The role of lipids in mechanosensation

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The ability of proteins to sense membrane tension is pervasive in biology. A higher-resolution structure of the Escherichia coli small-conductance mechanosensitive channel MscS identifies alkyl chains inside pockets formed by the transmembrane helices (TMs). Purified MscS contains E. coli lipids, and fluorescence quenching demonstrates that phospholipid acyl chains exchange between bilayer and TM pockets. Molecular dynamics and biophysical analyses show that the volume of the pockets and thus the number of lipid acyl chains within them decreases upon channel opening. Phospholipids with one acyl chain per head group (lysolipids) displace normal phospholipids (with two acyl chains) from MscS pockets and trigger channel opening. We propose that the extent of acyl-chain interdigitation in these pockets determines the conformation of MscS. When interdigitation is perturbed by increased membrane tension or by lysolipids, the closed state becomes unstable, and the channel gates.

Organisms use lipid bilayers, which are impermeable to ions and polar molecules, to compartmentalize. The exchange of molecules with the outside world occurs in a controlled manner via channels and transporters. In general, transporters respond to the presence of their substrates and energy, whereas channels respond to specific stimuli. Mechanosensitive channels gate in response to changes in the tension in the membrane bilayer. Mechanosensors are found in bacteria, archaea1,2 and eukaryotes3, in which they fulfill a variety of essential roles. The role of the human piezo channels in cardiovascular disease has recently attracted attention4. However, the best-studied essential roles. The role of the human piezo channels in cardiovascular disease has recently attracted attention4. However, the best-studied mechanosensors are found in bacteria, archaea1,2 and eukaryotes3, in which they fulfill a variety of essential roles. The role of the human piezo channels in cardiovascular disease has recently attracted attention4. However, the best-studied mechanosensors are found in bacteria, archaea1,2 and eukaryotes3, in which they fulfill a variety of essential roles. The role of the human piezo channels in cardiovascular disease has recently attracted attention4. However, the best-studied mechanosensors are found in bacteria, archaea1,2 and eukaryotes3, in which they fulfill a variety of essential roles. The role of the human piezo channels in cardiovascular disease has recently attracted attention4. However, the best-studied mechanosensors are found in bacteria, archaea1,2 and eukaryotes3, in which they fulfill a variety of essential roles.

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RESULTS
Lipids pack the voids in MscS
We covalently modified the MscS D67C single-cysteine mutant as previously described\(^1\) to yield MscS D67R1 (in which R1 denotes the MTSSL cysteine adduct). A MscS D67R1 crystal\(^2\) diffracted to 3.0-Å resolution (Table 1 and Supplementary Fig. 1). The protein structure is essentially identical to the 3.45-Å A106V open structure\(^3\). We located several additional residues and spin labels matching the measured pulsed electron–electron double resonance (PELDOR) distances\(^4\) (Supplementary Fig. 1a,b). We fitted difference (F\(_o\) – F\(_c\)) electron density at the transmembrane helices as alkyl chains (Fig. 1b and Supplementary Fig. 1c), but the results could not unambiguously differentiate between lipid and detergent\(^5\). Two alkyl chains penetrate into the ‘pocket’ formed by the arrangement of TM1, TM2 and TM3b in the heptamer while the third chain packs against TM3b (Fig. 1a). This is the first experimental evidence, to our knowledge, indicating that the voids in MscS are pockets that contain lipids or lipid-like molecules.

After detergent extraction and purification of the native MscS heptamer, we resolved up to five putative lipids bound to the MscS heptamer in the gas phase by nondenaturing MS\(^6\) (Fig. 2a). We observed a series of peaks with mass differences between 620 and 790 Da, ruling out n-dodecyl-β-D-maltopyranoside (DDM) (510 Da) or n-lauridylmethylamine-N-oxide (LDAO) (230 Da) adducts from the detergents used in sample preparation. The lowest-mass species was a lipid adduct of protein, not the protein alone (as seen for other membrane proteins\(^7\)). The mass range of small-molecule adducts was consistent with the two major types of \textit{E. coli} phospholipid (PL), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) but not cardiolipin (CL).

For two independent preparations of DDM-purified MscS D67R1, we extracted lipids and analyzed them by MS (Fig. 2b). Electrospray MS (ES-MS) and subsequent MS fragmentation detected seven lipid species, of which six were assigned (Fig. 2b). PG ionizes more readily than PE (Fig. 2b), thus preventing quantitative analysis. However, changes in the relative counts of species between samples were indicative of changes in the relative proportions of the phospholipids. PG 30:1 and particularly PE 14:0/14:0 and PE 16:1/14:0 were enriched relative to their natural abundance in \textit{E. coli}\(^8\). The differences in phospholipid composition in the protein sample were not a feature of \textit{E. coli} and were independent of detergent but specific to MscS (Supplementary Fig. 2).

We examined lipid extract from purified MscS by thin-layer chromatography (TLC) with staining by primuline to differentiate lipid from detergent and with ninhydrin (which does not stain PG or CL phospholipids) to identify PE (Fig. 2c). The plates indicated that PE was indeed the predominant phospholipid. A previous study using size-exclusion chromatography and inductively coupled plasma MS\(^9\) has estimated 2.6 to 3 unidentified phospholipids per MscS monomer. On the basis of TLC, we estimated approximately 0.5 PE molecules per monomer (consistently with the results from nondenaturing MS analysis; Fig. 2a). A protein sample that had been exposed to

Table 1 Data collection and refinement statistics

| MscS D67R1 | P2\(_1\)2\(_1\)2\(_1\) |
|---|---|
| Data collection | |
| Space group | |
| Cell dimensions | a, b, c (Å) |
| | α, β, γ (°) |
| | Resolution (Å) |
| | Rmerge | t / σt |
| | Completeness (%) | Redundancy |
| Refinement | |
| Resolution (Å) | 64.2–2.99 (3.07–2.99) |
| No. reflections | 66,747 |
| Rwork / Rfree | 24.4 / 26.3 |
| No. atoms | Protein, 13,733 |
| | Ligand, 203 |
| B factors | Protein, 114 |
| | Ligand, 103 |
| r.m.s. deviations | Bond lengths (Å), 0.008 |
| | Bond angles (°), 1.2 |

Data were collected from one crystal. *Values in parentheses are for highest-resolution shell.
Figure 2 Lipids pack in the pockets created by the TM helices. (a) Native MS of wild-type MscS. The expected theoretical weight of the heptamer is 223,727 Da. If this value is subtracted from the resolved first peak value of 224,344 Da (brown dot), then it yields a value of around 620 Da, consistently with a small lipid. Subsequent differences between peaks reveal additional lipid adducts. (b) ES-MS of phospholipid extracted from a sample of DDM-solubilized MscS. C.p.s., counts per second. (c) Thin-layer chromatogram of extracted lipids from DDM-solubilized MscS. The samples loaded are: lane 1, MscS-DDM (238 µg MscS protein); lane 2, MscS-DDM (280 µg lipids); lane 3, MscS-Fos-14 (305 µg MscS protein); lane 4, POPG (5 µg lipids); lane 5, POPE (2.4 µg lipids); lane 6, E. coli lipids (8 µg lipids); lane 7, DDM (5 µg lipids); and lane 8, Fos-14 (10 µg lipids). Asterisks highlight older sample. The result is representative of three separate experiments. Samples in lanes 1 and 3 were freshly purified, and that in lane 2 was several months old. PG, known to be present from MS in preparations 1 and 2, is not visible, owing to the overlap with the DDM, which is much more abundant in the detergent-solubilized material.

Lipid exchange between the pockets and bilayer

We performed multiscale molecular dynamics (MD) simulations (1-µs coarse grain (CG-MD) and 100-ns atomistic (AT-MD), 5 replications each) on both the closed and open structures (D67R1 mutated in silico to native) in a 4:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) lipid-bilayer model. We restrained the protein structure to prevent collapse of the pockets and distortion of TMs. In all simulations, lipids migrated to fill the TM pockets, and there were more lipids in the lower than the upper half of the pocket (Fig. 3a,b and Supplementary Fig. 3a,b). We observed strong local membrane curvature around MscS in both states (Fig. 3a,b), consistently with the proposal that the local membrane detergent for several months and subjected to multiple freeze-thaw cycles showed a lower PE/protein ratio (Fig. 2c and Supplementary Table 1). Therefore, we suggest that the precise lipid content may reflect differences in the purification and storage procedure.

Figure 3 Lipid exchange between the pockets and the bilayer. (a,b) Cut-away slices showing snapshots (at 100 ns) of atomistic simulations of the closed (a) and open (b) conformation of MscS in POPE/POPG (4:1) phospholipid bilayers. Movies of the lipid bilayer (with protein removed) are available as Supplementary Movies 1 and 2. (c) Comparison of the number of lipids that remain within 6 Å of the TM (i.e., residues 27 to 128, lighter bars) and the lower pocket (TM3b region, residues 106 to 122, darker bars) of closed (blue bars) and open state (magenta bars) MscS throughout the latter 0.5 µs of the CG-MD simulations. Error bars, s.d. of number of lipid contacts. (d) Single-tryptophan mutants of MscS probed with brominated phospholipids, with degree of quenching indicated by color shading. (e) Typical raw data of quenching experiments (selected mutants). Emission spectra are shown from MscS in 100% DOPC (black) or 100% BrPC (red). (f) Quantitative results of BrPC quenching, shown as fractional quenching by brominated lipid for TM3b mutants in DOPC (black bars; error bars, s.d. from n = 17 reconstitutions) and 80% DOPC/20% DOPG (white bars; error bars, s.d. from n = 4 reconstitutions). F0 and F represent intensity for samples containing 100% nonbrominated lipids or 100% brominated lipids, respectively.
environment around MscS is highly distorted. The distortion of the membrane bilayer around MscS (Fig. 3a, b and Supplementary Movies 1 and 2) highlights the limitations of a simple geometric representation of the bilayer as a flat sheet and illustrates the importance of characterizing the exact disposition of the lipids to understand tension transmission. Previous simulations have identified a smaller degree of curvature, but these simulations were shorter (<10 ns) and used a simpler POPC lipid bilayer. The N-terminal 24 residues of MscS, which are disordered, were excluded from our analysis, the mutants were functional (Supplementary Fig. 3f), but with 3 µM LPC 14:0 added to the trans compartment. (e) As in c, but with 10 µM LPC 14:0 added to the cis compartment. (f) As in c, but with 10 µM LPC 14:0 added to the trans compartment. Inset, recording at expanded time scale.

CG-MD showed that as MscS opens, the lipid content of the pocket decreases by approximately one lipid per pocket (Fig. 3a–c and Supplementary Fig. 3a,b), and the loss occurs between TM2 and TM3a (Supplementary Fig. 3d). The pockets (upper and lower regions) are more accessible to lipids of the cytosolic membrane leaflet (Fig. 3a,b and Supplementary Fig. 3a,b). The lipids in the pockets are mobile and are in continuous contact with the bulk membrane bilayer (Supplementary Movies 1 and 2), particularly the cytosolic leaflet (Fig. 3a–c).

The simulations suggested that one lipid contact (per subunit) persists in the lower part of the pocket (Fig. 3c). In the open state, these lipids are almost exclusively PE and overlap with alkyl chains in the crystal structure (Supplementary Fig. 3c). The protein proximity to these PE phospholipids remained essentially constant during the final 50 ns of AT-MD, in agreement with CG-MD findings and with the detection of PE phospholipids (Fig. 2a–c). AT-MD showed that the zwitterionic head groups of these phospholipids are coordinated primarily to charged residues on the loop that connects TMs 1 and 2 (Supplementary Fig. 3f). In the closed structure, TM3a helices are tightly packed, and there is no lipid penetration of the pore (Supplementary Fig. 3f), whereas in the open structure, the PE alkyl chains run parallel to the TM3b helix and terminate by contacting L105 and L109 (the pore-sealing residues) by penetrating between adjacent TM3a helices, thus linking the bilayer through TMs 1 and 2 to TM3a.

We introduced single tryptophan mutations into a tryptophan-free mutant of MscS, W16Y W240F W251F (MscS YFF), which has previously been shown to be stable and functional, albeit with a reduced pressure sensitivity in patch-clamp analysis. We chose A103W, V107W and L111W on TM3a and L115W, A119W and Q203W on the cytosolic domain (no lipid contact) from analysis, the mutants were functional (Supplementary Fig. 4a,b). We chose M47W, on the surface of TM1 (lipid exposed), L105W and L109 (the pore-sealing residues) by penetrating between adjacent TM3a helices, thus linking the bilayer through TMs 1 and 2 to TM3a.

The conformational state of MscS can be altered by perturbing the interactions between the phospholipid and the protein. (a) Fluorescence quenching. A119W (left) and M47W (right) were reconstituted into DOPC (top row) or BrPC (bottom row), shown in black. Brominated LPC (top) or nonbrominated LPC (bottom) was added (green), thus causing quenching or dequenching, respectively. (b) ES-MS of phospholipid extracted from DDM-solubilized MscS after treatment with LPC 14:0. Survey scan in positive-ion mode (465–500 m/z) showing the 490 m/z of the LPC 14:0. Survey scan in negative-ion mode (600–1,000 m/z) of MscS after treatment with LPC 14:0 (inset).

Only LPC 14:0 [M+Na+] 490.3 was observed.
Figure 5 A model for mechanosensation. (a) The phospholipids partition in the pockets and the lipid bilayer. As pressure is applied, the lateral tension increases, and as a result the phospholipids repartition (blue arrows) from the protein pockets to the bilayer, thus destabilizing the closed structure. The protein responds by undergoing a conformational change (orange arrow) to the open form. MscS is depicted as a simplified line diagram. PE and PG molecules are shown with black head groups, and those inside the pockets are highlighted with a green head group. (b) LPC (shown as a single chain with a yellow head group) first enters the bilayer and then the pockets from the cytoplasmic side; as a result, the lipid content (acyl chains) falls inside the protein pockets, thus destabilizing the closed structure. The protein undergoes a conformational change to a subconducting state. (c) Single-channel bilayer recordings showing that MscS D67C exhibits similar conductivity to that of wild-type protein when opened by addition of LPC 14:0 to the cis side (Fig. 4e).

or small membrane patches in which BrPC forms bilayers with similar properties to those of DOPC48,49. Upon incubation with brominated lipids, we observed strong quenching for the residues that face into the pockets (Fig. 3d,e and Supplementary Table 2). We observed the strongest quenching with A119W, but A103W and V107W also exhibited strong quenching (Fig. 3e); these results indicate that the tryptophan is close to the bromine atom on the lipid. L123W, which lies closer to the periphery of TM3b, exhibited weak quenching, possibly because it is close to the phospholipid head-group region and consequently is far from the bromine atoms at the 9,10 positions of BrPC. Similarly, the weaker quenching observed with the positive control M47W on TM1 (Fig. 3e and Supplementary Table 2) may be explained by the proximity of the tryptophan to lipid head groups. The three negative controls, L105W, Q203W and W240, exhibited very weak or no quenching. DOPC is not the natural lipid context for E. coli MscS, but it has been used successfully in previous studies36,50. We retested L111W, A119W and L123W in 1:1 PE/PG (an E. coli–like lipid composition) and found essentially the same results (Fig. 3f). These data demonstrate that the cavities predicted from crystal structures permit the exchange of phospholipids.

MscS is controlled by changes in protein-lipid interactions

MscS opened with LPC 18:1 has reported a single-channel conductance of 1 ± 0.2 nS (mean ± s.d. here and subsequently) (200 mM KCl, 90 mM MgCl2, 10 mM CaCl2, and 10 mM HEPES)16, a value similar to that obtained when transbilayer pressure differences are used for opening36,38,49,49. LPC has been proposed to work by increasing leaflet curvature, mimicking the patch-clamp pressure–induced opening5,16. We reconstituted A119W and M47W MscS in DOPC and then added brominated LPC 18:1 to a final concentration of 33% (LPC/DOPC molar ratio). Blue native gel analysis showed that the MscS heptamer remained intact (Supplementary Fig. 4b). Comparison of spectra obtained before and after addition of LPC indicated quenching (Fig. 4a). We observed dequenching when we reconstituted both mutants into BrPC and added nonbrominated LPC (Fig. 4a). Thus, LPC exchanges reversibly with lipids in the pockets. Because DDM-solubilized MscS incubated with LPC 14:0 partially dissociated (Supplementary Fig. 4c), we used gel filtration to select heptameric protein. MS analysis of the lipid extract from the heptameric fractions of MscS showed that LPC 14:0 remained associated with protein and displaced the E. coli PE lipids (Fig. 4b and Supplementary Fig. 4c–e).

There are no reports of MscS reconstituted into 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) planar lipid bilayers, possibly because this system offers no simple way to establish a pressure gradient. Over these longer traces, we observed multiple open channels (Supplementary Fig. 5d). The addition of LPC 14:0 to the trans compartment resulted in channel opening (G = 0.8 ± 0.2 nS) with frequent gating (of variable duration) rather than sustained opening (Fig. 4f). The statistically significant lower conductance with LPC 18:1 and LPC 14:0 (G = 0.8 ± 0.2 nS) compared to spontaneous opening (G = 1.2 ± 0.1 nS) indicates that,
at least in this system, LPC creates a subconducting (not fully conducting) state. D67C behaved as native in both planar lipid bilayers (Fig. 5c) and in downshock assays\textsuperscript{23}, D67R1 exhibited different behavior in planar lipid bilayers with no spontaneous opening and fluctuating and inconsistent behavior upon addition of LPC 14:0. MTSSL added to \textit{E. coli} expressing D67C MscS (according to a published approach\textsuperscript{53}), resulted in native behavior in a downshock assay (Supplementary Fig. 4f), thus suggesting that the modified protein remains functional and that the spin label particularly affects LPC gating.

**DISCUSSION**

**A model for pressure sensing by MscS**

The arrangement of the TM helices of MscS creates pockets open to the lipid bilayer (refs. 19,20,25,26 and Figs. 1a and 3a,b). The pockets are wedge shaped, with the wider end at the inner leaflet and the ‘sharp’ and narrow end in the outer leaflet. The TM1 and TM2 helices create large hydrophilic grooves that span the bilayer. Truly empty pockets between the TM1 and TM2 helices and TMs 3a and 3b are incompatible with a stable structure\textsuperscript{22,24}, and waters are unlikely to fill hydrophobic pockets. Doubts therefore exist regarding the validity of crystallography as a means to explore MscS function. Pulsed EPR of MscS, both in detergent solution and reconstituted into bilayers, has supported the presence of pockets\textsuperscript{23,24}. We demonstrated that specific phospholipids remain associated with MscS (Fig. 2a–c). A 3.0-Å crystal structure and MD showed that phospholipid (or lipid-like) molecules fill these pockets (Figs. 1 and 3a,b). The profile of the pockets suggested that lipids most easily gain access from the cytoplasmic face, as observed in the MD simulations (Fig. 3a,b, Supplementary Fig. 3a and Supplementary Movies 1 and 2). The role of the disordered residues (1–26) in these interactions is unknown—an important caveat to these results. That acyl chains fill hydrophobic grooves or pockets in membrane proteins is well known\textsuperscript{54}, and fluorescence quenching confirmed that lipids exchange with lipids in the pockets (Fig. 3e,f). MD calculations also showed lipids in pockets to be dynamic, both within each state and between states (Fig. 3a–c and Supplementary Fig. 3a–d).

The fitting of a two-state Boltzmann model to the observed plot of tension against the open probability of MscS embedded in liposomes has been used to derive a gating free energy of 29 kJ mol\textsuperscript{-1} and a change in cross-sectional area of 8.4 ± 0.4 nm\textsuperscript{2} (refs. 2,36). The structural data, however, suggested a cross-sectional area change of 3 nm\textsuperscript{2}, and this results in a poor fit to the experimental tension data (Supplementary Note). A second approach has used the protein cross-sectional area at the midpoint, the change in protein shape and the second derivative of the pressure profile to derive an equation for MscS gating\textsuperscript{55}. When parameters from the structural analysis of MscS are input into this formula, the calculations suggest that the open structure is more stable by 60 to 70 kJ mol\textsuperscript{-1} (Supplementary Note), a result that we believe to be unrealistic. These approaches, rooted in sound physical principles, assume that protein-lipid and lipid-lipid interactions do not change in response to pressure. We propose that, for MscS, the energy that arises from changes in lipid partition between the pockets and the bilayer is a previously missing component of tension-sensing models.

The composition of the annular membrane and the specific interactions of lipids with MscS are known to be key to transmission of the mechanical stimulus\textsuperscript{33,36}. Structural transitions of MscS upon gating\textsuperscript{19,20} have shown that the pockets are radically altered. Reliably estimating the change in volumes of the pockets is nontrivial. Use of a 2.5-Å probe indicated that upon opening each pocket is reduced by approximately 1,200 Å\textsuperscript{3}; modifying the probe radii yielded different results but consistently showed a reduction in pocket volume. We observed the loss of one lipid with two acyl chains upon gating in molecular dynamics simulations (Fig. 3c).

We propose that the conformational state of MscS is determined by the availability of lipids to fill the pocket; the closed structure, with larger pockets, needs at least one more phospholipid molecule per monomer than the open state to be stable (Fig. 3a,b). Biophysical measurements have established that lateral tension, not pressure per se, is the trigger for opening MscS\textsuperscript{36,57}. Lateral tension exerts a ‘pull’ on lipid acyl chains inside the protein pockets (which are in exchange with the bilayer); as tension increases, the equilibrium position of lipid molecules favors the bilayer, not the pockets. This reorganization of the lipids within and around the protein destabilizes the closed structure. MscS gates to the open form with smaller pockets that need less lipid (Fig. 5a); when the tension decreases, the process reverses. (An equivalent formulation is seen in low-tension lipids entering into pockets and favoring the closed form, whereas at higher tension the reduction of lipids in the pockets allows the protein to adopt the open state.) This ‘lipid moves first’ model is consistent with the exchange of lipids that we observed. In our model, the energy from reorganization of the acyl chains (i.e., between pockets and bilayer) (Fig. 5a) is central to MscS tension sensing.

We do not favor a ‘protein moves first’ model, because it would require strongly bound lipids as force transducers at the mobile elements of MscS (TMs 1, 2 and 3a). MD and biophysics experiments showed the phospholipids to be in exchange (not tightly bound). A further distinguishing feature of MscS gating, the rotational motion of TMs 1 and 2 (ref. 19), does not fit with lipids pulling the protein laterally.

LPC, a conical lipid, had been proposed to operate by the changing curvature in one leaflet, thus mimicking the curvature of patch clamping\textsuperscript{58}. This proposal means that insertion on the opposite (‘wrong’) side of a bilayer would lead to bilayer curvature in the wrong sense, i.e., curvature opposite to patch clamping\textsuperscript{58}. The curvature model of LPC action predicts that LPC will open the channel only when added to the side of MscS where curvature mimics patch clamping. We observed that LPC 14:0 opened MscS with the same conductivity irrespective of side of addition, a result inconsistent with a curvature mechanism. LPC has recently been suggested to directly create tension in the bilayer\textsuperscript{50}, consistently with addition from either leaflet. However, we observed that an LPC-opened structure has substantially lower conductance (Fig. 4c–f) than that opened by pressure\textsuperscript{56} or occurring spontaneously (Supplementary Fig. 5a,b); in short, LPC creates a subconducting state.

The packing of the head groups around the protein circumference\textsuperscript{54} limits the number of acyl chains that could intercalate into the pockets (Fig. 3a,b). However, lysolipids, with one acyl chain (not two) per head group will, we suggest, be unable to stabilize the closed structure as efficiently as a normal phospholipid (Fig. 5b). We propose that this causes the subconducting state observed in single-channel experiments (Fig. 4c–f). LPC can displace phospholipids from the pockets (Fig. 4a,b), a requirement for this model of LPC action. We observed stable opening when LPC was added at the cytoplasmic side but observed opening with frequent closures when LPC 18:1 (or even more so LPC 14:0) was introduced from the periplasmic side (Fig. 4d,f). Our biophysical data showing exchange of LPC with phospholipids (Fig. 4a) could not identify whether exchange occurs exclusively from the cytoplasmic face or from both faces. Although MD calculations of bilayers with LPC added were beyond the scope of this paper, the origin of lipids in the pockets is primarily the cytoplasmic bilayer leaflet (Fig. 3a,b and Supplementary Fig. 3a,b). Hence, we
hypothesize that LPC will interact with the pockets most effectively from the cytoplasmic face (Fig. 5b), and this underpins the difference in behavior depending on the side of LPC addition. The gating of MscS by LPC is thus a special case of our general model in which lipid interdigitation controls the channel’s behavior. Lipid-induced changes in structure have been observed in pore-forming toxins activated by host lipids.

Conclusion
Eukaryotic channels are now known to be modulated by pressure.27,31,32 The sensitivity of the TRAAK channel to membrane tension has been shown to result from movement of a specific lipid in and out of a binding site in the TM helices.37 In TRAAK, there is a substantial change in the cross-sectional area of the protein, but lipid reorganization is a noted component of the TRAAK gating energy. In our model, it is the lipid reorganization (lipids shifting between interacting with protein via the pockets and with the bulk lipid of the bilayer phase) that changes the relative stabilities of these conformational states and thereby controls mechanosensation. We did not identify a specific key binding site, as has been seen in TRAAK.37 Many mechanosensitive channels have been proposed to be activated by introduction of lysolipids (or equivalents), and other sensory channels, with ostensibly different gating signals, may share this property.34,60 We predict that where different conformational states of a membrane protein differ in their capacity for sequestering phospholipids, changes in membrane tension or introduction of lysolipids (along with other factors) will modify the transition between the states.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code PDB 5AJI.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.P. purified and spin-labeled MscS for lipid analysis, single-molecule analysis and crystallization; obtained and analyzed the new crystal structure; and participated in the single-molecule and lipid-analysis experiments. T.R. purified MscS and carried out and analyzed the fluorescence studies including synthesis of brominated lipids. A.C.E.D. wrote the MD-analysis software and performed and analyzed the simulations. K.R.M. performed and analyzed single-molecule experiments. A.R. made mutants of MscS and performed osmotic downshock assays. T.R. assisted in fluorescence experiments. T.K.S. performed lipidomic mass spectrometry. C.V.R. and J.G. carried out native mass spectrometry. P.M. performed TLC experiments. S.M., H.B., M.S.P.S., I.R.B. and J.H.N. conceived and supervised the study. All authors wrote, reviewed and approved the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Biophysics. Purification and crystallization. MscS D67R1 single-cysteine spin-labeled mutant was expressed, purified, spin labeled and crystallized as reported previously. The extent of spin-labeling efficiency was quantified by a fluorophore sensitivity method described previously. Briefly, the protein was concentrated by Vivaspin concentrators (Sartorius) with a 100-kDa cutoff, to 9–13 mg mL⁻¹ in the presence of 10% glycerol, 300 mM NaCl. Crystals of MscS D67R1 grew to a full size of 0.3 mm × 0.1 mm × 0.1 mm in 2 d. The best crystals (according to visual inspection) were obtained with Na citrate, pH 4.5, 0.07 M NaCl, and 30% v/v PEG 400 as precipitant. Before data collection, crystals were transferred into a solution containing 0.07 M sodium citrate, pH 4.5, 0.07 M NaCl, and 25% v/v PEG 400. Data for MscS D67R1 were collected at 100 K on a single crystal on ID14-4 at the European Synchrotron Radiation Facility (ESRF) (Grenoble, France) and indexed, integrated and merged by MOSFLM/SCALA, as implemented in CCP4 (ref. 62) (Table 1). The resolution of the data used in refinement was determined according to the procedure of Diederichs and Karplus, as implemented in the PDB REDO server. This method has been shown to yield more accurate models despite using weak data that are normally excluded by resolution cutoffs based on Rmerge or Rfactor criteria. The data and structure have been deposited under PDB 5AJL. Pocket velocities were measured with the CASTp server.

LPC treatment of purified MscS. Lyso-PC 14:0 was dissolved in 0.05% DDM, 50 mM sodium phosphate, pH 7.5, and 300 mM NaCl and added to 30% molar ratio (LPC 14:0/WT MscS monomer) purified in the same buffer as WT MscS in a microcentrifuge tube. The tube was placed on a rocker at RT for 5 min and subsequently sonicated for another 5 min for further incubation. The MscS-containing tube and LPC 14:0 was kept on ice for several minutes. The last three steps were repeated two more times. The sample was subjected to gel filtration (Superose 6 column, GE Healthcare) and run in a buffer containing 0.05% DDM, 50 mM sodium phosphate, pH 7.5, and 300 mM NaCl, without LPC 14:0. The fractions of the peak corresponding to the heptamer of WT MscS were collected and concentrated by the same method. For each 30 μM sample, 2 μL of 1 mg mL⁻¹ MscS D67R1 containing 300 mM NaCl was mixed with 8 μL of buffer or lipid mixture. Aliquots of 1 μL were taken for analysis of quenching.

Pore-volume determination. Pore-Walker was used for the determination of the pore diameter for MscS D67R1 and closed (PDB 2OAU) structures. For D67R1, the spin label was removed, and the residue was set back to native before analysis. In this calculation, only residues that were well resolved in both structures were taken into account and were aligned to the C-terminal end at residue 278, thus forming a total pore-axis length of approximately 105 Å (for example, a total number of 35 steps with each step being 3 Å long). Pore-disc surfaces were calculated for each step and were subsequently integrated along the full length of the pore axis, thus resulting in the determination of the total pore volume with OriginPro 8.0.

Lipid-volume determination. Lipid volumes were calculated for both truncated and full lipids from existing X-ray structures in the PDB database (Supplementary Table 3). Lipids with PE and PG head groups were included in our analysis because only these lipid types were detected in the in vivo data. Volume calculations were performed with Molspace (http://www.compbiochem.org/Software/molspace/Home.html), a plug-in of the VMD software routine.

Quenching of tryptophan fluorescence. Purification of the MscS tryptophan mutants generally followed a previously established protocol. Membranes were solubilized by incubation for 1 h at 4 °C in 0.9% DDM (Glycon) containing 50 mM sodium phosphate, pH 7.5, 300 mM NaCl, 10% glycerol, 50 mM imidazole, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Aggregates were removed by centrifugation at 3,000g for 10 min and filtration with a 0.2-μm syringe filter. MscS was then bound through its C-terminal His₆ tag to a prepacked 0.5-mL nickel–nitrilotriacetic acid (Ni-NTA)–agarose column (Sigma) and washed with 20 mL of washing buffer (50 mM sodium phosphate, pH 7.5, containing 0.05% DDM, 300 mM NaCl, 10% glycerol and 50 mM imidazole). After storage overnight at 4 °C, MscS was eluted with elution buffer (wash buffer containing 300 mM imidazole). Peak fractions were separated on a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) at 0.5 mL min⁻¹ with a buffer containing 0.03% DDM, 50 mM sodium phosphate, pH 7.5, and 150 mM NaCl. All mutants studied here purified as heptamers, as judged by size-exclusion chromatograms, and were proven to be functional in vivo, as assessed by osmotic downshift assays after induction with IPTG.

Quenching experiments of tryptophan fluorescence were performed as previously described. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti) was brominated by the stepwise addition of bromine (Sigma) to a solution of the lipid in chloroform cooled on ice until a faint yellow color of unreacted bromine persisted. Bromination of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG, Avanti) was performed by the same method. After a further 30 min, chloroform was removed under a nitrogen stream; this was followed by several rounds of dissolving and evaporation. The brominated lipid 1,2-di-(9,10-dibromo)stearyl-sn-glycero-3-phosphocholine (BrPC) was kept for several hours in a desiccator, dissolved again in chloroform and stored at −20 °C until further use. Bromination of DOPE was confirmed by NMR and MS (Supplementary Fig. 6). Films of lipids were formed in thin-walled glass tubes by drying the desired amount of DOPC or BrPC as chloroform solutions under a nitrogen stream. The remaining chloroform was completely removed by keeping the glass tubes under vacuum at 4 °C in a desiccator overnight. 2 μL of DOPC or BrPC was then suspended under a nitrogen atmosphere in 1.6 mL of buffer A containing 40 mM HEPES, pH 7.2, 100 mM KCl, 1 mM EGTA, and 15 mM sodium cholate by warming the tube for 20 s in warm water, vortexing for 5 min, and subjecting to ultrasonication for 10 min (Fisher Scientific, model FB1506D). MscS (1.27 nmol, determined by UV/vis spectroscopy with an extinction coefficient of εmax (MscS 1W) = 15.9 mM⁻¹ cm⁻¹) was added to lipid solution (100 μL, 127 nmol lipids) and incubated for 15 min at room temperature. MscS and lipids (25 μL) were added to measuring buffer (600 μL, in adequate buffer A without sodium cholate) in a 4 × 4 mm stirred quartz cell (Hellma). Measurements were performed after 5 min incubation (or after 15 min incubation in the case of L105W, because of slower equilibration) in an FLS920 fluorescence spectrometer (Edinburgh Instruments) with excitation at 295 nm and emission from 300 to 420 nm at 20 °C. Excitation and emission slits were set to 3 nm and 7 nm, respectively, and polarizers at 90° and 0°, respectively. The emission at 340 nm was used for analysis of quenching. This wavelength was chosen to avoid the effect of tyrosine fluorescence at low wavelengths (<305 nm), strong scattering at low wavelengths (<305 nm), Raman scattering (327 nm), and the higher noise at longer wavelengths, caused by low tryptophan fluorescence intensities. The emission at 340 nm was corrected with samples containing lipids but no MscS. Fractional quenching was calculated from the fluorescence intensities at 340 nm as FRQ = (Fₐ - Fₚ)/Fₚ, where Fₚ is the intensity for the sample containing 100% non-brominated lipids, and F is the intensity for the sample containing 100% brominated lipids. Experiments with mixtures of 80% DOPE and 20% DOPG or their brominated forms were performed in the same manner as with DOPC or BrPC. Brominated LPC (made as described for BrPC) or LPC were added (0.33 mol/mol of total lipid) to A119W MscS samples reconstituted in DOPC in the same manner as described above. MscS was reconstituted in BrPC according to the same procedure as described for DOPC.

The function of the studied mutant forms of MscS was assessed by an osmotic downshock assay, as previously described. The MscS constructs in the vector pTRc99A with an added C-terminal histidine tag were transformed into the E. coli strain MJF612 (ref. 71), and an overnight culture was grown in LB medium supplemented with 25 μg/mL ampicillin at 37 °C. The next day, the culture was diluted 100 times to fresh medium and grown to an OD₅₆₀₅₆₀ of 0.4. The cultures were then diluted ten times into LB medium with an additional 0.5 M NaCl and grown until an OD₅₆₀ of 0.3. For each sample, two of these cultures were grown, and one culture was supplemented with 0.3 mM IPTG at an OD₅₆₀ of 0.2. All samples were diluted 20 times into LB medium (shock) and into LB + 0.5 M NaCl (control). After serial dilutions into similar media, samples were grown on plates, and colonies were counted the next day. Survival was quantified as counted colonies for shock samples relative to control samples.
Analysis of lipid content. Native mass spectrometry. Native mass spectra were acquired with a Q-TOF 2 instrument (Micromass) equipped with a Z-spray source and modified to allow the transmission of high-molecular-weight species. Aliquots of MscS in dodecyl β-D-maltoside (DDM) were buffer exchanged with biospin-6 columns (Bio-Rad), first into 200 mM ammonium acetate, 0.02% DDM and then into 200 mM ammonium acetate, 0.05% lauryldimethylamine-oxide and electrospreyed from gold-coated nanospray capillaries in positive-ion mode. Optimized instrument parameters included collision-cell pressure capillary voltage, 1,500 V; sample cone, 200 V; extractor, 10 V; back pressure, 8.79 × 10−3 mbar; collision-cell pressure, 0.31 MPa; and collision energy, 150 V. Argon was used as the collision gas. All spectra were calibrated externally with a solution of cesium iodide (25 mg/mL). Spectra were acquired for approximately 100 scans and then processes (summed and then smoothed) with MassLynx V4.1 (Waters). Masses were calculated with software developed in house.

ES-MS and ES-MS-MS lipidomic analysis. Lipid removal from purified recombinant proteins was achieved by three successive vigorous extractions with ethanol to fully denature the proteins (final 90% (v/v))75. The pooled extracts were dried with nitrogen gas in a glass vial and reextracted with a modified Bligh and Dyer method75. To obtain a complete lipid extract from E. coli cells, cells were washed with PBS, suspended in PBS (100 µL), transferred to a glass tube containing chloroform/methanol (1:2, 375 µL) and vortexed. The sample was agitated vigorously for a further 10–15 min, made biphasic by the addition of CHCl3 (125 µL) and water (125 µL), vortexed again and centrifuged at 1,000g at room temperature for 5 min. The lower organic phase was transferred to a new glass vial, dried under nitrogen and stored at 4°C.

Lipid extracts were dissolved in chloroform/methanol (1:2, 15 µL) and acetonitrile/isopropanol/water (6:7:2, 15 µL) and analyzed with an ABIceox 4000 QTrap, a triple-quadrupole mass spectrometer equipped with a nanoelectrospray source. The samples were delivered with a Nanomate interface in direct infusion mode (~125 nL min−1). The extracts were analyzed in both positive- and negative-ion modes with a capillary voltage of 1.25 kV. MS-MS scanning (daughter, precursor and neutral-loss scans) were performed with nitrogen as the collision gas, with collision energies between 35 and 90 V. Each spectrum encompassed at least 50 repetitive scans.

Tandem mass spectra (MS-MS) were obtained with collision energies as follows: 35–65 V, PE in negative-ion mode, parent-ion scanning of m/z 196; 20–35 V, PS in negative-ion mode, neutral-loss scanning of m/z 87; and 40–90 V, for all glycerophospholipids (including PA, PG and CL) detected by precursor scanning for m/z 153 in negative-ion mode. MS-MS daughter-ion scanning was performed with collision energies between 35 and 90 V. Assignment of phospholipid species was based on a combination of survey, daughter, precursor and neutral-loss scans as well as previous assignments. The identity of phospholipid peaks was verified with the LIPID MAPS: Nature Lipidomics Gateway (http://www.lipidmaps.org/).

Thin-layer chromatography of lipid extracts. For quantification, lipid was extracted from purified MscS according to the method of Bligh and Dyer. Briefly, purified MscS was mixed with chloroform/methanol (1:2, 375 µL) v/v for 5 min. Chloroform (125 µL) was added and mixed, and this was followed by the addition of 1 M KCl (125 µL). After being vortexed for 1 min, the mixture was centrifuged at 300 g for 5 min. The lower phase, containing lipids, was dried under a nitrogen stream and dissolved in methanol (50 µL). The extracted lipids were spotted on SILICA 60 plates (Merck) and separated in a TLC tank presaturated with chloroform/methanol/1 M KCl (10:10:3) (v/v/v). The plate was air-dried and then stained with 0.5% phosphoric acid in acetone/water (80:20 (v/v)) to visualize lipids. Spots (lipid/detergent) were viewed with a UV transiluminator. Plates were then tested specifically for PE by staining in 0.1% ninhydrin in acetone/water (80:20 (v/v)); this was followed by heating until pink PE spots developed. The molar ratio of PE/MscS was determined by densitometric analysis of the lipid spots with Bio-Rad Image Lab software and compared with that of known POPE:POPG lipid bilayer. Each PE/PG bilayer was aligned with the transmembrane (TM) domain of the protein, and lipids overlapping the protein were removed. The system was solvated by approximately 16,000 water particles and charge neutralized, and the ionic concentration was set to −0.15 M NaCl. Each of the five CG-MD simulations was run for 1 µs.

CG to atomistic conversion and equilibration. The final coordinate sets from the CG simulation systems were converted to atomistic representations with a published protocol, yielding a system of ~300,000 atoms. Atomistic MD simulations were performed with the GROMOS96 (ref. 81) 53a6 force field and the SPC water model. Simulations used semi-isotropic pressure coupling with the Parrinello-Rahman barostat and the Berendsen thermostat at 310 K. The LINCS algorithm was used to constrain bond lengths. Long-range electrostatic interactions were modeled with the particle-mesh Ewald method, and a cutoff of 10 Å was used for van der Waals interactions. Each atomistic system was simulated for 100 ns. Visualization used in-built and custom scripted features for VMD 1.9.1 (ref. 68).

Analysis. To allow a degree of equilibration of protein-lipid interactions, only the latter half of each simulation trajectory was analyzed, i.e., from 0.5 to 1 µs of the CG and from 30 to 100 ns of the AT-MD simulations. The analysis of lipid contacts with MscS used a sampling time of 0.25 ns for AT and 2.5 ns for CG simulations. Contacts were assessed for the full TM domain (residues 27 to 128) and for the lower part of the pocket (TM helix 3, residues 106 to 122). Lipid metrics per residue were averaged across all seven polypeptide chains and the time in a given trajectory. Significant differences between states were calculated with the two-tailed Student’s t-test, with the null hypothesis of no difference, which was rejected for P ≤ 0.01.

Single-channel planar-lipid-bilayer recordings. Planar lipid-bilayer recordings of single MscS channels were carried out with bilayers of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) formed across an aperture (~70 µm in diameter) in a 25-µm-thick polytetrafluoroethylene (Teflon) film (Goodfellow), which separated the apparatus into cis and trans compartments. Bilayers were formed by first pretreating the aperture with hexadecane in n-pentane (1 µL, 10 mg mL−1) on each side. Both compartments were then filled with the electrolyte solution (200 mM KCl, 90 mM MgCl₂, 10 mM CaCl₂, and 10 mM HEPES, pH 7.5) and DPhPC in n-pentane (5 µL, 5 mg mL−1) was added to both sides to allow membrane formation when the electrolyte was raised above the aperture. The MscS channel was reconstituted by addition of 1 µL of a 50 µg mL⁻¹ solution of MscS in 0.05% DDDM, to the cis compartment of a planar bilayer apparatus containing 1 mL of electrolyte. This brought the detergent concentration below the CMC, thus causing protein aggregation and precipitation. At the same time, a few channels spontaneously inserted into the lipid bilayer. Fifteen minutes after the addition, assuming that one or a few channels had spontaneously inserted into the lipid bilayer, LPC was added to the cis or the trans compartment for channel activation, which was manifested by a jump in the ionic current (from 0 pA). We used LPC 14:0 (CMC of 43 µM at room temperature), LPC 18:1 and LPC 18:0 (0.4 µM at room temperature). Two independent preparations of DDM-purified WT MscS were used for the planar-lipid-bilayer recordings. Electrical currents were measured with two Ag/AgCl electrodes that were connected to the head stage of a patch-clamp amplifier (Axopatch 200B, Molecular Devices) operating in voltage-clamp mode. The cis compartment was connected to the grounding electrode, and the trans compartment was connected to the working electrode. The data were filtered by an analog low-pass four-pole Bessel filter at 2 kHz and digitally sampled at 10 kHz (Digitada 1440A digitizer, Molecular Devices). The data were analyzed and prepared for presentation with pClamp (version 10.2, Molecular Devices). In control experiments, LPC 14:0 and 18:1 were added to lipid bilayers at different concentrations, and bilayers were stable to LPC, however, higher concentrations resulted in bilayer rupture.

MD simulations. CG-MD simulations. Protein structures were converted to a coarse-grained (CG) representation with MARTINI v2.1 (ref. 77). During the CG simulations, an elastic network model was applied to the protein Ca atoms with a distance cutoff of 7 Å and a force constant of 10 kJ mol⁻¹ Å⁻². Simulations were performed with Gromacs v4.5.5 (ref. 79) (http://www.gromacs.org). Self-assembly simulations (50 ns) were used to produce five replicates of a 4:1 POPE:POPG lipid bilayer.
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