Transient Receptor Potential Melastatin 7 Ion Channels Regulate Magnesium Homeostasis in Vascular Smooth Muscle Cells
Role of Angiotensin II

Ying He, Guoying Yao, Carmine Savoia, Rhian M. Touyz

Abstract—Magnesium modulates vascular smooth muscle cell (VSMC) function. However, molecular mechanisms regulating VSMC Mg\(^{2+}\) remain unknown. Using biochemical, pharmacological, and genetic tools, the role of transient receptor potential membrane melastatin 7 (TRPM7) cation channel in VSMC Mg\(^{2+}\) homeostasis was evaluated. Rat, mouse, and human VSMCs were studied. Reverse transcriptase polymerase chain reaction and immunoblotting demonstrated TRPM7 presence in VSMCs (membrane and cytosol). Angiotensin II (Ang II) and aldosterone increased TRPM7 expression. Gene silencing using small interfering RNA (siRNA) against TRPM7, downregulated TRPM7 (mRNA and protein). Basal [Mg\(^{2+}\)], measured by mag fura-2AM, was reduced in siRNA-transfected cells (0.39±0.01 mmol/L) versus controls (0.54±0.01 mmol/L; P<0.01). Extracellular Mg\(^{2+}\) dose-dependently increased [Mg\(^{2+}\)], in control cells (E\(_{\text{max}}\) 0.70±0.02 mmol/L) and nonsilencing siRNA-transfected cells (E\(_{\text{max}}\) 0.71±0.04 mmol/L), but not in siRNA-transfected cells (E\(_{\text{max}}\) 0.5±0.01 mmol/L). The functional significance of TRPM7 was evaluated by assessing [Mg\(^{2+}\)], and growth responses to Ang II in TRPM7 knockout cells. Acute Ang II stimulation decreased [Mg\(^{2+}\)], in control and TRPM7-deficient cells in a Na\(^{+}\)-dependent manner. Chronic stimulation increased [Mg\(^{2+}\)], in control, but not in siRNA-transfected VSMCs. Ang II–induced DNA and protein synthesis, measured by \(^{3}\)[H]-thymidine and \(^{3}\)[H]-leucine incorporation, respectively, were increased in control and nonsilencing cells, but not in TRPM7 knockdown VSMCs. Our data indicate that VSMCs possess membrane-associated, Ang II–, and aldosterone-regulated TRPM7 channels, which play a role in regulating basal [Mg\(^{2+}\)], transmembrane Mg\(^{2+}\) transport and DNA and protein synthesis. These novel findings identify TRPM7 as a functionally important regulator of Mg\(^{2+}\) homeostasis and growth in VSMCs. (Circ Res. 2005;96:207-215.)

Key Words: cations ■ TRPM channels ■ vessels ■ aldosterone ■ angiotensin II ■ siRNA

Magnesium plays a major role in regulating vascular smooth muscle cell (VSMC) function. Increased intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]) causes vasodilation and attenuates agonist-induced vasoconstriction, whereas reduced [Mg\(^{2+}\)], has opposite effects, leading to hypercontractility and impaired vasorelaxation.\(^{1-4}\) Mg\(^{2+}\) also influences growth processes associated with remodeling and fibrosis, characteristic features of vascular damage in hypertension, atherosclerosis, and diabetes.\(^{5,6}\) At the subcellular level, these effects occur, at least in part, through Mg\(^{2+}\)-dependent regulation of mitogen-activated protein (MAP) kinases, tyrosine kinases, and reactive oxygen species, important signaling molecules involved in VSMC proliferation, fibrosis, and inflammation.\(^{7-9}\) Microarray studies demonstrated that changes in [Mg\(^{2+}\)], have potent modulatory actions on expression of various growth signaling molecules.\(^{10}\) We recently reported that altered [Mg\(^{2+}\)], influences cell cycle progression and VSMC growth by modulating cyclin-dependent kinases and MAP kinases.\(^{11}\)

Intracellular Mg\(^{2+}\) homeostasis is tightly regulated. In VSMCs, basal [Mg\(^{2+}\)], is maintained at 0.5 to 0.6 mmol/L.\(^{11,12}\) Although Mg\(^{2+}\) is the second most abundant intracellular cation and the predominant divalent cation, molecular mechanisms regulating cellular Mg\(^{2+}\) remain elusive.\(^{13}\) Some studies suggested that transmembrane Mg\(^{2+}\) transport occurs through the Na\(^{+}/\)Ca\(^{2+}\) antiporter,\(^{14}\) whereas others demonstrated that Mg\(^{2+}\) efflux is linked to Na\(^{+}/\)H\(^{+}\) exchange.\(^{15}\) Renal paracellular Mg\(^{2+}\) transport is mediated via paracellin-1.\(^{16,17}\) We reported that Mg\(^{2+}\) efflux is regulated by a Na\(^{+}\)-dependent Mg\(^{2+}\) exchanger linked to the Na\(^{+}/\)H\(^{+}\) antiporter in VSMCs.\(^{15,18}\)

Although studies of Mg\(^{2+}\) fluxes in mammalian cells have indicated the presence of functionally active plasma membrane Mg\(^{2+}\) transport mechanisms, proteins responsible for...
these fluxes have not been identified. Recent investigations suggested that two novel ion channels of the long or melastatin-related, transient receptor potential (TRPM) ion channel subfamily, TRPM6 and TRPM7, are critically involved in Mg²⁺ influx in epithelial and neuronal cells. TRPM6/7 are polypeptides with dual-function ion channel/protein kinases, characterized by six transmembrane spanning domains with an adjacent coiled coil region, a long, highly conserved cytoplasmic N-terminal region, and a cytoplasmic C terminus, which has enzymatic activity. TRPM6 and TRPM7, which have an overall amino acid sequence homology of 52%, harbor serine/threonine kinase domains in their C termini. TRPM6 is preferentially expressed in small intestine, colon, and kidney, participating in gastrointestinal and renal Mg²⁺ absorption. Mutations in TRPM6 cause hypomagnesemia with secondary hypocalcemia.

Expression of TRPM7 is widespread with transcripts in brain, spleen, lung, kidney, heart, and liver. It also expressed in lymphoid-derived cell lines, hematopoietic cells, granulocytes, leukemia cells, and microglia. In various cell lines, TRPM7 is regulated by intracellular levels of Mg-ATP and is strongly activated when Mg-ATP falls below 1 mmol/L. Studies in microglial and HEK293-transfected cells demonstrated that TRPM7 activity is also modulated through its endogenous kinase in a Ca²⁺, PKA-, and Src-dependent manner and is inactivated by PIP2 hydrolysis in cardiac fibroblasts.

To our knowledge nothing is known about the status of TRPM7 in the vasculature. It is unclear whether this cation channel influences Mg²⁺ transport in vascular cells and whether vasoactive agents regulate TRPM7. To gain insights into the putative role of TRPM7 in vascular Mg²⁺ homeostasis, we used a combination of biochemical, pharmacological, molecular and genetic approaches to investigate the presence and functional significance of TRPM7 in VSMCs. Our data demonstrate that VSMCs possess functionally active membrane-associated TRPM7 channels that are regulated by angiotensin II (Ang II) and aldosterone. Findings from this study identify for the first time that TRPM7 is a key modulator of vascular Mg²⁺ homeostasis and that it plays an important role in regulating VSMC function.

Materials and Methods

Cell Culture

The study was approved by the Animal and Human Ethics Committee of the Clinical Research Institute of Montreal and performed according to the recommendations of the Canadian Council for Animal Care. VSMCs from mesenteric arteries and aorta from Wistar Kyoto rats (WKY; Taconic Farms, Germantown, NY) and C57/B6J mice (Jackson Laboratory, Bar Harbor, Me) were isolated by enzymatic digestion and cultured as we described. VSMCs from healthy humans were derived from small arteries obtained from healthy humans were derived from small arteries obtained from healthy humans. VSMCs from C57/B6J mice (Jackson Laboratory, Bar Harbor, Me) were isolated from the gluteal biopsies as we detailed. Cells were maintained in DMEM containing 10% fetal calf serum (FCS). Low passaged cells (passages 2 to 7) were studied.

Reverse Transcriptase Polymerase Chain Reaction

Expression of TRPM7 gene was studied by reverse transcriptase polymerase chain reaction (RT-PCR). Primers for rat, mouse, and human TRPM7 are detailed in the Table. Cells were stimulated with vehicle (water), Ang II (10⁻⁷ mol/L), or aldosterone (10⁻⁷ mol/L) for 2 to 24 hours. Total RNA was extracted from cells (TRIzol Reagent). Reverse transcription was performed in 20 μL containing 2 μg RNA, 1.0 μL of 10 mmol/L dNTP, 4 μL of 5X first strand buffer, 1.0 μL oligo-(dT)₁₂–₁₈ primer (0.5 μg/μL), 1.0 μL of 200 μM M-MLV reverse transcriptase (GIBCO-BRL), 1.0 μL of rNasin (RNase inhibitor, 40 U/μL), 2 μL dithiothreitol (0.1 μmol/L), for 1 hour, 37°C. The reaction was stopped by heating at 70°C for 15 minutes. Two microliters of resulting cDNA mixture was amplified using specific primers (Table). TRPM7 amplification by PCR involved 95°C for 5 seconds, 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds, and extension for 5 minutes at 72°C. GAPDH amplification by PCR involved 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 45 seconds, and extension for 5 minutes, at 72°C. Amplification products were electrophoresed on 1% agarose gel containing ethidium bromide (0.5 μg/mL). Bands corresponding to RT-PCR products were visualized by UV light and digitized using Alphalager software. Band intensity was quantified using the ImageQuant software (version 3.3, Molecular Dynamics) software.

TRPM7 Protein Expression

Total protein was extracted from VSMCs as we described. Briefly, cells were washed with cold PBS and then harvested in HEPES buffer containing (in mmol/L), HEPES 10, pH 7.4, NaF 50, NaCl 50, EDTA 5, EGTA 5, Na pyrophosphate 50, containing triton-X 100 0.5%, phenylmethylsulfonyl fluoride (PMSF) 1mmol/L, leupeptin 1 μg/mL, and aprotinin 1 μg/mL. Cells were disrupted by brief sonication. Samples were then centrifuged (500g, 10 minutes, 4°C) to remove nuclei. For membrane and cytosol separation, samples were centrifuged at 100 000g for 1 hour at 4°C. The cell membrane was washed once with the buffer described above and then resuspended in buffer containing 100 mmol/L Tris-HCl, 300 mmol/L NaCl, 1% Triton X-100, and 0.1% SDS containing 2 mmol/L EDTA, 2 mmol/L PMSF, and 0.8 μg/mL leupeptin. Proteins (20 μg) were separated by electrophoresis on polyacrylamide gel (7.5%) and transferred onto a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (TBS-T) (1 hour, room temperature). Membranes were incubated with anti-TRPM7 antibody (1:750) (Abcam Inc) in TBS-T-milk at 4°C overnight with agitation. Washed membranes were incubated with horse-radish peroxidase-conjugated second antibody (1:2000) in TBS-T-Milk (room temperature, 1 hour). Membranes were washed and immunoreactive proteins detected by chemiluminescence. Blots were analyzed densitometrically (Image-Quant software, Molecular Dynamics).

Measurement of [Mg²⁺]i and [Ca²⁺]i in VSMCs

The selective fluorescent probes, mag fura-2AM and fura-2AM, were used to measure [Mg²⁺]i and [Ca²⁺]i, respectively, as described (see online data supplement available at http://circres.ahajournals.org). Responses to increasing concentrations of

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer Sequence</th>
<th>Predicted Size</th>
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<tr>
<td>Rat</td>
<td>5'-GCA AAT GAC TCC ACT CTC-3′</td>
<td>422</td>
</tr>
<tr>
<td>Mouse</td>
<td>5'-GATTCTCTTCTACCTCCAG-3′</td>
<td>422</td>
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<tr>
<td>Human</td>
<td>5'-CATCATTGCTGATGGATG-3′</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>5'-ATGAGCCCTTCCACATGCC-3′</td>
<td>435</td>
</tr>
</tbody>
</table>

S indicates sense; AS, antisense.
extracellular Mg\(^2+\) (0 to 5 mmol/L) were measured in cells incubated in Mg\(^2+\)-free, Ca\(^2+\)-containing modified Hanks’ buffer (in mmol/L, NaCl 137, NaHCO\(_3\) 4.2, NaHPO\(_4\) 3, KCl 5.4, KH\(_2\)PO\(_4\) 0.4, CaCl\(_2\) 1.3, glucose 10, and HEPES 5; pH 7.4). Cells were exposed to Mg\(^2+\)-free buffer for 15 to 20 minutes before addition of extracellular Mg\(^2+\). In some experiments, Ang II effects were assessed in Na\(^-\)-free Hanks’ buffer (Na\(^+\) isotonically replaced with N-methylglucamine). Sequences identical in human and mouse but that do not match other sequences in GenBank were used. siRNAs for knocking down TRPM7 were synthesized by QIAGEN Inc. The DNA target sequences of the annealed double strands were as follows: 5’-AAGCAGAGTGACCTGTTGAAGC-3’ (1884–1904), which has 100% homology to mouse gene only, and 5’-AAGCAGAGTGACCTGTTGAAGC-3’, which has 100% homology to both mouse and human gene. siRNA, with a nonsilencing oligonucleotide sequence (nonsilencing siRNA) that does not recognize any known homology to mammalian genes, was also generated as a negative control.

RNA Interference and Cell Transfection

High-performance purity grade (>90% pure) small interfering RNAs (siRNA) were generated against TRPM7. Sequences identical in human and mouse but that do not match other sequences in GenBank were used. siRNAs for knocking down TRPM7 were synthesized by QIAGEN Inc. The DNA target sequences of the annealed double strands that we used were as follows: 5’-AAGCAGAGTGACCTGTTGAAGC-3’ (1884–1904), which has 100% homology to mouse gene only, and 5’-AAGCAGAGTGACCTGTTGAAGC-3’, which has 100% homology to both mouse and human gene. siRNA, with a nonsilencing oligonucleotide sequence (nonsilencing siRNA) that does not recognize any known homology to mammalian genes, was also generated as a negative control.

Cells were seeded at a density of 1.6×10\(^5\) cells/well in 6-well plates and grown in DMEM containing 10% FCS and antibiotics. One day after seeding, cells were transfected with siRNA using RNAiFect Transfection Reagent (Qiagen Inc) according to the manufacturer’s instructions. Briefly, siRNA (5 μg) was mixed with EC-R buffer (Qiagen Inc) (100 μL) to which RNAiFect transfection reagent (15 μL) was added. After mixing (15 minutes), the lipoformulation was added dropwise onto the cells. Control cells were exposed to transfectant in the absence of siRNA. Forty-eight hours after transfection, gene silencing was monitored at the mRNA and protein levels by RT-PCR and Western blotting, respectively.

Measurement of DNA and Protein Synthesis by Ang II in TRPM7 siRNA-Transfected VSMCs

Cells were seeded, at an initial concentration of 1×10\(^4\) cells/mL, into 24-well multiwell plates and grown in DMEM containing 10% FCS and antibiotics. One day after seeding, cells were exposed to transfectant alone (control cells) or transfected with siRNA or nonsilencing siRNA as described earlier. Thirty-six hours after transfection, cells were stimulated with Ang II (10\(^{-11}\) to 10\(^{-6}\) mol/L, 24 hours) in the absence and presence of valserant, selective AT\(_1\) receptor blocker (10\(^{-5}\) mol/L). DNA and protein synthesis were evaluated by measuring incorporation of \(^3\)H-thymidine (5 μCi/mL) and \(^3\)H-leucine (2 μCi/mL, respectively, as we described. Briefly, after incubation, radioactive medium was removed, cells were washed with ice cold physiological buffered saline, incubated with trichloroacetic acid (TCA) (0.75 mol/L, 15 minutes), washed with cold TCA, and incubated with NaOH (0.2 mol/L for thymidine, 1 mol/L for leucine, 60 minutes, room temperature). Relative incorporation of \(^3\)H-thymidine and \(^3\)H-leucine was determined by liquid scintillation counting.

Statistical Analysis

Data are presented as mean±SEM. Groups were compared using one-way ANOVA or Student t test as appropriate. Tukey-Kramer correction was used to compensate for multiple testing procedures. A value of P<0.05 was significant.

Results

TRPM7 Expression in VSMCs

Presence of TRPM7 transcript was assessed by RT-PCR. GAPDH mRNA was used as an internal housekeeping gene and results were expressed as the ratio of TRPM7:GAPDH. As demonstrated in Figure 1A, mRNA for TRPM7 is present in VSMCs. Ang II and aldosterone significantly increased TRPM7 mRNA expression (Figure 1B). Maximal agonist-induced responses were obtained within 4 to 6 hours of stimulation.

To evaluate TRPM7 protein content in VSMCs, immunoblotting was performed using anti-TRPM7 antibody. α-Actin was used as an internal control. As shown in Figure 2A, SDS-PAGE analysis of total cell homogenate revealed a major band migrating at the 160- to 170-kDa position, corresponding to TRPM7. To establish whether TRPM7 is membrane-associated, we also probed for TRPM7 content in membrane-rich fractions. Figure 2B demonstrates that TRPM7 is present in VSMC membranes and that Ang II stimulation significantly increases membrane TRPM7 content (P<0.05 versus control). Daudi cell lysate (Abcam Inc) was used as a positive control.

Downregulation of TRPM7 Attenuates Basal [Mg\(^2+\)]\(_i\) and Abrogates Mg\(^2+\) Influx

The functional significance of TRPM7 in VSMC [Mg\(^2+\)]\(_i\), regulation was assessed in cells in which TRPM7 gene was silenced by siRNA. TRPM7 mRNA expression and protein abundance were markedly reduced in human and mouse VSMCs transfected with siRNA, but not in control cells or cells transfected with nonsilencing siRNA (Figure 3). Basal [Mg\(^2+\)]\(_i\), was significantly decreased (P<0.01) in siRNA-transfected cells (0.39±0.01 mmol/mL) compared with control (0.54±0.01 mmol/mL) and nonsilencing siRNA-transfected cells (0.51±0.02 mmol/mL). Results were not significantly different between control and nonsilencing transfected cells. Exposure of VSMCs to increasing concentrations of extracellular Mg\(^2+\) resulted in a significant [Mg\(^2+\)]\(_i\), rise (P<0.01) in control cells and in cells transfected with nonsilencing siRNA (Figure 4). Maximal [Mg\(^2+\)]\(_i\), responses were obtained at 3 mmol/L extracellular Mg\(^2+\), above which [Mg\(^2+\)]\(_i\), did not increase further. Exposure of siRNA-transfected cells to increasing concentrations of extracellular Mg\(^2+\) induced a modest, but nonsignificant increase, in [Mg\(^2+\)]\(_i\), (Figure 4).

To evaluate whether TRPM7 regulates Ca\(^2+\) homeostasis in VSMCs, [Ca\(^2+\)], effects of ionomycin were assessed in TRPM7 knockdown cells. Ionomycin induced a rapid [Ca\(^2+\)], rise as evidenced by increased fura-2 fluorescence. Basal [Ca\(^2+\)], was unaltered in siTRPM7-deficient cells (106±10 nmol/L versus control (94±4 nmol/L) and nonsilencing siRNA-transfected cells (102±6 nmol/L). Although ionomycin-mediated [Ca\(^2+\)], responses were slightly reduced in TRPM7-deficient cells, responses were not significantly different from control cells (Figure 1, online data supplement).
Essential Role of TRPM7 in Ang II–Stimulated VSMC Growth

To evaluate the functional significance of Ang II–regulated TRPM7, growth effects of Ang II were assessed in TRPM7 knockdown VSMCs. As demonstrated in Figure 6, Ang II dose-dependently increased incorporation of ³[H]-thymidine and ³[H]-leucine, indices of DNA and protein synthesis, respectively, in control and nonsilencing siRNA-transfected cells, but not in siRNA-transfected cells. Valsartan, a selective AT₁ receptor blocker, inhibited Ang II–mediated cell growth (Figure 2, online data supplement).

**Discussion**

In this study, we provide the first evidence that VSMCs possess TRPM7 channels, that TRPM7 is functionally active, and that this ion channel plays an essential role in regulating VSMC growth.

Figure 1. A, TRPM7 transcript is present in vascular smooth muscle cells (VSMC). mRNA was prepared from rat VSMCs (from aorta, Ao, and mesenteric arteries, Mes) and mouse VSMCs (from mesenteric arteries). TRPM7-specific forward and reverse primers amplified a product of the correct size but not in negative controls (NC) when cDNA was omitted. M indicates marker; PCR, polymerase chain reaction. B, Effects of Ang II and aldosterone on TRPM7 mRNA expression. mRNA was prepared from rat VSMCs exposed to Ang II or aldosterone (10⁻⁷ mol/L, Aldo) for 2 to 24 hours. Representative scans demonstrate amplified PCR products corresponding to TRPM7 and GAPDH. Bar graphs are mean±SEM of 3 to 5 experiments. Data are presented as TRPM7/GAPDH ratio. *P<0.05 vs Control (Cont) counterpart.
Mg\textsuperscript{2+} influx and maintaining intracellular Mg\textsuperscript{2+} levels. We also demonstrate that Ang II and aldosterone, which modulate vascular tone and structure, influence TRPM7 abundance. Finally, we report that Ang II–regulated TRPM7 is important in the long-term, but not in the short-term regulation of VSMC [Mg\textsuperscript{2+}], and that it plays a fundamental role in cell growth. Our findings suggest that TRPM7 is a highly regulated transmembrane Mg\textsuperscript{2+} transporter, critically involved in VSMC Mg\textsuperscript{2+} homeostasis and growth.

Transient receptor potential channels, particularly of the TRPC (for canonical TRP) and TRPV (for vanilloid TRP) subfamilies, have been demonstrated to be functionally important in regulating Ca\textsuperscript{2+} entry in VSMCs and endothelial cells.\textsuperscript{41–43} To our knowledge, nothing is known about TRPM7 status in vascular cells, although these ion channels have been well characterized in renal and gastric epithelial cells and in numerous mammalian immortalized cell lines.\textsuperscript{21,22,27,28,33} In the present study, we demonstrate that mouse, rat, and human VSMCs possess membrane-associated TRPM7.

Many vasoactive agents influence VSMC function, of which Ang II is particularly important. Ang II stimulates vascular contraction, growth, and inflammation, in part through Mg\textsuperscript{2+}-dependent processes.\textsuperscript{39,44} Aldosterone, a mineralocorticoid hormone that influences cellular Mg\textsuperscript{2+} metabolism,\textsuperscript{45,46} is increasingly being recognized as an important modulator of vascular function.\textsuperscript{47,48} In this study, we demonstrate that Ang II and aldosterone regulate TRPM7 mRNA and protein content. Exact mechanisms whereby these agonists control TRPM expression are unknown. However, both peptides stimulate activity of many transcription factors, which could play a role in de novo TRPM production.

To evaluate the functional significance of TRPM7 in VSMCs, we generated siRNAs to selectively reduce TRPM7 expression and used two independent assays to test siRNA...
activity and specificity. First, we questioned whether siRNA would attenuate TRPM7 content, and second, we used fluorescence digital imaging to test whether siRNA prevents Mg$^{2+}$ and Ca$^{2+}$ transmembrane transport. Using a lipid-based system for siRNA transfection, almost 100% TRPM7 gene silencing was obtained as evidenced by significantly reduced mRNA and protein expression. Previous studies reported that cultured cells rendered TRPM7-deficient via cre-lox–mediated destruction of the TRPM7 gene, undergo growth arrest and die after a few days in culture. In our investigations, cells remained viable for the duration of our studies. This may relate to the fact that TRPM7 was transiently downregulated and that experiments were performed 48 hours after transfection. Cell death was previously reported to occur 48 to 72 hours after TRPM7 gene silencing in TRPM7-deficient cell lines.

Basal [Mg$^{2+}$], was reduced in TRPM7-deficient cells, confirming the importance of TRPM7 in VSMC Mg$^{2+}$ homeostasis. Similar findings were observed in stably transfected 293-HEK cells expressing human TRPM7 mutants and in TRPM7 knockout cell lines. Increasing concentrations of extracellular Mg$^{2+}$ resulted in a marked [Mg$^{2+}$], rise in control cells. In contrast, in TRPM7-depleted cells (by siRNA), [Mg$^{2+}$], did not change significantly when exposed to high extracellular Mg$^{2+}$ levels. These findings suggest that TRPM7 facilitates transmembrane Mg$^{2+}$ transport in VSMCs, probably by promoting Mg$^{2+}$ influx through an ion channel mechanism, [Mg$^{2+}$], seems to be highly regulated, because [Mg$^{2+}$], plateaued despite increasing concentrations of extracellular Mg$^{2+}$ above 3 mmol/L. Possible reasons for this may relate first to the reciprocal interaction between intracellular Mg$^{2+}$ and channel activity and second to the presence of regulatory Mg$^{2+}$ efflux systems. There is now compelling evidence that intracellular Mg$^{2+}$ negatively influences TRPM7 channel activity. As [Mg$^{2+}$], increases, TRPM7 activity declines. This inhibitory feedback loop between excess intracellular Mg$^{2+}$ and channel activity may be important in maintaining [Mg$^{2+}$], within a physiological range and in protecting cells against Mg$^{2+}$ overload. It is also possible that as cellular Mg$^{2+}$ levels increase, Mg$^{2+}$ efflux systems, such as the Na$^{+}$/Mg$^{2+}$ exchanger, are activated.

Data from studies in cell lines demonstrate that TRPM7 has a unique permeation profile with a permeability sequence of Zn$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, Cd$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Sr$^{2+}$, Cd$^{2+}$, Ca$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Zn$^{2+}$. Because Ca$^{2+}$ plays a fundamental regulatory role in VSMC function, and because previous studies demonstrated that TRPM7 is a Ca$^{2+}$-permeable ion channel, we questioned whether TRPM7, in addition to regulating Mg$^{2+}$ influx, influences transmembrane Ca$^{2+}$ transport in VSMCs. Basal [Ca$^{2+}$], was unaltered in TRPM7-deficient cells, indicating that this ion channel may not be important in ambient Ca$^{2+}$ homeostasis in VSMCs. These findings are in contrast to neuronal-derived cells, where TRPM7 seems to be a major regulator of Ca$^{2+}$ influx. Ionomycin increased [Ca$^{2+}$], in both control and TRPM7 knockdown cells. Although responses...
were slightly attenuated in TRPM7-deficient cells, these effects were not significantly different from control cells. Taken together, our data indicate that TRPM7 channel is a major regulator of VSMC Mg\(^{2+}\)/H\(^{10}\) homeostasis and that it may be less important in maintaining Ca\(^{2+}\)/H\(^{11}\). Our findings confirm others demonstrating that TRPM7 channels are ion selective and that sensitivity for Mg\(^{2+}\) is greater than that for Ca\(^{2+}\).52,53

A major finding in our study relates to the temporal regulation of TRPM7 and Mg\(^{2+}\) by Ang II. Short-term exposure of cells to Ang II resulted in a rapid Mg\(^{2+}\), Mg\(^{2+}\) and TRPM7

Figure 5. TRPM7 plays a role in chronic, but not in acute, Ang II–mediated Mg\(^{2+}\) regulation. Ang II Mg\(^{2+}\) effects were assessed in VSMCs transfected with nonsilencing siRNA (NS-siRNA), siRNA against TRPM7, or in the presence of transfectant alone (Control). A, Acute Ang II–induced responses. Cells were exposed to Ang II for 5 minutes. B, Acute responses to Ang II (5 minutes) in Na\(^{+}\)-free conditions. C, Chronic Ang II–induced responses. Cells were exposed to Ang II for 24 hours. *P<0.05 vs basal counterpart; **P<0.01 vs basal counterpart; †P<0.05 vs control and NS-siRNA counterpart.

Figure 6. Essential role of TRPM7 in Ang II–stimulated VSMC growth. Effects of Ang II on \(^{3}\)H\()\)thyidine and \(^{3}\)H\())\)leucine incorporation in control, nonsilencing- (NS-siRNA), and siRNA-transfected cells. *P<0.05 vs other groups; **P<0.01 vs other groups; †P<0.05 vs NS-siRNA counterpart.
TRPM7, because intracellular Mg²⁺

However, long-term Ang II exposure resulted in a significant increase, which was abrogated in Na⁺-free conditions. Responses were similar in control and TRPM7 knockdown cells. However, long-term Ang II exposure resulted in a significant increase, which was not evident in TRPM7-deficient cells. These findings suggest that acute Ang II stimulation mediates transmembrane Mg²⁺ transport through Na⁺-dependent, TRPM7-independent pathways, whereas chronic stimulation leads to TRPM7-dependent Mg²⁺ influx. Putative mechanisms underlying these events could relate to initial activation of the Na⁺/Mg²⁺ exchanger by Ang II, resulting in Mg²⁺ influx, as we previously reported,

followed by activation of TRPM7 leading to Mg²⁺ influx and increased [Mg²⁺], (Figure 7). Upregulation of Ang II-stimulated TRPM7 may be due, in part, to increased TRPM7 content, as we demonstrated at the gene and protein levels. It is also possible that Ang II-induced [Mg²⁺], reduction stimulates TRPM7, because intracellular Mg²⁺ negatively influences TRPM7 channel activity.

At the functional level, we demonstrate that TRPM7 is important in Ang II-regulated growth of VSMCs. Incorporation of [³²P]H-thymidine and [¹⁴C]leucine was significantly increased by Ang II in control but not in TRPM7 knockdown cells, indicating that Ang II–stimulated DNA and protein synthesis in VSMCs require functionally active TRPM7. These processes are mediated through AT₁ receptors, because valsartan inhibited Ang II–induced effects. Although previous studies suggested that TRPM7 is involved in cell growth,

our findings here are the first to demonstrate that TRPM7/Mg²⁺-dependent pathways play a role in Ang II–regulated growth of VSMCs.

In summary, using a combination of biochemical, pharmacological, and genetic tools, we provide evidence that VSMCs possess functionally active TRPM7 ion channels that play an important role in modulating VSMC Mg²⁺ homeostasis and growth. Furthermore, we demonstrate that Ang II regulates VSMC [Mg²⁺], in a temporal and biphasic fashion such that acute Ang II stimulation mediates Mg²⁺ efflux through the Na⁺/Mg²⁺ exchanger, whereas chronic stimulation induces Mg²⁺ influx through TRPM7-sensitive pathways. To our knowledge, these are the first data to identify a putative regulator of transmembrane Mg²⁺ transport in VSMCs. These findings contribute to the further understanding of molecular mechanisms involved in Mg²⁺ homeostasis in vascular cells and to the signaling mechanisms underlying Ang II–mediated growth of VSMCs.

Acknowledgments

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References


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Methods

Measurement of \([\text{Mg}^{2+}]_i\) and \([\text{Ca}^{2+}]_i\) in VSMCs

The selective fluorescent probes, mag fura-2AM and fura-2AM were used to measure \([\text{Mg}^{2+}]_i\) and \([\text{Ca}^{2+}]_i\), respectively as described (1-4). Upon complexation with Mg\(^{2+}\), mag-fura 2 exhibits fluorescent changes and undergoes a shift in excitation wavelength. Mag-fura 2 has a binding constant for Mg\(^{2+}\) of 1.5 mmol/L (3). We fully characterized mag fura-2 specificity for Mg\(^{2+}\) in previous studies (4).

Washed cells were loaded with mag fura-2AM or fura-2AM (4 \(\mu\)mol/L) and incubated for 30 minutes at room temperature. \([\text{Mg}^{2+}]_i\) and \([\text{Ca}^{2+}]_i\) were measured in multiple cells simultaneously by fluorescence digital imaging system (Attofluor Ratiovision, Zeiss, West Germany) using an emission wavelength of 520 nm and alternating excitatory wavelengths of 343 nm and 380 nm (1-5). Video images of fluorescence at 520nm emission were obtained using an intensified charge-coupled device camera system with the output digitized to a resolution of 512 x 480 pixels. Images of fluorescence ratios were obtained by dividing, pixel by pixel, the 343 nm image after background subtraction by the 380 nm image after background subtraction.

\([\text{Mg}^{2+}]_i\) responses to increasing concentrations of extracellular Mg\(^{2+}\) (0-5 mmol/L) were measured in cells incubated in Mg\(^{2+}\)-free, Ca\(^{2+}\)-containing modified Hanks buffer (in mmol/L, NaCl 137, NaHCO\(_3\) 4.2, NaHPO\(_4\) 3, KCl 5.4, KH\(_2\)PO\(_4\) 0.4, CaCl\(_2\) 1.3, glucose 10 and HEPES 5, pH 7.4). Cells were exposed to Mg\(^{2+}\)-free buffer for 15-20 minutes before addition of extracellular Mg\(^{2+}\). \([\text{Ca}^{2+}]_i\) responses to ionomycin (10\(^{-6}\) mol/L) were determined in cells incubated for 15-20 minutes in modified Hank’s buffer containing Mg\(^{2+}\) (mmol/L, MgCl\(_2\) 0.5, MgSO\(_4\) 0.8).
References


Figure Legends

Figure 1. [Ca\textsuperscript{2+}]\textscript{i} responses in TRPM7-deficient VSMCs. Human VSMCs were transfected with non-silencing siRNA and TRPM7 siRNA as described in Methods. [Ca\textsuperscript{2+}]\textscript{i} was measured using fura-2AM (4 umol/L). Upper panel, representative fura-2AM tracings in control cells and cells transfected with non-silencing (NS) and TRPM7 silencing RNA. Cells were incubated for 15-20 minutes in Mg\textsuperscript{2+}-containing, Ca\textsuperscript{2+}-containing Hank’s buffer. Arrow indicates addition of ionomycin (10\textsuperscript{-6} mol/L). Lower panel, Bar graphs demonstrate maximal [Ca\textsuperscript{2+}]\textscript{i} effect of ionomycin in control and TRPM7-deficient cells. Results are means\pm SEM of 3 experiments with each experimental field comprising 18-26 cells. **p<0.01 vs basal counterpart.

Figure 2. Effects of valsartan, AT\textsubscript{1} receptor blocker, on Ang II-stimulated growth of VSMCs. Incorporation of \textsuperscript{3}H-thymidine and \textsuperscript{3}H-leucine was assessed in control, non-silencing siRNA- and siRNA-transfected VSMCs treated with Ang II (10\textsuperscript{-7} mol/L, 24 hours) with or without vasartan (val) (10\textsuperscript{-5} mol/L, 24 hours). **p<0.01 vs corresponding control, valsartan and valsartan+Ang II groups.
Figure 1

[Graph showing changes in VSMC [Ca^2+]i (nmol/L) with control, NS siRNA, and siRNA treatments.]

- Control
- NS siRNA
- siRNA

VSMC [Ca^2+]i (nmol/L) vs. time (60 secs) with arrows indicating changes due to treatments.

[Bar graph showing Basal and Ionomycin responses with control, NS siRNA, and siRNA conditions.]
Figure 2

**[H] Thymidine incorporation (% of control)

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**[H] Leucine incorporation (cpm of control)

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