditions can still be inhibited by spermine added to the internal solution (Suppl. Fig. 1a) [48,40].

Mg^{2+} efflux but not influx is sensitive to amiloride

It has been reported in other cell types that Mg^{2+} efflux is governed by the Na^+ - Mg^{2+} exchanger, thought to be encoded by SLC41A1, which is sensitive to submillimolar amiloride and imipramine [80,68,44,35]. Expression of SLC41A1 and SLC41A2, a closely related putative Mg^{2+} transporter, has been reported in Jurkat T cells [84,91]. Using RT-PCR, we compared SLC41A1 and A2 expression in Jurkat cells grown under low (8 μM) and normal (0.4 mM) Mg^{2+} . SLC41A1 mRNA levels were reduced in low Mg^{2+} , whereas SLC41A2 was unchanged (Fig. 4a). In order to determine if amiloride inhibits Mg^{2+} efflux, the cells were first grown in 1.4 mM $[\text{Mg}^{2+}]$, followed by a switch to 8 μM Mg^{2+} in the presence or absence of amiloride (Fig. 4b–d). Afterwards, preactivation index for each condition was derived from patch clamp recordings of TRPM7 currents. Depletion of Mg^{2+} in the presence of 300 μM amiloride was essentially prevented (Fig. 4b–d) after 1 – 2 days of incubation in low external Mg^{2+} . Imipramine, a tricyclic antidepressant drug that also inhibits Na^+ - Mg^{2+} exchanger was toxic to Jurkat T cells at 20 and 200 μM in a concentration-dependent manner, precluding further experiments (Suppl. Fig. 1c). Imipramine has been reported to cause apoptosis in human lymphocytes [41].

Next, we tested whether Mg^{2+} influx may also be amiloride-sensitive, reasoning that in high Mg^{2+} this exchanger may reverse direction and serve as a Mg^{2+} influx pathway, as reported for red blood cells [31]. The cells were grown in 8 μM Mg^{2+} for 1 day to deplete cellular Mg^{2+} , followed by a switch to 1.4 mM Mg^{2+} with or without amiloride for 1 day. We found no effect of 300 μM amiloride on Mg^{2+} loading as assessed by TRPM7 current pre-activation index (Fig. 4e). In agreement with a previous report, up to 300 μM amiloride did not block TRPM7 currents (data not shown)[38]. We conclude from these experiments that in Jurkat T cells Mg^{2+} efflux is likely occurring through Na^+ - Mg^{2+} exchanger SLC41A1, whereas Mg^{2+} influx occurs through an unrelated, amiloride-insensitive influx pathway. These experiments also confirmed that the observed current preactivation in cells grown in low Mg^{2+} represents cytoplasmic Mg^{2+} depletion, rather than a direct effect of external Mg^{2+} removal.

Divalent metal cation cytotoxicity under conditions when TRPM7 channels are active or inactive

TRPM7 channels have been reported to conduct divalent metal cations such as Ni^{2+} , Zn^{2+} , Ba^{2+} , Sr^{2+} , Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} and Cd^{2+} (e.g. [60,53,36,55,16,69,2]). In most cases, divalent cation permeability has been measured during acute application and/or in cells overexpressing TRPM7 heterologously using 10 mM or higher concentrations. The metal ion permeability sequence determined from patch clamp measurements was: $\text{Zn}^{2+} > \text{Ni}^{2+} \gg \text{Ba}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} \text{ Mn}^{2+} \text{ Sr}^{2+} \text{ Cd}^{2+} \text{ Ca}^{2+}$ with no permeation of La^{3+} and Gd^{3+} [60]. Uptake of the majority of these metal cations is known to be toxic to cells, including lymphocytes [29,10,36,57,1,89]. Our finding that removal or supplementation of extracellular Mg^{2+} can effectively switch on and off TRPM7 channel currents in intact cells (Figs. 1, 3), led us to investigate whether influx of permeant divalent cations through TRPM7 under these conditions can be quantitated by cytotoxicity using the trypan blue exclusion assay. We reasoned that if TRPM7 is indeed a major Mg^{2+} entry pathway in Jurkat T cells, then the cytotoxicity by divalent cations would follow the permeability sequence determined for TRPM7. On the other hand, inhibiting TRPM7 channels by Mg^{2+} -loading or pharmacological blockade would be expected to rescue cells from cytotoxicity by eliminating the uptake pathway. To address this question we grew Jurkat T cells in the presence of 0.42 mM Ca^{2+} and 0.4 mM of divalent metal cation for 1 day. Afterwards, we quantitated cell viability by trypan blue dye exclusion [25]. In cells grown in normal RPMI ($[\text{Mg}^{2+}] = 0.4 \text{ mM}$) (Fig. 5a) and in the absence of external Mg^{2+} (Fig. 5b), with TRPM7 channels activated, we found the cytotoxicity sequence to be $\text{Cd}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Ni}^{2+} > \text{Mn}^{2+} \gg \text{Ba}^{2+} > \text{Sr}^{2+} = \text{Mg}^{2+}$ (Fig. 5a). In the normal medium there was some reduction in cell killing by Mn^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} but not Cd^{2+} (Fig. 5a). Neither Ba^{2+} nor Sr^{2+} were noticeably cytotoxic (Fig. 5a, b). We then compared metal cytotoxicity in low (8 μM) and high (1.4 mM) $[\text{Mg}^{2+}]$ medium (Fig. 5c). Even in cells grown in elevated Mg^{2+} , cytotoxicity caused by Mn^{2+} , Ni^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} persisted under conditions in which TRPM7 channels are expected to be inactive due to inhibition by Mg^{2+} (Figs. 1, 2). In order to mimic the conditions in our electrophysiological experiments (Figs. 1–3) we incubated Jurkat T cells in 8 μM and 0.4 mM Mg^{2+} for 24 hours before adding divalent metal cations for another 24 hours. As shown in Fig. 5d, results were essentially similar to Fig. 5a, b except for a ~10% reduced viability in cells treated with 8 μM Mg^{2+} for 48 hours. These experiments show that cytotoxicity by Mn^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} uptake is modestly reduced when TRPM7 channels are closed but Cd^{2+} cytotoxicity is unaffected.

Effects of external Ca^{2+} on Mg^{2+} depletion and divalent metal cytotoxicity

TRPM7 channel I-V relation is strongly dependent on extracellular divalent cation concentrations. Upon removal of all external Ca^{2+} and Mg^{2+} , the I-V relation is transformed from outwardly rectifying (see Fig. 1a, b and 3a) to semi-linear [47,42]. This is thought to represent removal of Ca^{2+} and Mg^{2+} tonic pore blockade. It is likely that in the presence of Ca^{2+} (0.42 mM in our experiments) Ca^{2+} competes with Mg^{2+} and other permeant divalent cations for the conduction pathway and may thus hamper their entry [42,53,62]. In order to investigate whether reduced external Ca^{2+} modifies divalent metal cation entry, we first investigated if Mg^{2+} depletion and loading can proceed in the absence of Ca^{2+} . We incubated Jurkat T cells in Chelex-RPMI with 8 μM Mg^{2+} and 1.4 mM Mg^{2+} without added

Ca^{2+} . Incubation in low Mg^{2+} resulted in highly preactivated TRPM7 currents, whereas incubation in elevated Mg^{2+} resulted in virtually no preactivation (Fig. 6a, b). These results were essentially the same as what we observed in the presence of 0.42 mM $[\text{Ca}^{2+}]$ (Fig. 1), and demonstrate that Mg^{2+} depletion and loading do not require external Ca^{2+} . We then repeated metal cytotoxicity experiments similar to Fig. 4 in the absence of added Ca^{2+} but found that cell viability in divalent-free RPMI dropped to around 30%, making interpretation of cytotoxicity measurements challenging (Fig. 6c). Cell viability reached almost 100% when 0.42 mM $[\text{Ca}^{2+}]$ was added (Fig. 6c). Interestingly, 0.4 mM MgCl_2 alone without Ca^{2+} was also sufficient to completely rescue the cells (Fig. 6c). We then measured metal cytotoxicity in 40 μM $[\text{Ca}^{2+}]$ (roughly 10-fold lower than normal RPMI) and found significantly improved cell survival, reaching ~60% (Fig. 5d). 40 μM $[\text{Ca}^{2+}]$ is sufficient to block most of the inward monovalent TRPM7 current [42,62]. The cytotoxicity profile of Cd^{2+} , Zn^{2+} , Ni^{2+} and Co^{2+} however, remained essentially the same as in the absence of Ca^{2+} (Fig. 6c), suggesting that TRPM7 pore occupancy by Ca^{2+} and competition with other divalent cations does not significantly influence entry of these metals. The only exception was Mn^{2+} , which was somewhat less cytotoxic in 40 μM Ca^{2+} compared to no Ca^{2+} (35.2 vs. 26.3% survival; Fig. 6d). Additionally, we found that similar to Ca^{2+} , Mg^{2+} and Sr^{2+} alone also increased cell survival (Fig. 6d). In control experiments we compared cell viability in the presence of 10 and 100 μM HEDTA finding no significant difference, and concluding that contamination by divalent metals is negligible after Chelex treatment (Suppl. Fig. 1b). By contrast, addition of 100 μM EDTA modestly decreased cell viability compared to 100 μM HEDTA, which could be due to toxic effects of EDTA to cells irrespective of its chelating efficiency.

Effects of TRPM7 blockers and knockout on divalent metal cytotoxicity

In patch-clamp experiments, we tested NS8593 blockade, which was voltage-independent and 20 μM was sufficient to eliminate TRPM7 currents entirely without affecting the CRAC current in agreement with previous reports (Suppl. Fig. 1e–h) [16,23]. By contrast, we found that 400 μM La^{3+} blocks the inward component of TRPM7 currents reversibly, while leaving the outward component intact (Suppl. Fig. 1d). NS8593 was originally identified as a small conductance Ca^{2+} -activated K^+ channel (SK) inhibitor [78]. SK2 channels are highly expressed in Jurkat T cells, and 20 μM NS8593 would be sufficient to block SK channels completely (IC_{50} for SK channels is 60 nM) [78,37,20]. Therefore, we used 1 μM NS8593 as a negative control to eliminate the contribution of SK channels (Fig. 7a). To test the involvement of TRPM7 channels in entry of metal cations we maintained Jurkat T cells in the presence of either 20 μM NS8593 (Fig. 7a) or 400 μM $\text{La}(\text{NO}_3)_3$ (Fig. 7b), concentrations sufficient for significant reduction of TRPM7 channel activity. We found, however, that cytotoxicity by divalent metal cations persisted even under these conditions (Fig. 7a, b). We also tested the effect of TRPM7 channel blocker FTY720 (fingolimod) and its inactive analog FTY720 phosphate [65,79] and, as expected, found that 3 μM FTY720 reversibly inhibited TRPM7 currents whereas FTY720 phosphate at 10 μM had no effect (Suppl. Fig. 1i, j). We tested these compounds in cytotoxicity assays but found that FTY720 was itself somewhat toxic to Jurkat T cells, unlike FTY720 phosphate (Fig. 7c). From these series of experiments we conclude that divalent metal cations (Zn^{2+} , Cd^{2+} , Mn^{2+} , Co^{2+}) can enter and accumulate in Jurkat T cells, but largely not through TRPM7 channels. Notably,

Cd^{2+} , which is only sparingly permeant through TRPM7 was the most cytotoxic cation. Conversely, Ba^{2+} and Sr^{2+} , cations highly permeant through TRPM7, did not cause noticeable cell damage (see below). Martineau et al [57] evaluated Cd^{2+} cytotoxicity under Mg^{2+} depletion in MC3T3-E1 osteoblast cell line and found that it stimulated Cd^{2+} entry. The authors argued that this reflected increased TRPM7 channel activity. We did not detect any effect of Mg^{2+} loading or NS8593 on Cd^{2+} cytotoxicity, which suggests that Cd^{2+} entry pathway in this cell type, primarily occurs through a pathway unrelated to TRPM7. La^{3+} reduced Cd^{2+} and Zn^{2+} cytotoxicity (Fig. 7b), however, this may simply suggest that Cd^{2+} and Zn^{2+} entry pathways are sensitive to 400 μM La^{3+} , since La^{3+} may block other ion transport pathways (e.g. [88]).

In view of the fact that TRPM7 channel blockers are relatively non-specific, we also examined if TRPM7 serves as a divalent metal cation entry using HAP1 wildtype and TRPM7 CRISPR knockout lines [22,15,17]. Co^{2+} , Ni^{2+} and Mn^{2+} cytotoxicity was reduced in TRPM7 knockout cells compared to WT, however, Cd^{2+} and Zn^{2+} cytotoxicities were unaffected (Fig. 7d). Western blot analysis confirmed a significant reduction of TRPM7 protein in HAP1 KO cells compared to WT (Fig. 7e). These observations suggest that similar to Jurkat, Cd^{2+} and Zn^{2+} entry pathways are distinct from TRPM7 in HAP1 cells. By contrast, Co^{2+} , Ni^{2+} and Mn^{2+} entry, in part, involves TRPM7.

Cellular uptake of non-toxic metal cations evaluated by inhibition of TRPM7 channels

TRPM7 channels are inhibited not only by internal Mg^{2+} but also by other divalent and trivalent metal cations such as Zn^{2+} , Ba^{2+} , Mn^{2+} and La^{3+} [46,48]. We took advantage of this property to evaluate whether the metal cations that are non-toxic when applied externally (Fig. 4) also cannot enter the cell cytosol. We measured TRPM7 preactivation index in cells incubated in 8 μM Mg^{2+} or 1.4 mM Ba^{2+} and found no significant difference, which suggested that Ba^{2+} did not accumulate in these cells (Fig. 8a). Internal 1.4 mM Ba^{2+} readily inhibited TRPM7 currents in cells incubated in the absence (Fig. 8b) and presence (Fig. 8 c) of 1.4 mM external Ba^{2+} . Similarly, in cells incubated in 1.4 mM Sr^{2+} , preactivation index was the same as in Mg^{2+} -depleted cells (Fig. 7d). Unlike with 1.4 mM Mg^{2+} , incubation in 1.4 mM Sr^{2+} did not reduce the preactivation index (Fig. 8e). These experiments demonstrate that the non-toxic metal cations Ba^{2+} and Sr^{2+} , despite being highly permeant through TRPM7 channels [60], do not accumulate in Jurkat T cells. Ba^{2+} and Sr^{2+} applied internally through the patch pipette resulted in cell death after several minutes of recording (unpublished observations).

Blockade of TRPM7 and Mg^{2+} loading

In order to test if the blockade of TRPM7 channels will reduce Mg^{2+} entry, the cells were grown in the presence of La^{3+} and NS8593 to prevent TRPM7 channel activity. Initially the cells were grown for 1 day in 8 μM Mg^{2+} (Mg^{2+} depletion step) after which the medium was switched to 1.4 mM Mg^{2+} (Mg^{2+} loading) (Fig. 9a). We reasoned that if Mg^{2+} loading occurs through TRPM7 channels, then cells treated with La^{3+} and NS8593 would have large preactivated currents, whereas untreated cells will be fully loaded with Mg^{2+} and show no preactivation. La^{3+} blocks TRPM7 currents primarily at negative membrane potentials (Suppl. Fig. 1d) whereas NS8593 block is voltage-independent (Suppl. Fig. 1e, f). Fig. 8b

shows TRPM7 I-V relations obtained during Mg^{2+} depletion and loading in the presence of NS8593 and La^{3+} . We found that in the presence of 20 μM NS8593 Mg^{2+} loading was largely prevented (Fig. 9b, c). However, neither 3 nor 400 μM La^{3+} had an effect on Mg^{2+} loading (Fig. 9b, d). These results suggest that TRPM7 participates in Mg^{2+} loading, however, this question requires further investigation in view of lack of La^{3+} effect (see Discussion). Our results also suggest that blockade of SK2 channels with 1 μM NS8593 (Fig. 9c) or CRAC channels with 3 μM La^{3+} (Fig. 9d) have no effect on Mg^{2+} loading.

Discussion

TRPM7 and the closely related TRPM6 have been reported to participate in both cellular and body homeostasis of Mg^{2+} as well as other divalent cations like Zn^{2+} [92]. TRPM7 channels are highly expressed in primary T lymphocytes and lymphocytic cell lines (e.g. [13,79]. We were interested in TRPM7 channel function in intact cells and its role as a divalent metal entry pathway.

The goals of the present study were: 1) to characterize the behavior of TRPM7 channels in Jurkat T cells during growth in the presence of low and high extracellular Mg^{2+} concentrations 2) to evaluate whether entry of divalent metal cations Mg^{2+} , Zn^{2+} , Cd^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , Ba^{2+} , Sr^{2+} occurs via TRPM7 channels. We used two experimental approaches: patch-clamp recording of endogenous TRPM7 channel activity and cell viability measurements. Additionally, we evaluated metal ion cytotoxicity in HAP1 wildtype and TRPM7 CRISPR knockout cells.

We measured endogenous TRPM7 channel activity in Jurkat T cells grown under varying Mg^{2+} concentrations and found that the dose-response relationship had an IC_{50} of 54 μM and Hill coefficient of 0.9. TRPM7 channel activity was quantified by determining the preactivation index (I_0/I_{max}) for each cell, which reflects the fraction of channels open in an intact cell at a given Mg^{2+} concentration. The external Mg^{2+} dependence was close to what we previously observed by directly changing $[Mg^{2+}]$ on the cytoplasmic side [13,14]. Thus, intracellular $[Mg^{2+}]$ appeared to be similar to extracellular $[Mg^{2+}]$, since we saw only modestly increased Mg^{2+} in the cytoplasm due to the negative resting membrane potential. At negative membrane potentials of -55 to -70 mV in Jurkat T cells (CTL and JAK, unpublished observations), increased Mg^{2+} entry would be expected to shift the dose-response curve leftwards, but this was not observed.

External Mg^{2+} concentration dependence of TRPM7 channel activity (Fig. 1) closely resembled the dependence of splenic T-cell proliferation on Mg^{2+} [3] obtained under similar conditions. This suggests that Mg^{2+} dependence of proliferation may be mediated by a process not too far removed from increased cytoplasmic Mg^{2+} . Possibly, cytoplasmic Mg^{2+} elevations result in increased screening of $PI(4,5)P_2$ phospholipid negative charges [48,90]. We have reported that Mg^{2+} dependence of TRPM7 reflects electrostatic screening of this membrane phospholipid [48,90].

The target of Mg^{2+}_o during T-cell proliferation is not currently known and it is possible that gradual reduction of TRPM7 current in the cell due to increasing cytoplasmic Mg^{2+}

mediates increased proliferation. The mechanism might be the same as adding Mg^{2+} and other inhibitory cations internally, namely, PIP_2 screening by Mg^{2+} entering from outside. Another possibility would be a more hyperpolarized membrane potential in T cells with inhibited TRPM7 channels. This would potentiate Ca^{2+} influx through Orai1 channels. It is well known that Ca^{2+} is a second messenger required for efficient T-cell proliferation. Several studies have demonstrated that extracellular Mg^{2+} deficiency can result in reductions in cellular ATP [61,87]. Given that 80–90% of cellular Mg^{2+} is bound to ATP [71], this would be expected to have profound effects on various enzymes, including TRPM7 kinase. Interestingly, prolonged growth in low Mg^{2+} increased the maximum current amplitude, which represents the total number of TRPM7 channels in the cell (Fig. 2b,c). This was not accompanied by increased TRPM7 mRNA expression (Fig. 2d). Previously, TRPM7 mRNA expression was reported not to depend on Mg^{2+} [30]. We also found that viability of Jurkat T cells is supported equally by Ca^{2+} and Mg^{2+} (Fig. 6c). The mechanisms of external Mg^{2+} effect on native T-cell proliferation and survival and channel expression will be addressed in our future studies.

In effect, growing cells in low micromolar $[Mg^{2+}]$ for 1–2 days results in pre-activated TRPM7 current, whereas growing them in elevated Mg^{2+} (1.4 mM) completely eliminates the current in intact cells (Fig. 1). This raises questions about the interpretation of results obtained under high Mg^{2+} growth conditions (~10 mM), which would be expected to eliminate TRPM7 currents. Importantly, the majority of divalent metal cation cytotoxicity studies were done in normal culture medium, which would render TRPM7 channels in intact cells largely inactive (see Fig. 1e, f). Our approach was to test the metal cations in low Mg^{2+} to maximize TRPM7 channel activity (Fig. 5).

Growing Jurkat T cells in Chelex-RPMI containing 400 nM or 8 μ M free Mg^{2+} was sufficient to almost fully activate TRPM7 by depletion of cytoplasmic Mg^{2+} (Fig. 1). Mg^{2+} depletion and loading did not depend on external Ca^{2+} (Figs. 1 and 6). The time course of Mg^{2+} depletion was slow, reaching a maximum at ~12 hours (Fig. 2). This is significantly slower than the 10–20 minutes required for full depletion in HEK293 cells overexpressing SLC41A1 [44]. The slower depletion rate in Jurkat cells may be due to lower expression levels of endogenous SLC41A1 under our experimental conditions (Fig. 4a). Increased SLC41A1 expression in response to low Mg^{2+} treatment has been reported [28,56], however, we found a reduction in its mRNA levels in low Mg^{2+} -grown cells (Fig. 4a). We found no change in SLC41A2 expression in response to Mg^{2+} changes in agreement with [28]. In addition to Mg^{2+} , both SLC41A1 and A2 were reported to transport other divalent metal cations and may have a role in their cellular entry [28,27].

We tested other divalent metals in Chelex-RPMI containing 0.4 mM of metal cation. The goal here was to examine the potency of the metal ions in killing cells. We assumed that only those ions that enter the cells would effectively kill. The sequence of killing potency (Figs. 5, 7) did not match the divalent cation permeability sequence of TRPM7 reported by several studies. Specifically, Cd^{2+} was the most potent in cell toxicity assays (Figs. 5–7) yet it conducts through TRPM7 rather poorly [60]. Conversely, Ba^{2+} and Sr^{2+} , which are highly permeant through TRPM7 did not reduce cell viability (Figs. 5, 6). Ni^{2+} and Co^{2+} , highly permeant through TRPM7, had only moderate killing effect. Mg^{2+} loading, which would be

expected to eliminate TRPM7 current, TRPM7 blockers (La^{3+} , NS8593, FTY720) [86,16,65] and TRPM7 knockout did not rescue cells from killing by Cd^{2+} (Figs. 5, 7). Moreover, divalent metal toxicity persisted essentially unchanged in no Ca^{2+} and 40 μM Ca^{2+} , even though Ca^{2+} would be expected to compete with other divalent cations in the TRPM7 conduction pathway [53] (Fig. 6). In summary, these findings point to the existence of metal entry pathways that are distinct from TRPM7. Our findings are in agreement with [32] who reported that Zn^{2+} , Ni^{2+} and Cd^{2+} are toxic to Jurkat T cells.

In *C. elegans* cells, Ni^{2+} influx occurs through TRPM channels GON-2 and GTL-1. Toxicity by this ion was greatly reduced under Mg^{2+} loading conditions ($[\text{Mg}^{2+}]_o = 40 \text{ mM}$) or in animals with inactivating mutations in GON-2 and GTL-1 [81]. Interestingly, the authors proposed that even though TRPM7 ortholog GON-2 conducts Mg^{2+} and other cations better than GTL-1, the feedback Mg^{2+} inhibition of the former makes it a significant cation entry pathway during acute, rather than continuous perturbations of ionic homeostasis. The relevance of this idea to our study may be that cellular divalent metal ion homeostasis, which we have examined here, is probably set not only by TRPM7 but also by other pathways that are not inhibited by Mg^{2+} .

One question arising from these findings is: if TRPM7 channels are fully active why do they not provide a more substantial metal cation entry pathway in Jurkat T cells? Even if we assume that Cd^{2+} enters the cell through a different pathway which conducts this ion better than TRPM7, this cannot explain why Ba^{2+} and Sr^{2+} do not enter the cell even at 1.4 mM. It should be noted in this context, that previous studies describing metal cation entry through overexpressed and native TRPM7 employed higher concentrations (10 – 100 mM). It is therefore possible that at 0.4 mM or 1.4 mM these cations do not permeate sufficiently to enter the cell in significant quantities. Nevertheless, 0.4 mM Mg^{2+} (present in normal RPMI) does enter the cell, as evidenced by significantly inhibited TRPM7 currents (Fig. 1). One possibility is that highly permeant divalent ions, while entering initially, quickly inhibit TRPM7 channels from inside and this feedback inhibition limits their further entry. Point mutants of TRPM7 with reduced Mg^{2+} sensitivity may shed light on this possibility [34,90]. Certainly, various metal cations may enter Jurkat T cells through unrelated pathways. Jurkat T cells do not express TRPM6, making TRPM7 the only Mg^{2+} -inhibited channel. Ion channels present in Jurkat T cells have been extensively studied over the past forty years and the only well documented inward currents are TRPM7 and CRAC. In our experiments using 3 and 400 μM La^{3+} , CRAC currents would be expected to be blocked already at 3 μM [70,4], ruling out this channel as a possible pathway of divalent cation entry (Fig. 7b). Additionally, CRAC currents are not normally active in intact cells, requiring prior Ca^{2+} store depletion [63,49].

Based on our experiments, several features of the putative divalent metal entry pathway in Jurkat T cells are becoming evident: it is not sensitive to amiloride, La^{2+} and NS8593 and likely insensitive to external Mg^{2+} or Ca^{2+} . Further investigations will be required to identify the main divalent cation influx pathway in Jurkat cells.

In regards to the role of TRPM7 as a Mg^{2+} entry pathways in Jurkat T cells, we found that NS8593, for the most part prevented Mg^{2+} loading at a concentration sufficient to block

TRPM7 (Fig. 9). Surprisingly, La^{3+} , which only blocks the inward component of TRPM7 current (Suppl. Fig. 1d) was ineffective (Fig. 9). It is thought that the inward current detected in patch clamp experiments reflects divalent cation entry through TRPM7 and TRPM6 [60,82,43]. Our results suggest that both inward and outward currents through TRPM7 channels are required for cellular Mg^{2+} loading. A less likely explanation is that NS8593 at 20 μM can inhibit unrelated (and unknown) Mg^{2+} entry pathways. Interestingly, some cells could be loaded with Mg^{2+} even in the presence of NS8593, which indicates that an alternative Mg^{2+} influx pathway is functional in these cells (Fig. 9c). Future experiments will address the nature of differences between La^{3+} and NS8593 effects on Mg^{2+} loading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TRPM7	transient receptor potential melastatin 7
[Mg^{2+}]_o	external magnesium concentration
[Mg^{2+}]_i	internal magnesium concentration
CRAC	calcium release-activated calcium
SLC41A1	2 solute carrier family 41, members 1, 2

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Fig. 1. TRPM7 channel activity in Jurkat T cells grown under varying $[\text{Mg}^{2+}]_o$.

a, b. TRPM7 current-voltage relations obtained at break-in (I_0) and after dialysis with Mg^{2+} -free internal solution (I_{MAX}). Cells were grown in Chelex-RPMI supplemented with 8 μM Mg^{2+} for 1 day (**a**) or 1.4 mM Mg^{2+} for 2 days (**b**). **c, d.** Time dependence of TRPM7 current amplitude in the same cells as **a** and **b**, respectively. **e.** Scatter plot of TRPM7 current preactivation index (I_0/I_{MAX}) obtained from cells grown for 1–3 days at the indicated external Mg^{2+} concentrations. Each symbol represents current magnitude measured at 83.42 mV from one cell. Data points for 400 μM Mg^{2+} represent cells grown in normal RPMI. **f.** Dose

response relationship obtained from data shown in **e**. Each symbol represents a mean $\pm$ SEM. Numbers of cells for each Mg²⁺ concentration are in parentheses. The data were fitted with the Hill equation with IC₅₀ of 54 μ M and n= 0.9.

Fig. 2. TRPM7 current amplitudes during Mg^{2+} loading and depletion. Time course of Mg^{2+} depletion.

Dependence of TRPM7 preactivation index (**a**) and maximum amplitude (**b**) on the duration of Mg^{2+} depletion (up to 60 hrs). **c**. Maximum TRPM7 current amplitudes in Jurkat T cells incubated in 1.4 mM and 8 μM [Mg^{2+}] for 1 day. 3 cells prior to 1.4 Mg^{2+} were exposed to 8 μM Mg^{2+} . In **a–c**, Chelex-RPMI was supplemented with 0.42 mM [CaCl_2]. TRPM7 current amplitudes were measured at 83.77 mV (**a**, **b**) and 83.42 mV (**c**). **d**. RT-PCR of total RNA isolated from Jurkat T cells incubated in 8 μM and 0.4 mM Mg^{2+} in the presence of 0.42 mM CaCl_2 for 1 day. Primers for TRPM7 and GAPDH were used with predicted amplicon sizes of 734 and 555 bp, respectively. In lanes marked –RT here and in Fig. 4a, reverse transcriptase was omitted from the reaction.

Fig. 3. Perforated-patch recording of TRPM7 currents in Mg^{2+} -depleted intact Jurkat T cells. Jurkat T cells were grown in Chelex-RPMI supplemented with $8 \mu\text{M} [\text{Mg}^{2+}]_o$ as in Fig. 1a, b for two days. **a.** Preactivated TRPM7 current-voltage relation obtained in perforated-patch configuration (red) and after inhibition by internal Mg^{2+} and Ca^{2+} upon break-in (black). **b.** Time dependence of TRPM7 current amplitude in the same cell. A representative recording of $n=4$ cells is shown.

Fig. 4. Sensitivity of Mg^{2+} efflux and influx to amiloride.

a. Expression of SLC41A1 and SLC41A2 in Jurkat T cells. RT-PCR with primers specific for SLC41A1 and A2 was performed with RNA isolated from cells maintained in 8 μ M and 0.4 mM Mg^{2+} for 1 day. Predicted amplicon sizes were 694 and 374 bp for SLC41A1 and A2, respectively. **b–d.** Jurkat T cells were first grown in 1.4 mM Mg^{2+} then transferred to 8 μ M Mg^{2+} containing RPMI for 1 (**a, c**) or 2 days (**d**) before performing whole-cell recordings. **a.** Top: superimposed time courses of TRPM7 current development in cells incubated in the presence (red) and absence (black) of amiloride for 1 day. **c, d.** Scatter plots of preactivation indices obtained from cells grown in control and 300 μ M amiloride-containing media for 1 day or 2 days **e.** After growing in 8 μ M Mg^{2+} for 1 day, cells were transferred to 1.4 mM Mg^{2+} in the presence and absence of 300 μ M amiloride. Asterisks indicate significant differences by Student's t test, $p < 0.01$.

Fig. 5. Viability assay of cells grown in the presence of divalent metal cations.

Viabilities of Jurkat T cells were tested in the presence of 0.4 mM Zn^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} , Ba^{2+} , Sr^{2+} , after maintaining in normal RPMI containing 0.4 mM Mg^{2+} (a) or in Chelex-RPMI without Mg^{2+} (b). Asterisks in a and b denote comparisons to Mg^{2+} control (black bar) ($p < 0.05$). c. Comparison of metal cation toxicities in cells grown in 8 μM and 1.4 mM Mg^{2+} added to Chelex-RPMI. In the control only Mg^{2+} at indicated concentrations was added. d. Cell viability measurements similar to c except with 24 hr preincubation in 8 μM or 0.4 mM Mg^{2+} containing Chelex-RPMI before addition of toxic metal cations for 24 hrs.

0.42 mM CaCl_2 was added to Chelex-RPMI in **b–d**. Double asterisks in **c** and **d** denote two-sample t-test comparisons for the same metal under low and high Mg^{2+} conditions ($p < 0.01$). Graphs **a–d** represent means of 3 independent experiments each.

Fig. 6. Effect of external Ca^{2+} on Mg^{2+} depletion and cell viability.

Jurkat T cells were incubated in Chelex-RPMI for 1 day without Ca^{2+} supplementation at 1.4 mM and 8 μM Mg^{2+}_o concentrations. **a.** TRPM7 current-voltage relations at break-in (black) and after maximum current development (red) for cells incubated in 1.4 mM (top) and 8 μM $[Mg^{2+}]_o$ (bottom) in the absence of added Ca^{2+} . **b.** Preactivation indices (I_0/I_{max}) of cells treated as in **a.** Asterisk represents $p < 0.05$, two-sample Student's test. **c.** Cell viability assay for cells in the absence of added Ca^{2+} . **d.** Cell viability assay for cells grown in 40 μM $CaCl_2$ supplemented Chelex-RPMI. In **c** and **d**, double asterisks ($p < 0.01$) represent comparisons with 0 and 40 μM Ca^{2+} , respectively.

Fig. 7. Effects of TRPM7 channel blockade and knockout on divalent metal cytotoxicity. Jurkat T cells were grown in the presence of 0.4 mM metal ion and NS8593 (**a**), La³⁺ (**b**), FTY720 and FTY720-P (**c**). Chelex-RPMI was **d**. Divalent metal toxicity (0.4 mM) in WT and TRPM7 CRISPR knockout HAP1 cells. **e**. Western blot analysis of TRPM7 protein in anti-TRPM7 immunoprecipitates from whole cell lysate of WT and TRPM7 KO HAP1 cells. Actin was used as an internal control. Chelex-RPMI was used in **a**, **b** and Chelex-advanced RPMI in **c**. In **d**, Chelex-RPMI supplemented with 1.4 mM CaCl₂ was used. Asterisks denote statistical significance for pairwise comparisons for each metal cation under two conditions.

Fig. 8. Ba^{2+} and Sr^{2+} uptake evaluated by inhibition of TRPM7 channels.

Jurkat T cells were treated as shown for each panel in the scheme and x axis labels. **a.** TRPM7 preactivation indices for cells grown in 8 μM [Mg^{2+}] and 1.4 mM [Ba^{2+}] without Mg^{2+} . The difference was not significant ($p=0.39$ from Student's t test and $p=0.25$ from Wilcoxon rank sum test). **b.** Inhibition of TRPM7 current by internal Ba^{2+} in a cell grown in 1.4 mM [Ba^{2+}]. **c.** Inhibition of TRPM7 current by internal Ba^{2+} in a cell grown in 8 μM Mg^{2+} and no Ba^{2+} . **d.** TRPM7 preactivation indices in cells grown in 8 μM [Mg^{2+}] and 1.4 mM [Sr^{2+}] without Mg^{2+} . The difference was not significant ($p=0.25$ from Student's t test, $p=0.37$ from Wilcoxon rank sum). **e.** Comparison of preactivation indices in cells grown in 1.4 mM [Mg^{2+}] vs. 1.4 mM [Sr^{2+}], $*p<0.0001$ from Student's t test, $p=0.0159$ from Wilcoxon rank sum test. In **a–e** internal solutions contained no Mg^{2+} .

Fig. 9. Effect of TRPM7 blockers on Mg^{2+} loading.

Experiments similar to Fig. 4 were performed in the presence of La^{3+} and NS8593. **a.**

Experimental paradigm used to measure Mg^{2+} loading. **b.** TRPM7 current-voltage relations obtained (break-in ($t=0$) and I_{max}) in cells incubated in 8 μM , followed by 1.4 mM Mg^{2+} in the presence of NS8593 or La^{3+} . **c.** TRPM7 preactivation indices in cells grown in 1.4 mM [Mg^{2+}] with NS8593. **d.** TRPM7 preactivation index in cells grown in 1.4 mM [Mg^{2+}] with La^{3+} . In **c** and **d** 1 μM NS8593 and 3 μM La^{3+} were used as negative controls since at these concentrations TRPM7 channels are not affected.