Small membrane permeable molecules protect against osmotically induced sealing of t-tubules in mouse ventricular myocytes

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Submitted 2 November 2015; accepted in final form 16 May 2016

Uchida K, Moench I, Tamkus G, Lopatin AN. Small membrane permeable molecules protect against osmotically induced sealing of t-tubules in mouse ventricular myocytes. Am J Physiol Heart Circ Physiol 311: H229–H238, 2016. First published May 20, 2016; doi:10.1152/ajpheart.00836.2015.—Cardiac t-tubules are critical for efficient excitation-contraction coupling but become significantly remodeled during various stress conditions. However, the mechanisms by which t-tubule remodeling occur are poorly understood. Recently, we demonstrated that recovery of mouse ventricular myocytes after hyposmotic shock is associated with t-tubule sealing. In this study, we found that the application of Small Membrane Permeable Molecules (SMPM) such as DMSO, formamide and acetamide upon washout of hyposmotic solution significantly reduced the amount of extracellular dextran trapped within sealed t-tubules. The SMPM protection displayed sharp biphasic concentration dependence that peaks at ~140 mM leading to >3- to 4-fold reduction in dextran trapping. Consistent with these data, detailed analysis of the effects of DMSO showed that the magnitude of normalized inward rectifier tail current ($I_{K1,\text{tail}}$), an electrophysiological marker of t-tubular integrity, was increased ~2-fold when hyposmotic stress was removed in the presence of 1% DMSO (~140 mM). Analysis of dynamics of cardiomyocytes shrinking during resolution of hyposmotic stress revealed only minor increase in shrinking rate in the presence of 1% DMSO, and cell dimensions returned fully to prestress values in both control and DMSO groups. Application and withdrawal of 10% DMSO in the absence of preceding hyposmotic shock induced classical t-tubule sealing. This suggests that the biphasic concentration dependence originated from an increase in secondary t-tubule sealing when high SMPM concentrations are removed. Overall, the data suggest that SMPM protect against sealing of t-tubules following hyposmotic stress, likely through membrane modification and essentially independent of their osmotic effects.

T-TUBULES form an extensive network of interconnected plasma membrane invaginations within cardiomyocytes and may contribute up to ~30–65% to the total surface membrane (25). This network is a central feature of normal adult ventricular cardiac myocytes and contains critical elements underlying efficient excitation-contraction coupling (for reviews, see 4, 11). In healthy cardiomyocytes, t-tubules allow for propagation of action potential deep inside the cell and ensure nearly synchronous release of Ca$^{2+}$ from SR and thus spatially and temporally uniform contraction (3, 5). Numerous studies have shown, however, that the function of t-tubular network becomes compromised during various experimental and clinical conditions, including heart failure (for review, see 12). Disruptions in the t-tubule network, also commonly referred to as t-tubule remodeling, constitute a spectrum of structural changes that range from dilations (18, 20, 32, 35) to tight constrictions (27, 30) and loss of t-tubules (for review, see 12), ultimately leading to changes in electrical properties of t-tubules and aberrant Ca$^{2+}$ handling through multiple mechanisms. For example, it has been shown that action potential propagation is impaired in partially constricted t-tubules (30), which can lead to nonsynchronous SR Ca$^{2+}$ release (8) and ultimately defective contraction (10). In more extreme cases, segments of t-tubules can be constricted so tightly that action potential propagation is fully abolished in the regions previously occupied by these segments, further exacerbating the latency in Ca$^{2+}$ release (8). Additionally, we have previously shown that sealed t-tubules remain functionally active albeit being electrically disconnected from the rest of the sarcolemma (21). Importantly, we showed that the process of t-tubular sealing itself is associated with a massive influx of Ca$^{2+}$-trapped in sealed t-tubules which is likely due to depolarization of sealed t-tubules. However, the mechanistic understanding of the causes of t-tubular structural changes still remains unclear, which is in part due to the paucity of appropriate tools to address the problem.

We have recently shown that many essential features of t-tubular remodeling can be reproduced in a model of acute hyposmotic shock (22). In particular, we found that constriction of t-tubules occurs nearly exclusively at the time of resolution of hyposmotic stress (i.e., during shrinking of the cell). This led us to a general hypothesis that the aberrant relaxation of the stretched sarcolemmal membrane is a key step underlying the loss of t-tubular integrity. While this hypothesis does not provide molecular details, it can serve as a guide. In particular, membrane stretch and membrane relaxation are

NEW & NOTEWORTHY

The t-tubular network is essential for efficient excitation-contraction coupling but its integrity is compromised in various pathophysiological conditions. The current study shows that DMSO and other small membrane permeable molecules are able to provide significant protection to the t-tubule network against hyposmotic stress.

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surely modulated by numerous factors and processes (e.g., composition of lipids, membrane cytoskeletal interactions).

While testing various pathways that may regulate this relaxation process, we serendipitously discovered that the commonly used solvent DMSO can significantly protect against t-tubular remodeling when applied at relatively low concentration (%; corresponds to ~140 mM) upon resolution of hyposmotic stress. We also show that other small membrane permeable molecules (SMPM), including the well-known detubulating agent formamide, also display a similar protective effect. At high concentrations, DMSO was also found to resemble formamide as removal of either molecule in the absence of hyposmotic stress resulted in effective t-tubule sealing. These results suggest that SMPM have similar modes of action that can lead to divergent t-tubule effects under different conditions. Detailed analysis of the actions of SMPM on t-tubular sealing strongly suggests that the observed protective effect against hyposmotic shock is likely due to their specific modification of membrane bilayer.

**MATERIALS AND METHODS**

**Animals**

All experiments involving mice were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition; The National Academic Press, Washington, DC) and protocols approved by the veterinary staff of the University Committee on Use and Care of Animals at the University of Michigan.

**Solutions**

All solutions were filtered using a 0.22-μm filter. Osmolarity was measured using Vapro Osmometer 5520 (Wescor, ELITEchGroup, France; mean ± SD).

- **Modified Tyrode (Tyr) solution.** Modified Tyrode (Tyr) solution was composed of (in mM) 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.3 CaCl₂, 0.16 NaH₂PO₄, 3 NaHCO₃, 5 HEPES, 10 glucose, pH adjusted to 7.35 with NaOH (solution osmolarity: 273 ± 6 mOsm/l).

- **Myocytes storage solution (C solution).** Myocytes storage solution (C solution) was composed of (in mM) 122 NaCl, 5.4 KCl, 4 MgCl₂, 0.16 NaH₂PO₄, 3 NaHCO₃, 15 HEPES, 10 glucose, 5 mg/ml of bovine serum albumin, 1.38 mg/ml taurine, pH adjusted to 7.35 with NaOH (solution osmolarity: 289 ± 3.5 mOsm/l).

- **Hyposmotic Tyrode (0.6 Na) solution.** Hyposmotic Tyrode (0.6 Na) solution was prepared as Tyr but with 60% of NaCl (82.2 mM) (solution osmolarity: 182 ± 4 mOsm/l).

- **KINT (pipette solution used for patch-clamp experiments).** KINT was composed of 140 KCl, 2 EGTA, 0.2 CaCl₂, 10 HEPES, 5 ATP, pH adjusted to 7.35 with KOH.

**Source of the Chemicals**

Chemicals were obtained as follows: urea was from Difco Laboratories; sucrose was from Acros Organics; HEPES was from Calbiochem; KCl, NaHCO₃, and NaH₂PO₄ were from Mallinckrodt Chemicals; tetramethylrhodamine dextran (3,000 mol wt; anionic form) was from Life Technologies. All other chemicals and reagents were from Sigma or Sigma-Aldrich.

**Isolation of Ventricular Myocytes**

Myocytes were isolated from the hearts of adult (~2–6 mo old) C57BL/6 mice of either sex as described in Moench and Lopatin (21) and used for experiments within 1–8 h postisolation.

**Dextran Trapping Assay and Confocal Imaging**

Ten milligrams of tetramethylrhodamine dextran was dissolved in 1 ml PBS and used as a stock (stored at ~20°C).

Isolated cardiomyocytes were first preincubated (~2 min) in Tyr immediately before application of hyposmotic 0.6 Na solution. One microliter of dextran stock solution was added to 10 μl of cells pelleted by centrifugation in 0.6 Na solution approximately 2 min prior to washout of 0.6 Na. Hyposmotic stress lasted 7–9 min and was stopped by washing with Tyr containing dextran for 5 min, and extracellular dextran was then washed out using Tyr solution. The cells were further washed and stored in C solution prior to imaging. Control myocytes were treated identically to test cells except that they were exposed to Tyr solution instead of 0.6 Na solution.

Confocal imaging was performed using an Olympus FV-500 microscope (Microscopy and Image Analysis Laboratory, University of Michigan, Ann Arbor). Images were analyzed using ImageJ software (http://imagej.nih.gov) followed by further analysis in Microsoft Excel. Images of myocytes were manually outlined and mean intracellular fluorescence of trapped dextran per unit area calculated. The data were corrected for background fluorescence observed in the control group.

**Electrophysiological Measurements**

Ionic currents were recorded in the whole cell configuration of patch-clamp technique at room temperature essentially as described by Cheng et al. (6) with a few minor modifications. In particular, the resistance of the patch pipettes (RP) varied from 2 to 4 MΩ when filled with KINT solution. After establishing whole cell configuration, series resistance (Rs > RP) was compensated to an effective value of ~1–3 MΩ. Currents were filtered at 2 kHz. During recordings, the myocytes were continuously superfused with Tyr solution using a flow chamber. Membrane capacitance (Cm) was measured with the aid of Clampex (Molecular Devices, Sunnyvale, CA) built-in algorithm using 5 mV depolarizing voltage steps from a holding potential of ~75 mV.

The current at the end of depolarizing voltage step (I<sub>K,end</sub>) and following inward rectifier tail current (I<sub>K1,tail</sub>) were analyzed essentially as described by Cheng et al. (6). Briefly, ionic currents were recorded in response to a 400-ms voltage step to +50 mV followed by repolarization back to a holding potential of ~75 mV. The I<sub>K1,tail</sub> Current was fitted using a single-exponential function A·exp(−t/τ) + C. In a fitting procedure ~15 ms of the current traces was excluded from the fit to minimize a contribution from capacitative currents. The amplitude of exponential component (A) was then recalculated to zero time using measured time constant. Finally, the ratio I<sub>K1,tail</sub>/I<sub>K,end</sub> was calculated and presented as normalized tail current F<sub>K1,tail</sub>.

**Measurements of Myocytes Dimensions**

Myocytes were placed in a flow chamber and imaged using Nikon TE2000-S inverted microscope at 5-s intervals with the aid of CoolSnap EZ camera (Photometrics, Tucson, AZ) and NIS-Elements D 3.00 software (Laboratory Imaging, Prague, Czech Republic). Time series of images were then analyzed using custom-made application (programmed in Delphi 2009, Embarcadero Technologies, San Francisco, CA) to measure changes in length and width of the myocytes.

In a subset of cardiomyocytes (n = 3 hearts), the cells were imaged and patched to simultaneously record cell size, C<sub>m</sub>, and I<sub>K1,tail</sub> current. Imaging was performed using a MD500 microscope eyepiece camera and the AmScope 3.7 software (AmScope, Irvine, CA). The images were imported into ImageJ and the myocyte outline was manually traced to measure the cross-sectional area.

Importantly, in all experiments (electrophysiological and imaging) DMSO and other agents were completely washed out using normal
DMSO concentration led to an increase in trapped dextran after resolution of hyposmotic stress. Further increases in absence of DMSO, suggesting that most of t-tubules remain intensity of t-tubular fluorescence compared with that in the concentration, there is a 4-fold reduction \( (P < 0.001) \) in the intensity of t-tubular fluorescence compared with that in the absence of DMSO, suggesting that most of t-tubules remain open after resolution of hyposmotic stress. Further increases in DMSO concentration led to an increase in trapped dextran (Fig. 1).

RESULTS

DMSO Prevents Stress-Induced Trapping of Extracellular Dextran in T-Tubules

In the course of the study aimed to understand the mechanisms underlying sealing of t-tubules in response to osmotic challenges (22), we have found that DMSO, present in the washing Tyr solution as a result of addition of a number of DMSO soluble agents, displays a strong and nearly instant protective effect (Fig. 1). The magnitude of t-tubule sealing was quantified using a fluorescent dextran trapping assay as previously described (22). Briefly, in this assay t-tubules are first prefilled with fluorescent dextran molecules during cell swelling (by hyposmotic solution), which is then followed by washout of the hyposmotic solution, leading to sealing of t-tubules and thereby trapping the dextran within the cell (Fig. 1, A and B). Detailed analysis of the action of DMSO (when it is present only in the washout solution) shows that the protective effect displays a clear biphasic dose-response relationship with 1% DMSO being the most effective (Fig. 1C). At this concentration, there is a 4-fold reduction \( (P < 0.001) \) in the intensity of t-tubular fluorescence compared with that in the absence of DMSO, suggesting that most of t-tubules remain open after resolution of hyposmotic stress. Further increases in DMSO concentration led to an increase in trapped dextran (Fig. 1C).

Surprisingly, DMSO was significantly less effective in protecting t-tubules when present continuously during both swelling and shrinking phases. Specifically, in a separate set of experiments (Fig. 2) dextran trapping was reduced only by \( \sim 36\% \) in the continuous presence of DMSO vs. \( \sim 69\% \) when applied only upon resolution of stress (see DISCUSSION for a likely explanation).

T-Tubules in DMSO Protected Cells Display Altered Electrophysiological Properties

It has been shown that the integrity of t-tubule system can be quantitatively assessed by an electrophysiological approach using measurements of \( I_{K_{1,tail}} \) current (6, 7). Therefore, we have applied this technique to quantify the protective effects of DMSO described above (Fig. 3). Prior to patching, cardiomyocytes were treated as in the dextran trapping experiments and stored in DMSO-free C solution. The cells were subsequently plated onto the glass bottom of the recording chamber continuously perfused with normal Tyrode solution to further ensure washout of any residual DMSO and to exclude any direct effects that DMSO may have on ionic currents (23).

As it has been shown before (22) the amplitude of \( I_{K_{1,tail}} \) current is significantly reduced in mouse ventricular myocytes that were acutely stressed with hyposmotic 0.6 Na solution (Fig. 3, A and B; 0.6 Na), consistent with most t-tubules being sealed and not contributing to the measured current. In this study, the amplitude of normalized \( I_{K_{1,tail}} \) current was re-

Fig. 1. Effects of post-shock acute application of DMSO on t-tubular sealing. A: timing of the application of fluorescent dextran and DMSO relative to the timing of hyposmotic challenge induced by application of 0.6 Na solution for \( \sim 7 \) min. Extracellular fluorescent dextran is applied \( \sim 2 \) min before removal of 0.6 Na solution and DMSO is applied exactly at the time of its washout by normal Tyr solution. After \( \sim 5 \) min myocytes are transferred to Tyr solution and the amount of trapped dextran measured using confocal microscopy. B: proposed mechanism of trapping of extracellular dextran. T-tubules become sealed (lumens are highly constricted) at the time of cell shrinking thus trapping previously added extracellular dextran. C: quantification of the amount of fluorescent dextran trapped in sealed t-tubules in the presence of varying concentrations of DMSO during cell-shrinking phase. T-tubules are most protected against sealing at 1% DMSO. \( n = 40, 40, 33, 38, \) and 38 for 0% to 10% DMSO groups, respectively. ***\( P < 0.001 \).

Fig. 2. Effect of timing of DMSO application on dextran trapping. A: protocols describing the application of 1% DMSO. 0.6 Na: protocol is characterized by maximum dextran trapping. DMSO-A: in this protocol 1% DMSO was present all the time, i.e., before, during and after hyposmotic challenge with 0.6 Na solution. DMSO-W: in this protocol 1% DMSO was present only during the washing phase of the hyposmotic challenge with 0.6 Na solution. B: representative confocal images of ventricular myocytes (from the groups described in A) containing trapped dextran. The cell border is outlined for clarity. Scale bars = 20 \( \mu \)m. C: quantification of the amount of trapped dextran in the three groups as in A using fluorescent confocal microscopy. Myocytes were imaged identically and the measured fluorescence is presented in arbitrary units for relative comparison. \( n = 31, 26, 25, \) and 24 for 0.6 Na, DMSO-A, DMSO-W, and control myocytes (used for background correction), respectively. ***\( P < 0.001 \).
Fig. 3. Electrophysiological effects of DMSO application. A: integrity of t-tubules was assessed using so-called \( I_{K,tail} \) current (inset: METHODS). Whole cell currents recorded from different groups of myocytes were normalized at the end of depolarizing voltage step (inset; ○) in order to highlight the differences in the magnitude and kinetics of the \( I_{K,tail} \) current. Zero current is indicated by the horizontal dashed line. Ctrl, control myocytes; 0.6 Na, myocytes treated with 0.6 Na hyposmotic solution; DMSO-A, myocytes were exposed to 1% DMSO all the time, i.e., before, during, and after hyposmotic challenge with 0.6 Na solution; DMSO-W, myocytes were exposed to 1% DMSO only during the washing phase of the hypotonic challenge with 0.6 Na solution. B: quantification of normalized \( I_{K,tail} \) currents in myocytes treated as described above. \( n = 34, 34, 35, \) and 24 for Ctrl, 0.6 Na, DMSO-A, and DMSO-W myocytes, respectively. C: quantification of the time constant (τ) of \( I_{K,tail} \) current in Ctrl and DMSO-W treated myocytes above. \( n = 34 \) and 35 myocytes, respectively. D: relationship between cell cross-sectional area (CSA) and membrane capacitance (\( C_m \)). The regression lines are as follows: \( C_m = 0.0385 \times \text{CSA} + 32.7, \) \( R^2 = 0.846 \) for Ctrl (\( n = 17 \)); \( C_m = 0.0306 \times \text{CSA} + 14.6, \) \( R^2 = 0.809 \) for 0.6 Na (\( n = 17 \)); \( C_m = 0.0277 \times \text{CSA} + 43.9, \) \( R^2 = 0.883 \) for DMSO-A (\( n = 19 \)); \( C_m = 0.028 \times \text{CSA} + 37.2, \) \( R^2 = 0.826 \) for DMSO-W (\( n = 18 \)). E: average of individual \( C_m \) normalized by the CSA (\( C_m^{\text{CSA}} \)). *P < 0.05, **P < 0.01, ***P < 0.001.

Reduced >3.5 fold from 0.332 ± 0.027 pF/\( \mu \)m\(^2\) in control myocytes to 0.095 ± 0.016 after treatment with 0.6 Na solution (\( P < 0.001 \)). Unexpectedly, \( I_{K,tail} \) current was reduced even further, by −6-fold to 0.056 ± 0.010 when 1% DMSO was present during the whole hypotonic stress procedure (Fig. 3, A and B; DMSO-A). However, \( I_{K,tail} \) current was reduced only −2-fold, to 0.184 ± 0.016 (\( P < 0.001 \)), when 1% DMSO was applied only during resolution phase of the stress (Fig. 3, A and B; DMSO-W), suggesting that a significantly larger portion of the t-tubules remains electrically accessible compared with that in two other (0.6 Na and DMSO-A) test groups.

One of the other important features of \( I_{K,tail} \) current is its kinetics as it also reflects the magnitude of t-tubule remodeling (6). Inspection of current traces in Fig. 3A shows that myocytes treated with 1% DMSO applied upon resolution of stress display slower decline of \( I_{K,tail} \) current. Accordingly, quantification of the data (Fig. 3C) shows that the time constant of \( I_{K,tail} \) current is increased nearly 2-fold, from 74.0 ± 2.5 ms to 144.0 ± 8.0 ms (\( P < 0.001 \)), in control and 1% DMSO-W treated myocytes, respectively. Combined with the partial preservation of \( I_{K,tail} \) current amplitude described above, the data provide further evidence that DMSO-treated cells still have electrophysiological defects that are consistent with partial t-tubule constrictions. It is tempting to suggest that the time constants of \( I_{K,tail} \) currents in other test groups, 0.6 Na and DMSO-A, are significantly larger than that observed in DMSO-W-treated cells but unfortunately, the smaller amplitude of \( I_{K,tail} \) currents in those test groups makes estimation of the time constant unreliable (data not presented).

The membrane capacitance, \( C_m \), was also measured to further characterize t-tubule remodeling. In order to minimize potential bias in measured average \( C_m \) between various data sets which may arise due to the large dependence of \( C_m \) on cell size (31), cardiomyocytes were also imaged and the cross-sectional area (CSA) measured. There was a broad spread of cardiomyocyte CSA ranging from ~2,200 to ~8,000 \( \mu \)m\(^2\) with average CSA of 4,545 ± 346 \( \mu \)m\(^2\) (\( n = 17 \)) in control cells. Prior exposure of cardiac myocytes to hypotonic solutions, with or without added DMSO, had no effect on the average CSA: 4,165 ± 304 \( \mu \)m\(^2\) (\( n = 17 \)), 4,086 ± 338 \( \mu \)m\(^2\) (\( n = 18 \)), and 4,450 ± 302 \( \mu \)m\(^2\) (\( n = 19 \)), for 0.6 Na, DMSO-W, and DMSO-A conditions, respectively.

Measured values of \( C_m \) and CSA for individual myocytes as well as corresponding linear regression fits for all groups are shown in Fig. 3D. The data show significant reduction in measured \( C_m \) in myocytes treated with 0.6 Na solution compared with that in control cells. However, the expected restoration of \( C_m \) in DMSO-W group towards control values is less evident, in particular, due to the large scatter in the individual data. In order to take advantage of known cell size, individual \( C_m \) data were normalized to the corresponding CSAs and averaged normalized \( C_m \) (\( C_m^{\text{CSA}} \)) for each group compared (Fig. 3E).

Control cells show a \( C_m^{\text{CSA}} \) value of 0.047 ± 0.005 pF/\( \mu \)m\(^2\) that is nearly identical to the value reported by Pavlovic et al. (26) for isolated mouse ventricular myocytes (Fig. 3E). Furthermore, cells treated with 0.6 Na solution show a ~27% reduction in \( C_m^{\text{CSA}} \) compared with control cells, 0.034 ± 0.004 pF/\( \mu \)m\(^2\) (\( P < 0.01 \)), similar to the reduction in \( C_m \) that we observed previously (22). In line with the measurements of \( I_{K,tail} \) currents, \( C_m^{\text{CSA}} \) was significantly preserved to 0.038 ± 0.005 pF/\( \mu \)m\(^2\) (\( P < 0.05 \)) and 0.038 ± 0.004 pF/\( \mu \)m\(^2\) (\( P < 0.01 \)), for DMSO-W and DMSO-A groups, respectively, when compared with cells treated with 0.6 Na solution. Again, the data are consistent with DMSO preserving the integrity of t-tubular system.
Evidence Against Osmotic Effects of 1% DMSO in T-Tubule Protection

Despite the seemingly low concentration, 1% DMSO increases the osmolarity of Tyr solution by ~130 mOsm/l. Therefore, we examined if this increased osmolarity could account for the protective effects of DMSO described above. Figure 4A shows a representative time course of changes in the cell width, highlighting quick swelling and shrinking phases as well as a characteristic response to withdrawal of 1% DMSO (Fig. 4A; arrow). Consistent with the previous findings, the change in the dimensions of the myocytes was primarily due to changes in the width (~14%) of the cells (Fig. 4B) (1, 2, 22, 29). Importantly, the cell width returned to its prestretched value in both control and DMSO-treated myocytes: 99.7 ± 0.3% and 100.0 ± 0.3% (P ~ 0.2) in the absence and presence of 1% DMSO, respectively. The focus of this study, however, was largely on the kinetics of cell shrinking. The data in Fig. 4C show that the rate of shrinking (τoff) was slightly faster (but not statistically significant; P ~ 0.12) in the presence of 1% DMSO, consistent with increased osmolarity of washing solution. In order to confirm that this small increase in the rate of shrinking is not due to differences in cell size of the corresponding populations (i.e., the rates of osmotic response may correlate with the cell size) we have also compared swelling rates in both DMSO-treated and non-treated cells. The data in Fig. 4D indicate the absence of this correlation. Also, the minor effect of 1% DMSO on the rate of shrinking is in striking contrast to significant protective changes in the level of dextran trapping (Fig. 1C) and the amplitude of \( P_{K,L} \) (tail current) (Fig. 3B).

It should be noted that an increased rate of shrinking in the presence of 1% DMSO would be predicted to increase t-tubule sealing. To confirm that the protection by DMSO-W (404 ± 13 mOsm/l) is not due to the increased osmolarity of the washout solution, a series of dextran trapping experiments was performed using Tyr solutions with added 60 mM NaCl (362 ± 1 mOsm/l) and 120 mM sucrose (354 ± 3 mOsm/l) instead of DMSO. As shown in Fig. 4D (left), washout with either hypotonic solutions did not reduce dextran trapping. Rather, in both conditions a trend toward increased dextran trapping (P = 0.08 and P = 0.07, respectively) is observed, confirming that the mechanism of protection by DMSO is not due to its bulk osmotic effects.

It remained possible that the low level of dextran trapping in the presence of 1% DMSO (Fig. 1C) could be explained by transient reopening of constricted t-tubules upon DMSO withdrawal which is associated with transient swelling of the cells due to diffusion of accumulated DMSO out of the cells. However, additional experiments (Fig. 5) designed to test this hypothesis argue strongly against this possibility. In particular, if t-tubules transiently reopen and close again upon DMSO withdrawal then adding dextran prior to this step (but not at the time of washout of hypotonic stress) should lead to its trapping. However, this effect was not observed (Fig. 5; protocol 4). Alternatively, applying and removing DMSO to cells that already have dextran trapped in sealed t-tubules does not lead to its release (Fig. 5; protocol 5). Consistent with these data, postponing DMSO withdrawal does not affect dextran trapping as well (Fig. 5; protocol 3). Collectively, the data lead to its release (Fig. 5; protocol 3). Collectively, the data.
confirm that the protective effect of 1% DMSO occurs during the trapping process, within the first few minutes following resolution of hyposmotic stress, and the reduced level of dextran trapping is not associated with the following removal of DMSO.

**Efficacy of Osmolytes Against Hyposmotic Stress is Correlated with their Membrane Permeability**

In order to elucidate the mechanism of t-tubule protection by DMSO, the effects of several other osmolytes on t-tubular integrity were tested (Fig. 6). Each osmolyte was added to the washout solution at the concentration matching the osmolarity of Tyrode solution containing 1% DMSO (solution osmolarity: 410 ± 3 mOsm/l). Interestingly, highly membrane permeable formamide (solution osmolarity: 376 ± 2 mOsm/l) and acetamide (solution osmolarity: 398 ± 4 mosM/l) showed a significant protective effect similar to that observed with DMSO (Fig. 6A). In contrast, structurally similar urea (solution osmolarity: 385 ± 4 mOsm/l) showed no significant protective effects. The results in Fig. 4D demonstrated that application of relatively membrane impermeable NaCl and sucrose also showed no significant protection, suggesting that the high membrane permeability of the osmolytes may underlie the protection against hyposmotic stress. In order to compare relative membrane permeabilities of DMSO, formamide, acetamide, and urea, we measured the changes in the width of the myocytes in response to the application of high concentrations (~700 mM) of each agent (Fig. 6C). As expected, application of highly permeable DMSO (solution osmolarity: 966.7 ± 7 mOsm/l), formamide (solution osmolarity: 794 ± 10 mOsm/l), and acetamide (solution osmolarity: 912 ± 6 mOsm/l) led to fast but transient shrinking of the cells and thus is characterized by relatively small amplitude of the changes in the cell width. A clear feature of the formamide effect was that the steady-state width of the cell in the continuous presence of the agent was measurably larger than the original value, consistent with observations made by Kawai et al. (16). In contrast, application of urea (solution osmolarity: 926 ± 8 mOsm/l) led to significant and quasi-steady-state shrinking of the cells which was quickly (within a few minutes) followed by cell death. These data are consistent with significantly lower membrane permeability of urea compared with that of DMSO, formamide, and acetamide. Similar results were also observed with n = 3–9 more cells in each group (the data were not quantified).

**At High Concentrations DMSO Acts as a Detubulating Agent**

A loss of protective effect of DMSO at high concentration (10%, which corresponds to ~1.4 M; Fig. 1) suggests an additional mechanism of its action on the integrity of t-tubular system. Formamide at ~1.4 M (5.6%) concentration has been used as a tool to detubulate cardiomyocytes for many years (16). Accordingly, we hypothesized that in the experiments presented in Fig. 1, the increase in trapped dextran at 10% DMSO may be due to a secondary (to that induced by washout of 0.6 Na hyposmotic solution) detubulation that occurs when DMSO is finally withdrawn. To test this hypothesis, we first compared the efficacy of DMSO (10%) and formamide (5.6%) in inducing t-tubule sealing using the classical protocol [a brief application of the drug followed by its withdrawal (16); Fig. 7A]. Indeed, the data show that DMSO seals t-tubules as effectively as formamide, and the magnitude of dextran trapping induced by both agents is similar to that produced by application of 0.6 Na solution alone (Fig. 7A). These data strongly support the above hypothesis. Specifically, even if t-tubules were to be fully protected by DMSO at the time of washout of hyposmotic 0.6 Na solution, they would become sealed and dextran trapped since it is still present in t-tubules at the time of withdrawal of 10% DMSO (Fig. 1A). In order to further confirm this suggestion, we performed experiments similar to those described in Fig. 5 where we studied similar effects of DMSO but at lower concentration (1%). In this regard, the data in Fig. 7B show that if extracellular dextran is removed before withdrawal of 10% DMSO the amount of trapped dextran becomes even lower than that observed in experiments using 1% DMSO (Figs. 1C and 5B). Altogether, these data reveal a truly strong protective effect of DMSO at the time of removal of hyposmotic 0.6 Na solution and explain the biphasic concentration dependence of DMSO effects on t-tubular sealing presented in Fig. 1C.

Experiments with formamide (5.6%) showed similar results (Fig. 7B), suggesting that it likely protects t-tubules by the same mechanisms as DMSO.

To further illustrate the true magnitude of protection against hyposmotically induced t-tubule sealing, the data from exper-
FORMAMIDE, ALTERNATIVELY, IN /H9262 SHADeD THick LINE DEpICTS THE TRUE PROTECTIVE EFFECT OF DMSO AND FORMAMIDE AGAINST HYPOSOMATICALLY INDUCED DETUBULATION.

However, the mode of action of DMSO in partly from its ability to counteract the freezing-induced osmotic stress (37). However, the data in Figs. 6 and 7 show that the SMPM is quite different (Fig. 6) which would likely intracellular pathway ultimately leading to t-tubule protection. However, the data in Figs. 6 and 7 show that the SMPM is quite different (Fig. 6) which would likely intracellular pathway ultimately leading to t-tubule protection.

DISCUSSION

In this paper, we show that significant protection against t-tubular sealing in response to hyposmotically induced membrane stretch can be achieved by application of DMSO, one of the most common solvents in biological research, as well as other SMPM such as formamide and acetamide.

Proposed Mechanism of Protection by SMPM

DMSO and other SMPM may have a variety of targets that can potentially modulate t-tubule integrity. In particular, SMPM could act on targets located at both the plasma membrane and within the cardiomyocyte. However, a number of key observations in this study put significant constraints on the potential mechanisms of their action. For example, the protection by DMSO against hyposmotically induced sealing of t-tubules is strongest when it is applied at the time of cell shrinkage (Fig. 2) and the effect is fast. This would be more consistent with DMSO acting directly on a membrane target or the lipid bilayer itself rather than through activation of some intracellular pathway ultimately leading to t-tubule protection. Also, despite very similar structures, the chemical nature of the tested SMPM is quite different (Fig. 6B) which would likely translate to differential effects due to different specificity to the key target. However, the data in Figs. 6 and 7 show that DMSO, formamide, and acetamide produce nearly identical level of protection.

FORMAMIDE has been used as a cryoprotectant, which stems partly from its ability to counteract the freezing-induced osmotic stress (37). However, the mode of action of DMSO in t-tubule protection is unlikely to be simply through counteracting changes in the osmotic pressure. For example, the increase in the rate of cell shrinkage due to added DMSO (Fig. 4C) is expected to trend toward increased rather than decreased t-tubular sealing. Consistent with this prediction, the data in Fig. 4D indeed show a trend toward an increase in the amount of dextran trapping when osmolarity of washout solutions is increased by adding NaCl or sucrose instead of DMSO. The osmotic effects of DMSO and formamide at higher concentrations are indeed strong and in fact do lead to sealing of t-tubules (Fig. 7A) but only upon withdrawal of the agents, not at the critical time of resolution of hyposmotic stress. In this regard, the data in Fig. 7 reveal strong protective effects of SMPM at high concentrations when the data are corrected for secondary detubulation caused by their withdrawal. Clearly, increased osmolarity of washout solution does not explain the protective effects of SMPM.

It follows from above that the most unifying property of the agents displaying protection is their membrane permeability, which would translate to their ability to intercalate into the lipid bilayer of the membrane. In this regard, the ability of DMSO to influence membrane fluidity may explain its protective effects when this agent is present throughout the experiment (Fig. 2). For example, Gurvenenko and Anwar (13) have demonstrated using molecular dynamics simulations that DMSO penetrates into the membrane and acts as a spacer between lipids, ultimately leading to membrane thinning. In addition to the in silico findings, work by Hochmuth et al. (14) using laser tweezer pulled membrane tethers has demonstrated that high concentrations of DMSO have significant effects on membrane tension. In particular, DMSO at concentrations 1% and 5% reduced the apparent surface tension and the bending modulus for the membrane, which would be expected if membrane expansion occurs. These observations demonstrate that DMSO directly affects the biomechanical properties of the plasma membrane in a way that the membrane becomes more flexible, which is in agreement with the in silico results (13).
PROTECTION AGAINST SEALING OF CARDIAC T-TUBULES

With this in mind, it is tempting to speculate that the enhanced membrane fluidity may minimize its misfolding in the spatially restricted space of t-tubules after the relief of membrane stretch to limit t-tubule sealing.

T-tubules are significantly more protected against sealing when DMSO is applied during washout of hyposmotnic stress rather than when being constantly present. This important finding suggests that the concentration gradient of the SMPM plays a significant role. In this regard, the bilayer couple model may explain these results (33, 34). This model posits that each leaflet of the bilayer can be modified independently of the other leaflet and this asymmetric modification of the membrane can lead to significant structural changes of the membrane (33). In particular, if SMPM expand the outer side of the membrane bilayer relative to the inner side, for example due to the existence of a concentration gradient of SMPM, then the bilayer would protrude outward, and vice versa. According to this model, SMPM should produce an outward protruding force on the membrane bilayer during their application which would then counteract some of the inwardly directed osmotic forces produced by the removal of hyposmotnic solution. It has been shown that inwardly directed osmotic forces may induce invaginated plasma membrane vacuoles in other cell types (17, 19, 28), and it is tempting to speculate that the same osmotic forces underlie t-tubule sealing in cardiomyocytes.

The data in this study show that SMPM can act as both detubulating and protective agents against hyposmotnic stress (Fig. 7). These seemingly contradictory findings can again be easily explained by the bilayer couple model described above. Specifically, it is known that in the classical formamide-induced detubulation (16) t-tubule sealing occurs at the time of washout of formamide. At this time, formamide concentration is greater inside the cell which in turn would produce an invaginating force upon the membrane according to bilayer couple model. This is also consistent with a recently discovered phenomenon of detubulation of cardiac myocytes in the presence of a number of cationic amphiphilic drugs (CADs) (24). CADs selectively accumulate in the inner leaflet due to their high affinity for anionic phosphoinositides and thus may induce a similar invaginating force. However, further experiments would be necessary to test this model.

Additional important information about the mechanism of DMSO protection can be gleaned from the analysis of \( I_{K1,tail} \) currents and membrane capacitance. For example, the data in Fig. 3 show that in myocytes protected by 1% DMSO the amplitude of \( I_{K1,tail} \) currents is significantly reduced compared with normal cells, and the time constant of \( I_{K1,tail} \) current decline is significantly increased. This suggests the presence of a significant number of partially (weakly) constricted t-tubules. However, these t-tubule constrictions are likely large enough (in diameter) to allow most of the initially trapped dextran to escape to the extracellular space before myocytes are imaged. The significantly smaller protective effect of 1% DMSO on membrane capacitance (Fig. 3, D and E) when compared with that on dextran trapping or amplitude of \( I_{K1,tail} \) currents is likely a reflection of the differences in the measured parameters used to estimate the magnitude of t-tubular constrictions. In particular, it should be noted that the measurements of \( C_M \) used in this study (performed using the algorithm provided in Clampex software) in principle depend on the length of the analyzed current traces. Specifically, it is clear that partially constricted t-tubules are still connected to the outside solution and contribute the same capacitance as normal t-tubules. However, the time constant of the capacitive current originating from constricted t-tubules may be significantly longer than the analyzed segment of the current ultimately leading to reduced measured \( C_M \). Therefore, measured \( C_M \) should be treated as apparent \( C_M \) and the data interpreted accordingly.

Physiological Connections

The results of this study have important physiological implications since the hyposmotnic swelling applied in this study is likely relevant in a number of pathological conditions including ischemic stress. For example, it has been estimated that during ischemia, accumulation of metabolic byproducts can increase the intracellular osmolarity by as much as 69 mOsm/l relative to the extracellular fluid (15). This estimate is very close to the ~90 mOsm/l gradient between the Tyrode and 0.6 Na solutions used in this study. This ischemic swelling has been validated in vitro using isolated adult ventricular cardiomyocytes (9). Cardiomyocytes swell in volume by as much as 40% after 30 min of simulated ischemia, a magnitude similar to that observed in cardiomyocytes exposed to 200 mOsm/l hyposmotnic solutions (9). This massive cell swelling is rapidly reversed upon reperfusion. Thus the volume changes a cell experiences during ischemia-reperfusion may be mimicked by the hyposmotnic stress used in our current study. Accordingly, our findings suggest that DMSO may protect against ischemia-reperfusion stress by attenuating constriction of t-tubules but whether the volume changes during ischemic stress can lead to sealing of t-tubules in the first place still remains unknown. In this regard, an alternative hypothesis has been suggested that t-tubule remodeling following ischemia-reperfusion may be mediated by calpain-mediated degradation of the t-tubular structural protein, junctophilin-2 (36). However, further experiments would be necessary to test the above hypotheses and to confirm the t-tubular protective action of SMPM in ischemic conditions.

Cellular dysfunction consistent with constricted t-tubules has also been observed in cardiac myocytes isolated from failing hearts. For example, Sacconi et al. (30) reported AP propagation failure in t-tubules that were still accessible by extracellular membrane specific dye di-8-ANEPPS. Importantly, these findings highlight the limitations of using di-8-ANEPPS alone to characterize t-tubule remodeling as this assay is not well suited for characterization of the functional defects that arise from partial constriction of t-tubules. Constriction of t-tubules in failing myocytes was also associated with altered Ca\(^{2+}\) release (8), which is consistent with our previous finding of aberrant t-tubular Ca\(^{2+}\) handling in myocytes exposed to hyposmotnic stress (21).

In conclusion, the results of this study show that sealing of cardiac t-tubules in response to hyposmotnic stress in cardiac ventricular myocytes can be significantly attenuated by a brief application of various SMPM which likely exert their protective effect through direct action on the cellular membrane. However, further work is required to determine the specific properties of SMPM contributing to their protection against hyposmotically induced t-tubule sealing. Furthermore, understanding the mechanisms of SMPM action on cardiac t-tubules

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00836.2015 • www.ajpheart.org
could be important in identifying novel agents and protocols for their application with strong protective effects against t-tubular sealing in various disease conditions.

ACKNOWLEDGMENTS

We thank Olga Zolova for assistance with myocytes preparation in the late stages of the study and Azadeh Nikouee for help with some osmolarity measurements.

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Preliminary results of this work were presented in Abstract/Poster form at the Annual Meeting of Biophysical Society (Uchida K, Moench I, Lopatin AN. Biophysical J 108: 266a, 2015).

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-069052 and HL-127023 (A. N. Lopatin); National Institute of General Medical Sciences T32 Training Grant GM-008322 (K. Uchida); and the Department of Molecular and Integrative Physiology and the Endowment for Basic Science at the University of Michigan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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