Peer Review File

Manuscript Title: Deformation and curvature-gating of the PIEZO1 channel in lipid membranes

Reviewer Comments & Author Rebuttals

Editorial Note: The figure numbering below has been changed to reflect the published version of the manuscript.

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Yang et al. have obtained a novel, flattened conformation of the mechanosensitive Piezo1 ion channel. Using cryo-EM analysis of Piezo1 proteins reconstituted in liposomes, they identify both the canonical curved and novel planar conformations of the channel. Comparing these structures, the authors identify structural changes in domains of the protein, including shifts in the blade, inner face of the protein, cap, and pore domains, between the two conformations. These structural changes may underlie a deformation mechanism of mechanical force detection.

This is a novel and exciting progression in Piezo research, supporting earlier predictions about how the Piezo conformation could become more planar under tension. It also aligns with functional work demonstrating the tension sensitivity, rotation of the Piezo cap domain, and roles of the plug and latch domains in Piezo mechanosensation. This work will open new avenues of investigation in Piezo research, but also provides much anticipated evidence for a novel, protein-deformation-based mechanism of mechanosensitivity. These findings will be of broad interest in mechanosensory biology and ion channel research, and this study is therefore fully appropriate for publication in Nature.

A weakness of the work is that it does not definitively identify the gating mechanism of the channel, as the moderate (6.81 angstrom) resolution of the planar structure is insufficient to support firm conclusions about structural changes in the pore and other Piezo subdomains. Still, this is a very impressive study, and there are few minor revisions that I believe should be incorporated in the final manuscript:

1. Discussion about structural changes that may be linked to the Piezo gating mechanism should further emphasize that the moderate planar structure resolution hinders confirmation of the gating mechanism. Furthermore, the phrase “dynamic gating” in the title should be removed.

2. The structural changes of the beam domain observed in the planar Piezo structure, including kinking and sliding, are not features of a lever, and I ask that the authors reconsider this description.

3. In the tension sensitivity calculation, the method of determining the mechanical force and work values is not intuitive or obvious and should be clarified in the methods.

4. Residue V2476 has been functionally implicated as part of the Piezo channel gate in Zheng et al.,

2019, where mutation of this residue is shown to cause loss of inactivation. Reference to this work, as well as discussion of the other residue implicated in that paper, L2475, should be included in the discussion of the shifting pore amino acid locations between the planar and curved Piezo structures.

Referee #2 (Remarks to the Author):

This manuscript by the laboratories of Drs. Xiao and Li described the structures of PIEZO1 reconstituted into proteoliposome. The relatively small size of proteoliposome imposes certain membrane tension to constrain the conformation of the reconstituted PIEZO1 and allows capturing PIEZO1 in both curved and flattened conformations. The homogeneity of proteoliposome size enabled averaging of sufficient number of particles deformed in the similar way to produce structures of both curved and flattened PIEZO1 with sufficient resolution. The efforts of reconstituting PIEZO1 into proteoliposomes, cryo-EM data collections and processing is a tour de force. The results are very impressive, clearly represent a major step in understanding the gating mechanism of PIEZO channels in response to force transduced through membrane tension. The capturing of flattened PIEZO1 in proteoliposome is particularly interesting, revealing a key feature of PIEZO channel that it can be flattened, as has been speculated. This is also rather challenging to achieve. It provides a nice structural base for the understanding of force induced channel gating. Overall, this is a beautiful study and a major step forward in mechanistic understanding of PIEZO channel gating.

Piezo channel has been visualized previously in liposome, as cited in the reference 10. However, the size of the proteoliposome in this previous study is much larger. In this manuscript, authors cleverly constrained the sizes of liposome to much smaller, making it possible to impose some tensions to the protein via membrane and relative homogenous sizes allow sorting of sufficient number of particles of homogeneous conformation for averaging. They also captured the PIEZO reconstituted in the membrane in opposite orientation, resulting a flattened structure. In this regard, this study clearly is a major step forward from the previous eLife paper (reference 10).

The current version of manuscript is very descriptive, lacking connections to the mechanism of channel gating or did not provide a mechanistic picture of how the flattening induce gate opening. There are some mechanistic descriptions and discussions, but an overall picture is not emerged or is lost in the rather extensive detailed descriptions. Certainly, the limited resolution the flattened channel prevents a precise description of the channel gating. But with the main chain movement, it is totally reasonable to hypothesize the gating mechanism. The description of structural changes from curved to flattened becomes quite complicated when described in detail. It would be very helpful to summarize the structural movement with some cartons. Overall, the writing in the current version is quite difficult to follow.

What is the pore profile of the channel in curved and flattened state? I understand that the resolution of flattened structure is insufficient to model side chains. But considering that the main chain is quite well defined, it is still possible to calculate a “presumptive” pore profile from the flattened structure and compare it with that from the curved structure and/or from detergent solubilized structure. This will facilitate a better understanding of the channel gating, and the

proposed gating through “lateral plug”.

About image processing: did authors use symmetry? I assume not, as it was not mentioned. Did authors try symmetry expansion? The near symmetric feature of liposome may form the deformation into a symmetric fashion. I wonder if it is possible to look into the similarity between different monomers within the trimer? This may provide idea of how asymmetric membrane distortions may influence channel gating?

I also feel that the reference 10 is not emphasized enough in the current version. Reference 10 proposed a mechanistic picture of how Piezo may deform membrane of liposome. While it is cited, but the citation is only for the structural aspect but not the mechanism proposed in that reference. For example, reference 10 already proposed that the bean will be tilted when the channel is flattened by membrane tension. Here, it is nicely show that the bean, while tilting but bend in the middle. Such finding can be nicely connected or contrasted with the previous hypothesis.

Minor

Line 115: “We calculated the curvature …” This sentence is almost impossible to understand. My guess is that the authors want to say that they measured/calculated curvature … of the same liposome. And they did so for liposome off different sizes. It is better to separate this sentence to two or three so that the meaning can be easily understood.

Line 123: Similarly, “Rpiezo1 is smaller than the Rliposome” but “becomes larger”, what becomes larger? Is the difference become that is larger or the Rpiezo1 becomes larger?

Line 148 – 150: I do not understand what this sentence trying to say.

Line 155: “4 pairs of phospholipids”, do authors mean by “pair”? Looking at the figure, it seems that there are only 4 lipids identified.

Line 198: “.. undergo dynamic regulation …”. It is hard to state that the lipid has regulatory role. As lipid protein interactions are rather fluid, lipid may simply adapt the conformation of the protein.

These are minor points. However, since the manuscript is largely descriptive, it is important to make the description easier to follow and making figures easier to follow.

Yifan Cheng

Author Rebuttals to Initial Comments:

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Response: We thank the reviewer for thoroughly reviewing the paper and the highly supportive and constructive comments.

1. Discussion about structural changes that may be linked to the Piezo gating mechanism should further emphasize that the moderate planar structure resolution hinders confirmation of the gating mechanism. Furthermore, the phrase “dynamic gating” in the title should be removed.
Response: In light of the reviewer’s suggestion, we have specified in the discussion section that “despite that the moderate resolution of the flattened structure at its ion-conducting pathway might not allow a conclusive assignment of its functional state, we observe that the drastic deformation of the mechanosensing blade is converted into the modest change of the transmembrane gate and the lateral plug gate respectively through the motion of the extracellular cap and the intracellular beam (Fig. 4g)” (lines 265-270). We have also stressed this issue in the result section by stating that “On the basis of the ~10 Å dilation of the gating residue L2469 from the fully closed pore of PIEZO2 to the flattened state of PIEZO1 (Fig. 4d-f), the flattened structure might likely represent an open transmembrane pore. Nevertheless, given that PIEZO1 can undergo rapid inactivation and the membrane tension in the D-shaped proteoliposomes is expected to persist, we could not exclude the possibility that the flattened structure might represent an inactivating state. In line with this scenario, the modest dilated V2476 has been previously proposed to form an inactivation gate (reference 34). Regardless of the exact functional state of the flattened structure, the dynamic decoupling of the cap and the blade might result in gating of the central transmembrane pore (Fig. 4g)” (lines 225-230).

Given that there is a clear conformational change of the main chain of the pore-lining IH upon transition from the curved to the flattened state (Fig. 4d-f), we hope the reviewer would agree with us that it is reasonable to include “dynamic gating” in the title.

2. The structural changes of the beam domain observed in the planar Piezo structure, including kinking and sliding, are not features of a lever, and I ask that the authors reconsider this description.
Response: We agree with the reviewer that the beam domain undergoes both kinking and sliding motions. Intriguingly, as illustrated in the Supplementary Movie 2, the beam is first slid toward the center, allowing the previously proposed pivot residues L1342 and L1345 to be positioned underneath the CTD triangular plane. A kinking motion is then occurred at the residue S1341, right before the proposed pivot position, resulting in the distal part rotating ~30° downward (Fig. 4b). Therefore, we consider the motion feature is consistent with a lever-like motion. We have included the detailed description of the motion feature of the beam in the revised version of the manuscript (lines 183-189).

3. In the tension sensitivity calculation, the method of determining the mechanical force and work values is not intuitive or obvious and should be clarified in the methods.
Response: In light of the reviewer’s comment to better present the method of determining the mechanical force and work values, we have adapted the previously determined force-displacement curve by Lin et al., 2019 and the free energy equations in Fig. 3c to help guide the calculation process and obtain the key values.

4. Residue V2476 has been functionally implicated as part of the Piezo channel gate in Zheng et al., 2019, where mutation of this residue is shown to cause loss of inactivation. Reference to this work, as well as discussion of the other residue implicated in that paper, L2475, should be included in the discussion of the shifting pore amino acid locations between the planar and curved Piezo structures.
Response: We have cited the work done by Zheng et al.,2019 in the revised version of the manuscript by stating that “Nevertheless, given that PIEZO1 can undergo rapid inactivation and the membrane tension in the D-shaped proteoliposomes is expected to persist, we could not exclude the possibility that the flattened structure might represent an inactivating state. In line with this scenario, the modestly dilated V2476 has been previously proposed to form an inactivation gate (reference 34)” (lines 225-228).

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This manuscript by the laboratories of Drs. Xiao and Li described the structures of PIEZO1 reconstituted into proteoliposome. The relatively small size of proteoliposome imposes certain membrane tension to constrain the conformation of the reconstituted PIEZO1 and allows capturing PIEZO1 in both curved and flattened conformations. The homogeneity of proteoliposome size enabled averaging of sufficient number of particles deformed in the similar way to produce structures of both curved and flattened PIEZO1 with sufficient resolution. The efforts of reconstituting PIEZO1 into proteoliposomes, cryo-EM data collections and processing is a tour de force. The results are very impressive, clearly represent a major step in understanding the gating mechanism of PIEZO channels in response to force transduced through membrane tension. The capturing of flattened PIEZO1 in proteoliposome is particularly interesting, revealing a key feature of PIEZO channel that it can be flattened, as has been

speculated. This is also rather challenging to achieve. It provides a nice structural base for the understanding of force induced channel gating. Overall, this is a beautiful study and a major step forward in mechanistic understanding of PIEZO channel gating.

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Response: We thank the reviewer for the constructive comment for improving the manuscript. Following the reviewer's suggestion, we have made extensive changes of the writing and figure presentation to provide a mechanistic picture of how the flattening induce gating opening in the revised manuscript, including 1) describing the structural changes upon transition from the curved to the flattened state by following two themes: flattening the peripheral blade and beam to control the intracellular lateral plug gate, while detaching and rotating the Cap from the flattened blade to gate the transmembrane pore; 2) using a schematic diagram in the new Fig. 4g to illustrate the two-themed structural movement; 3) moving the description of the structural determination into Method section; 4) moving some of the description of the curved structure into the Extended Data Fig. 3. We hope that the reviewer will find the revised version of the manuscript easier to follow.

What is the pore profile of the channel in curved and flattened state? I understand that the resolution of flattened structure is insufficient to model side chains. But considering that the main chain is quite well defined, it is still possible to calculate a "presumptive" pore profile from the flattened structure and compare it with that from the curved structure and/or from detergent solubilized structure. This will facilitate a better understanding of the channel gating, and the proposed gating through "lateral plug".
Response: We thank the reviewer for the constructive suggestion. In the revised new Fig. 4d,e we have included the alignment of the central pore region and the pore radius profile of the fully closed PIEZO2, the curved and flattened PIEZO1 structures, but specifically stated in the figure legend that "The pore radius of the flattened structure is presumptive as it lacks precise side chain density". Indeed, these comparisons clearly show the dilation of the transmembrane gate residues, consistent with the shift of the main chain position of the gating residues.

About image processing: did authors use symmetry? I assume not, as it was not mentioned. Did authors try symmetry expansion? The near symmetric feature of liposome may form the deformation into a symmetric fashion. I wonder if it is possible to look into the similarity between different monomers within the trimer? This may provide idea of how asymmetric membrane distortions may influence channel gating?
Response: In data processing, all the 3D refinement and 3D classification were performed with C3 symmetry. We have now specified this message in the Method section in the revised version of the manuscript (lines 421-422). We agree with the reviewer that it will be interesting to obtain the structure without adding symmetry, which might allow us to analyze how asymmetric membrane tension might influence structural deformation and channel gating. Unfortunately, when we performed 3D refinement without symmetry, we could only obtain very low-resolution structures. The flexibility of the three blades might be the main reason limiting the resolution. Meanwhile, the blades are largely extended in the lipid membrane. The strong signal from the membrane may also influence the accurate alignment. We had also tried to extract each single blade for 3D alignment as we previously did for solving the structure of PIEZO2, but did not succeed in improving the resolution.

I also feel that the reference 10 is not emphasized enough in the current version. Reference 10 proposed a mechanistic picture of how Piezo may deform membrane of liposome. While it is cited, but the citation is only for the structural aspect but not the mechanism proposed in that reference. For example, reference 10 already proposed that the bean will be tilted when the channel is flattened by membrane tension. Here, it is nicely show that the bean, while tilting but bend in the middle. Such finding can be nicely connected or contrasted with the previous hypothesis.
Response: In light of the reviewer's concern, we have introduced the studies of reference 10 and 11 done by Mackinnon and colleagues in the introduction paragraph by stating that "PIEZO1 reconstituted in liposome vesicles has been shown to curve the residing membrane and undergo reversible flattening at biologically relevant forces, leading to the intriguing proposal that Piezo channels might utilize a structure-based membrane dome mechanism for accounting for their extraordinary mechanosensitivity (reference 10,11)".

The lever-like tilting at the middle of the beam has specifically described in our previous studies (reference 8,30,31), but not by reference 10. In the present study, the beam in the flattened structure is bent at the residue S1341, right before the previously proposed pivot residues L1342 and L1345 (Fig. 4b). The lever-like motion supports our previous proposal that the intracellular beam might function as a lever to convert large conformational changes of the peripheral blade into a smaller motion of the central region (reference 8,30,31).

Minor

Line 115: "We calculated the curvature …" This sentence is almost impossible to understand. My guess is that the authors want to say that they measured/calculated curvature … of the same liposome. And they did so for liposome off different sizes. It is better to separate this sentence to two or three so that the meaning can be easily understood.
Response: We have changed the description as "For proteoliposomes of different sizes, we calculated the curvature radius of the PIEZO1-residing pole (R_{Piezo1}) and the opposite pole of the same liposome ($R_{liposome}$)" (lines 91-93).

Line 123: Similarly, “Rpiezo1 is smaller than the Rliposome” but “becomes larger”, what becomes larger? Is the difference become that is larger or the Rpiezo1 becomes larger?
Response: We have changed the description as “In contrast, for liposomes with Rliposome larger than 11 nm, $R_{Piezo}1$ is smaller than the $R_{liposome}$” (lines 89-91).

Line 148 – 150: I do not understand what this sentence trying to say.
Response: We have moved the description regarding the previous Fig. 2c to the revised Extended Data Fig. 3d and the related description to the figure legend. “The PIEZO1 map derived from proteoliposomes contains extra patches of densities in-between the blades and in parallel to the inner membrane layer. These densities are noticeable even at a high contour level of 4 and consist of the modeled α1-helix of the clasp domain (Clasp$^{\alpha1}$), the α-helix preceding TM33 (TM33$^{pre\text{-}\alpha1}$), and potentially unmodeled intracellular loop regions that are stabilized by interacting with lipid layers. This layer of membrane-parallel structure might help to stabilize the bowl-shaped structure of PIEZO1 in lipid membranes”.

Line 155: “4 pairs of phospholipids”, do authors mean by “pair”? Looking at the figure, it seems that there are only 4 lipids identified.
Response: We apologize for the confusion. We have identified a total of 8 phospholipids in each blade termed “Blade lipids”, which are illustrated and labeled in the revised Fig. 2d. These 8 lipids are located in both sides of the blade (with 4 at each side). To avoid confusion, we have changed “4 pairs of phospholipids” as “8 phospholipids”.

Line 198: “.. undergo dynamic regulation …”. It is hard to state that the lipid has regulatory role. As lipid protein interactions are rather fluid, lipid may simply adapt the conformation of the protein.
Response: We have changed the description as “Furthermore, we have identified the pore lipid but not the other 8 blade lipids (Extended Data Fig. 5d and 6h), suggesting that phospholipids might adapt the conformational change for interaction” (lines 149-151).

These are minor points. However, since the manuscript is largely descriptive, it is important to make the description easier to follow and making figures easier to follow.
Response: As described above in response to the first major point of the reviewer, we have made extensive changes of the main text and figures. We hope the reviewer will now find the revised version of the manuscript better to follow.