Assessment of Potential Stimuli for Mechano-Dependent Gating of MscL: Effects of Pressure, Tension, and Lipid Headgroups†

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ABSTRACT: MscL is a mechanosensitive channel of large conductance that serves as an "emergency relief valve", protecting bacteria from acute hypoosmotic stress. Although it is well-accepted that the MscL protein and an adequate membrane matrix are necessary and sufficient for the function of this channel, the exact role of the membrane has yet to be elucidated. Here, we address the role of the membrane matrix through in vitro reconstitution of the MscL protein in defined lipid bilayers. We have applied Laplace's law to visualized membrane patches where we can measure patch curvature as described in previous studies. Here, by comparing patches with different curvatures, we demonstrate that the MscL channel senses tension within the membrane and that the pressure across it plays no detectable role as a stimulus. In addition, gating only occurs when the smallest radius of curvature is nearly achieved, suggesting that the lateral tension rather than membrane curvature is the important biophysical parameter. Finally, we have examined the contribution of specific headgroups by measuring their effect on the membrane tension required to gate the channel. We have found that the addition of neither anionic nor endogenous lipids to a non-native membrane effected a leftward shift in the activation curve. In fact, the major endogenous lipid of the *Escherichia coli* membrane, phosphatidylethanolamine, led to a channel activity at a higher tension threshold, suggesting that this lipid effects altered activity through changes in the biophysical properties of the membrane, rather than through an MscL-specific interaction.

Mechanosensitive channels that gate in response to mechanical stress applied to the cell membrane or to cytoskeletal elements are proposed to play a central role in a variety of physiological processes including touch, hearing, balance, circulation, and osmoregulation (*1*). The best-studied mechanosensitive protein is MscL, a bacterial channel that plays a vital role in osmoregulation, serving as a biological "emergency release valve" upon hypoosmotic insult (*2*).

Structural studies, combined with mutagenesis and modeling, have led to models for the MscL-in vivo closed state and the structural changes that occur upon gating (*3*−*8*). In brief, the channel is a homopentamer in which each subunit spans the membrane twice with the N- and C-termini residing in the cytoplasm, the first transmembrane domain (TMD1)[1] forms the constriction point of the closed channel, while the second transmembrane domain (TMD2) interacts with the lipid matrix. The size of the open pore, estimated by conductance and molecular sieving experiments (*9*), is thought to be greater than 30 Å. An early study relying on molecular modeling and limited disulfide trapping studies suggested a model for gating in which the transmembrane domains tilt within the membrane and TMD1 lines the pore (*4*, *10*). Subsequent studies have refined this model to further suggest that, upon gating, when viewed from the periplasmic perspective TMD1 undergoes a relatively large clockwise rotation (*7*, *8*).

While much of the previous work has centered on the structural changes that occur upon gating, less is known of the way in which MscL senses mechanical forces. From reconstitution studies, it is clear that the channel senses pressure or tension directly; no additional proteins are necessary (*11*). Previous studies demonstrated that a mechanosensitive channel in *Saccharomyces cerevisiae* directly senses tension within the membrane rather than pressure across it (*12*). Many studies of MscL, including those determining the energetics of the channel (*13*−*15*), assumed that MscL also exclusively sensed membrane tension; however, the theory had not been directly tested. In addition, although several lines of evidence suggest that the channel senses changes in the biophysical properties of the bilayer (*16*, *17*), it is unclear if specific protein−lipid interactions are important for normal MscL activation. While interactions with specific headgroups have been predicted by molecular dynamic simulations (*15*, *18*) and by fluorescence spectroscopy estimation of the binding affinity of different lipids to MscL with tryptophan substitutions (*19*), direct evidence that any of the putative interactions actually play a role in the ability of MscL to sense mechanical perturbations is lacking.

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[1] Abbreviations: TMD, transmembrane domain; OG, octyl-β-glucopyranoside; DOXX, for lipids, where DO is 1,2-dioleoyl-*sn*-glycero-3- and XX is one of the following headgroups, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidylethanolamine (PE); T, tension with a sole exception where it is noted to be temperature.

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Here, we utilize techniques that allow us to directly measure the influences of pressure, tension, and lipid headgroups on MscL gating. We have employed a technique used previously by other researchers (*20*, *21*) to visualize the patch-clamped membrane and measure its radius of curvature. Knowing this value, the pressure across the membrane, and making use of Laplace's law, we calculate the tension within the membrane of the patched membrane. It is by using this approach that we are able to determine the influence of specific biophysical parameters on the activation of the MscL mechanosensitive channel. The data presented show that MscL indeed senses tension in the membrane and the pressure across it plays no appreciable role as a stimulus. In addition, gating only occurs when the smallest radius of curvature is nearly achieved, suggesting that the lateral pressure within the membrane, rather than membrane curvature, is the salient biophysical stimulus. In addition, reconstituting the channel into lipids with various headgroups, including anionic lipids and those found in *Escherichia coli* inner membranes, strongly suggests that if there are specific interactions between the channel and lipid headgroups, they do not play a significant role in the ability of MscL to detect membrane tension.

MATERIALS AND METHODS

Molecular Biology and Protein Purification. The *E. coli mscL* structural gene was modified by PCR to encode a hexahistidine tag appended to the carboxyl-terminus to yield $MscLH_6$; this construct was expressed from the moderate copy-number vector pB10b, under the control of an inducible promoter, in the *mscL*-null *E. coli* strain PB104 (*22*). Host cells were grown at 37 °C in Luria−Bertani medium containing ampicillin (100 μg/mL) and IPTG (1 mM) to induce expression of the channel protein. Cells were harvested while in logarithmic growth ($\sim$0.6 OD_{600}) and washed with KMg buffer (50 mM KPi, pH 7.2, 5 mM $MgSO_4$, and 1 mM DTT).

To harvest the tagged protein, the cells were resuspended to 20% w/v in KMg buffer plus 1 mM PMSF, 1 mg/mL lysozyme, and 0.5 μg/mL DNaseI and incubated at room temperature with gentle mixing. The suspension was then subjected to two passages through a French pressure cell at 16 000 psi to complete the lysis. This and most further steps were performed at 4 °C. The crude lysate was cleared by centrifugation for 10 min at 6000*g*, and the total cell membrane fraction was collected by centrifugation at 200 000*g* for 30 min. This membrane fraction was solubilized in extraction buffer consisting of 300 mM NaCl, 50 mM KPi, pH 8.0, 20 mM imidazole, pH 8.0, and 2% octyl-β-glucopyranoside (OG) in a glass dounce and cleared by centrifugation for 20 min at 230 000*g*.

$MscLH_6$ was enriched from the membrane homogenate by metal-chelation chromatography using Ni-NTA resin (Qiagen). Briefly, washed resin equilibrated with extraction buffer and 1% OG was batch-incubated, at room temperature, for 30 min with the homogenate and transferred to a column. The column was washed first with extraction buffer and 1% OG and finally with elution buffer (50 mM NaPi, pH 7.2, 300 mM NaCl, 1 mM PMSF, and 1% OG). $MscLH_6$ was eluted at 200 mM imidazole and found to be essentially homogeneous by SDS−PAGE. Total protein in the eluted fraction was quantified by the modified Bradford assay (Pierce).

Reconstitution. Lipid vesicles suitable for reconstitution were prepared as outlined previously (*23*). Lipids, 1,2-dioleoyl-*sn*-glycero-3-XX (DOXX), where XX is one of the following headgroups: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidylethanolamine (PE), were utilized (Avanti). Briefly, the lipids, dissolved in chloroform and stored under argon at −20 °C, were shell-dried under argon in a 13 mm glass tube. Where lipids were to be combined, they were mixed as a chloroform solution at this step. This lipid film was resuspended to 20 mg/mL in buffer (10 mM TrisCl, pH 7.2, 1 mM EDTA, and 1 mM EGTA), and lipid vesicles formed by bath sonication to near-clarity. $MscLH_6$ was combined with lipid vesicles to a 1:500 protein-to-lipid mass ratio. Detergent was removed by dialysis against 2 × 1000 vol of buffer (100 mM NaCl, 0.2 mM EDTA, 5 mM TrisCl, pH 7.2, and 0.02% NaN_3) containing CALBIOSORB beads (Calbiochem) to sequester the detergent. The resultant proteoliposomes were collected by a 20-minute centrifugation at 30 psi in an Airfuge (Beckman-Coulter Instruments). The pellet was resuspended to 1.3 mg/μL in 5% ethylene glycol and 10 mM MOPS, pH 7.4, and desiccated overnight under vacuum at 4 °C.

Desiccated proteoliposomes were rehydrated for at least 2 h in Buffer A (150 mM KCl, 0.1 mM EDTA, 10 μM $CaCl_2$, and 5 mM HEPES, pH 7.2) at a lipid concentration of 90 mg/mL. Unilamellar blisters, suitable for access by patch-clamp electrode, were induced in the proteoliposomes by incubation in Buffer A and 30 mM $MgCl_2$.

Biophysical Characterization. Excised patches were studied at room temperature under symmetrical buffer conditions in Buffer A and 30 mM $MgCl_2$. Recordings were performed at a relatively low potential, +20 mV (electrode), in an attempt to avoid possible partitioning of negatively charged lipids to a single leaflet of the bilayer. Data were acquired at a sampling rate of 20 kHz with a 5 kHz filtration using an AxoPatch 200B amplifier in conjunction with Axoscope software (Axon). A piezoelectric pressure transducer (World Precision Instruments) was used to measure the pressure applied to the system.

To facilitate visualization of the membrane patch, the tip of the electrode was fused to an angle that placed it parallel to the focal plane while in the recording chamber. Images of the membrane patch were captured using a Plan Apo 100× (NA 1.40), DIC H (Nikon) oil-immersion objective coupled with a Nikon DXM CCD camera and 5× relay lens calibrated by optical micrometer. The patch radius was determined from the captured images using a commercial computer-assisted design package IntelliCAD 2000 (CADopia).

Channel open probability (P_O) was determined by I/I_{max} for a 3 s window corresponding to each patch image (black squares, Figure 2A) and fit to a Boltzmann curve either as a function of pressure $P_O(P)$, or tension $P_O(T)$ using the Origin software suite (Microcal). Patch tension (T) was calculated from the transmembrane pressure (P) and patch radius (r) data using Laplace's relationship; $T = (1/2)Pr$.

RESULTS

MscL Senses Tension in the Membrane, Not the Pressure across It or Increased Membrane Curvature. We have

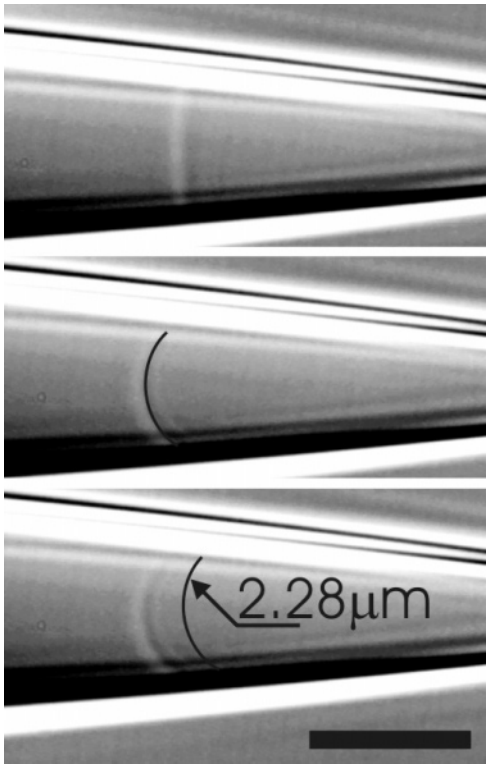

FIGURE 1: Morphometric analysis of the membrane patch permits the calculation of tension by Laplace's law. Shown is the quiescent patch (top) before application of pressure, the pressure-saturated patch (middle), and measurement of patch radius (bottom) from which the patch tension is determined. The scale bar represents 5 μm.

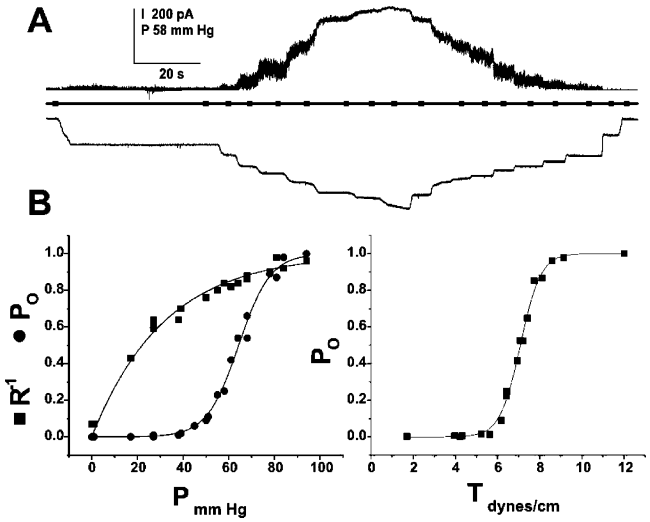

FIGURE 2: A typical electrophysiological record of *E. coli* MscL reconstituted in a DOPC/DOPS (1:1) membrane highlighting the increase in open probability (P_O) as a function of pressure. (A) A trace that achieved saturation, allowing P_O to be determined. The average I_{patch} was determined over 3 s windows (black bars, center line) corresponding to each patch image. P_O was determined as I/I_{max}. (B) Analysis of data from panel A showing, left panel, that the patch radius (■) is essentially "saturated" by the time and that $P_O(P)$ (●) achieves 0.5, and right panel, a Boltzmann fit of $P_O(T)$.

utilized an approach previously employed to determine the energetics of the MscL channel (*16*, *17*). As described in Materials and Methods, and shown in Figure 1, the patched membrane from reconstituted systems was visualized and the radius of curvature was determined. Simultaneously, electrophysiological recordings were obtained. Patch image data were correlated with the electrophysiological data by using the "tag" function of the Axon software. A typical recording, in this case obtained from a DOPC/DOPS (1:1) membrane, is shown in Figure 2A. Simultaneous measurements of the radius of curvature and the probability of opening (P_O) derived from electrophysiological recordings are shown in Figure 2B, left panel. When we use these data and Laplace's law (Tension = Pressure·radius/2), the tension within the membrane can be calculated and P_O plotted as a function of tension (Figure 2B, right panel). Shown in Figure 2B, left panel, and consistent with a previous report (*17*), the MscL channel appears to gate after the curvature of the membrane has nearly achieved its smallest value (i.e., while the channel P_O increases from 0.5 to 0.9, the patch radius changes by less than 8%). We observed that, although the patch radius can vary between patches, MscL activity was consistently seen only after the membrane patch had nearly achieved its smallest radius, strongly suggesting that curvature of the membrane is not a primary stimulus.

If the channel senses pressure across the membrane, then pressure at which the $P_{1/2}$ occurs should be constant. If, on the other hand, the channel senses tension within the membrane, and the radius from independent patches is found to be variable, no strong correlation will exist. In one combination of lipids (DOPC/DOPE, 1:1), such a variability in patch radius was observed (Figure 3, top panel). These three independent patches also showed variability in the amount of pressure required to open the channel (Figure 3, middle panel). However, when tension is calculated and plotted (Figure 3, bottom panel), the three curves converge, yielding approximately the same $T_{1/2}$. These data strongly suggest that the sole stimulus for gating of the MscL channel is tension within the membrane.

Previous studies have utilized an approach in which the slope parameter (α) of the Boltzmann fit of $P_O(P)$ has been used to calculate the free energy (ΔG) of channel gating ($\Delta G_o = kT(P_{1/2}\alpha)$ (where T is temperature); see Hamill and Martinac (*1*)). Here, we compared, using a similar analysis, the determination of ΔG from $P_O(P)$ and $P_O(T)$ (Figure 3, middle and lower panels, respectively) and found a 2-fold reduction in variance when tension was used as the reporter of system energy. Hence, while the former approach using pressure is facile and provides a reasonable approximation, we find that the latter approach, although tedious, may provide a more stringent approach for determining the energy requirements for MscL gating.

Negatively Charged and Endogenous Lipid Headgroups Do Not Significantly Decrease the Tension Required for MscL Gating. Although the zwitterionic PC is not a lipid headgroup found in bacterial membranes, previous studies have demonstrated that the MscL channel is functional when reconstituted in DOPC (*24*). Because this reflects an entirely artificial environment for the MscL protein, we chose to utilize this lipid and heterologous system as a standard in which to test the effect of different lipid headgroups on the system. Our observation of the amount of intramembrane tension required to gate the channel in this lipid environment is consistent with that previously reported (*17*) (Figure 4).

There are several charged amino acids near the predicted lipid−aqueous interface at both the periplasmic and cytoplasmic interface of the transmembrane domains; many bear

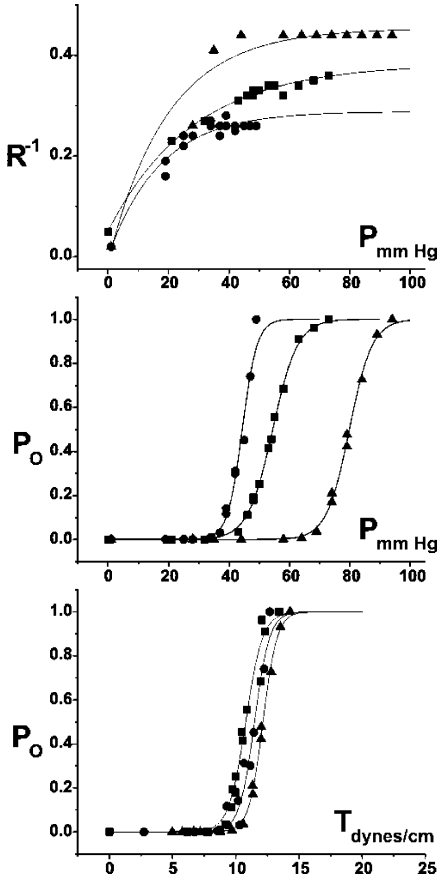

FIGURE 3: MscL senses tension within the membrane; the pressure across it plays no detectable role as a gating stimulus. A comparison of Boltzmann analysis of $P_O(P)$ and $P_O(T)$ highlighting the disparity between $P_O(P)$ and $P_O(T)$. A reconstitution of *E. coli* MscL in DOPC/DOPE (1:1) membranes reveals that a membrane patch may achieve disparate radii upon deformation by a pressure gradient (top panel). Subsequent analysis (middle panel) that defines P_O as a function of transmembrane membrane pressure suggests that the membrane patch radius defines the channel activity. However, refinement of the analysis (bottom panel) to account for membrane tension by using Laplace's law shows that tension is the salient force affecting MscL activity.

positive charges. We therefore tested the possibility that adding lipids with negatively charged headgroups would decrease the membrane tension required for channel gating. However, addition of DOPS at a 1:1 ratio with DOPC did not decrease the tension required to gate the channel (Figure 4A). We therefore tested addition of the bacterial endogenous negatively charged lipids, PG headgroups, which are abundant in the native *E. coli* bacterial membrane. Here again, no decrease was observed in the tension required to gate the channel (Figure 4A). These data suggest that negatively charged headgroups do not form specific interactions with the channel protein leading to a decrease in the tension required for mechanosensitive gating.

E. coli cells contain an additional component headgroup in their inner membranes, PE. Previous studies have suggested that these headgroups may interact with the channel protein. We therefore increased the ratio of DOPE in the reconstituted system. A modest increase in DOPE (1/4 of total lipids) yielded no significant change in gating tension (not shown). However, at ratios 1:1 and higher, a significant increase in gating tension was observed (Figure 4B). Higher percentages of the nonbilayer forming PE containing lipids

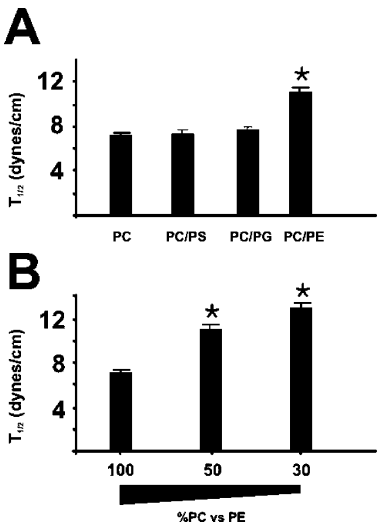

FIGURE 4: Negatively charged and endogenous lipid headgroups do not appear to affect tension sensing of the MscL channel. A comparison of lipid composition (A) indicates that, at a 1:1 ratio, neither anionic (PS) nor endogenous (PG) lipids observably affect the activity of MscL. The presence of PE, however, significantly increases the energy required to open MscL. (B) This effect was explored by titration of PE vs PC. Although no significant effect was detected at lower complements of PE, higher ratios showed a clear effect on the gating tension. Data are $n \geq 3$; error bars represent the SEM; *, $p < 0.001$ in *t*-test when compared to DOPC data.

were not tolerated in the protocol used for patch clamp; "blisters" required for patch formation were not formed. This observation could suggest that PE lipids, which prefer the nonlamellar inverse hexagonal phase, are not accommodated within blistered membranes. However, the observations that PE, which is perhaps the most likely to partition and be excluded from the patch, did indeed change the tension required to gate the channel, and that the observed changes are dependent upon PE concentration, demonstrate that the lipids are not excluded from the blisters. In sum, the data suggest that, while there may be subtle effects of PE headgroups directly interacting with the channel, influences due to changes in the biophysical properties of the membrane are overwhelming and lead to greater tensions required for channel gating.

DISCUSSION

Previous experiments involving reconstitution of the MscL protein have largely used the poorly defined and heterogeneous azolectin lipids (*23*, *25*) or POPC (*24*, *26*) and have concentrated on the spatial parameters and energetics of channel gating (*17*) or biophysical changes in the lipid lateral profiles that influence these energetics (*24*, *26*). Here, we have expanded these studies to determine what, if any, influence pressure across the membrane or specific headgroups may play in the sensitivity of the channel.

The mechanosensitive channel activity in *S. cerevisiae* has been well-studied (*12*). By obtaining a whole-cell clamp configuration of yeast of different sizes, and therefore different radius of curvature, Gustin determined that the *S. cerevisiae* mechanosensitive channel senses and responds exclusively to the tension in the membrane rather than the pressure across it. Early reconstitution experiments with MscL relied on these findings and the consistency of the

results obtained to speculate that this was also true for this channel (*17*). However, it was unclear if the consistency was truly due to the channel sensing membrane tension or simply due to a low variability in patch radius. In addition, it was theoretically possible that the channel sensed some combination of both, tension in the membrane and pressure across it. Here, we report several membrane patches that have quite different radius of curvature and found that, while they give different pressure profiles for gating, when tension is calculated they all gate at the same tension. Hence, it appears that pressure across the membrane plays no detectable role in MscL channel gating; membrane tension is by far the primary stimulus.

Negatively charged lipids have been shown to play a vital role in the functionality of the hyperosmotically regulated bacterial transporter OpuA (*27*). Studies in several other systems have led to the hypothesis that specific interactions between negatively charged lipids with several other transporters and the modulation of these interactions by monovalent cations, especially K^+, lead to the regulation seen in hyperosmotic conditions (*28*). More pertinent, a recent study has used tryptophan substitutions and fluorescence spectroscopy to estimate the relative binding affinity of lipids to the MscL channel (*19*). The authors conclude that anionic lipids bind to basic residues at the cytoplasmic region of TMD2. Analysis of mutants was consistent with the notion that the binding was to a specific site containing basic residues within this subdomain, RKK, starting at position 104 in the *E. coli* MscL. The proposed lipid−protein interactions apparently decrease channel activity because removal of this binding site by site-directed mutagenesis led to a gain-of-function (slowed growth) cellular phenotype, which was equated to an overactive channel. Unfortunately, some aspects of the data are difficult to interpret because of the use of *Mycobacterium tuberculosis* MscL channel, which has been shown to be less functional in all heterologous systems tested (*29*), and the fact that activity of mutated channels was inferred from whole-cell physiological approaches rather than direct electrophysiological measurements. A further complication is that the proposed anionic lipid binding site, RKK, is not conserved; for example, it is REE in the *Pseudomonas fluorescence* MscL, which has been shown to have similar channel properties and mechanosensitivity as the *E. coli* MscL (*30*). Here, we demonstrate that addition of exogenous anionic lipids (PS) or the primary endogenous anionic lipid (PG) to the MscL reconstitution system has no detectable influence on the amount of membrane tension required to gate the channel. Hence, if anionic lipid binding sites do exist in MscL, they play no detectable role in the ability of the channel to sense membrane tension.

Numerous additional studies have indirectly implied specific lipid−protein interactions may occur with MscL independent of anionic lipids and that these interactions are critical for the normal function of the protein. For example, molecular dynamic simulations have indicated numerous hydrogen bond formations between PE headgroups and the MscL protein (*18*). The simulations also suggest that, while the structure of MscL in PE lipids is similar to the crystal structure, the structure in PC is significantly different (*15*). Furthermore, a genetic study in which loss-of-function MscL channels were isolated suggested that residues in the lipid-facing TMD2 at the lipid−aqueous interface played a vital role in the functionality of the channel (*31*). More recently, a similar genetic study isolating suppressors of gain-of-function mutants (*32*), combined with residue-substitution scanning experiments (*33*), identified residues in this region as candidates for lipid interaction. Finally, a cysteine scan of the entire TMD2 domain found residues that modified MscL function, many being found at the lipid−aqueous interface (*5*). While much of the data from the above studies implied lipid−protein interactions, because the experiments were often not designed to test for these interactions, other interpretations are possible. Here, by manipulating the lipid composition, we more specifically address the question of whether PE, the predominant lipid headgroup in the *E. coli* cytoplasmic membrane, influences the gating tension of the MscL channel. In contrast to the predictions made by the above studies, we found that addition of PE actually increased the amount of tension required for channel activation. We believe that this finding is most likely due to a change in biophysical properties of the membrane. The addition of PE lipids would presumably increase both membrane thickness and lateral pressures within the membrane. Both of these factors have been proposed to decrease channel activity (*24*). Hence, it seems likely that if PE binds the MscL channel, any influence it has on gating is extremely subtle and easily masked by other effects due to changes in the biophysical properties of the membrane.

Several mechanosensitive channels show functional similarities to MscL, although sequence homology is not obvious. For example, amphipaths, which intercalate asymmetrically within the membrane, have been shown to influence the gating of MscL (*24*) as well as the apparently nonrelated *E. coli* MscS channel (*34*). It has also been previously noted that the ratio of the threshold pressure required to gate MscL divided by the threshold pressure for MscS is a constant; this observation has led to the use of MscS as an internal control for studying MscL mutants and homologues (*35*). These data suggest that these two channels sense a common stimulus. From the data presented here, it seems unlikely that the stimulus is a change in membrane curvature or that it is influenced by interactions with specific lipid headgroups. The results with amphipaths, instead, may be the result of the protein somehow sensing decreases in the lateral pressure profile below the lipid headgroups within the bilayer (see refs *36*−*38* for predictions of membrane lateral pressures). The observation that some mammalian mechanosensitive channels, specifically members of the two-pore domain channel family, are also influenced by amphipaths (*39*) suggests that such a mechanism may be found even in channels from higher organisms.

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