Energetics of gating MscS by membrane tension in azolectin liposomes and giant spheroplasts

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Introduction

Mechanosensitive (MS) ion channels are molecular sensors that detect and transduce signals across prokaryotic and eukaryotic cell membranes into electrical or chemical intracellular signals.¹⁻³ In bacteria, MS channels belonging to the MsCL and MscS families protect bacterial cells from hypo-osmotic shock by alleviating osmotic stress.⁴ The canonical MscS channel of E. coli, is a representative of a very large and diverse family of MS channels found in prokaryotic cells as well as in fungal and plant eukaryotes. This channel functions in weakly hypo-osmotic environments⁷⁻⁹ since it requires less membrane tension to open compared with MsCL.¹⁰⁻¹¹

MscS is a homo-heptameric protein of 211 kDa¹² and possesses three distinctive domains: (i) a transmembrane domain consisting of three α-helices, TM1, TM2, and TM3, (ii) an intermediate cytoplasmic domain composed exclusively of β-strands, and (iii) a C-terminal cytoplasmic vestibulum consisting of a mix of α-helices and β-strands (Fig. 1).¹²⁻¹³ The channel pore is formed by TM3 helices, which create a hydrophobic constriction on the cytoplasmic side formed by residues L105 and L109.¹² Upon membrane stretch the MscS pore opens to 16 Å in diameter.¹⁴ In addition to the channel pore seven 14 Å portals within the cytoplasmic vestibulum¹²⁻¹⁵ control diffusion of ions and solutes across the channel and determine its weak preference for anions over cations.¹⁶⁻¹⁷

MscS has functionally been studied for many years in both giant spheroplasts and liposomes.¹⁰⁻¹¹,¹⁴,¹⁶⁻¹⁸⁻²³ A number of MscS studies have reported a significant difference for the activation of MscS by membrane tension in these two preparations.¹⁰⁻¹¹,²³ Given that the origin of this difference has not been well understood we examined it in this study using a linear force model of MS channel gating²⁴ to assess the energetics of MscS channel gating in both preparations. Herein we discuss our findings and propose future research directions.

Results

The objective of our study was to compare the gating parameters ΔA and γ between MscS channels reconstituted in liposome membranes and MscS channels recorded in native membranes of giant spheroplasts (Fig. 2A). This was performed by analyzing channel Boltzmann distribution functions, which describe the dependence of channel activity on membrane tension (Fig. 2B). For that we employed the linear force model,²⁴ which assumes that the distortion of the cell membrane in response to an external force can be described as a conformational transition between closed and open channel states. Fitted values of ΔA and γ for the midpoint Pₙ = 0.5 were determined from the Boltzmann distribution functions shown in Figure 2B using initial values of ΔA = 7 nm² through to ΔA = 9 nm² with 10% tolerance (Fig. 3). The choice of this range of ΔA values is based on dimensions of the non-conductive and open channel structures of MscS (Fig. 4).

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Fig. 1

Fig. 2B
from which we estimated the area change between the two MscS conformations as $\Delta A = 7.8 \text{ nm}^2$.

The analysis of Boltzmann distribution functions indicated that the area change $\Delta A$ was the same for MscS channels recorded in both preparations. The comparison of the membrane tension $\gamma$, however, showed a significant difference between the MscS channel activities in these two preparations (Fig. 3). On average, for the same $\Delta A$ value, $\gamma$ for MscS channels in spheroplast preparations was found to be much higher compared with liposomes, as previously reported. For MscS channels in liposome preparations, the value range of membrane tension $\gamma$ was found to be between $6 \text{mN/m}$ and $14 \text{mN/m}$, corresponding to an area change of $7 \text{nm}^2$ to $9 \text{nm}^2$. For the same range of area change, the value range of membrane tension $\gamma$ for spheroplast preparations was found to be between $9 \text{mN/m}$ and $20 \text{mN/m}$. The difference in membrane tension values between liposome and spheroplast preparations as obtained from curve fitting corresponds well to the experimental data obtained from patch-clamp recordings of MscS channels in these two preparations (Fig. 2). For the midpoint, i.e., $P_o = 0.5$, the average membrane tension of liposome preparations ($n = 5$) was recorded as $6.3 \pm 0.8 \text{mN/m}$ whereas the average membrane tension of spheroplast preparations ($n = 5$) was recorded as $17.8 \pm 2.2 \text{mN/m}$. From these values, the intrinsic energy difference between open and closed states in the absence of applied force $\Delta u = \gamma \Delta A$ amounts to $49 \times 10^{-21} \text{J} (-12 \text{k}_B \text{T})$ and $139 \times 10^{-21} \text{J} (-34 \text{k}_B \text{T})$, which indicates about a three times larger energy barrier between the closed and open states for MscS in spheroplast patches.

### Discussion

In this study we applied the two-dimensional linear force model (Eq. 2) to the gating of MscS channels by membrane tension in the lipid bilayer of azolectin liposomes and the native membrane of bacterial giant spheroplasts. Our results show that the change in area occupied by a closed compared with an open MscS channel, $\Delta A$, did not depend on the membrane environment in which the MscS channels were functioning. This is to be expected since $\Delta A$ is inherently linked to the protein channel structure and is usually little affected by the lipid composition of the membrane bilayer.

However, as previously reported, the lipid composition of the membrane bilayer did significantly affect the response of MscS to membrane tension. Given that $\Delta A = 7.8 \text{nm}^2$ in both liposome and spheroplast patches and the average membrane tension of liposome and spheroplast preparations of $6.3 \pm 0.8 \text{mN/m}$ and $17.8 \pm 2.2 \text{mN/m}$, respectively, we could calculate using Eq. (3) the values of $K_{o}$ of $12.8 \text{mN/m}$ for the liposome membrane and $36.3 \text{mN/m}$ for the spheroplast membrane (for details see Supplementary Information). Although both values are much lower than area elasticity constant $K_{o}$ values for a typical lipid bilayer ($10^2 - 10^3 \text{mN/m}$) resulting most likely from the ionic composition of our recording solutions given that $K_{o}$ values were reported to vary with solution conditions, $K_{o}$ for the spheroplast membrane is three times larger than the area elasticity constant for liposome membranes. In addition to the physical properties of the bilayer, such as $K_{o}$ (Eq. 3), affecting MscS gating other factors such as membrane protein content, a direct interaction of a particular type of lipid
with the channel protein or remnants of the peptidoglycan cell wall could be responsible.

The *E. coli* inner membrane consists of phosphatidylethanolamine (PE: 70–80%), phosphatidylglycerol (PG: 20–25%), and cardiolipin (CL: 5–10%), whereas soybean azolectin is between 16–24% phosphatidylcholine with the remainder of the phospholipids being a mixture of phosphatidylethanolamine and phosphatidylserine with phosphatic acid as minor components. If an interaction with a specific lipid(s) within the *E. coli* cell membrane is responsible for the parallel leftward shift of the Boltzmann function, a likely candidate lipid is the anionic lipid (double negative charge) cardiolipin, which represents between 5 and 10% of the *E. coli* cytoplasmic membrane. The reason for this is 3-fold; first, MscS is known to co-localize with cardiolipin at the poles of *E. coli* cells. Second, preliminary results demonstrate that cardiolipin may directly modulate MscS channel activity. Third, cardiolipin is found in the inner mitochondrial membrane where it has been extensively shown to interact with numerous membrane proteins including Cytochrome C oxidase and stomatin-like proteins. The mechanism and nature of the interaction between MscS and cardiolipin may reveal interesting aspects regarding the physiological role of cardiolipin in general regardless of whether the lipid directly modulates channel behaviour or not. Given the importance and localization of this lipid to the inner mitochondrial membrane these insights may be applicable to its role in eukaryotes and not just bacteria. The aim of future experiments is to elucidate the exact molecular mechanisms underlying the large disparity in the required tension for gating of MscS in liposomal membranes compared with the native membrane of *E. coli* spheroplasts. This includes examining the potential interaction of lipids such as cardiolipin with MscS to determine whether they play an integral role in this process.

**Materials and Methods**

**Giant spheroplast preparation**

Giant spheroplasts were generated from MJF465 *E. coli* strain transformed with the pQE-60lacI mscS plasmid with some minor modifications to the original method. Modifications included induction of MscS protein expression in the single cell filaments ("snakes") with 0.5mM IPTG for 75 min at 18 °C and shaking at 150rpm. The spheroplast preparation requiring lysozyme and EDTA treatment of filaments, which followed the harvest of filaments by centrifugation (1500 × g, 5min, 4 °C) and re-suspension of the pellet in 2.5ml of 0.8M sucrose was performed following the original procedure. Aliquots of the spheroplast preparation were stored at -20 °C for use in patch-clamp experiments over several months.

**Protein purification and liposome reconstitution**

Competent M15 *E. coli* cells containing pREP4 [Qiagen] and pRARE [Merck] were transformed with the pQE-30 [Qiagen] MscS-6xHis fusion expression plasmid. Cell culture in antibiotic media, induction of protein expression with IPTG, cell harvesting and disruption by French press as well as detergent solubilization and protein extraction followed by protein purification and thrombin cleavage of the 6x-His tag were performed as previously described. Functionality of the collected protein was checked using the patch clamp technique with the purified protein being stored at 4 °C. For patch clamp experiments the MscS protein was reconstituted into azolectin liposomes (Boltzmann curves for five individual experiments on the left) and for MscS in spheroplast membranes (Boltzmann curves for five individual experiments on the right) are shown with their corresponding best-fit plots calculated using Eq. 2. Note the large difference in membrane tension required for the activity of MscS channels recorded in liposomes compared with giant spheroplasts. The dotted line represents the average midpoint tension for liposomes (6.3 ± 0.8 mN/m) and for spheroplasts (17.8 ± 2.2 mN/m).

**Patch-clamp recording**

An aliquot (1.5–3.0 µl) of giant spheroplasts or an aliquot (2–4 µl) from the rehydrated liposomes was taken and added to the recording chamber. In giant spheroplasts the MscS channel
activity was examined in inside-out patches obtained by quick air exposure of the tip of the patch pipette. The spheroplasts were placed in a recording chamber containing 250 mM KCl, 90 mM MgCl₂, and 5 mM HEPES (pH 7.2). In proteoliposomes, which were placed in the recording chamber containing 200 mM KCl, 40 mM MgCl₂, 5 mM HEPES (pH 7.2), liposome patches were sampled from liposomes that settled on the bottom of the recording chamber and formed unilamellar blisters. In recordings from both giant spheroplasts and liposomes pipette solution contained 200 mM KCl, 40 mM MgCl₂, 5 mM HEPES (pH 7.2). Negative pressure (suction) was applied to patch pipettes using a syringe and recorded in millimeters of mercury (mmHg) by monitoring the pressure using a pressure gauge (PM 015R, World Precision Instruments, Sarasota, FL). Borosilicate glass pipettes (Drummond Scientific Co., Broomall, PA) were pulled using a pipette puller (PP-83, Narishige, Tokyo, Japan) to a diameter corresponding to a pipette resistance within the 3.0–5.0 MΩ range. Single-channel currents arising from activation of MscS channels were amplified with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), and data were acquired at a sampling rate of 5 kHz with 2-kHz filtration and analyzed using pCLAMP10 software (Molecular Devices, Sunnyvale, CA).

**Channel gating by mechanical force**

The linear force model discussed by Sukharev and Corey with regard to the gating of MS channels by unidirectional mechanical force assumes two fundamental conformational channel states, closed and open, separated by an energy barrier. The model links the open probability of a channel, which is an equilibrium state between the two conformational states, to the mechanical force acting on the channel using a Boltzmann distribution function, which describes a sigmoidal dependence of on applied force by the following expression:

\[
P_o = \frac{1}{1 + \exp\left[-\frac{(f - b + \Delta u)}{k_B T}\right]}
\]

where is the force acting on the channel, is the displacement of the gating swing when the channel opens (if the channel is gated by a tether acting as an elastic spring transmitting the force unidirectionally), is an intrinsic energy difference between open and closed states in the absence of applied force, and is thermal energy (~4 × 10⁻²¹ J at room temperature). For channels activated by membrane tension (stretching the membrane in two dimensions of the membrane plane), such as MscS, the free energy term in Eq. (1) is replaced by \(-\gamma\Delta A + \Delta u\), i.e.

\[
P_o = \frac{1}{1 + \exp\left[-\frac{\gamma\Delta A + \Delta u}{k_B T}\right]}
\]

where is membrane tension acting in both directions of the membrane plane, and \(\Delta A = A_o - A_c\) is the change in the in-plane area of the channel upon opening with \(A_o\) and \(A_c\) representing areas of the open and the closed channel, respectively. At \(P_o = 0.5\) when the channel is open or closed 50% of the time \(\gamma = \Delta u/\Delta A\). As pointed out by Sukharev and Corey in this model mechanosensitive channels do not possess an intrinsic threshold required for their activation because the channel open probability changes continuously with the applied force or membrane tension. Consequently, the channels can sense stimulus energies < 1 k_BT. To open an MS channel, the product \(\gamma\Delta A\) is usually several k_BT. For example, a change of ~17 k_BT is required for MscSP, an MscS-like channel from the marine bacterium *S. pomeroyi* expressed in *E. coli*, to change \(P_o\) from 0.05 to 0.95.

Furthermore, tension in general is the energy excess per unit area created by any type of stress. A lateral two-dimensional stretch of a membrane generates an expanded area. The tension in this instance is given by Eq. (3):

\[
\gamma = -K_A (\Delta A / A)
\]

where is the area elasticity constant and \(\Delta A / A\) is the proportional area change of the membrane. Note that the spring constants for two-dimensional materials have units of N/m, as for one-dimensional springs, but the stretch \(\Delta A / A\) is a dimensionless ratio, so that lateral tension is also measured in N/m or J/m². The area elasticity constant for a typical lipid bilayer range between 10² and 10³ mN/m depending on the cholesterol content of the bilayer, although \(K_A\) may also vary with solution conditions. Lytic tensions range approximately between 3 and 30 mN/m, consistent with maximal expansion of bilayers being only 2–4% before rupture.

In this study we used the two-dimensional linear force model to evaluate the energetics of MscS channel gating in giant spheroplasts compared with the channel gating in liposome preparations.
made of azolectin consisting of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol as the main phospholipids having the carbon chain length C16:0, C18:0, C18:2, C18:3 of which C18:2 comprised 60%.

Computer modeling

Data obtained from patch-clamp recordings of MscS channels in liposome and spheroplast preparations were analyzed using the curve fitting toolbox of MATLAB software. MATLAB (MATTrixLABoratory) is a high-level interactive environment for numerical computation, visualization and programming. The curve fitting toolbox of MATLAB is an easy-to-use setting for fitting curves or surfaces to data and supports linear as well as nonlinear regression with the option of using in-built or custom equations, as desired.

For our analyses, the Boltzmann distribution function (Eq. (2)) was introduced as the custom equation and the nonlinear least squares method was used to fit the experimental values of open probability (P0) and pressure (p) expressed in mmHg obtained from patch-clamp recordings of MscS channels. For interested readers the details on how one calculates membrane tension γ in mN/m from the negative pressure applied to a patch pipette, given in mmHg, can be found in Supplementary Information.

For 5 individual experiments each of liposome and spheroplast preparations, fitted values of tension (γ) and change in area (ΔA) for the mid-point, i.e., P0 = 0.5, were obtained using initial values of ΔA = 7nm2, 8 nm2 and 9 nm2, with 10% tolerance to the initial values. The best fits were shortlisted using both numerical methods i.e., computing goodness-of-fit statistics (Adjusted R-square, SSE, confidence bounds etc.) and graphical methods such as residuals analysis and prediction bounds. The fitted values obtained for liposome and spheroplast preparations are plotted in Figures 3A and 3B respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplementary material may be found here: http://www.landesbioscience.com/journals/channels/article/28366/

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