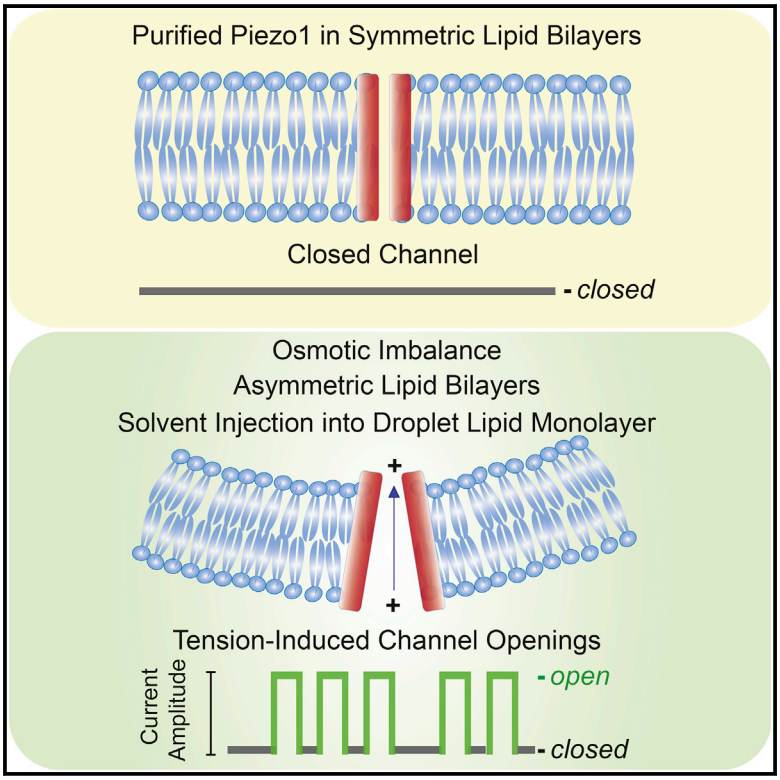
Cell Reports

Piezo1 Channels Are Inherently Mechanosensitive

Graphical Abstract



Highlights

- Purified wild-type and mutant Piezo1 channels are active in lipid bilayers
- Piezo1 and MscS, but not KcsA, are active in bilayers with osmotic gradient
- Piezo1 and MscS, but not KcsA, respond to perturbations in membrane tension
- Piezo1 is activated in the absence of other cellular components

Authors

Ruhma Syeda, Maria N. Florendo, Charles D. Cox, Jennifer M. Kefauver, Jose S. Santos, Boris Martinac, Ardem Patapoutian

Correspondence

ruhma@scripps.edu

In Brief

Piezo proteins transduce physical forces and are activated by mechanical indentation and stretching of cell membranes. By reconstituting these channels in lipid bilayers, Syeda et al. show that Piezo1 channels are stimulated in the absence of other cellular components via a variety of methods, including osmotic imbalance and membrane perturbation.

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Piezo1 Channels Are Inherently Mechanosensitive

Ruhma Syeda,[1,6,*] Maria N. Florendo,[1] Charles D. Cox,[2] Jennifer M. Kefauver,[1] Jose S. Santos,[3,5] Boris Martinac,[2,4] and Ardem Patapoutian[1]

[1]Howard Hughes Medical Institute, Molecular and Cellular Neuroscience, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, CA 92037, USA
[2]Victor Chang Cardiac Research Institute, Lowy Packer Building, 405 Liverpool Street, Darlinghurst, NSW 2010, Australia
[3]Section of Neurobiology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA
[4]St Vincent's Clinical School, University of New South Wales, Darlinghurst, NSW 2010, Australia
[5]Present address: Dart NeuroScience, 12278 Scripps Summit Drive, San Diego, CA 92131, USA
[6]Lead Contact
*Correspondence: ruhma@scripps.edu
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SUMMARY

The conversion of mechanical force to chemical signals is critical for many biological processes, including the senses of touch, pain, and hearing. Mechanosensitive ion channels play a key role in sensing the mechanical stimuli experienced by various cell types and are present in organisms from bacteria to mammals. Bacterial mechanosensitive channels are characterized thoroughly, but less is known about their counterparts in vertebrates. Piezos have been recently established as ion channels required for mechanotransduction in disparate cell types in vitro and in vivo. Overexpression of Piezos in heterologous cells gives rise to large mechanically activated currents; however, it is unclear whether Piezos are inherently mechanosensitive or rely on alternate cellular components to sense mechanical stimuli. Here, we show that mechanical perturbations of the lipid bilayer alone are sufficient to activate Piezo channels, illustrating their innate ability as molecular force transducers.

INTRODUCTION

Hudspeth and colleagues established in the 1970s that hearing is enabled by direct activation of mechanosensitive (MS) ion channels (Corey and Hudspeth, 1979). Since then, a role of MS channels has been established in a variety of cell types, including touch neurons, pain neurons, muscle cells, endothelial cells of blood vessels, and red blood cells, to name a few (Chalfie, 2009; Guharay and Sachs, 1984; Nilius and Honoré, 2012; Ranade et al., 2015). However, the molecular identity of these MS channels has remained elusive. To define an ion channel as a physiologically relevant mechanosensor, various criteria have to be met (Arnadóttir and Chalfie, 2010; Ranade et al., 2015): (1) the gene encoding the mechanosensitive protein must be expressed in mechanosensitive cells, (2) deletion of the gene should abolish mechanosensitivity of the cells while leaving other cellular functions intact, (3) the candidate mechanosensitive protein should contain a pore-forming subunit for rapid ion conduction, and (4) mutations of amino acids in critical domains should alter the pore properties of the MS currents.

Very few ion channels satisfy all these criteria. Even in those rare cases, it is still unknown whether these channels are inherently mechanosensitive or depend on various cellular components to sense mechanical force. Cellular components that could be required for gating MS channels include auxiliary subunits, extracellular/intracellular tether proteins, specialized lipid domains such as rafts, or small molecules that are released during mechanical stimulation (Anishkin et al., 2014; Gillespie and Walker, 2001; Teng et al., 2015; Zhang et al., 2015). For example, *Drosophila* mechanically activated channel NOMPC (no mechanoreceptor potential C) has been recently shown to require cytoskeleton connection to confer mechanosensitivity (Zhang et al., 2015). Therefore, the ultimate evidence that an ion channel is inherently MS is to demonstrate its mechanosensitivity in a cell-free environment (Berrier et al., 2013; Brohawn et al., 2014b; Najem et al., 2015; Sukharev et al., 1994). This force transmission directly from lipids to proteins is termed the "force from lipids" concept (Anishkin et al., 2014; Martinac et al., 1990; Teng et al., 2015).

Based on these criteria, bacterial MscS and MscL and mammalian TRAAK and TREK channels are classified as mechanosensitive ion channels. Both MscS and MscL respond to extracellular osmotic challenges to prevent cell lysis (Booth, 2014; Martinac et al., 2014). In contrast the physiological role of TRAAK in mechanotransduction is not completely understood, but it is widely expressed in neurons and is involved in mechanical and thermal nociception in mice (Noël et al., 2009). Various studies in cellular and cell-free environments have established MscS, MscL, TRAAK, and TREK to be inherently mechanosensitive channels (Bass et al., 2002; Battle et al., 2009; Berrier et al., 2013; Brohawn et al., 2014a, 2014b; Dong et al., 2015; Perozo et al., 2002).

In vertebrates, cation-selective Piezo channels are required for mechanotransduction in a number of biological processes (Ranade et al., 2015; Woo et al., 2015) and are necessary and sufficient for the mechanosensitivity of various cells (Coste et al., 2010, 2012). Heterologous expression of Piezos in cells exhibit large mechanically activated currents, and recent reports show

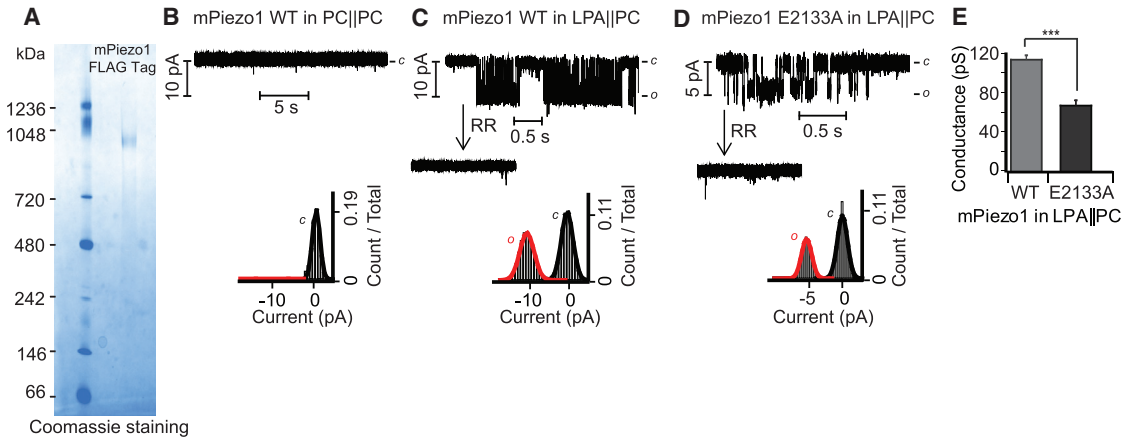


Figure 1. Reconstitution of Piezo1 in Droplet Lipid Bilayers

(A) Purified Piezo1-FLAG separated on Bis-Tris native gel and visualized by Coomassie staining (representative gel from n = 5).

(B–D) Single-channel current recordings in 500 mM KCl, V = −100 mV, and the all-point histograms of purified Piezo1-WT (B and C) and Piezo1-E2133A mutant (D). Channel openings are downward deflections where *c* represents closed and *o* represents open. The insets below show the channel block upon RR injection (40 μM final).

(E) Single-channel conductance comparison of Piezo1-WT versus Piezo1-E2133A in LPA||PC lipid bilayers under similar conditions (500 mM KCl, 10 mM HEPES [pH 7.4] at V = −100 mV). The two groups in question are significantly different as determined by two-tailed unpaired t test: ***$p < 0.001$. Error bars represent SEM. See also Table S1.

that Piezo1 responds to lateral membrane tension in cellular membranes (Coste et al., 2012; Cox et al., 2016; Lewis and Grandl, 2015; Ranade et al., 2015). Indeed, the roles of various cellular components that modulate Piezo channel activity are also emerging (Anishkin et al., 2014). For example, overexpressing a pathogenic mutant of Polycystin-2 (Peyronnet et al., 2013) and depletion of second messenger phosphoinositides (PIP or PIP2) inhibits mechanically activated currents in Piezo-expressing cells (Borbiro et al., 2015). Also, the integral membrane protein STOML3 strongly potentiates Piezo1 and Piezo2 responses to mechanical stimuli (Poole et al., 2014). Furthermore, Piezo1 sensitivity to mechanical indentation is dynamically modified by disruption of the cytoskeleton (Gottlieb et al., 2012) and cytoskeletal element filamin A (Retailleau et al., 2015). Although Piezos satisfy all the criteria required to be MS channels (Ranade et al., 2015), whether the channel is inherently mechanosensitive or whether it requires cellular components to sense mechanical forces is still unknown.

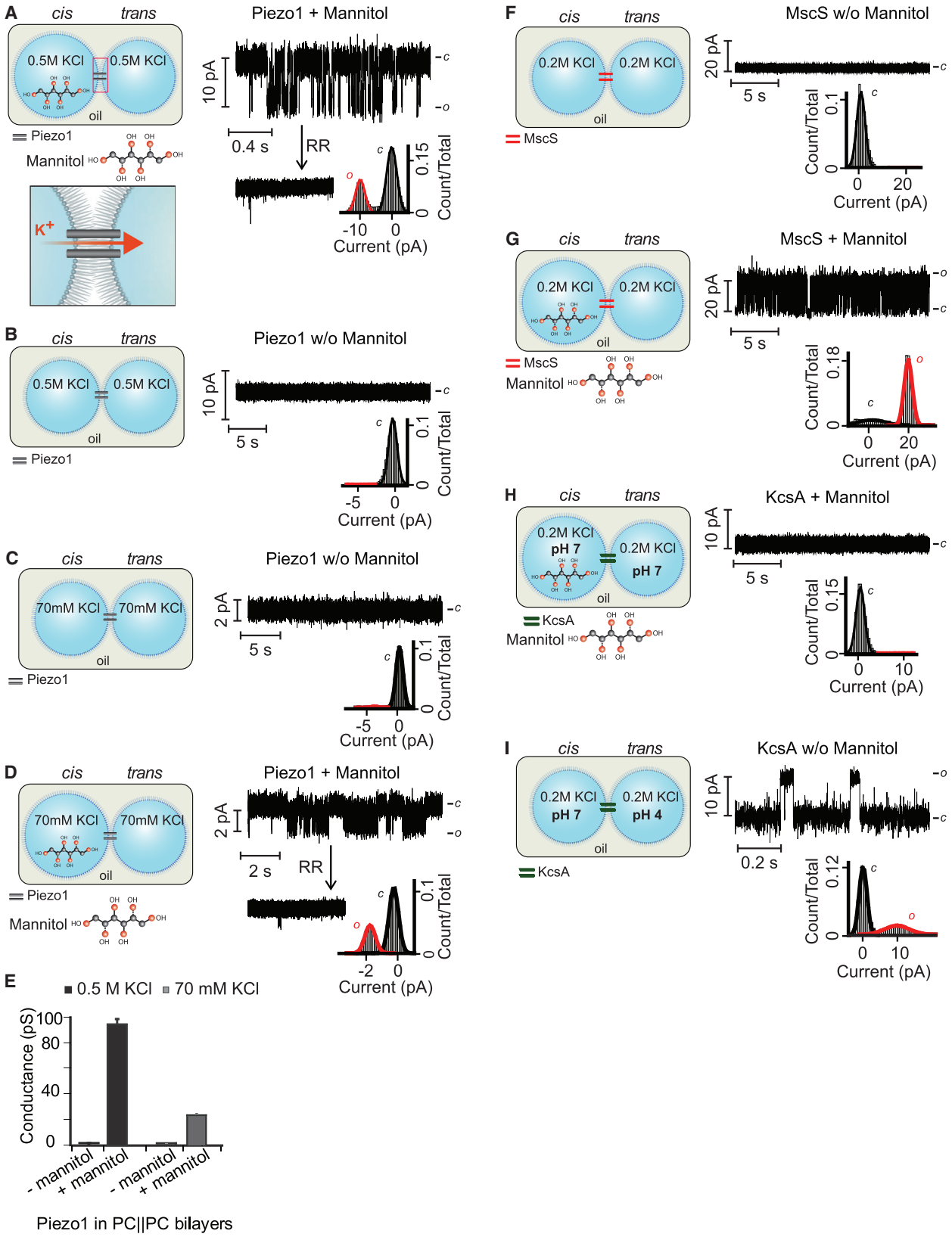
We had shown that purified GST (glutathione S-transferase)-fused Piezo1 reconstituted in asymmetric droplet bilayers (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine [DPhPC] doped with 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid [DOPA] on one monolayer) gave rise to constitutive Piezo1-dependent activity (Coste et al., 2012). We also showed that Piezo1 channel activity was not observed in symmetric DPhPC bilayers but that the application of agonist Yoda1 produced channel activity (Syeda et al., 2015). These experiments suggest that membrane properties play an important role in Piezo1 gating, but they do not directly address whether Piezo1 is inherently mechanosensitive. The chemical activation of Piezo1 in symmetric bilayers raises the possibility of using mechanical forces on symmetrical bilayers to probe whether Piezo1 is mechanosensitive in a cell-free system. Here, we use various manipulations of the droplet-bilayer system to provide evidence that Piezo1 directly senses mechanical forces within the membrane.

RESULTS AND DISCUSSION

Evidence that Currents Recorded in Droplet Bilayers Are Derived from Piezo1 Ion Channels

Previously, we used a GST tag to purify Piezo1 ion channels (Coste et al., 2012). Recently, cryo-electron microscopy studies showed that Piezo1 forms a trimer (Ge et al., 2015). This study also showed that GST-tagged Piezo1 can form an artificial dimer of Piezo1 trimers, in addition to a trimer (Ge et al., 2015). To avoid GST-induced dimerization, we used a Piezo1-FLAG construct for overexpression in HEK293T cells, followed by affinity-tag purification (Ge et al., 2015). The identity and homogeneity of the purified protein was assayed by Coomassie blue staining of a native gel (Figure 1A). A single prominent protein band with a molecular weight of ~900 kDa was detected, consistent with trimeric Piezo1 (Figure 1A) (Ge et al., 2015). This purified Piezo1 sample was reconstituted into droplet lipid bilayers for subsequent investigation of its mechanosensitivity.

The FLAG-tagged Piezo1 behaved similarly to the GST fused protein described earlier: Piezo1 activity was not observed when reconstituted in symmetrical DPhPC (phosphatidylcholine||phosphatidylcholine; PC||PC) bilayers (n = 19; Figure 1B) (Coste et al., 2012; Syeda et al., 2015). However, when PC bilayers were doped with lysophosphatidic acid (LPA) on the *cis* monolayer, discrete ruthenium-red (RR)-sensitive channel activity was detected (Figure 1C). RR blocked the currents exclusively from the *trans* droplet; hence, the channels are oriented so that the *cis* droplet represents the intracellular side of the protein (Coste et al., 2012). The calculated single-channel conductance (γ) of Piezo1-FLAG in asymmetric LPA||PC bilayers



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was 114 ± 4 pS in 500 mM KCl (n = 6; Table S1). We also calculated the γ of Piezo1-FLAG in DOPA||PC bilayers (γ = 117 ± 5 pS in 500 mM KCl; n = 4; data not shown). Both of these conductance values are in agreement with what we previously calculated for Piezo1-GST-fused protein in asymmetric DOPA||PC bilayers (γ = 118 ± 15 pS; 500 mM KCl, n = 6) (Coste et al., 2012). Furthermore, when similar ionic conditions are compared, the conductance of Piezo1 in cells and droplet lipid bilayers are in agreement (Ranade et al., 2015). Thus, the identity of the tag or a specific asymmetric lipid composition did not change Piezo1 functional properties.

A striking feature of Piezo1 activity in whole-cell recordings is rapid inactivation. However, the recordings from lipid bilayers did not recapitulate such Piezo1 kinetics. This may suggest that specific partners or cellular structures are necessary for Piezo1 inactivation. To quell any remaining concerns that we are recording ionic currents from Piezo1 in bilayers, we assayed the activity of Piezo1 mutant (E2133A) that exhibits reduced γ (~50% of wild-type [WT]) in the cellular assay. The Piezo1 E2133A exhibited a γ of 67 ± 5 pS in LPA||PC bilayers (n = 7) compared to WT Piezo1, γ = 114 ± 4 pS (500 mM KCl; Figures 1D and 1E; Table S1) (Coste et al., 2015). Thus, the electrical activity recorded in droplet lipid bilayers arose from Piezo1 channels.

Activation of Piezo1 upon Stimulation by an Osmotic Gradient

Next, we asked whether reconstituted Piezo1 could be acutely activated by mechanical stimuli. We tested the effect of osmotic stress generated by an osmolyte (mannitol) gradient by supplementing the *cis* droplet with 500 mM mannitol. Under these osmotic stress conditions, single or multiple channels sensitive to RR were observed (n = 10) (Figure 2A; Table S1). Piezo1 exhibited a γ = of 97 ± 4 pS, and open probability (P_o) = 0.5 ± 0.06. Importantly, no Piezo1 channel activity was observed in the presence of mannitol in both droplets or in its absence (n = 9) (Figure 2B). Lipid bilayers are permeable to water. Diffusion of water across the membrane would cause monolayer stretch in one droplet, as well as changes in ionic strength. One mechanistic possibility is that Piezo1 responds to decreased local ionic strength as a consequence of water movement across the membrane, similar to what was observed for volume-regulated anion channels (Syeda et al., 2016). We ruled out that the ionic strength is the cause of Piezo1 activation by recording channel activity in the presence of a reduced ionic concentration (symmetrical 70 mM KCl). Discrete single or multiple channels (n = 7; Table S1) were observed under an osmotic gradient in 70 mM KCl, but no channel activity was observed in the absence of osmotic stress (n = 7) (Figures 2C and 2D). As expected, γ was reduced in these ionic conditions (24 ± 2 pS), but the P_o was unaffected (0.45 ± 0.08) when compared to 500 mM KCl. These data show that Piezo1 is gated in response to osmotic stress (Figure 2E).

To validate our osmotic stress assay, we examined the activity of other ion channels to serve as positive and negative controls. We tested the well-characterized bacterial mechanosensitive channel MscS. Purified MscS was reconstituted into PC||PC bilayers (200 mM KCl), and the activity was recorded in the presence and absence of 500 mM mannitol (Figures 2F and 2G). In the presence of mannitol, distinct MscS single or multiple channels (n = 6; Table S1) were observed with a γ of 630 ± 30 pS; no activity was observed without osmotic stress (n = 4). The MscS activity induced is "flickery" in nature, in accordance with previous reports in pure lipid bilayers (Ridone et al., 2015), and did not display noticeable inactivation (Cox et al., 2013).

As a negative control, we used the bacterial K^+ channel KcsA (a non-mechano-activated channel) to demonstrate that not every channel reconstituted in droplet bilayers is active under osmotic stress. Indeed, KcsA is not activated by osmotic stimulus while the intracellular side faces pH = 7.0 (n = 8) (Figures 2H and S1). Whereas discrete channel activity exhibiting γ = 114 ± 7 pS in 200 mM KCl was observed when pH = 4.0 is applied, a relevant KcsA-activating stimulus (LeMasurier et al., 2001) (n = 7; Figures 2I and S1; Table S1).

Activation of Piezo1 by Solvent Injection Assay

To complement the osmotic stress data, we tested whether Piezo1 was activated following direct expansion of one droplet monolayer. To achieve this, we injected 30 nL of solvent (500 mM KCl) into the *cis* droplet, which resulted in an area increase of ~15%–30%. The differential droplet area will induce changes in the bilayer tension profile (Supplemental Information). We first tested the effect of injection on PC||PC lipid bilayers and show that the characteristics of the bilayers were maintained after injection (n = 10) (Figures 3A and 3B). Similar injection protocols repeated in the presence of Piezo1 (Figure 3C) resulted in single- or multiple-channel activity with the expected γ = 98 ± 3 pS in 500 mM KCl (n = 8; Figures 3D and 3E; Table S1). As a validation for this assay, we repeated the injection protocols with reconstituted MscS and KcsA. MscS displayed discrete channel openings after solvent injection and exhibited a γ of 630 ± 40 pS in 200 mM KCl (n = 5; Figures 3F and 3G; Table S1). No activity was observed for KcsA (n = 6; Figures 3H and 3I). How much tension is being applied to the membrane in the solvent injection assay? Measurements derived from confocal images estimated the bilayer tension to be 3.4 ± 0.8 mN/m (n = 4), with >75% coming from the *cis* monolayer (Figures 3 and S2). Interestingly, this is in close agreement with

Figure 2. Activation of Purified Piezo1 by Osmotic Stress Stimulation

(A–D) Experimental illustrations of droplets with and without an osmotic gradient in the presence of either 500 mM KCl or 70 mM KCl and the single-channel recordings of Piezo1 in PC||PC bilayers at V = −100 mV. *c*, closed; *o*, open.

(E) Single-channel conductance comparison of Piezo1 for the indicated experimental conditions. The two groups in question are significantly different as determined by two-tailed unpaired t test: $p < 0.001$. Error bars represent SEM.

(F and G) Single-channel recordings of MscS in PC||PC bilayers in the absence and presence of osmotic stress with 200 mM KCl at V = 30 mV.

(H and I) Single-channel recordings of KcsA in the indicated pH solutions in the absence and presence of osmotic stress with 200 mM KCl at V = +100 mV.

See also Figure S1 and Table S1.

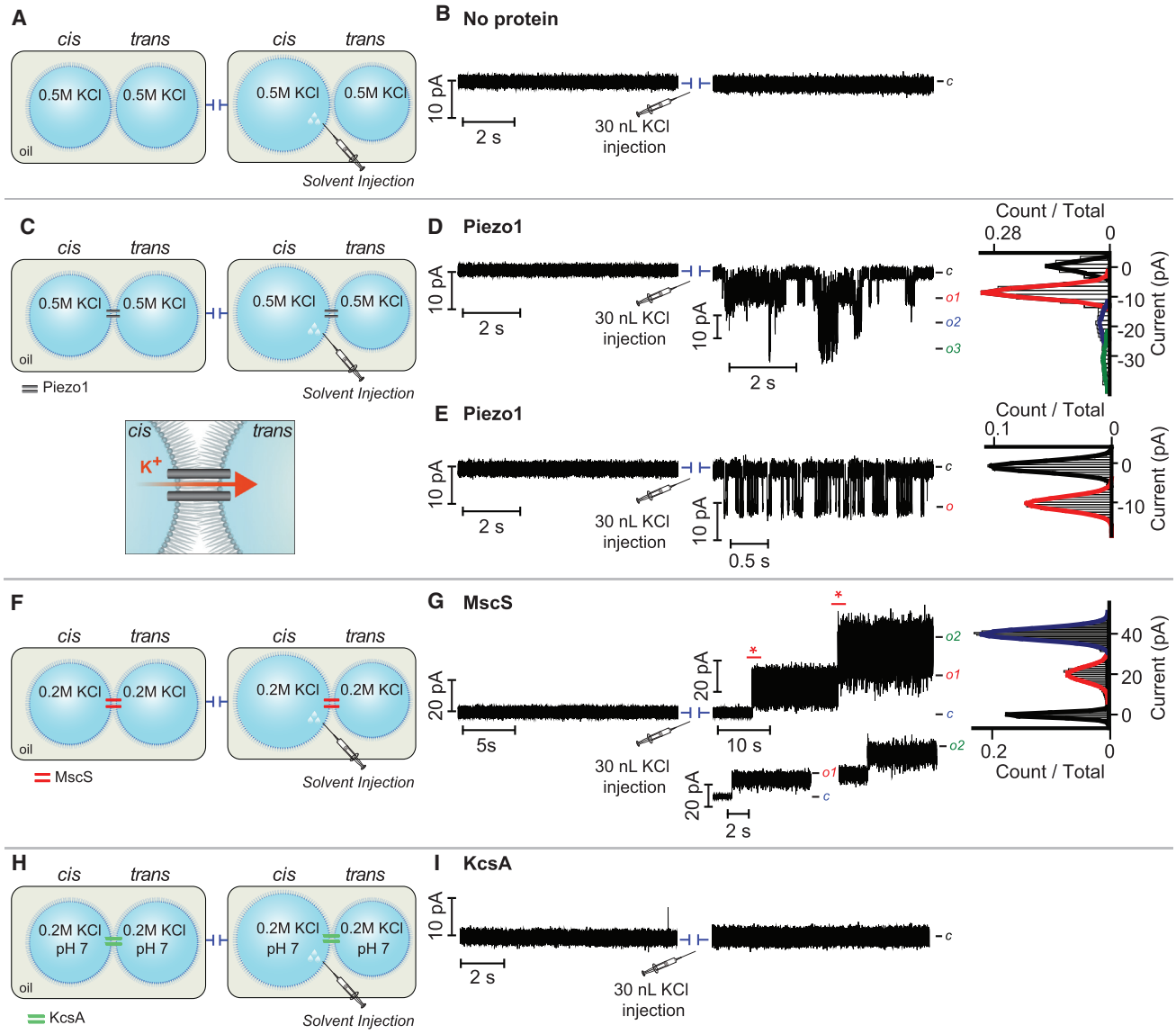

Figure 3. Activation of Piezo1 by Solvent Injection in the Lipid Monolayer

(A and B) Illustration of the solvent injection assay and electrical recordings in the absence of protein before and after the injection protocols. *c*, closed.

(C–I) Illustration of the solvent injection assay and examples of electrical recordings in the presence of (C–E) Piezo1 in 500 mM KCl, V = −100 mV; (F and G) MscS in 200 mM KCl, V = 30 mV; and (H and I) KcsA in 200 mM KCl, V = 100 mV. *o*, open.

See also Figure S2, Table S1, and Supplemental Experimental Procedures.

previously reported values for the tension threshold of both MscS (3–4 mN/m) (Nomura et al., 2012, 2015) and Piezo1 activation (~2–3 mN/m) (Cox et al., 2016; Lewis and Grandl, 2015).

In summary, we establish that Piezo1 mechanosensitivity in droplet lipid bilayers follows the force-from-lipids paradigm by responding to mechanical forces in the lipid bilayer without the requirement for any other cellular components (Kung, 2005; Martinac et al., 1990; Teng et al., 2015). The methods used here to study Piezo1 activation (membrane asymmetry, osmotic stress, and solvent injection) are quite distinct in nature. However, they evoke asymmetric changes in the transbilayer pressure profile. Our data provide evidence that Piezo1 detects forces—in particular, tension—imparted by the lipid bilayer alone. This is consistent with a recent report that Piezo1 responds to lateral membrane tension in cells (Lewis and Grandl, 2015). Furthermore, mechanosensitivity of Piezo1 is also preserved in membrane blebs, which are largely free of cytoskeleton (Cox et al., 2016). However, our data do not argue that other factors are not involved in modulating Piezo mechanosensitivity. For example, the cytoskeleton and associated proteins, such as STOML3, regulate the mechanosensitivity of Piezo1 (Borbiro et al., 2015; Gottlieb et al., 2012; Poole et al., 2014). Regardless, our studies establish that, like the bacterial MS channels, the mechanosensitivity of Piezo1 is inherent, and no other proteins or

second-messenger signals are required for the baseline mechanosensitivity of Piezo1.

EXPERIMENTAL PROCEDURES

Mouse Piezo1-FLAG Tag Purification

The mouse Piezo1-FLAG construct and mouse Piezo1-FLAG E2133A mutant were both assembled using the QuikChange XL Site-Directed Mutagenesis Kit from Agilent Technologies. Using designed primers and Piezo1-ires-pcDNA3.1 as a template, the FLAG sequence was inserted as a C-terminal fusion to Piezo1, creating the Piezo1-FLAG construct. The Piezo1-FLAG E2133A mutant was assembled by changing the amino acid at position E2133 of the Piezo1-cFLAG-ires-pcDNA3.1 construct from "E" to "A" (codons GAG to GCG). The plasmids were transformed to XL10-Gold competent cells. Positive clones were screened and verified by full-length sequencing. HEK293 cells were used for the production of protein because of its easily transfectable nature and its ability to efficiently produce proteins. We specifically used the HEK293T cell line, which is derived from HEK293s and includes a simian virus 40 (SV40) T antigen for increased protein production of vectors. 48 hr after transfection, HEK293T cells were collected from four to six confluent 500-cm^2 dishes and incubated in a homogenizing buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EGTA, 5 mM EDTA, 20 μg pepstatin, and a protease inhibitor cocktail for 15 min. This cell suspension was homogenized using a glass pulverizer and forced through a 27.5G needle ten times. After homogenization, the mixture was centrifuged at 4°C for 15 min at 3,500 rpm. The supernatant was collected and centrifuged at 4°C for 45 min at 53,000 rpm. A second centrifugation step was performed to wash the membrane pellet using the same conditions as the previous spin.

The protein was extracted from the membrane pellet by using the glass pulverizer and extraction buffer: 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EGTA, 1% CHAPS, 0.5% PC, iodoacetamide (4 mg/mL), and a cocktail of protease inhibitors. The protein was purified by ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, catalog #A2220). The resin was prepared in a batch mode, as per the user manual and technical bulletin. The extracted protein was incubated with the resin overnight, with rotation at 4°C. The protein was eluted with 3×FLAG peptide (100 μg/mL) in the elution buffer: 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.6% CHAPS, 0.1% PC, and a protease inhibitor cocktail. Two thirds of this sample was used for proteoliposome, while the rest was analyzed using a native gel. Similar purification protocols were performed for the mutant E2133A.

NativePAGE Novex Bis-Tris Gel

The eluted protein samples were analyzed using a non-denaturing 3%–12% NativePAGE Novex Bis-Tris gel electrophoresis in accordance with the user manual (Invitrogen). Each sample was mixed with NativePAGE Sample Buffer and NativePAGE 5% G-250 Sample Additive and then run on a gel at 150 V for 2 hr. After electrophoresis, the native gel was visualized using Coomassie G-250 staining.

MscS Purification

MscS protein containing a C-terminally linked 6xHis-tag was expressed in *E. coli* and purified using immobilized-metal affinity chromatography similar to a previously reported protocol (Vásquez et al., 2007). Briefly, cells were disrupted using a French press (French Cellular Press, Thermo Scientific), and membranes were solubilized overnight at 4°C in a PBS-based buffer (pH 7.5) containing 8 mM DDM, 10% glycerol, and PMSF. Solubilized supernatant was incubated with cobalt resin (Talon, Clontech) for 3 hr at 4°C subsequent to a pre-wash with PBS (pH 7.5) supplemented with 1 mM DDM (eight times the resin volume). The protein was finally eluted with 300 mM imidazole, 1 mM DDM, and 10% glycerol in PBS buffer (pH 6). Protein was desalted using buffer exchange and the use of an Amicon filter (Millipore, Amicon Ultra-15 Centrifugal Filter Device).

Formation of Proteoliposomes

Proteoliposomes were formed by incorporating purified Piezo1 (WT or E2133A), MscS, or KcsA in asolectin liposomes. First, asolectin liposomes (5 mg/mL) were prepared by resuspending dried asolectin lipids (L-α-phosphatidylcholine, Avanti Polar Lipids, catalog #840054) in 200 mM KCl, 10 mM HEPES (pH 7.2). The liposomes were then extruded through a 0.1-mm filter (Whatman Nuclepore Track-Etch membrane). Asolectin liposomes (400 μL at 5 mg/mL) were semi-permeabilized for 1 hr at room temperature with rotation, by adding 18 μL DDM (200 mM stock). Purified proteins (5–10 μL) were added in the semi-permeabilized liposomes (5 mg/mL) and incubated with rotation at room temperature for 1 hr. This proteoliposome sample (protein mass:lipid mass ratio of ~1:400) was then subjected to a Slide-A-Lyzer dialysis cassette with a molecular cutoff weight of 3.5 KDa (Thermo Scientific, product #66330) to eliminate excess CHAPS detergent and FLAG peptide (in the case of Piezo1), which has molecular weights of 1.0 kDa for FLAG and 2.8 kDa for 3xFLAG peptide. The dialysis was performed at 4°C against 500 mL of 200 mM KCl, 10 mM HEPES (pH 7.2). The dialysis buffer was replaced in full after 6 hr and again after 12 hr. The dialyzed sample was ultracentrifuged in a TLA rotor to pellet the proteoliposomes at 60,000 × *g* for 1 hr at 12°C. The proteoliposomes were resuspended in 40 μL of detergent-free buffered solution (200 mM KCl, 10 mM HEPES [pH 7.4]). The proteoliposome sample served as the starting material for subsequent protein reconstitution in droplet lipid bilayers.

Reconstitution of Protein in Droplet Lipid Bilayers

The purified proteins (Piezo1-FLAG WT and mutant E2133A, MscS, and KcsA) were incorporated into droplet lipid bilayers to test their functionality. Droplet lipid bilayers were formed as described previously (Syeda et al., 2008, 2014), with the following modifications. The droplets (~100–200 nL) were dispensed to the tips of two Ag/AgCl electrodes (0.1-mm diameter) in a hexadecane medium. After 5–10 min of incubation, droplets were manipulated to join together. The *cis* droplet contained purified protein (WT-Piezo1, E2133A-Piezo1, MscS, or KcsA) and was connected to the grounded electrode, while the *trans* droplet was connected to the commanding potential electrode so that, at negative applied potentials, cations move from the *cis* to the *trans* side. We actively titrated the proteins in the droplet solution to exclusively get single channels; hence, some of the bilayers were silent and did not evoke channel activity, even in the presence of applied stimulus. It is likely that these bilayers were devoid of any channel protein at the droplet interface. The lipid bilayers were formed between two droplets containing (in mM) 0.5 DPhPC liposomes, 500 KCl (200 or 70 KCl, as indicated in the text), 20 HEPES (pH 7.4) in a hexadecane medium. For the formation of asymmetric bilayers, the liposomes contained 90% DPhPC + 10% LPA in the *cis* droplet, whereas the symmetric bilayers were made with 100% DPhPC in both droplets. We also tested asymmetric bilayers composed of 10% 1-stearoyl-2-hydroxy-sn-glycero-3-PC (LPC) and 90% DPhPC in the *cis* droplet but did not achieve stable bilayers to reconstitute and record protein activity (n = 11; data not shown). Additionally, LPA was preferred because of its direct comparison with DOPA's head-group chemistry, as shown previously (Coste et al., 2012). For mannitol gradient experiments, the dried lipids were re-suspended in either 300 mM or 500 mM mannitol, 500 KCl, 10 mM HEPES (pH 7.4). For low-ionic-strength experiments, 500 mM KCl was replaced by 70 mM KCl.

Solvent Injection Assay

The solvent injection experiments were performed by injecting potassium chloride solution (~30 nL) using a nano-injector at the speed of 46 nL/s, as suggested by nano-injector protocols (World Precision Instruments). The nano-injector was steadily brought in contact with the *cis* droplet near the bilayer interface before expelling the volume into the 100-nL droplet. The nano-injector was removed from the bilayer system prior to the electrical recordings. These injection protocols caused a ~30-s disruption in electrical recordings. We also observed that ~70% of the droplets undergo coalescence within 2 min of injection, presumably due to high lytic tension and decreased lipid density in the *cis* monolayer. Nonetheless, the injection assay provides a 30-s to 2-min time frame in which to record reliable channel activity with a sufficient number of events to construct all-point current histograms.

Data Analysis

All the electrical recordings conducted in droplet bilayers were acquired at 10 kHz and online filtered at 2 kHz. Additional offline filtering of 1 kHz was

applied for the purpose of analysis and display. The recordings were performed from at least five different protein purification trials (Figure 1A). Statistical significance was evaluated using an unpaired two-tailed Student's t test for comparing the difference between two samples. Single-channel conductance (γ) was calculated for each experiment by fitting a Gaussian curve to the all-point current histograms. The current amplitudes obtained from the histograms were divided by the applied voltage to calculate γ. The conductance is plotted as mean ± SEM from the indicated number of experiments (n).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.033.

AUTHOR CONTRIBUTIONS

R.S. and A.P. designed the experiments and wrote the manuscript. R.S., M.N.F., and J.M.K. conducted Piezo1 purification, with help from J.S.S. R.S. conducted electrical recordings and performed analysis. C.D.C. and B.M. provided MscS protein, analyzed bilayer tension data, and wrote the manuscript.

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