Modeling and simulations
The extrapolated motion protocol, which allowed us to generate the library of 86,000 models and was briefly described previously (Akitake et al., 2007; Anishkin et al., 2008a, b), will be presented in detail in a separate paper (unpublished data). The criteria for automated model selection based on experimentally determined spatial proximities are given in the Materials and methods. Here we only present the details and parameters of equilibrium and steered MD simulations used in this work.

Candidate models of the resting and inactivated states satisfying the constraints were equilibrated and refined in MD simulations with explicit medium. To ensure better equilibration of the lipid bilayer and adjustment to the channel, we used POPC membrane from the previously equilibrated model of the resting MscS embedded in the membrane (Anishkin et al., 2008a) and replaced the protein structure with one of the two conformations selected from extrapolated motion protocol results as described in Materials and methods. Slightly overlapping lipids were displaced toward the bulk of the membrane. Simulation cell setup and all of the molecular modifications (protein embedding, overlapping lipid displacement, etc.) were performed using the Visual Molecular Dynamics (VMD) package (Humphrey et al., 1996). The final simulation cell contained ~170,000 atoms in total, including ~30,000 protein atoms (1,960 residues), 220 POPC molecules, ~37,000 TIP3P waters, and 65 potassium and 72 chloride ions (corresponding to a 200-mM salt solution). The whole system was energy minimized with a restrained protein backbone for 10,000 steps using the conjugate energy gradient algorithm. The minimized system was simulated for 3 ns with the protein backbone harmonically restrained near the modeled positions with a spring constant of 1 kcal/mol/Å for 3 ns. A flexible hexagonal cell was simulated with periodic boundary conditions under 1 bar of pressure and a lateral tension of 40 dyne/cm. All simulations were performed using NAMD2 (Kalé et al., 1999) with the CHARMM27 force field (MacKerell et al., 2004), the particle mesh Ewald method for long-range electrostatics estimation (Darden et al., 1993), a 10-Å cutoff for short-range electrostatic and Van der Waals forces, and a Langevin thermostat set at 310 K.

To explore the structural changes in MscS under increased crowding in the cytoplasm, we have approximated the pressure of the large macromolecules by a force acting on the C-terminal half of the cytoplasmic cage (residues 183–280). To speed up the simulation, the force was applied only to nonhydrogen backbone atoms of that region (393 atoms per monomer, 2,751 per whole channel). The force was always acting in the same direction, toward the midplane of the bilayer. The magnitude of force was set for slow exponential growth from 0.001 to 0.1 kcal/mol/Å/atom over the course of 20-ns simulations. To prevent the net displacement of the protein and membrane under the force, we have restrained the center of mass of nonhydrogen backbone atoms of TM1–TM2 helices at the level of lipid bilayer (residues 12–54 and 70–89) in the direction normal to the membrane midplane. Note that only the mean position of the center of mass of this region was restrained, whereas any relative motions within (e.g., helices tilting or kinking) were not affected. All of the simulation conditions and settings were the same as in the relaxation simulations described in Materials and methods. The starting conformations of the protein (resting and inactivated) were taken from the final step of the symmetry-annealing of the relaxed structures.

Visualization of the channel structures was performed using the VMD package (Humphrey et al., 1996). All of the structural analyses (relative position of the protein domains, in-plane area of the channel in the membrane, and estimation of the cytoplasmic cage volume) were performed using custom-written Tcl scripts for VMD.

Figure S1. Detailed view of side chain packing at the TM3b–β domain interface. (A and B) Depicted are the crystal conformation (PDB ID 2OAU; A) and the modeled resting state with TM3b and β domains separated (B).
Figure S2. The scheme representing the functional cycle of WT MscS. The compact resting state is occupied at low subthreshold tensions. It is characterized by the upright positions of peripheral (TM1–TM2) helices forming the buried contact with the pore-forming TM3s. This state is stabilized by D62–R131 salt bridges between the TM1–TM2 loops and the upper hemisphere of the cage. The application of super-threshold tension drives the population of resting channels in two different paths. The first is opening, which leads to hydration of the hydrophobic gate associated with a larger (~15 nm²) in-plane expansion of the TM domain (Akitake et al., 2007) and likely preserves TM2–TM3 contacts. The second inactivation pathway, triggered by tension of a similar threshold, leads to the detachment of the peripheral helices from the central TM3 core, accompanied by a kinking of TM3 helices at G113, which is stabilized by an association between the β domains of the cage (purple) and the TM3b segments. Inactivation is associated with a smaller in-plane expansion of the TM domain (Kamaraju et al., 2011). The return to the resting state is predicted to include straightening if G113 kinks, detachment of β domains from the TM3b segments, and reformation of D62–R131 bridges. This transition is predicted to result in an ~12 Å displacement of the gate upward, toward the midplane of the membrane.

Figure S3. The scheme representing the altered resting state in the N117V and N167V mutants with stabilized TM3b–β domain interactions. Tight association of these domains imposes TM3 kinks at G113. Simulations suggest that in the absence of tension these kinks still permit formation of TM2–TM3 buried contact, which engages the gate and even formation of salt bridges between the TM1–TM2 loops (D62) and the upper hemisphere of the cage (R128 or R131). These mutants open at considerably higher tension than WT, produce a highly unstable open state (which may have a somewhat different conformation from WT), and willingly enter the inactivated state without opening. The recovery from the inactivated state back to the altered resting state in these mutants is especially fast, apparently because the TM3 helices do not have to straighten and undergo the upward displacement (see Fig. S1). Although WT MscS is unable to transition from the open conformation directly to the inactivated one, currently we have no such information about these mutants.
Figure S5. The crowding effect of 1 M (24 vol%) trehalose on WT and G168D MscS opening, closing, and inactivation. (A and B) Under symmetric ramps of pressure, both the opening and closing of WT (A) and G168D (B) MscS are modulated by the presence of 1 M trehalose (red traces). The shift toward higher tensions required to open and close the channel is comparable with that which occurred during identical protocols in the presence of 5% Ficoll (Fig. 3 A). The notable difference is the more pronounced drop in maximum current caused by the higher resistance of a 1-M trehalose solution. (C and D) Pulse-step-pulse protocols were then used to investigate the tension-driven inactivation of WT (C) and G168D (D) before (black traces) and after (red traces) exposure to 1 M trehalose in a similar manner as was performed with 10% Ficoll (Fig. 4, A and B). Differences in amplitude of pulses in the beginning and end of the traces indicate the degree of inactivation (arrows). Although trehalose, possibly permeable through the cage portals, is able to alter channel opening and closing in the same manner as cage-impermeable Ficoll 400, it has minimal effect on inactivation. Under similar tension, Ficoll drives the entire population of WT MscS into the inactivated state (Fig. 4; see also Results).

Figure S4. The change in MscS inactivation and recovery rates in control and in the presence of 25 (7.5 vol%) and 40 mM (12 vol%) PEG 3350 on the cytoplasmic side of the excised patch. The comb pressure protocol includes a prolonged (30 s) conditioning step of pressure, typically chosen at the midpoint of activation (p0.5), and a train of interspersed saturation pulses indicating the fraction of active channels. In control, MscS shows decaying activation to four of the pulses 6 s apart. 25 mM PEG 3350 makes the channel open only once. 40 mM PEG 3350 activates only ~3% of the population, which immediately inactivates. The rate of recovery is visibly decreased by 25 mM PEG 3350. This experiment allows us to separate the previously described processes of crowding-promoted adaptation (Grajkowski et al., 2005) from tension- and crowding-dependent inactivation.
Table S1

Summary of gating phenotypes for several mutants with altered contact region between the C-terminal segment (TM3b) of the gate-forming TM3 helix and the β domain of the MscS cage

| Mutant         | pMscS/pMscL | Midpoint | Open state stability | Ramp response | Inactivation | Recovery | τ of recovery |
|---------------|-------------|----------|----------------------|---------------|--------------|----------|--------------|
|               | mN/m        |          |                      |               |              |          |              |
| WT            | 0.6         | 7.8      | WT like              | WT like       | WT like      | WT like  | 1.3 ± 0.6    |
| A98S          | 0.45        | 5.8      | Unstable             | WT like       | Less than WT | -        | -            |
| G113A         | 0.6         | 7.8      | Very stable          | WT like       | Less than WT | Fast     | -            |
| N117A         | 1           | 13       | WT like              | WT like       | Less than WT | Fast     | 0.5 ± 0.02   |
| G121A         | 0.6         | 7.8      | Very stable          | WT like       | Less than WT | Slow     | 229 ± 8.4    |
| F151R         | 0.6         | 7.8      | Very stable          | Reduced WT    | Slow         | 3.4 ± 1.9 |
| F151D         | 0.6         | 7.8      | Very stable          | WT like       | Less than WT | -        | -            |
| N167D         | 1           | 13       | Unstable             | WT like       | Less than WT | Fast     | 1.9 ± 0.6    |
| G168D         | 0.6         | 7.8      | Very stable          | WT like       | Less than WT | -        | -            |
| N117V/N167V   | 1           | 13       | Unstable             | None          | More than WT | Fast     | 0.6 ± 0.2    |
| F151R/N167D   | 1           | 13       | Unstable             | None          | More than WT | Fast     | -            |

The above mutants were generated and scrutinized via patch clamp to investigate the deviations from WT MscS they produced. The given midpoint and recovery values are based on a minimum of three independent measurements where possible. Omissions in recovery information are the result of either a lack of adaptation, thus prohibiting inactivation measurements (A98S, G113A, F151D, and G168D), or extreme gating tensions, which destabilize the patch (N117A and N117V/N167V).

REFERENCES

Akitake, B., A. Anishkin, N. Liu, and S. Sukharev. 2007. Straightening and sequential buckling of the pore-lining helices define the gating cycle of MscS. Nat. Struct. Mol. Biol. 14:1141–1149. http://dx.doi.org/10.1038/nsmb1341

Anishkin, A., B. Akitake, and S. Sukharev. 2008a. Characterization of the resting MscS: modeling and analysis of the closed bacterial mechanosensitive channel of small conductance. Biophys. J. 94:1252–1266. http://dx.doi.org/10.1529/biophysj.107.110171

Anishkin, A., K. Kamaraju, and S. Sukharev. 2008b. Mechanosensitive channel MscS in the open state: modeling of the transition, explicit simulations, and experimental measurements of conductance. J. Gen. Physiol. 132:67–83. http://dx.doi.org/10.1085/jgp.200810000

Anishkin, A., A.L. Milac, and H.R. Guy. 2010. Symmetry-restrained molecular dynamics simulations improve homology models of potassium channels. Proteins. 78:932-949. http://dx.doi.org/10.1002/prot.22618

Darden, T., D. York, and L. Pedersen. 1993. Particle mesh Ewald: An N-Log(N) method for Ewald sums in large systems. J. Chem. Phys. 98:10089–10092. http://dx.doi.org/10.1063/1.464397

Grajkowski, W., A. Kubalski, and P. Koprowski. 2005. Surface changes of the mechanosensitive channel MscS upon its activation, inactivation, and closing. Biophys. J. 88:3050–3059. http://dx.doi.org/10.1529/biophysj.104.05546

Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: visual molecular dynamics. J. Mol. Graph. 14:35–38: 27–28. http://dx.doi.org/10.1016/0263-7855(96)00018-5

Kalè, L., R. Skeel, M. Bhandarkar, R. Brunner, A. Garsoy, N. Krawetz, J. Phillips, A. Shinozaki, K. Varadarajan, and K. Schulten. 1999. NAMD2: Greater scalability for parallel molecular dynamics. J. Comput. Phys. 151:283–312. http://dx.doi.org/10.1006/jcph.1999.6201

Kamaraju, K., V. Bely, I. Rowe, A. Anishkin, and S. Sukharev. 2011. The pathway and spatial scale for MscS inactivation. J. Gen. Physiol. 138:49–57. http://dx.doi.org/10.1085/ jgp.2011110606

MacKerell, A.D. Jr., M. Feig, and C.L. Brooks III. 2004. Improved treatment of the protein backbone in empirical force fields. J. Am. Chem. Soc. 126:698–699. http://dx.doi.org/10.1021/ja036959e