The Journal of Physiology

The LRRC family of BK channel regulatory subunits: potential roles in health and disease

Vivian Gonzalez-Perez, Yu Zhou, Matthew A Ciorba, and Christopher J Lingle
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Criss Hartzell (Referee #1); Michael J Shipston (Referee #2)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Lingle,

Re: JP-TR-2021-281952 "The LRRC family of BK channel regulatory subunits: potential roles in health and disease" by Vivian Gonzalez-Perez, Yu Zhou, Matthew A Ciorba, and Christopher J Lingle

Thank you for submitting your Topical Review to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert referees. You are now invited to address the comments of the reviewers and submit a revised version for further consideration.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

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I hope you will find the comments helpful and have no difficulty in revising your manuscript within 4 weeks.

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Yours sincerely,

Ian D. Forsythe
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EDITOR COMMENTS

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This topical review manuscript has a particular focus on the potential role of LRRC family of the BK channel in a number of distinct areas where the roles of BK channels are often less well appreciated than in some other systems, which could provide a complementary insight in addition to existing reviews.

It has been assessed by two referees. The major concerns raised by the referees include imprecise/inconsistent language, poor figure quality, ambiguous abbreviations/nomenclature and presentation, speculative 'conclusions', incomplete abstract summary, new figure inclusion, structural change to section III, introduction clarification, perspective inclusion.

On the point of speculative 'conclusions', speculations are sometimes allowed for the topical review in The Journal of Physiology as long as they are reasonably justified and are based on the up-to-date experimental evidence and current understandings of the field.

Senior Editor:

Your manuscript has received some detailed and thorough consideration from the referees and RE and while there are some serious issues raised, all are of a nature that can be addressed by a thorough rewrite. This will broaden the appeal of your review and help present these important ideas to the widest audience.

Finally, please re-write your abstract to clear present the findings of your review. It should not be a 'review plan' (as in 'this review is about.....') but is should inform the readers of the key findings and come to a clear final concluding sentence.

REFEREE COMMENTS

Referee #1:

This manuscript seems to be written for BK channel cognoscenti who have already read the primary papers. I don't work on BK channels, but I have a very strong and broad background in ion channels. Yet, this review was a struggle for me to read. The language was imprecise, the figures poorly and confusingly labelled, abbreviations not defined, nomenclature poorly used, conclusions speculative, and the rationale and interpretation of experiments incompletely and cryptically presented.

The manuscript was excessively wordy. For example, the introductory sentences (lines 61-68) are redundant and could be re-written to be more concise: a suggestion: Potassium channels of large conductance (BK or KCa1.1, encoded by the KCNMA1 gene (Butler et al., 1993)) are ubiquitously expressed and contribute to rapid repolarization in excitable cells or K+ fluxes in non-excitable cells (Bailey et al., 2019; Contet et al., 2016). A notable property of these channels is that they can be activated independently by two physiological signals: increases in submembrane Ca2+ even in the absence of depolarization, or depolarization in the absence of Ca2. Although my language may be more boring, it conveys the same information in 76 words vs 110.

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Figure 1. The authors should provide more details of the docking of LRRC26 and BK. Is this done entirely manually? What are the criteria for placing LRRC26 in this precise orientation (besides the non-competition with β). Are there any interfacial contacts that would support this model? Have the authors performed any MD of this complex and can the free energy of binding be estimated? The authors state clearly that this is speculative - but it should be made clear whether this is a shot in the dark or something more substantial.

Figure 2A. The solid and dotted lines are not identified in the legend. Are all these curves in zero Ca or just the solid curves?

Line 172. Gene names are not primarily used, the γ terminology is frequently used (for example lines 203-211, 255-257). It's confusing.

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Line 197: "injected LRRC26 subunits" Are subunits or cDNA injected?

Figure 3B - Do these times refer to the time in zero Ca? Why does the effect take so long to develop? Also, the explanation of the figures is really obscure. The Figure says 0 Ca at the top and G/V with 10μM Ca at the bottom. It takes some mental gymnastics between the legend, text, and figure to understand what the authors are saying. This is written for someone who is familiar with the original paper rather than someone new to the discussion.

Figure 3. All panels are really annoying. The solid and dotted curves are not clearly labelled.

Figure 3F. The mallotoxin results are enigmatic to me. With BK, malltoxin acts like LRRC26 and shifts the GV to the left. With BK+WT LRRC26, mallotxin has no effect. With BK+F273S, mallotoxin irreversibly shifts (part of) the GV to the right. Do I understand correctly? It would be nice to have a logical mechanistic interpretation of these results. The effect of mallotoxin on BK alone and BK+WT LRRC26 suggest that mallotoxin binds to the same site and has the same effect as LLRC26. But, if so, why would it abolish the effect of the mutant? If mallotoxin irreversibly disrupts F273S interaction with BK, one would expect it to have the same effect malltoxin has on BK channels alone.

Line 199-200. This argument assumes that BKα and LRRC26 have the freedom to assemble in different stoichiometries (4:1, 2:1, 1:1...). Is it possible that LRRC26 can only assemble in a 4:4 complex with BK and other stoichiometries such as 1 LRRC26 per BK tetramer is not permissible? In that case, the biphasic GV curves would just reflect the ratios of channels with LRRC26 and those without. Sorry, I haven't read the original paper. Have the authors measured stoichiometry biochemically? The experiment with the β2/γ1 chimera needs more explanation to make it understood by someone who does not know the original paper.

Line 237. "When LRRC26 is coexpressed with BK subunits in Xenopus oocytes and the cytosolic face of excised patches is continuously exposed to Ca2+-free solutions, the initial -120 mV gating shift undergoes a gradual transition from low voltage-activated BK channels to those exhibiting no shift at all." This statement is really confounding because the language is imprecise (a problem throughout the manuscript). In this sentence, it is not at all clear what causes the -120 mV shift: exposure to zero Ca or co-expression of LRRC26. Also, there are two shifts happening (the shift to the left caused by LRRC26 and the shift to the right with time at -140 mV and zero Ca). I would say (more precisely): Co-expression of LRRC26 with BK caused a -120mV shift in the GV curve recorded in 10μM Ca compared to BK alone. However, when cells were held at -140 mV in zero Ca between GV curves, the GV curve shifted to the right with time. When cells were held at +80 mV, this did not occur. (Am I correct or did I misunderstand?)

Line 240. What is the evidence that zero Ca causes LRRC26 dissociation as implied in this sentence ("one component corresponding to shifted LRRC26-containing BK channels and the other to BK α subunits alone")? Line 245 suggests that evidence for dissociation is lacking. I found that the argument (or implication) that the effects of voltage on the "shift" produced by LRRC26 can be explained by changes in binding affinity to be weak and not supported well by data. This problem reappears at the end of the manuscript (line 505) where a major speculation is built on weak data.

Line 269. What exactly does "some reduction in co-IP" mean. Please be (semi-)quantitative.

Line 299 "which appears to be exclusively expressed in sperm" and Line 308. Line 299 should be changed to read "which was initially thought to be exclusively expressed in sperm" or delete this altogether because it is irrelevant before line 308.

Line 323. Why suddenly change to the gene name KCNMA1 when throughout you have been referring to BKα.

Line 339. Nephropathies? Please be specific - this global term is not illuminating.

Line 353. From this paragraph, especially the last sentence, I would conclude that these are not subunits of the BK channel. Delete this paragraph and clean up line 120 to say there are 4 γ subunits. Add one sentence saying that LRTMs may belong to the same family, but have not been found to have any effect on BK or slo3.

Line 372. "Unclear" - explain why.

Line 412. In contrast to glandular epithelia? Well, let's see: salivary gland has serous and mucus cells, at least. I don't think this statement is accurate.

Line 452. UC is not defined.

Line 472. "G/V curves identified by paxilline subtraction?" Give me a break. Not only is the wording imprecise and sloppy, it requires that the reader knows what paxilline is and what the process of "paxilline subtraction" is.

Line 475. Whoa! Do I have everything mixed up? It says: BKα+LRRC26 has Vh=-15mV and BKα alone has Vh=-130mV. So, I go back to Fig. 2A. The solid and dotted lines are not identified, but from the text on line 156 ("LRRC26 produces a shift in the BK current of -140mV"), I presume the solid line is +LRRC26. If that is the case, it seems in BMSCs the effect of LRRC26 is the opposite to what was described for LNCap cells.

Line 514. LVA and HVA are not defined.

Line 523. Speculative, yes. Compelling, no.

Line 569. If critical phenotypic analysis has not been done, why are we even talking about this question? Straw man.

Line 582. " CFTR-/- mice are also vulnerable to DSS-induced colitis (Laroui et al., 2012), although this may be associated with knockdown of TMEM16A." I don't get it. Does knockout of CFTR cause downregulation of TMEM16A expression?

Referee #2:

In this topical review the authors, who have made very substantial contributions to the field, present an engaging and provoking review that has the potential to have broad impact and relevance to the wider readership of the Journal of Physiology. While the fundamental properties of the LRRC family of accessory subunits of the important BK channel potassium channels have been reviewed recently, including by the authors in an Annual Review of Physiology, this topical review emphasises the potential role of this family in a number of distinct areas of non-excitable cell physiology where the role of BK channels perhaps less well appreciated than in some other systems.

While the review is written in an engaging and accessible style some structural changes in particular to section III and abstract would significantly enhance the review for the general reader.

Major

1) The abstract reads more as an insight into LRRC26 physiology and does not do justice to the range of topics including cancer, epithelial, vascular and other cell function) covered in the main review. While it is true most is known about this subunit the broader context as implied by title is somewhat lost on the general reader- a point also as in minor point2 regarding graphical abstract.

2) Inclusion of a figure better illustrating the range of systems/physiology that LRRCs may be implicated in controlling would be helpful

3) Sections IIIC and IID feel out of place and in this reviewers opinion would be much better in section II. This would provide the final viewpoint on changes in interaction between LRRC and BK and changes in LRRC expression as potential mechanisms underlying pathology?

4) In the introduction (p4, line 80) the authors suggest that there may be 2 or 3 'distinct' families of regulatory subunits: beta subunits, LRRC gamma and as written a 'distinct' LINGO family. However, the general reader would not appreciate that the LINGO family are also LRR containing single transmembrane pass proteins. For completeness including the relationship with LINGO1 and at least highlighting that is highly expressed in brain and confers rapid inactivation on BK channels in the broader discussion. A dendrogram might also be useful here

5) The review needs a brief conclusion/summary perspectives & challenges section

Minor:

1) Abstract line 40-41: Id avoid the use of phrase "Here in a rather speculative..." This could be very off putting for general Journal of Physiology reader who might think its not worth reading further - that would be a real shame!

2) Graphical abstract figure suggests that only role is in enterocytes. A simpler and broader graphical abstract is warranted as per major point 2

3) Figure 2A: clarification of dotted and solid lines required as not clear from text legend or inset legend. i.e dotted are BK expressed alone in HEK293 and KD of LRRC26 in LNCAP respectively etc

4) In section on time dependent shifts in G/V curves (lability of effect of LRRC26 on page 9 line 235) it would be helpful to clarify if this phenomenon has only been observed in Xenopus oocytes as not observed in native cells or HEK cells ie suggest microenvironment of Xenopus oocyte patch is important. If lability aonly seen in oocytes this also has implications for discussion on potential chane sin affinity as discussed on line 278-279.

5) Is anything know about where mallotoxin binds as potential disruption of mutated LRRC might support competition for similar sites?

6) In sections C & D the authors allude to the promiscuity of LRRC 52 with KCNU1 but that LRRC 38 has no effect on KCNU1. Would be useful to clarify if LRRC26 and 55 also regulate KCNU1 and more general issue of promiscuity for example I relation to LRRC 26 and cancer?

7) Page 12 in section C the implication that low levels of LRRC and BK could be functionally important could be expanded for the general reader by reiterating the large conductance of these channels - you don't need many to be active to have large influence on K+ flux. This is also relevant to discussion about changes in association in lines 523-529

8) In implications of low affinity interaction section some indications of mechanisms that might control this would be useful - eg might polybasic domain interact with negatively charged phospholipids so that change in lipid environment may be

important and/or interactions with other peptide residues whose charge may be modified - eg by phosphorylation etc

9) Line 569 - what sort of 'critical phenotypic analysis hasn't been performed to understand changes in phenotype

10) A significant number of references in bibliography are not complete: e.g Chen et al 2021 ; Dudem 2021; Gonzalez-Perez 2021, Hu et al 2021; Lorca 2018; Nystrom 2021;

REQUIRED ITEMS:

-Your MS must include a complete "Additional information section" with the following 4 headings and content:

Competing Interests: A statement regarding competing interests. If there are no competing interests, a statement to this effect must be included. All authors should disclose any conflict of interest in accordance with journal policy.

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It must be stated that all authors approved the final version of the manuscript and that all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding: Authors must indicate all sources of funding, including grant numbers. If authors have not received funding, this must be stated.

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Acknowledgements: Acknowledgements should be the minimum consistent with courtesy. The wording of acknowledgements of scientific assistance or advice must have been seen and approved by the persons concerned. This section should not include details of funding.

-Author profile(s) must be uploaded via the submission form. Authors should submit a short biography (no more than 100 words for one author or 150 words in total for two authors) and a portrait photograph of the two leading authors on the paper. These should be uploaded, clearly labelled, with the manuscript submission. Any standard image format for the photograph is acceptable, but the resolution should be at least 300 dpi and preferably more. A group photograph of all authors is also acceptable, providing the biography for the whole group does not exceed 150 words.

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END OF COMMENTS

Confidential Review **13-Oct-2021**

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On the point of speculative 'conclusions', speculations are sometimes allowed for the topical review in The Journal of Physiology as long as they are reasonably justified and are based on the up-to-date experimental evidence and current understandings of the field.

Regarding speculative aspects of the manuscript, we cautioned the editors before embarking on this effort that, given the state of current research, we felt that any suggestions regarding how LRRC subunits are directly related to disease would necessarily be highly speculative. Although KO animals may generate a pathological phenotype, which is essentially the reason that the review was solicited, that doesn't necessarily illuminate how that may occur in naturally occurring diseases. The KO data do indicate that any downregulation or loss of expression of LRRC26 has the potential to contribute to naturally occurring pathology. But then the issue is, how might such downregulation occur? The functional data on LRRC26 does support the view that down-regulation of the LRRC26 effect can be observed by manipulations that may influence BK-LRRC26 interaction affinity, and that downregulation of LRRC26 has been observed in humans in association with gene methylation. We feel that highlighting these considerations may help point to new experiments that can either support or invalidate these suggestions. Without these suggestions, I think this review would serve no purpose other than rehashing information that is already out there, although perhaps not pulled together in this fashion. We have removed a speculative structural proposal, since it did not add anything to the major points of the review.

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We do appreciate the referee's guidance with specific and detailed comments. We hope that the manuscript is now easier to read, and more accessible for the general readers, with or without ion channel expertise.

The issue of speculative conclusions os touched on in comments above to the Reviewing Editor.

The manuscript was excessively wordy. For example, the introductory sentences (lines 61-68) are redundant and could be re-written to be more concise: a suggestion: Potassium channels of large conductance (BK or KCa1.1, encoded by the KCNMA1 gene (Butler et al., 1993)) are ubiquitously expressed and contribute to rapid repolarization in excitable cells or K+ fluxes in non-excitable cells (Bailey et al., 2019; Contet et al., 2016). A notable property of these channels is that they can be activated independently by two physiological signals: increases in submembrane Ca2+ even in the absence of depolarization, or depolarization in the absence of Ca2. Although my language may be more boring, it conveys the same information in 76 words vs 110.

Implemented as suggested, along with other deletions from intro. The manuscript has been shortened by deletion of sections involving LTRM1 and LTRM2 and by deletion of section concerned with why other phenotypes have not been observed for LRRC26 KO. Overall length is about the same, since it was additional explanations to address issues raised were required.

Line 109 and 113 and 167: Terminology: are LRRCs domains or subunits. Are LRRs domains or subunits?

Reworded to explicitly designate LRR's as generic domains common to many proteins, and LRRCs as BK-related subunits.

Line 120. "6 loosely related family members, with 3 pairs of more closely related proteins" is confusing. 3 pairs = 6, so are there a total of 12 members? Or do the 3 pairs constitute the 6? If so, the qualifiers "loosely" and "closely" seem contradictory. Table 1 only lists 4. And, why are they paired?

This section is entirely re-written. Consideration of LRTM1 and LRTM2 has been minimized.

Figure 1 Legend. The authors should give the URL for Alpha-Fold and the version number of the model. The authors have not defined VSD or PGD. Relevant information is provided.

Figure 1. The authors should provide more details of the docking of LRRC26 and BK. Is this done entirely manually? What are the criteria for placing LRRC26 in this precise orientation (besides the non-competition with β). Are there any interfacial contacts that would support this model? Have the authors performed any MD of this complex and can the free energy of binding be estimated? The authors state clearly that this is speculative - but it should be made clear whether this is a shot in the dark or something more substantial. We have removed the candidate docking from the manuscript. We have done this with a combination of ClusPro2.0, which provides a set of candidate poses based on rigid docking of candidate protein partners, and then Rosetta, which optimizes positioning based on side-chain optimization minimizing steric clashes. Although we think the most likely crevice of occupancy is correctly revealed, these methods are admittedly imperfect and inclusion of this speculative proposal serves no purpose here.

Figure 2A. The solid and dotted lines are not identified in the legend. Are all these curves in zero Ca or just the solid curves? The figure legend has been rewritten.

Line 172. Gene names are not primarily used, the γ terminology is frequently used (for example lines 203-211, 255-257). It's confusing. We have now removed the use of γ terminology, except in the initial definitions. Although the gamma nomenclature can have advantages for its brevity, for a general audience, the gene names are more appropriate.

Line 186. The phrase "abolishes the 'gating shift'" is bizarre. KO of LRRC26 causes a shift compared to the wild type current. To say that KO abolishes a shift makes this sound like a double negative. That bit of jargon has been removed in the re-write.

Line 197: "injected LRRC26 subunits" Are subunits or cDNA injected? Wording changed to reflect fact that ratio of injected LRRC26:BKα cRNA was varied.

Figure 3B - Do these times refer to the time in zero Ca? Why does the effect take so long to develop? Also, the explanation of the figures is really obscure. The Figure says 0 Ca at the top and G/V with 10μM Ca at the bottom. It takes some mental gymnastics between the legend, text, and figure to understand what the authors are saying. This is written for someone who is familiar with the original paper rather than someone new to the discussion.

The legend and text have been extensively rewritten and information on the top of Figure panels A-C has been added. Yes, the times refer to cumulative time in 0 Ca^{2+}. We are uncertain what the referee is referring to in regards to the effect taking so long. Our hypothesis is that the time course of the shift in panel B reflects time until the last LRRC26 on a BK channel dissociates from the channel. At the single channel level, one observes that a channel is fully shifted and then at some random time in 0 Ca^{2+}, the channel will undergo a change in gating to the unshifted situation. Although this issue is beyond this presentation, in our analysis of the time course of the shift occurring when patches are exposed to 0 Ca^{2+}, we noted that the time course is best fit with an exponential raised to a power, with n=4 the limiting value in a limited set of patches. This is consistent with the idea that the LRRC26+BK channels among different patches may have different average numbers of LRRC26 subunits. However, only when the last LRRC26 subunit dissociates (or loses function), does the gating shift occur.

Figure 3. All panels are really annoying. The solid and dotted curves are not clearly labelled. Legend and labelling of figures have been edited. Text has been edited, with more detail about the experiments and their implications.

Figure 3F. The mallotoxin results are enigmatic to me. With BK, malltoxin acts like LRRC26 and shifts the GV to the left. With BK+WT LRRC26, mallotxin has no effect. With BK+F273S, mallotoxin irreversibly shifts (part of) the GV to the right. Do I understand correctly? It would be nice to have a logical mechanistic interpretation of these results. The effect of mallotoxin on BK alone and BK+WT LRRC26 suggest that mallotoxin binds to the same site and has the same effect as LLRC26. But, if so, why would it abolish the effect of the mutant? If mallotoxin irreversibly disrupts F273S interaction with BK, one would expect it to have the same effect malltoxin has on BK channels alone.

We hope the revisions have clarified these results. The explanation underlying the mallotoxin rightward shift with the F273S construct is that mallotoxin dislodges F273S from the channel. The position of the G/V under that condition is then identical to the effect of mallotoxin on BK alone. When mallotoxin is then washed off, the G/V returns to that expected for BK alone. We now also point out that the Guan et al. (2017) paper shows that the approximately 20% occupancy (relative to WT LRRC26) by F273S is markedly reduced to near 5% by mallotoxin, directly indicating that mallotoxin dislodges F273S from the BK channel, with no effect on WT LRRC26.

Line 199-200. This argument assumes that BKα and LRRC26 have the freedom to assemble in different stoichiometries (4:1, 2:1, 1:1...). Is it possible that LRRC26 can only assemble in a 4:4 complex with BK and other stoichiometries such as 1 LRRC26 per BK tetramer is not permissible? In that case, the biphasic GV curves would just reflect the ratios of channels with LRRC26 and those without. Sorry, I haven't read the original paper. Have the authors measured stoichiometry biochemically? The experiment with the β2/γ1 chimera needs more explanation to make it understood by someone who does not know the original paper.

The experimental support for how stoichiometry has been determined is now expanded. For the referee, attempts to measure stoichiometry from co-IP experiments by Jiusheng Yan's group (personal communications) have noted that LRRC26 dissociates from BK during IP, such that proteomic determinations reveal something more than one LRRC26 per BK channels, but not four. Crosslinking experiments have not been attempted. We know of no biochemical method that would address the issue of the stoichiometry of the functional effects.

Line 237. "When LRRC26 is coexpressed with BK subunits in Xenopus oocytes and the cytosolic face of excised patches is continuously exposed to Ca2+-free solutions, the initial -120 mV gating shift undergoes a gradual transition from low voltage-activated BK channels to those exhibiting no shift at all." This statement is really confounding because the language is imprecise (a problem throughout the manuscript). In this sentence, it is not at all clear what causes the -120 mV shift: exposure to zero Ca or co-expression of LRRC26. Also, there are two shifts happening (the shift to the left caused by LRRC26 and the shift to the right with time at -140 mV and zero Ca). I would say (more precisely): Co-expression of LRRC26 with BK caused a -120mV shift in the GV curve recorded in 10μM Ca compared to BK alone. However, when cells were held at -140 mV in zero Ca between GV curves, the GV curve shifted to the

right with time. When cells were held at +80 mV, this did not occur. (Am I correct or did I misunderstand?). The reviewer's synopsis is correct. The section has now been edited to make the description more precise and concise. Some additional explanatory sentences are added to elaborate on alternative explanations for the loss of gating shifts. For most of these experiments, Ca^{2+} is not elevated, so is not producing channel activation. LRRC26 produces a -120 to -140 negative shift in BK gating.

Line 240. What is the evidence that zero Ca causes LRRC26 dissociation as implied in this sentence ("one component corresponding to shifted LRRC26-containing BK channels and the other to BK α subunits alone")? Line 245 suggests that evidence for dissociation is lacking. I found that the argument (or implication) that the effects of voltage on the "shift" produced by LRRC26 can be explained by changes in binding affinity to be weak and not supported well by data. This problem reappears at the end of the manuscript (line 505) where a major speculation is built on weak data.

As the reviewer is noting, there is no direct evidence that zero Ca is causing LRRC26 dissociation. At present, this is simply one plausible explanation, with the other being that the effect produced by LRRC26 has been permanently disabled (at least over the time scale of our recordings). The idea that dissociation seems plausible and is a reasonable working hypothesis builds from several independent pieces of evidence that we subsequently summarized in various sections. However, we have tried to clarify this better in our revisions and attempt to tie the various bits of information together. To summarize, first, as one titrates BKα with LRRC26, one observes an increase in the shifted LVA fraction relative to the unshifted HVA fraction. Thus, the two component Boltzmann's arise from relative fractional occupancy of BK channels by LRRC26. Second, the loss of the gating shift in 0 Ca^{2+} recapitulates the two component Boltzmann behavior one would expect if LRRC26 was being removed. Yes, this could be LRRC26 becoming "inactive", but simply bathing patches in elevated Ca^{2+} does not restore the gating shift. Third, various mutations in the LRRC26 TM and cytosolic piece can also result in two component Boltzmann behavior and this is, in many cases, associated with a diminished coassembly of the mutated LRRC26 construct with BKα. The published gels are not quantitative, but there are some valuable clues. For example, in the Li et al., 2016 paper the P270V and F273S single mutations each cause a large reduction in LRRC26 that can be pulled down from Co-IP with BKα. Although no densitometry comparisons are provided, while BKα bands appear unchanged in the transfections, the LRRC26 bands are reduced to perhaps less than 20% of control levels. Yet, recordings of currents from cells transfected with either the P270V or F273S constructs show that approximately 75% of the BK currents in such cells still show LVA-gating behavior, while the remainder appears to be unshifted. This result is consistent with the idea that the mutated subunits bind more weakly to BKα. However, consistent with the all-or-none behavior, a fractional occupancy of LRRC26 binding sites of about 0.2 predicts that the fraction of channels with LVA gating will be about 0.6 (Gonzalez-et al., 2014)

Line 269. What exactly does "some reduction in co-IP" mean. Please be (semi-)quantitative. Most of the Western blots from the Yan lab have not been analyzed by densitometry. Levels for co-IP for the P270V and F273S constructs seem to be conservatively much less than 50% of control and perhaps as much as 10%. The implications of this reduction in assembly are explained more fully as stated in the response immediately above. In the Guan et al., 2017 paper, densitometry numbers are provided for F273S, which shows that its level of association with BK is reduced by about 80% compared to LRRC26, with both expressed under identical conditions. Mallotoxin further reduces the LRRC26 presence down to 5%.

Line 299 "which appears to be exclusively expressed in sperm" and Line 308. Line 299 should be changed to read "which was initially thought to be exclusively expressed in sperm" or delete this altogether because it is irrelevant before line 308. Edited as suggested

Line 323. Why suddenly change to the gene name KCNMA1 when throughout you have been referring to BKα. Changed to BKα

Line 339. Nephropathies? Please be specific - this global term is not illuminating. Specific details are now provided.

Line 353. From this paragraph, especially the last sentence, I would conclude that these are not subunits of the BK channel. Delete this paragraph and clean up line 120 to say there are 4 γ subunits. Add one sentence saying that LRTMs may belong to the same family, but have not been found to have any effect on BK or slo3. Yes, good suggestion.

Line 372. "Unclear" - explain why. Done. Explanation given.

Line 412. In contrast to glandular epithelia? Well, let's see: salivary gland has serous and mucus cells, at least. I don't think this statement is accurate. Oops. This was definitely NOT accurate. The mention of salivary glands was not necessary at that point, so has been deleted.

Line 452. UC is not defined. Done

Line 472. "G/V curves identified by paxilline subtraction?" Give me a break. Not only is the wording imprecise and sloppy, it requires that the reader knows what paxilline is and what the process of "paxilline subtraction" is. For the general reader, the details of how BK currents were identified and the G/V curves generated are not essential. Therefore mention of paxilline is omitted. This passage also inappropriately introduced the terminology, V_h, without defining it and without usage earlier in the document. This is now fixed.

Line 475. Whoa! Do I have everything mixed up? It says: BKα+LRRC26 has Vh=-15mV and BKα alone has Vh=-130mV. So, I go back to Fig. 2A. The solid and dotted lines are not identified, but from the text on line 156 ("LRRC26 produces a shift in the BK current of -140mV"), I presume the solid line is +LRRC26. If that is the case, it seems in BMSCs the effect of LRRC26 is the opposite to what was described for LNCap cells. The text used V_h=~(single wavy dash to signify “approximately”)130-150 mV, so it is shifted +145 to +165 mV. It now reads “V_h ~ +130-150 mV” for the mASMCs and “Vh ~ -15 to -25 mV” for the BSMCs. I presume the Journal may have their own ways of handling the formatting for “approximate”.

Line 514. LVA and HVA are not defined. These are now defined much earlier when gating shifts observed by Yan and Aldrich is first mentioned.

Line 523. Speculative, yes. Compelling, no.

We hope we have developed a more thoroughly reasoned case, but we would still agree that it remains a speculative topic, but one worth future investigation.

Line 569. If critical phenotypic analysis has not been done, why are we even talking about this question? Straw man. We have deleted this section, although we suspect many readers will be wondering about this.

Line 582. " CFTR-/- mice are also vulnerable to DSS-induced colitis (Laroui et al., 2012), although this may be associated with knockdown of TMEM16A." I don't get it. Does knockout of CFTR cause downregulation of TMEM16A expression? Because the relevant section was deleted, this is no longer mentioned.

Referee #2:

In this topical review the authors, who have made very substantial contributions to the field, present an engaging and provoking review that has the potential to have broad impact and relevance to the wider readership of the Journal of Physiology. While the fundamental properties of the LRRC family of accessory subunits of the important BK channel potassium channels have been reviewed recently, including by the authors in an Annual Review of Physiology, this topical review emphasises the potential role of this family in a number of distinct areas of non-excitable cell physiology where the role of BK channels perhaps less well appreciated than in some other systems.

While the review is written in an engaging and accessible style some structural changes in particular to section III and abstract would significantly enhance the review for the general reader.

Major

1) The abstract reads more as an insight into LRRC26 physiology and does not do justice to the range of topics including cancer, epithelial, vascular and other cell function) covered in the main review. While it is true most is known about this subunit the broader context as implied by title is somewhat lost on the general reader- a point also as in minor point2 regarding graphical abstract. Perhaps Matt is coming up with something.

2) Inclusion of a figure better illustrating the range of systems/physiology that LRRCs may be implicated in controlling would be helpful. This has been addressed in two ways. We include a list of organ systems potentially affected by LRRC26 in the Summary Figure. Furthermore, the modified Figure 1 gives an overview of candidate organs that may be affected by each of the LRRC subunits.

3) Sections IIIC and IID feel out of place and in this reviewers opinion would be much better in section II. This would provide the final viewpoint on changes in interaction between LRRC and BK and changes in LRRC expression as potential mechanisms underlying pathology?

We concur with the reviewer on these topics. However, also influencing by referee #1, we are simply removing section IIIC concerned with why other phenotypic changes have not been observed. Section IIID has now been moved to section II. Because the original section IIID pertains to information in the original IIIB, the change in order also required some editing of each.

4) In the introduction (p4, line 80) the authors suggest that there may be 2 or 3 'distinct' families of regulatory subunits: beta subunits, LRRC gamma and as written a 'distinct' LINGO family. However, the general reader would not appreciate that the LINGO family are also LRR containing single transmembrane pass proteins. For completeness including the relationship with LINGO1 and at least highlighting that is highly expressed in brain and confers rapid inactivation on BK channels in the broader discussion. A dendrogram might also be useful here

Our desire was really to minimize consideration of any regulatory subunits other than the set of 4 LRRCs. I think that for present purposes just pointed out that other families exist, with appropriate citations is sufficient. Anything else, including that LINGO contains a very different sort of LRR topology, may only tend to muddle the account, with going into much more detail.

5) The review needs a brief conclusion/summary perspectives & challenges section

This is now provided.

Minor:

1) Abstract line 40-41: Id avoid the use of phrase "Here in a rather speculative..." This could be very off putting for general Journal of Physiology reader who might think its not worth reading further - that would be a real shame!

Abstract is rewritten.

2) Graphical abstract figure suggests that only role is in enterocytes. A simpler and broader graphical abstract is warranted as per major point 2

We are unclear regarding “suggests that only role is in enterocytes”. Perhaps “only role is in goblet cells” was intended. Table 1 was intended to highlight various loci in which different LRRC subunits may be important. However, we’ve also amended Figure 1 to highlight a few other tissues of importance.

3) Figure 2A: clarification of dotted and solid lines required as not clear from text legend or inset legend. i.e dotted are BK expressed alone in HEK293 and KD of LRRC26 in LNCAP respectively etc

This has been addressed in my answers to Referee #1.

4) In section on time dependent shifts in G/V curves (lability of effect of LRRC26 on page 9 line 235) it would be helpful to clarify if this phenomenon has only been observed in Xenopus oocytes as not observed in native cells or HEK cells ie suggest microenvironment of Xenopus oocyte patch is important. If lability aonly seen in oocytes this also has implications for discussion on potential chane sin affinity as discussed on line 278-279.

This section has hopefully been clarified, since it was clearly a concern to both referees.

5) Is anything know about where mallotoxin binds as potential disruption of mutated LRRC might support competition for similar sites?

This is definitely an interesting question, but nothing is known. As yet, we don't even know the specific interactions between LRRC26 and BK, although the computational docking may allow candidates that can be probed experimentally.

6) In sections C & D the authors allude to the promiscuity of LRRC 52 with KCNU1 but that LRRC 38 has no effect on KCNU1. Would be useful to clarify if LRRC26 and 55 also regulate KCNU1 and more general issue of promiscuity for example I relation to LRRC 26 and cancer? The issue of promiscuity has now been briefly expanded in Section C. Although a potential relationship to cancer is intriguing, that seems outside anything we currently know about.

7) Page 12 in section C the implication that low levels of LRRC and BK could be functionally important could be expanded for the general reader by reiterating the large conductance of these channels - you don't need many to be active to have large influence on K+ flux. This is also relevant to discussion about changes in association in lines 523-529

This is an important point and it is now highlighted in regards to the challenges that are faced in trying to determine presence and properties of a channel population expressed at low abundance in a very circumscribed loci.

8) In implications of low affinity interaction section some indications of mechanisms that might control this would be useful - eg might polybasic domain interact with negatively charged phospholipids so that change in lipid environment may be important and/or interactions with other peptide residues whose charge may be modified - eg by phosphorylation etc

A sentence raising these possibilities is now included. For the referee's benefit, in our earlier work, we have extensively tested whether application of PIP2 (various forms) and ATP to the cytosolic face of patches might prevent, stabilize, or reverse the gating shift in 0 Ca^{2+}, but those results were uniformly negative.

9) Line 569 - what sort of 'critical phenotypic analysis hasn't been performed to understand changes in phenotype We have chosen to omit this section.

10) A significant number of references in bibliography are not complete: e.g Chen et al 2021 ; Dudem 2021; Gonzalez-Perez 2021, Hu et al 2021; Lorca 2018; Nystrom 2021;

Now addressed. Not sure Endnote is handling format of online manuscripts properly, but I am using most up-to-date J.Physiology style

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REQUIRED ITEMS:

-Your MS must include a complete "Additional information section" with the following 4 headings and content:

Competing Interests: A statement regarding competing interests. If there are no competing interests, a statement to this effect must be included. All authors should disclose any conflict of interest in accordance with journal policy.

Author contributions: Each author should take responsibility for a particular section of the study and have contributed to writing the paper. Acquisition of funding, administrative support or the collection of data alone does not justify authorship; these contributions to the study should be listed in the Acknowledgements. Additional information such as 'X and Y have contributed equally to this work' may be added as a footnote on the title page.

It must be stated that all authors approved the final version of the manuscript and that all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Dear Dr Lingle,

Re: JP-TR-2021-281952R1 "The LRRC family of BK channel regulatory subunits: potential roles in health and disease" by Vivian Gonzalez-Perez, Yu Zhou, Matthew A Ciorba, and Christopher J Lingle

I am pleased to tell you that your Topical Review article has been accepted for publication in The Journal of Physiology, subject to any modifications to the text that may be required by the Journal Office to conform to House rules.

PLEASE NOTE
There are some very minor corrections needed (see editor/reviewer comments below) - these can be attended to at proof stage.

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

This revised manuscript has satisfactorily addressed the original concerns raised by the referees. It would be an excellent review paper if the authors take consideration of the minor comments suggested by the referee. These include:

1. inconsistent positioning of LRRC subunit in summary figure and figure 1, which potentially causes confusion to readers;

2. inconsistent referencing of specific terms: e.g. Line 59: LRRC52 regulation of large conductance K+ channels (it would be better to use 'BK channels' for consistency); Line 64: that may impact LRR-dependent pathologies (would 'LRRC' instead of 'LRR' be better for consistency?); Line 380: LRR subunit regulation of channels (would 'LRRC subunit' be better for consistency?); Line 567: high voltage-activated (it needs to be rephrased); Font size in page 30 and 31 is not consistent.

Senior Editor:

Thank you for the revisions. There are a couple of minor issues that should be dealt, but can be done in the proofs.

I look forward to reading your article In Press.

REFEREE COMMENTS

Referee #1:

The authors have greatly improved their review and they have addressed all of my concerns.

Referee #2:

The authors have adequately addressed my concern including new Figures and graphical abstract.

As indicated in my original review, and as authors reiterate, some aspects are more speculative but this is the advantage of this review in that it has the potential to highlight areas of physiology in which these channels may play an important role that have received relatively little attention to date.

I have just 2 minor issues:

1) Positioning of LRRC compared to BK tetramer is displayed differently between Summary Figure and Figure 1 - to avoid confusion a consistent approach should be taken

2) There are still inconsistencies in referencing - in particular flipping between abbreviated and full length journey names etc. This needs to be looked at carefully.

1st Confidential Review **07-Dec-2021**