A mechanosensitive ion channel regulating cell volume

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Hua SZ, Gottlieb PA, Heo J, Sachs F. A mechanosensitive ion channel regulating cell volume. Am J Physiol Cell Physiol 298: C1424–C1430, 2010. First published March 24, 2010; doi:10.1152/ajpcell.00503.2009.—Cells respond to a hypotonic challenge by swelling and then returning toward the resting volume, a process known as the regulatory volume decrease or RVD. The sensors for this process have been proposed to include cationic mechanosensitive ion channels that are opened by membrane tension. We tested this hypothesis using a microfluidic device to measure cell volume and the peptide GsMTx4, a specific inhibitor of cationic mechanosensitive channels. GsMTx4 had no effect on RVD in primary rat astrocytes or Madin-Darby canine kidney (MDCK) cells but was able to completely inhibit RVD and the associated Ca2+ uptake in normal rat kidney (NRK-49F) cells in a dose-dependent manner. Gadolinium (Gd3+), a nonspecific blocker of many mechanosensitive channels, inhibited RVD and Ca2+ uptake in all three cell types, demonstrating the existence of at least two types of volume sensors. Single-channel stretch-activated currents are present in outside-out patches from NRK-49F, MDCK, and astrocytes, and they are reversibly inhibited by GsMTx4. While mechanosensitive channels are involved in volume regulation, their role for volume sensing is specialized. The NRK cells form a stable platform from which to screen drugs that affect volume regulation via mechanosensory channels and as a sensitive system to clone the channel.

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CELL VOLUME REGULATION is essential for viability since metabolism and the resulting changes in osmotic pressure are universal, and environmental challenges are commonplace (1, 21). How does a cell know if its volume has changed? One common model uses mechanosensitive ion channels (MSCs), either cation selective (8, 9, 25) or anion selective (30, 33) and activated by membrane tension in a swollen cell. Proposed effector proteins that restore volume after a perturbation include transporters of neutral osmolytes such as the KCl cotransporter (13, 40), amino acid transporters (20), paired anion/cation channels, or the cytoskeleton operating in a three-dimensional mode (34).

In the traditional interpretation of cell volume regulation as a membrane-bound process (33, 35), cell swelling causes an increase in membrane tension that activates mechanosensitive channels, allowing an influx of Ca2+ and Na+. Most cells do show an influx and elevation of intracellular Ca2+ before regulatory volume decrease (RVD), suggesting that it serves as a second messenger for effector proteins (15). Ca2+ elevation can be triggered directly by Ca2+ passing through MSCs (8) or arriving through the Na+/Ca2+ exchanger (31). Channels selective for particular ions cannot regulate volume by themselves since volume regulation requires transport of net neutral solutes. In bacteria, the nonselective MSCs protect against extreme osmotic stress (22) because they open before lysis. Despite the presumptive role of MSCs as sensors of animal cell volume, channel activation has only been occasionally observed in patch-clamp experiments (8, 25) and there is no direct evidence linking MSCs to volume regulation. The hypothesis that MSCs play a role in volume regulation began as an explanation of why MSCs are present in all cells (8). Supporting the idea of MSCs having a sensory function for RVD, the lanthanides such as Gd3+ and La3+ prove capable of inhibiting both the channels (17, 43), swelling-induced Ca2+ elevation, and volume regulation (4, 9). However, the lanthanides are nonspecific reagents and may have multiple targets.

In contrast, the cationic MSCs are specifically inhibited by GsMTx4, a 4-kDa inhibitory cysteine knot (ICK) peptide (28) found in tarantula venom (6). The peptide has unusual pharmacological properties as it functions in both the D and L enantiomeric forms (39). If these mechanosensitive channels serve as sensors for volume regulation, then RVD should be blocked by GsMTx4, and we have tested this prediction in three different cell types. Only one cell type, normal rat kidney (NRK-49F), responded to GsMTx4 with a loss of RVD and Ca2+ uptake, while astrocytes and Madin-Darby canine kidney (MDCK) cells were unaffected. In contrast, Gd3+ inhibited both responses in all three cell types, consistent with its observed nonspecific interaction with MSCs and other membrane-bound proteins and lipids. The ability of GsMTx4 to inhibit RVD in NRK cells by inhibiting the channels, Ca2+ uptake, and volume regulation suggests that in this cell type the channels do in fact serve as physiological sensors of cytoplasmic osmotic stress. The MSCs in other cell types apparently serve different functions.

MATERIALS AND METHODS

Cell volume measurement. Cell volume was measured at room temperature using an impedance-based microfluidic sensor (3) that measures the resistance of a small chamber containing the cells. As cells swell, they displace saline and thus increase the chamber resistance. The cells were grown as monolayers on coverslips, and these solutions, and the cell volume was monitored in real time. The ratio of volume change to the resting volume was calculated as previously described (18). A fresh cell culture was used for each trace, and the data represent the first challenge since we have found that RVD rapidly disappears with repeated stimulation (3). Results are plotted as means ± SE of n traces.

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Cytosolic Ca\(^{2+}\) measurements. Cells were washed and incubated in isotonic solution containing 6 μM Fluo-4 AM (Invitrogen, Carlsbad, CA) at 37°C for 15 min for astrocytes, and 40 min for MDCK and NRK cells. The cells were then washed and kept in the dark at room temperature for 15 min before the experiment. The microfluidic chamber was also used for fluorescence measurements using a Zeiss upright microscope (Axio Imager M1, Zeiss), and the data were recorded using a charge-coupled device camera (AxioVision MRm, Zeiss). We used the filter set 38 HE EGFP (excitation: 470 ± 40 nm; dichroic filter: 495 nm; emission: 525 ± 50 nm, Zeiss). Time-lapse experiments were recorded with AxioVision software (Zeiss). The latencies for the Ca\(^{2+}\) increase were quantified as the time from solution change until reaching 10% of (peak-baseline) Ca\(^{2+}\).

Patch clamp. An Axopatch 200B (Axon Instruments) was used to control patch voltage. Data were collected (National Instruments) using QUBIO software. Currents were sampled at 10 kHz and low-pass filtered at 2 kHz through a four-pole Bessel filter. All potentials are defined with respect to the extracellular surface.

Electrodes were pulled on a HEKA pipette puller (PIPs), painted with Sylgard 184 (Dow Corning), and fire polished. Electrodes were filled with potassium solution of (in mM) 140 KCl, 5 EGTA, 3 MgCl\(_2\), and 10 HEPES, pH 7.4. Electrode resistances ranged from 3 to 8 MΩ. Bath saline contained (in mM) 140 NaCl, 5 KCl, 3 MgCl\(_2\), 1 CaCl\(_2\), and 10 HEPES, pH 7.4. Pressure pulses were generated with a high-speed pressure clamp (ALA Scientific, Farmingdale, NY). GsMTx4 was perfused over the patch at the indicated concentration using an ALA Scientific perfusion system. Offline data analysis was done with free QUB software (www.qub.buffalo.edu). Data were averaged for a minimum of five pressure pulses.

Cell culture. Cells were grown to 80–95% confluence on glass coverslips in 35-mm petri dishes. Primary astrocytes were isolated from rat brain and stored frozen as previously described (37) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penicillin and streptomycin. NRK-49F cells obtained from American Type Culture Collection were cultured in DMEM containing 5% calf serum and 1% penicillin and streptomycin. MDCK epithelial cells were grown in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin.

Solution and reagents. Isotonic and hypotonic solutions both contained (in mM) 75 NaCl, 5 KCl, 1 CaCl\(_2\), 2 MgCl\(_2\), and 10 HEPES titrated to pH 7.4. The isotonic solution (320 mosM) was prepared by adding mannitol to the hypotonic solution (187 mosM), keeping the ionic strength fixed. To remove extracellular Ca\(^{2+}\), CaCl\(_2\) was replaced with MgCl\(_2\). The conductivity of all solutions was gently titrated to equality using NaCl, and the final osmolarity was measured with an osmometer (model 303, Advanced Instrument). Gadolinium chloride (Sigma-Aldrich, St. Louis, MO) was prepared as a 10 mM stock solution in water. The enantiomeric forms of GsMTx4 were perfused over the patch at the indicated concentration using an ALA Scientific perfusion system.

![Figure 1](http://ajpcell.physiology.org/)

Fig. 1. Effects of GsMTx4 on cell volume regulation of normal rat kidney (NRK-49F) cells (A and B), primary rat astrocytes (C), and Madin-Darby canine kidney (MDCK) cells (D). The chamber was perfused with isotonic solution (Iso) for 10–15 min and then switched to hypotonic solution (Hypo) at the times indicated. The ratio of the volume change to the resting cell volume, ΔV/V₀, is plotted in A–D. Both GsMTx4 L enantiomer (5 μM) and D enantiomer (2.5 μM) blocked regulatory volume decrease (RVD) in the NRK cells (control and L-form, n = 5; D-form, n = 3). The cells responded in a dose-dependent fashion (B) with an apparent K_d of 100–200 nM. In primary rat astrocytes (n = 5) and MDCK (n = 6) cells, 5 μM GsMTx4 in both isotonic and hypotonic solutions had no significant effect on RVD (C and D).
GsMTx4 were made and purified according to previously published protocols (26, 39).

RESULTS

Effects of GsMTx4 on RVD. Cells were initially tested for RVD in the sensing chamber by perfusing an isotonic solution (320 mosM) followed by a hypotonic solution (187 mosM). To test the effect of GsMTx4, we perfused fresh cultures (to avoid the adaptation seen in repeated stimulation) with isotonic saline containing 5 μM GsMTx4 for 10–15 min followed by a hypotonic challenge in saline also containing 5 μM GsMTx4.

In NRK cells, GsMTx4 eliminated RVD and the cells continued to swell as long as the stimulus was applied (Fig. 1A). The D enantiomer of GsMTx4 (2.5 μM) also blocked the RVD in NRK cells as shown in Fig. 1A. However, GsMTx4 had no effect on RVD in either astrocytes or MDCK cells (Fig. 1, C and D). In NRK cells, the dose-response behavior of RVD to GsMTx4 (Fig. 1B) indicated a $K_d$ of 100–200 nM in agreement with published results on the sensitivity of MSCs to GsMTx4 in patch-clamp experiments (37). GsMTx4 had no significant effect on the steady-state volume of challenged MDCK or astrocytes, although it slightly slowed the kinetics of RVD in both cell types.

While all three cell types have cationic MSCs that are inhibited by GsMTx4 as shown later in this section, the results above suggest that cationic MSCs do not universally function as volume sensors. MSC currents that are visible in inside-out or cell-attached patch-clamp recordings appear generally unresponsive to osmotic stress in the bath, suggesting that they

Fig. 2. Sensitivity of RVD to Gd$^{3+}$ for NRK-49F (A), primary rat astrocytes (B), and MDCK cells (C). Gd$^{3+}$ (100 μM) in isotonic and hypotonic solutions inhibited RVD in all three cell types, causing a slow, extended, increase in volume (NRK, n = 4; astrocytes, n = 3; MDCK, n = 3).

Fig. 3. The dependence of cell volume regulation on extracellular Ca$^{2+}$ for NRK-49F (A), primary rat astrocytes (B), and MDCK cells (C). The removal of Ca$^{2+}$ suppressed RVD in all three cell types (NRK, n = 4; astrocytes, n = 4; MDCK, n = 3).
are well protected by the cytoskeleton (24, 34). The effect of GsMTx4 on mild slowing of the RVD kinetics in astrocytes and MDCK cells may be an effect of GsMTx4 on Ca\textsuperscript{2+} - activated K\textsuperscript{+} channels (19, 32).

Since the lanthanides are known to inhibit RVD (9), swelling-induced Ca\textsuperscript{2+} uptake (2), and MSC activity (43), we tested the cells’ sensitivity to 100 \( \mu \)M Gd\textsuperscript{3+}. In agreement with previously published work (25), Fig. 2, A–C, shows that Gd\textsuperscript{3+} inhibited RVD and Ca\textsuperscript{2+} uptake (data not shown) in the three cell types by mechanisms that may include sensors and/or effectors.

**Extracellular Ca\textsuperscript{2+} entry is required for RVD.** Most cells require an influx of extracellular calcium (23) for RVD, and cationic MSCs that are permeable to Ca\textsuperscript{2+} are a likely source. We tested the effect of extracellular Ca\textsuperscript{2+} concentration on the swelling-induced Ca\textsuperscript{2+} uptake. As expected (37), replacing extracellular Ca\textsuperscript{2+} with Mg\textsuperscript{2+} suppressed the swelling-induced Ca\textsuperscript{2+} increase in all three cell types (Fig. 3, A–C). These results are consistent with previous findings for astrocytes (8) and MDCK (32). Inward Ca\textsuperscript{2+} current via L-type channels has been reported in NRK-49F cells (10), although there are no data available in the literature on RVD in NRK cells. Removing extracellular Ca\textsuperscript{2+} without chelation did not completely abolish RVD, probably resulting from contamination with 50–100 \( \mu \)M Ca\textsuperscript{2+} present in other salts.

**MSCs are coupled to RVD via Ca\textsuperscript{2+} entry in NRK cells.** If Ca\textsuperscript{2+} entry in a hypotonic challenge occurs via MSCs, it should be blocked by GsMTx4. In NRK cells a hypotonic challenge led to an elevation of Ca\textsuperscript{2+} with a striking 30 ± 8.6-s latency (Fig. 4, A–C). The Ca\textsuperscript{2+} elevation was inhibited by 5 \( \mu \)M GsMTx4 (Fig. 4, D–F). Thus, for NRK cells the simple picture of cell swelling increasing membrane tension and opening cationic MSCs causing Ca\textsuperscript{2+} influx seems correct. The long latency may reflect the time required for cell swelling to cause fracturing of the cytoskeleton that normally protects the channels from osmotic stress (38).

In both astrocytes and MDCK cells, a hypotonic challenge increased intracellular Ca\textsuperscript{2+} (Fig. 5, A and C), but in these cells, GsMTx4 (5 \( \mu \)M) had no effect on swelling-induced Ca\textsuperscript{2+} elevation (Fig. 5, B and D). This observation suggests that the swelling-induced Ca\textsuperscript{2+} flux did not enter through cationic MSCs. In apparent contradiction, the latency for Ca\textsuperscript{2+} elevation in these cells was significantly shorter than for the NRK cells (\( P < 0.0001 \)), as though the volume sensors in those cells were not cationic MSCs and were unprotected.

**Effect of GsMTx4 on stretch-activated currents.** Both MDCK and NRK cells showed mechanosensitive channel activity in outside-out patch recordings, and these currents were inhibited by GsMTx4 (Fig. 6). The same effect of GsMTx4 was observed in primary astrocytes and reported previously (28). The patches were perfused with bath solution to determine the baseline activity (Fig. 6, A and B, black line). GsMTx4 (3 \( \mu \)M) dissolved in bath solution was perfused over the patch (Fig. 6, A and B, red line) to demonstrate inhibition. This reaction was reversible when the peptide was washed out with bath saline (Fig. 6, A and B, blue line). The channels in both cell types were nonselective cation channels reversing near 0 mV (data not shown). The response for three patches is shown in Fig. 6C, determined by averaging channel currents during the pressure pulse in the presence and absence of GsMTx4.

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**Fig. 4.** Response of intracellular Ca\textsuperscript{2+} to hypotonic challenges in NRK-49F cells. A and B: Ca\textsuperscript{2+} Fluo-4 images before (A) and after (B) hypotonic challenge in control solutions. C: typical traces of the time dependence of the Ca\textsuperscript{2+} increase from representative cells marked in A and B are plotted as the fractional change in fluorescence (\( I - I_0/I_0 \)). Similar responses were observed in \( \sim 80\% \) of cells. D–F: application of 5 \( \mu \)M GsMTx4 in both isotonic and hypotonic solutions eliminated the swelling-induced Ca\textsuperscript{2+} elevation (same conditions as in A–C).
DISCUSSION

In all three cell types, RVD required extracellular Ca\(^{2+}\) and was blocked by Gd\(^{3+}\). However, only in NRK cells did the L and D enantiomers of GsMTx4 block both Ca\(^{2+}\) uptake and RVD. GsMTx4 is known to inhibit cationic MSCs in a variety of cell types including rat astrocytes, chick heart, skeletal muscle, and human smooth muscle (6, 11, 37). Although the physiological function of these channels remains uncertain, the general function is not volume regulation.

The influx of Ca\(^{2+}\) through MSCs seems to couple channel activation to the effectors that are responsible for RVD. The effectors in NRK cells are not known, although their sensitivity to GsMTx4, RVD, and extracellular Ca\(^{2+}\) provides a useful tool to screen candidates. Since RVD requires an influx of Ca\(^{2+}\) to trigger a release from intracellular stores, the MSCs that mediate Ca\(^{2+}\) entry must be close to the Ca\(^{2+}\) release sites so the Ca\(^{2+}\) concentration will rise significantly above the mean.

Most work on cell volume regulation has focused on membrane-based models that utilize transport mechanisms across membranes to sense and determine cells volume, consistent with a process for the early transient stage of volume regulation before the involvement of three-dimensional cytoskeleton. However, attached cells under osmotic stress are not spheres so that the stress in the cytoskeleton dominates the response. The role of the cytoskeleton in determining the steady-state cell volume appears to have been underestimated (12) and this can account for the continued swelling when volume regulation is inhibited. When highly swelled cells were returned to normal media there was undershoot in the volume, suggesting that the cells did lose diffusible osmolytes during the RVD (control data in Figs. 1–3). However, the NRK cells treated with GsMTx4 (Fig. 1A) and cells treated with Gd\(^{3+}\) (Fig. 2, A–C) returned to the resting volume, suggesting no net gain or loss of osmolytes during the challenge.

There is a 1:1 correlation between cytoskeletal volume and cell volume. The response to a hypotonic challenge begins with an influx of water, with the cell volume lagging as it increases only with the integral of the flux. Recent atomic force microscopy experiments show that most of the osmotic stress of attached cells is borne by the cytoskeleton (34). Swelling of the cytoskeleton may lead to breakage of cross-links and subsequent reformation in a new pattern of interaction (7) with a lower osmotic pressure. The osmotic pressure of a gel is inversely related to the cross-link density (41). The swelling-induced Ca\(^{2+}\) elevation could have a significant effect on cytoskeleton cross-linking (14), so that the cytoskeleton itself may serve as one of the effectors. During the initial phase of

Fig. 5. GsMTx4 has little effect on swelling-induced Ca\(^{2+}\) elevation in astrocytes and MDCK cells. A: time dependence of swelling-induced intracellular Ca\(^{2+}\) in astrocytes as taken from representative cells. B: time dependence of swelling-induced Ca\(^{2+}\) elevation in the presence of 5 μM GsMTx4. C and D: swelling-induced Ca\(^{2+}\) elevation in MDCK cells (as per A and B).

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swelling, cross-links in the cortex can break, transferring stress to the bilayer and activating MSCs. The physiological function of MSCs may be to detect weakness in the local cytoskeleton.

The pronounced latency of the Ca$^{2+}$/H11001 increase with NRK cells (Fig. 4), also observed with cells in cultures of astrocytes and MDCK cells (cf. Fig. 5), is a signature of multiple reactions preceding Ca$^{2+}$/H11001 entry. Some of the delay will arise from the fact that the cell volume is the integral of the flux, but that will not explain the differences in latency between cell types. We hypothesize that the latency reflects the time required for delamination of the membrane from the cytoskeleton as described for blebs (7), and this will vary with cell type, shape and metabolic status. The short latency of astrocytes and MDCK cells to a hypotonic challenge implies that if they use membrane-bound sensors, they are partly delaminated at rest. The cell volume sensors employed in astrocytes and MDCK cells are clearly different from the MSCs observed in patch-clamp recordings since the channels in the patch experiments are blocked by GsMTx4 (Fig. 6A). This subtle variability in the sensor of cell volume may explain why cloning of the endogenous sensors has proven so difficult (16). While cationic MSCs are likely to be members of the transient receptor potential superfamily, there are clearly many variations (29).

The ready characterization of MSCs in NRK cells using only cell volume and/or intracellular Ca$^{2+}$ probes creates a screen for pharmacological agents that are active on that class of MSCs observed in patch-clamp recordings since the channels in the patch experiments are blocked by GsMTx4 (Fig. 6A). This subtle variability in the sensor of cell volume may explain why cloning of the endogenous sensors has proven so difficult (16). While cationic MSCs are likely to be members of the transient receptor potential superfamily, there are clearly many variations (29).

The ready characterization of MSCs in NRK cells using only cell volume and/or intracellular Ca$^{2+}$ probes creates a screen for pharmacological agents that are active on that class of MSCs. The MSCs have been implicated as drug targets for the treatment of muscular dystrophy (36), atrial fibrillation (5), glial tumors (27), and other diseases that may involve defects in the distribution of mechanical stress. The NRK screen could be exploited to clone the GsMTx4-sensitive channels by using randomized RNAi libraries (42).

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DISCLOSURES

P. A. Gottlieb and F. Sachs are stockholders in Rose Pharmaceuticals.

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