Differential regulation of transient receptor potential melastatin 6 and 7 cation channels by ANG II in vascular smooth muscle cells from spontaneously hypertensive rats

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MAGNESIUM (Mg2+), the second most abundant intracellular cation, is critically involved in regulating vascular smooth muscle cell (VSMC) function (34, 44). Through its Ca2+ antagonistic properties Mg2+ negatively modulates intracellular free Ca2+ concentration ([Ca2+]i), a major determinant of VSMC contraction/dilatation. Mg2+ influences VSMC growth and apoptosis through its modulatory actions on MAP kinases, tyrosine kinases/phosphatases, gene expression, and cell cycle proteins (1, 38, 45a). Changes in [Mg2+]i are associated with endothelial dysfunction, inflammatory responses, altered vascular tone, and structural remodeling, characteristic features of hypertensive vascular damage (20, 44a). In particular low Mg2+ conditions contribute to increased vascular tone, enhanced responses to vasoconstrictor agents, blunted vasodilatation, oxidative stress, vascular remodeling, and increased blood pressure (14, 37).

Accumulating evidence indicates reduced [Mg2+]i, in vascular and circulating cells from experimental models of hypertension and essential hypertensive patients (37). Intracellular Mg2+ deficiency contributes to vascular dysfunction in hypertension, as normalization of [Mg2+]i improves vascular tone, attenuates oxidative stress and inflammation, regresses vascular remodeling, and prevents development of hypertension (14, 39).

Despite the fact that intracellular Mg2+ is the most abundant divalent intracellular cation and that it modulates over 320 enzymes, processes regulating cellular Mg2+ homeostasis remain elusive. Some studies suggested that transmembrane Mg2+ transport occurs through the Na+/Ca2+ exchanger linked to the Na+/H+ antipporter (21, 32), while others demonstrated that Mg2+ efflux is linked to Na+/H+ exchange (35). Renal Mg2+ transport is mediated via paracellin-1, a paracellular process (30, 43). We reported that Mg2+ efflux is regulated by a Na+-dependent Mg2+ exchanger linked to the Na+/H+ antiporter in VSMCs (33, 35, 36).

Until recently, specific proteins mediating transcellular Mg2+ influx were unknown. In 2001, the mitochondrial Mg2+ transporter Mrs2 was suggested as a candidate transporter (46). In 2003, Mrsp2 was identified as an essential component of the major electrophoretic Mg2+ influx system in mitochondria (11). More recently, transient receptor potential melastatin (TRPM) 6 and 7 were found to be master regulators of cellular Mg2+ influx. Confirmation of the importance of TRPM6 and TRPM7 in Mg2+ homeostasis is evidenced by findings that mutations in TRPM6 cause hypomagnesemia with secondary hypocalcemia (3, 27, 41), that thiazide-induced Mg2+ wasting
is linked to TRPM6 downregulation (19), and that mutant TRPM7 in dwarf zebrafish is associated with defective skel-letonogenesis, altered mineral metabolism, and kidney stone for-mation (5).

TRPM6 and TRPM7, cloned and characterized by two independent groups (24, 28), are novel dual-function proteins, comprising an ion channel related to the TRP proteins fused with a Ser/Thr kinase (16, 29). These proteins are the only known examples of a kinase domain incorporated in the same molecule with an ion-channel pore (2, 26). They are Mg2+-permeable channels that facilitate Mg2+ influx (15, 40). TRPM6 expression is restricted mainly to epithelial cells, whereas TRPM7 expression is widespread (12). In various cell lines, TRPM7 is negatively regulated by intracellular levels of Mg ATP (6, 18), and activity is modulated through its endog-enous kinase in a cAMP-, PKA- and Src-dependent manner (10, 31) and is inactivated by PIP2 hydrolysis in cardiac fiброblasts (25). The only known substrate for TRPM7 kinase is annexin I (4). We recently demonstrated that TRPM7 is present and functionally active in VSMCs and that it is a major regulator of Mg2+ influx (9).

Because vascular [Mg2+], homeostasis is altered in hyper-tension (37), we questioned whether TRPM6 and TRPM7 may play a role in this process. Here, we sought to determine whether TRPM6 and TRPM7 are differentially regulated in VSMCs from Wistar Kyoto (WKY) and spontaneously hyper-tensive rats (SHR). Findings from our study demonstrate that whereas TRPM6 is equally expressed in VSMCs from WKY and SHR, TRPM7 abundance and activity are reduced, ANG II-induced expression of TRPM7 is blunted and [Mg2+]i is attenuated in SHR. These novel data suggest that downregulation of TRPM7-mediated Mg2+ transport may play an important role in intracellular Mg2+ deficiency in VSMCs from SHR.

MATERIALS AND METHODS

Cell culture. The study was approved by the Animal and Human Ethics Committee of the University of Ottawa and carried out according to the recommendations of the Canadian Council for Animal Care. VSMCs from mesenteric arteries from 16-wk-old WKY and SHR were isolated by enzymatic digestion and cultured as we have described (38). Cells were maintained in DMEM containing 10% FCS. Low passaged cells (passages 2–7) were used.

RT-PCR. Expression of the TRPM6 and TRPM7 genes was studied by RT-PCR. Primers are detailed in Table 1. Cells were stimulated with vehicle (water) and ANG II (10−7 mol/l) for 2–24 h. 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 5 × first-strand buffer, 1.0 μl oligo-(dT)12–18 primer (0.5 μg/μl), 1.0 μl of 200 U/μl M-MLV reverse transcriptase (GIBCO-BRL, Carlsbad, CA), 1.0 μl of rRNAin (RNase inhibitor, 40 U/μl), 2 μl dithiothreitol (0.1 mol/l), for 1 h, 37°C. The reaction was stopped by heating at 70°C for 15 min. Two microliters of resulting cDNA mixture was amplified using specific primers (table). TRPM6 and TRPM7 amplification by PCR involved: 95°C for 5 s, 35 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s and extension for 5 min at 72°C. GAPDH amplification by PCR involved 94°C for 5 min, 30 cycles of 94°C, 30 s, 57°C for 30 s, 72°C for 45 s and extension for 5 min, at 72°C. Amplification products were electrophoresed on 1% agarose gel containing ethidium bromide (0.5 μg/ml). Bands corre-sponding to RT-PCR products were visualized by UV light and digitized using AlphaImager software. Band intensity was quantified using the ImageQuant (version 3.3, Molecular Dynamics) software.

TRPM6 and TRPM7 protein expression. Total protein was extracted from VSMCs as we described (38). Briefly, cells were washed with cold PBS and then harvested in HEPES buffer containing (in mmol/l), 10 HEPES, pH 7.4, 50 NaF, 50 NaCl, 5 EDTA, 5 EGTA, 50 Na pyrophosphate, containing Triton X-100 0.5%. 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were disrupted by brief sonication. Samples were then centrifuged (500 g, 10 min, 4°C) to remove nuclei. Proteins (20 μg) were separated by electrophoresis on polyacrylamide gel (7.5%) and trans-ferred onto a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (TBS-T) (1 h, room temperature). Membranes were incubated with anti-TRPM6 antibody (1:500) (kind gift from T. Gudermann) and anti-TRPM7 antibody (1:750) (Abcam Cambridge, MA) in TBS-T-milk at 4°C overnight with agitation. Monoclonal antibody to alpha-actin (Sigma-Aldrich, St. Louis, MO) was used as an internal control. Washed membranes were incubated with horse- radish peroxidase-conjugated secondary antibody (1:2000) in TBS-T-Milk (room temperature, 1 h). Membranes were washed, and immu-noresponsive proteins detected by chemiluminescence. Blots were ana-lyzed densitometrically (Image-Quant software, Molecular Dynamics, Sunnyvale, CA).

Assessment of annexin-I activity. Annexin-I activation was assessed by determining translocation from the cytosol to the membrane. Cells, stimulated with ANG II (10−7 mol/l) for 10 min, were fractionated to obtain cytosol- and membrane-rich fractions. Homog-enates were centrifuged at 50,000 g for 30 min at 4°C, thereby isolating cytosolic fraction in the supernatant. The particulate fraction was incubated under constant shaking for 30 min at 4°C in lysis buffer containing 1% Triton X-100 and centrifuged at 50,000 g for 30 min at 4°C. Western blotting was performed as described above using anti-annexin-I antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:500).

Measurement of [Mg2+]i, in VSMCs. The selective fluorescent probe, mag-fura-2 AM, was used to measure [Mg2+]i. Cells were washed with modified Hank’s buffered saline solution containing (in mmol/l): 137 NaCl, 5.4 KCl, 4.2 NaHCO3, 3Na2HPO4, 0.4 KH2PO4, 1.3 CaCl2, 0.5 MgCl2, 0.8 MgSO4, 10 glucose, 5 N2-Hydroxethyl-piperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4, and loaded with mag-fura-2 AM (4 μmol/l), which was dissolved in dimethyl sulfoxide with 0.02% pluronic acid. Cells were incubated for 30 min at room temperature and then washed with warmed buffer and incubated for a further 15 min to ensure complete deesterification. Under these loading conditions, the ratiometric (343/380 nm) fluorescence cell images were homogeneous, indicating that there was no significant intracellular compartmentalization of mag-fura-2. The coverslip-containing cells was placed in a stainless steel chamber and mounted on the stage of an inverted microscope (Axiovert 135, Zeiss, AJP-Regul Integr Comp Physiol • VOL 290 • JANUARY 2006 • www.ajpregu.org
West Germany) as previously described (35). \([\text{Mg}^{2+}]\), was measured in basal and in ANG II-stimulated cells (10^{-7} \text{ mol/l}, 2 h).

Statistical analysis. Data are presented as means ± SE. Groups were compared using one-way ANOVA or Student’s t-test as appropriate. Tukey-Kramer’s correction was used to compensate for multiple testing procedures. \(P < 0.05\) was significant.

RESULTS

**mRNA expression of TRPM6 and TRPM7.** Basal mRNA expression of TRPM6 was similar in VSMCs from WKY and SHR (Fig. 1). Basal TRPM7 mRNA content was significantly reduced in VSMCs from SHR compared with WKY (Fig. 2). Exposure of cells to ANG II for 2–24 h induced a slight increase in TRPM6 in WKY and SHR cells. However, significance was not achieved. On the other hand, ANG II time-dependently increased TRPM7 expression in WKY cells, but not in SHR VSMCs. In all experiments, GAPDH was used as an internal housekeeping gene, and data are expressed as the TRPM6/7 ratio relative to GAPDH expression.

**TRPM6 and TRPM7 abundance in VSMCs.** Western blot analysis demonstrated the presence of TRPM6 and TRPM7 in VSMCs. Basal expression of TRPM6 tended to be lower in WKY cells compared with SHR, although significance was not achieved (Fig. 3). Basal content of TRPM7 was significantly reduced in SHR vs. WKY VSMCs (Fig. 4). ANG II induced a modest, nonsignificant increase in TRPM6 expression in WKY and SHR (Fig. 3). TRPM7 abundance was time-dependently increased by ANG II in WKY, but not in SHR cells (Fig. 4).

**Activation of annexin-1.** Translocation of annexin-1, the only known specific substrate of TRPM7, was assessed as an index of TRPM7 activity. As shown in Fig. 5, annexin-1 translocation from cytosol to membrane was attenuated in SHR cells compared with WKY, both in basal and ANG II-stimulated conditions.

**VSMC \([\text{Mg}^{2+}]\), in SHR and WKY.** Basal and ANG II-stimulated \([\text{Mg}^{2+}]\), as assessed by mag fura-2 methodology, was significantly lower in VSMCs from SHR compared with WKY (Fig. 6). Compared with basal \([\text{Mg}^{2+}]\), ANG II-stimulated \([\text{Mg}^{2+}]\), was elevated in VSMCs from WKY but not in cells from SHR (Fig. 6).
DISCUSSION

Major findings from the present study demonstrate that 1) VSMCs possess both TRPM6 and TRPM7 cation channels, 2) TRPM7 is the predominant TRPM in VSMCs and 3) ANG II influences TRPM6/7 regulation. In addition, we provide evidence that TRPM6 is equally abundant in VSMCs from WKY and SHR, whereas basal and ANG II-stimulated TRPM7 expression and activity are attenuated in SHR. These novel findings suggest that TRPM6 and TRPM7 are differentially regulated in VSMCs and that TRPM7 may be the major Mg\(^{2+}\)-permeant cation channel responsible for transcellular Mg\(^{2+}\) transport in VSMCs. Downregulation of TRPM7 may be responsible, at least in part, for reduced VSMC [Mg\(^{2+}\)]\(_i\) in SHR. Such processes may contribute to altered regulation of VSMC Mg\(^{2+}\), which may contribute to vascular dysfunction in hypertension.

TRPM6 shows \(50\%\) homology with TRPM7 and has a restricted expression pattern predominantly present in epithelia (8, 42). TRPM6 is preferentially expressed in the small intestine, colon and kidney, participating in gastrointestinal and renal Mg\(^{2+}\) absorption (13, 27, 28, 40). Here, we demonstrate for the first time that TRPM6, both at the gene and protein levels, is present in VSMCs and that its expression is regulated by ANG II. Unlike TRPM7, TRPM6 was not differentially expressed in cells from WKY and SHR. The exact functional significance of vascular TRPM6 remains unclear, and its role in Mg\(^{2+}\) transport in VSMCs still awaits clarification. Because expression patterns were equal between WKY and SHR, we propose that vascular TRPM6 may not be critically involved in altered VSMC Mg\(^{2+}\) homeostasis in hypertension.

Expression of TRPM7 is widespread with transcripts in brain, spleen, lung, kidney, heart, and liver (17, 24). It is also expressed in lymphoid-derived cell lines, hematopoietic cells, granulocytes, leukemia cells and microglia (10, 29), and we recently reported that TRPM7 is present and functionally active in human and mouse VSMCs (9). Here, we show that TRPM7 is also present in rat VSMCs and that it is differentially expressed in cells from WKY and SHR. Basal TRPM7 content, both at the mRNA and protein levels, was lower in SHR compared with WKY. Stimulation by ANG II increased TRPM7 abundance in WKY, but not in SHR. These findings indicate diminished vascular TRPM7 expression and blunted regulation of TRPM7 by ANG II in SHR.

To investigate whether altered TRPM7 status translates to changes in TRPM7 activity, we examined annexin-1, the only known specific downstream target of TRPM7 (4). Annexin-1 is a Cu\(^{2+}\)- and phospholipid-binding protein that translocates to the cell membrane upon activation (23). It influences cell growth and differentiation and plays a role in anti-inflammatory actions of glucocorticoids (23). Within 10 min of ANG II stimulation, annexin-1 translocated from the cytosol to the cell

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Fig. 4. TRPM7 protein content in VSMCs from WKY and SHR. Cells were stimulated with ANG II for 4–24 h. Results are presented as the ratio between TRPM7 and \(\beta\)-actin content. Results are means \(\pm\) SE of three experiments. *\(P<0.05\) vs. Veh counterpart. +\(P<0.05\) vs. WKY counterpart.

Fig. 5. Annexin-1 translocation in VSMCs from WKY and SHR. Cells were stimulated with ANG II (10\(^{-7}\) mol/l), and annexin-1 expression was determined by immunoblotting in the membrane and cytosolic fractions. Results are presented as the annexin-1 content in membrane fraction:cytosolic fraction. Results are expressed as means \(\pm\) SE. *\(P<0.05\) vs. basal, **\(P<0.01\) vs. WKY counterpart.

Fig. 6. Intracellular free Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)) in VSMCs from WKY and SHR in basal and ANG II-stimulated conditions. Cells were exposed to ANG II (10\(^{-7}\) mol/l) for 2 h. Results are expressed as means \(\pm\) SE of 3–6 experiments. *\(P<0.05\), **\(P<0.01\) vs. WKY counterpart, +\(P<0.05\) vs. basal counterpart.
membrane, indicating activation. This acute response was temporally disassociated from changes in TRPM7 expression, which was evident a few hours after stimulation. However, TRPM7 is rapidly activated through an autophosphorylation process (26). Hence, rapid annexin-1 stimulation may be linked to acute-phase activation of TRPM7, followed by a more prolonged activation state. The exact kinetics between annexin-I and TRPM7 await clarification. Activation of VSMC annexin-1, measured as a cytosolic/membrane translocation, was lower in SHR vs. WKY. Although this phenomenon may be due to multiple factors, we propose here that it is related to TRPM7 downregulation in SHR. This is supported by the findings that both TRPM7 expression and annexin-1 translocation are attenuated in basal conditions in SHR vs. WKY. Furthermore, in WKY, but not in SHR, ANG II-induced upregulation of TRPM7 was associated with an increase in annexin-1 activity.

Considering that TRPM7 is a Mg\(^{2+}\)-permeant channel responsible for transcellular Mg\(^{2+}\) transport (28) and an important mediator of cell growth (7, 39), it is possible that altered cellular Mg\(^{2+}\) homeostasis and abnormal VSMC function in hypertension may be related to defective TRPM7 expression/activity. We previously demonstrated that TRPM7 knockdown by siRNA in VSMCs was associated with reduced [Mg\(^{2+}\)]\(_i\) and altered cellular growth, which was reversible when Mg\(^{2+}\) was normalized (9). Here, we show that basal and ANG II-stimulated [Mg\(^{2+}\)]\(_i\) is markedly reduced in VSMCs from SHR. This may be due, at least in part, to downregulation of TRPM7 and consequent decreased transmembrane Mg\(^{2+}\) transport. It is also possible that defective TRPM7 activity contributes to vascular dysfunction in hypertension independently of changes in [Mg\(^{2+}\)]\(_i\). However, this awaits further clarification.

In conclusion, findings from the present study demonstrate that TRPM6 and TRPM7 are present in rat VSMCs, that TRPM6 is similarly expressed in WKY and SHR, and that TRPM7 content and activity are reduced in SHR. Furthermore, we show that ANG II influences TRPM6 and TRPM7 and that ANG II-induced effects are blunted in SHR. Together with the observation that VSMC [Mg\(^{2+}\)]\(_i\) is reduced in SHR, we suggest that downregulation of TRPM7 may play a role in altered vascular Mg\(^{2+}\) homeostasis in SHR. These novel findings provide new insights into putative mechanisms underlying cellular regulation of Mg\(^{2+}\) in hypertension.

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