BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site

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August 24, 2022

Dr. Jiusheng Yan
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Re: 202213237

Dear Dr. Yan,

Thank you for submitting your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" to JGP. Your manuscript has now been seen by 3 reviewers, whose comments are appended below. You will see that all three reviewers find observations in your paper of interest and of potential importance. I agree. However, they have raised multiple concerns that should be addressed prior to further consideration of the manuscript at JGP. We think these requests, if done, will substantially strengthen the paper and increase its potential impact. Below, I summarize what are the major issues that need to be addressed, some of which were discussed among the reviewers and myself during a Reviewer Consultation Session. Some of these comments are rather extensive, but the hope is that these comments may help provide a clear understanding of the concerns.

1. Both Revs. #2 and #3 feel that it is important that additional examples of raw current traces be included along with the graphs and I agree.

2. Both the Methods and Figure Legends are lacking key experimental details. This would include info on amplifier used, sampling rate, filtering, was there a particular time point for tail current measurements. Some figure legends lack info on peptide concentration, and the method of perfusion is inadequately described. How long were applications of different peptide concentrations in order to ensure steady-state? You will note other such queries in the Reviews below, so please check the Method section to be sure all necessary information to reproduce the expts is provided.

3. As mentioned by Rev. #1, the issue of whether gamma1 even assembles with the BK-TM construct needs to be addressed, along with other constructs were no functional effect of gamma1 might arise from lack of assembly. Given that this is routinely done in your lab, this should pose no problem. Whatever the outcome of that result, it won't impact on the significance of the paper, but is important information. Given that the triple mutation also appears to reduce gamma affinity (perhaps), it would not be surprising if gamma1 does not coassemble with BK-TM.

4. Also pertinent to Fig. 1, the reviewers had concerns about the apparent Ca-dependence of the gamma1 effects, given that 10 micromolar is only a little above the effect Kd for activation. The data presented leave open the question of the basis for this gamma effect. Does gamma reduce the maximal Ca-dependent gating shift, or is gamma simply shifting the apparent Kd? Using something like 100 micromolar Ca (or a series of Ca concentrations) would better address this. Is this necessary for the points your paper are trying to make? We do think it is important in terms of developing an understanding of gamma and BK-Ca dependence, and certainly impacts on interpretation of your results with the 5D5N mutation. Because of coupling of the VSD and CTD, one might imagine that gamma acting on the TM might influence Ca-dependence and vice versa mutation of 5D5N might also impact on the behavior of the TM. Pertinent to the GV shifts, as you know, zFVh is generally considered a better indication of the energetics of a given shift. The Reviewers discussed this and feel that in this case Vh is probably the most appropriate "Data Descriptor Parameter" (as coined by one reviewer). However, it may be worth mentioning the limitations of Vh in the Methods. Although, in general, most of your GVs seem to involve fairly parallel shifts along the voltage axis, that doesn't apply to all panels (and it may become an issue particularly with more elevated Ca). For any additional experiments you do, it would only be necessary to focus on gamma1.

5. In Figure 3, a much more extensive evaluation of tail currents needs to be done including display of tail currents on a time base that readers can readily see and a sample GV derived from currents before and after peptide. Is there an unblocking hook in the tails, and where is the tail amplitude being measured? Also, In the example of 3A, the control outward currents at +140 mV appear to be almost twice the amplitude of the inward current at -120 mV. Such extreme rectification seems problematic and is not a feature of WT BK currents. Is there a bandwidth or sampling problem? Also, the issue was raised that work from the Aldrich lab (Li et al) and also other labs has shown that fast blocking mechanisms can lead to a slowing of deactivation. If the gamma peptides and TAT were acting similarly, that would lead to an increase in apparent tail amplitude, and an apparent gating shift, depending on how the tails are being measured. We think the 5D5N mutation argues that there really is a shift effect of the peptides, but Fig. 3 needs to more rigorously address the separation of block and activation. Also it may be worth considering that the panels in Fig. 3B-E may be compromised by the possibility that the outward current may reflect the simultaneous occurrence of both an activating effect and the blocking effect, given that you are not at saturating activation at

+140 mV and 0 Ca2+. As such, the time course difference may not be the optimal way of asserting they are two separate phenomena. Also, is there a reason for the somewhat unusual concentration scale on panels 3B,C, and G?

6. The peptide results including the test of electrostatic interactions, are certainly provocative, but have suffered from the attempt to examine all three gamma subunits, rather than focusing more in depth on gamma1. Do gating shifts depend exclusively on net basic charge? The one mutation of the gamma1 peptide is suggestive but doesn't allow a conclusion. Would a completely scrambled gamma1 peptide have essentially the same effect? Does sequence matter at all? Although the Reviewers and myself do not doubt that the peptide effects are likely to be mediated by electrostatics, it is typical in the pages of JGP that, in order to assert such a conclusion, control experiments assessing the effect of osmotic strength or low ion strength would be included.

7. Finally, there was a sense among reviewers that you have missed an opportunity to put together a more compelling discussion of your work. It is difficult to know exactly what to suggest. However, this might include a more measured assessment of the arguments for and against whether the peptides and gamma subunits are acting by the same mechanism and at the same site. Although you have touched upon that to some extent, the current presentation seems limited. My sense was that it could have been summarized more strongly what the similarities and differences between the peptide vs gamma subunit effects are. My take is that perhaps about 25-30% of the gamma1 effect involves a potential mechanism similar to that of the peptide, based on your observations that both 1 M K and 5D5N each only partially affects the gamma1 effect while completely precluding the peptide effect. Yet, it seems problematic that, in the presence of a full gamma1 gating shift, the gamma1 peptide can still produce essentially its full gating shift. Although there are probably many potential explanations of that observation, to me it suggests that the peptide shift effect is entirely independent of gamma1. I think it also remains an open question whether the effects of the 5D5N mutations really reflect a disruption of binding to that site, or some other indirect effect. In the Reviewer Consultation session, the topic was also raised that the TAT peptide of course intercalates into membranes and perhaps the gamma peptides might do the same, somehow altering coupling between the CTD and TM domains. Overall, here, I am simply pointing out that we feel the manuscript would benefit from some time spent on developing a more balanced Discussion of the complex set of phenomenology in your paper. At this point, our sense is that your results do not necessarily lend themselves to definitive conclusions, but are suggestive. You are certainly entitled to put forward your preferred explanations, but balanced consideration of other possibilities is welcome. It might also be useful for readers if you discussed whether, based on the BK channel structures, any of the charged arginines are likely to reach the calcium bowl, assuming a partially extended gamma1 C-terminus.

Although I have tried to highlight and elaborate on what we felt were the most substantive concerns, there are other points raised in the Reviewer comments below that need to be addressed. Overall, I would like to reiterate that there was considerable interest in the phenomenology in your paper.

We would be pleased to receive a suitably revised manuscript that addresses these concerns, which will be re-reviewed, most likely by some or all of the original referees. Based on the scope of the requested changes, we typically anticipate that the revision process will take no longer than 6 months. However, we understand you may need additional time to work on your resubmission to JGP. We therefore ask that you simply keep us informed as to a realistic submission timeline that is appropriate for your particular circumstances. In addition, please do not hesitate to contact me (via the editorial office) if you feel that a discussion of the reviewers' and editors' comments would be helpful.
Please submit your revised manuscript via the link below along with a point-by-point letter that details your responses to the editors' and reviewers' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). If the article is eventually accepted, it would include a 'revised date' as well as submitted and accepted dates. If we do not receive the revised manuscript within one year, we will regard the article as having been withdrawn. We would be willing to receive a revision of the manuscript at a later time, but the manuscript will then be treated as a new submission, with a new manuscript number.

Please pay particular attention to recent changes to our instructions to authors in sections: Data presentation, Blinding and randomization and Statistical analysis, under Materials and Methods, as shown here: https://rupress.org/jgp/pages/submission-guidelines#prepare. Re-review will be contingent on inclusion of the required information (including for data added during revision) and demonstration of the experimental reproducibility of the results (i.e., all experimental data verified in at least 2 independent experiments).

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Thank you for the opportunity to consider your manuscript.

Sincerely,

Christopher Lingle, Ph.D.

On behalf of Journal of General Physiology

Journal of General Physiology's mission is to publish mechanistic and quantitative molecular and cellular physiology of the highest quality; to provide a best-in-class author experience; and to nurture future generations of independent researchers.

Reviewer #1 (Comments to the Authors):

In the paper "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+ bowl site", Yan and colleagues explore the electrophysiological effects of calcium regulatory (RCK) domains of the BK channel on its subunit dependent modulation. The authors find that calcium binding to the 2 RCK domains of the channel differentially affect the subunit dependent shifts of the conductance voltage (GV) relationships, with the Ca-bowl (RCK2) site exhibiting a more pronounced effect. Synthetic peptides derived from positively charged C-terminal fragments of the subunits appear to block and modulate BK channel gating and the channel-peptide interaction is proposed to be at least partially electrostatic in nature. However, the modulation by peptides tethered to the subunit appears to involve non-electrostatic interactions as well. The experimental observations are important and the data is nicely illustrated. However, there are several aspects of the paper which could be improved. Below are some suggestions to strengthen the work.

The authors should consider performing (semi-)quantitative IP (or comparable) experiments to explore the biochemical association of γ subunit and BKα-TM - it would help discern if the loss of modulatory effects of the γ subunit is due to lack of association or lack of modulation. The same suggestion would apply to BK (D362A/D367A/5D5N) mutant and γ3 association.

As it stands, much of the data (in Figs.1 and 2) and their descriptions are presented in a very phenomenological way. It would be very useful to interpret the data in the context of established gating models of the BK channel. From channel biophysics standpoint, it is not entirely surprising that effects of the γ subunits are Ca dependent. The interdependence of the calcium and γ subunit effects on the BK channel could arise (at least partly) from their convergent allosteric effects on the channel pore. The authors should discuss this and explain why they seem to favor the possibility of a physical interaction between the C-terminal tail of γ and the RCK domains. The authors might also want to compare/contrast their observations with γ subunit regulation of pH dependence of the related Slo3 channel (Leonetti, et al. PNAS 2012). The authors also might consider exploring the effect of γ subunit on Cd2+ modulation (on intact WT BK channels) which appears to act via the RCK1 site- based on the data/hypothesis presented here, one would predict that the effect of γ subunit remains similar with and without Cd2+ ions.

The peptides used in this study seem to have 2 effects on BKα - gating modification and blocking. That mutations in the RCK2 site seem to selectively abolish modulation and not blocking (Fig. 5), suggest that the 2 effects of the peptides might be mechanistically different. Nevertheless, it would be very helpful if the authors very clearly described in the main text how the GV curves were obtained particularly in the presence of peptides (steady state vs tail currents). A voltage-dependent block of the peptides (arising due to charge of the peptides or an open-state preference) will affect the shifts of macroscopic GV curves because the normalizing conductances (Gmax¬) are different. Similarly, the I_tails and their kinetics will also be affected because of concurrent unblocking and channel closure. The authors should discuss these possibilities without which it is not really clear, how large the gating shifts caused by the peptides truly are. It would also help if the authors showed the deactivation kinetics of the channel in the presence of the peptides on an expanded time scale (particularly for Fig. 3F and G and also for Fig. 5E).

In experiments comparing the functional effects of 1M and 140mM KMeSO3, there is also a substantial difference in osmolarity of the 2 solutions. It might be useful to perform experiments with solutions of comparable osmolarity to support the claims.

The authors should cite Gonzales-Perez V, et al PNAS 2018 (Regulatory γ1 subunits defy symmetry in functional modulation of BK channels) particularly in context of Fig. 2A while describing the effect of γ1 subunits on the D362A/D367A/5D5N mutant.

In Table 1, while reporting the number of experimental replicates (n), I would request to clearly distinguish number of patches vs number of cells. This is an important point to consider because current data seem to indicate that the association between BK and γ subunits might be weak and contribute to cell-cell variabilities, despite the 1:1 ratio of the cDNAs maintained through the transfection strategy used in this study.

Reviewer #2 (Comments to the Authors):

In this work, Chen et al. propose that the intracellular C-terminal region of BK is required for BK channel modulation by the γ1 subunit. They discovered positively charged peptides to be BK channel modulators and proposed a role for the high-affinity Ca2+-bowl site in modulation by positively charged peptides and auxiliary subunits. The modulation by positively charged peptides is an interesting topic by itself. The work is of interest to BK channel researchers and the ion channel's research community in general. However, I have some major concerns that I believe must be addressed before accepting for publication in JGP.

Main concerns
1. My primary concern is the Ca2+ concentration used. The authors refer to the Ca2+ concentration used as 'high' throughout the text, but the concentration used was just 10 µM. I agree that concentration can cause a significant shift in the G-V curve, but it is actually about half the maximum shift caused by Ca2+ because the kD for the high affinity Ca2+ binding sites is in the same range as the concentration used. Here are some kD values found in the bibliography:
Horrigan and Aldrich (2002, JGP) estimated kD to be in the 7.4-11.2 µM range.
Cox et al. (1997, JGP) estimated kD as 10 µM for the closed state.
Carrasquel et al. (2015, JGP) estimated the apparent kD to be 9.9 µM.
Lorenzo et al. (2019, eLife) estimated the apparent kD to be 6 µM.
When the kD for the individual high-affinity Ca2+ binding sites was estimated, the reported values are roughly in the same range (kD(RCK1) = 13 - 24 M and kD(RCK2) = 3 - 5 M) (Sweet and Cox, 2008, JGP; Bao et al., 2002, JGP; Xia et al., 2002, Nature), with the lowest values reported by Lorenzo et al. In none of these cases can 10 M saturate either site.
Given that there is strong evidence in all of these studies that kD is allosterically changed by the voltage sensor domain or pore states, and that the presence of gamma could definitely change the state of the pore, I wonder if the different shift is due to a change in the kD of the different sites or the apparent kD.
If the Ca2+ concentration used is at least saturating to the two high affinity Ca2+ binding sites, the article's conclusions may be more robust.
2. I missed the representative records in all the figures but Figures 4 and 5. They should be added to improve clarity.
3. In figure 4, is the scale below the BKα representative record common to the rest of the representative records? What is the value of the vertical line?
4. In Figure 5, a zoom-in of the tails may aid in visualization.
5. The authors discovered that the positively charged C-terminal of gamma1-3 or the HIV TAT peptide cause a G-V shift to the left. Do the authors speculate that these peptides interact with the Ca2+ bowl? If this is the case, does the shift disappear when the peptides are tested on the Ca2+ bowl mutant? Can the authors rule out the possibility that these peptides interact with the membrane, altering the electric field profile? It is a reasonable hypothesis given that HIV TAT and other positively charged peptides are known to interact and even cross membranes.
6. It can be confusing for non-specialists what the authors mean by "shifts in the BK channel's voltage-gating toward hyperpolarizing direction". Perhaps more typical wording regarding the half activation voltage would be clearer.
7. In the first paragraph of the result, the V1/2 of BKα alone is mentioned, but it is not shown in Fig. 1A.

Minor concerns
1. Line 3 of Introduction: Should be written 'neuron' instead 'neutron'?
2. Some details should be explained in Methods: '1% penicillin and streptomycin' refers to 1% w/v or 1% dilution from a stock solution? If the latter, what was the stock concentration?
3. Also in Methods: Some details about the electrophysiology rig should be stated. What amplifier (200B?), filters, and AD converters were used? What were the sampling rate and the filter?

Reviewer #3 (Comments to the Authors):

General comments

Chen et al. set out to study how Slo1 gamma subunits and select positively charged peptides alter electrophysiological properties of heterologously expressed Slo1 BK channels. The experiments appear straightforward and the results are of good quality. I don't see anything wrong with the conclusions but I don't find the conclusions presented in the discussion section of this version particularly satisfying either.

• The study utilizes the macroscopic GV V1/2 value as the primary data description parameter, which could be influenced by multiple gating characteristics. One must wonder the exclusive reliance of this data description parameter provides enough insights about the underlying mechanisms. There are so many ways to alter those V1/2 values.

• The results presented do raise multiple follow-up questions. Some readers many wonder what may happen in WT/mutant heteromultimers. Others may want to whether/how the gamma C terminus approaches the Ca2+ bow sensor sites.

• The manuscript does show clearly that some short peptides containing positively charged residues noticeably alter functional characteristics of the Slo1 BK channel. The authors failed to say why the findings are important - I am sure that some readers would want to know. Is it possible that other divalent cation binding proteins are altered by short positively charged peptides?

Specific comments

• Page 3-4. "... "all-or-none"..." It is probably appropriate to cite Gonzalez-Perez et al. 2014 also.

• Fig. 1.
Others may disagree but I do think it is important to show some raw/primary data sweeps somewhere. After all, this the results

in Fig. 1A are fundamental to the whole study? Further, it is unclear how long the pulses were, how frequently the pulses were applied, leak/capacititive current corrections, etc.
Something is wrong with the BKalpha alone fit curves (green dashed curves) shown in B, C, an D (also in many other figures)? The curves appear to saturate at >1. Hopefully, this potential issues does not affect the mean V1/2 value presented.

• Fig. 2
There must be a better way of organizing all the GV curves (I hope). In Fig. 1B-D, the most pertinent comparison was between the two dashed curves and the two solid curves. Here I don't see any obvious logic/rationale for the colors and it took me a long time to go through each graph. Maybe filled symbols vs. open symbols, etc?

The authors should clearly define "Triple mutant".

(Also in other figures) I would like to see all the data points plotted whenever possible (instead of just the mean and SEM values).

I don't see much (or any) value in doing null hypothesis testing in this study. But if the authors really want to show the P-values for some reason, please show the exact values instead of showing asterisks.

• P6 "...about 43 and 75 mV..."
It may be good to state explicitly that the gamma 1 number is from the two component fitting procedure here.

• P7 "... indicates in increase in the channel's open probability ..."
This may be true. But the authors should discuss the possibility that the single-channel current size may be affected. If you screen some of the negative charges near the channel mouth, it is conceivable that the unitary current size may change?

• Fig. 3
The authors should show the tail currents on a faster time scale so that the readers could verify that the tail currents kinetics do slow down.
How reversible are the peptide effects? Also see below.
Ideally different concentrations of the peptides could/should be investigated so that some inferences about the concentration dependence of the "on" and "off" time courses can be made; more mechanistic inferences, such as the number of peptides required for the effects observed, can be made. I realize that this may be difficult because the peptides appear to have multiple effects.
I am not sure exactly how the GV curve data are constructed. Are the data with peptides normalized to the control/no peptide data or are they normalized within themselves? What is "Gmax"? I am assuming that all the data are presented are paired data sets - before peptide and after peptides.

• P8 "... concentration of 20 μM ..."
See above about the concentration dependence.

• Fig. 4, comparison of V1/2 values, P8
As the authors describe (based on the results in Fig. 3), the gamma1 peptide has multiple and complicated effects on the channel. What do the V1/2 values with the peptide tell us? I don't think it is clear. Changes in V1/2 from the tail current measurements by the peptide may or may not reflect the "voltage dependence of the channel activation" (P8). Either the authors should somehow tease out the activation effect somehow or put a clear disclaimer/qualifier?
The TAT peptide has more presumably positively charged residues. It should have produced a bigger shift?

• P8 "...to be related to the positive charges..."
Or simply the peptide size/length?

It does not look like the effects depend on the peptide sequence that much then?

• Fig. 5
Perhaps show the original sweeps in a supplementary figure or something?
Somehow, the gamma2 peptide effect is less affected by the 5D5N mutation? Any speculation?

• Fig. 6
Some illustrative traces are in order here.
Are the effects other than the "gating/activation" effects diminished with the 1 M KMeSO3 solution?
I expect that many readers would like to see results with a lower ionic strength solution also. The decrease in the Debye-Hückel length from the normal solution to the 1 M solution used is not expected to be that much. A much bigger change is expected if a (very) low ionic-strength solution is used
Similar ionic-strength experiments with 5D5N are clearly in order.
Perhaps a few osmolarity control experiments?

• P9 "... in different manners..."
Clearly identical. They could still be mediated by electrostatic interactions - different ranges/scales? Manipulations of bulk ionic strengths may or may not alter the phenomena you want to study.

• P10 "... may affect BK channels' voltage dependence of channel..."
Would this really happen? When the peptide is applied from the extracellular side, the intracellular concentration may not be high enough to do anything. The authors could easily check this using whole-cell measurements.

Responses to reviewers' comments

Thank you for submitting your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" to JGP. Your manuscript has now been seen by 3 reviewers, whose comments are appended below. You will see that all three reviewers find observations in your paper of interest and of potential importance. I agree. However, they have raised multiple concerns that should be addressed prior to further consideration of the manuscript at JGP. We think these requests, if done, will substantially strengthen the paper and increase its potential impact. Below, I summarize what are the major issues that need to be addressed, some of which were discussed among the reviewers and myself during a Reviewer Consultation Session. Some of these comments are rather extensive, but the hope is that these comments may help provide a clear understanding of the concerns.

Response: We greatly appreciate the editor and reviewers' thorough, thoughtful, and constructive reviews of this manuscript. We added new plots and data and thoroughly revised the manuscript to address the concerns.

1. Both Revs. #2 and #3 feel that it is important that additional examples of raw current traces be included along with the graphs and I agree.

Response: Additional examples of raw current traces were included in the figures, see Figures 1D, 2A, 4E, 5A, and 6A.

2. Both the Methods and Figure Legends are lacking key experimental details. This would include info on amplifier used, sampling rate, filtering, was there a particular time point for tail current measurements. Some figure legends lack info on peptide concentration, and the method of perfusion is inadequately described. How long were applications of different peptide concentrations in order to ensure steady-state? You will note other such queries in the Reviews below, so please check the Method section to be sure all necessary information to reproduce the expts is provided.

Response: We have added details, including instruments, sampling rate, filtering, leak subtraction, tail current measurements, and peptide concentrations and application methods in the Methods and related Figure Legend parts. For the peptide application and current recording, we usually recorded the current amplitude around 1 min after application of peptides, since the peptides effect reaches steady state in ~30s.

3. As mentioned by Rev. #1, the issue of whether gamma1 even assembles with the BK-TM construct needs to be addressed, along with other constructs were no functional effect of gamma1 might arise from lack of assembly. Given that this is routinely done in your lab, this should pose no problem. Whatever the outcome of that result, it won't impact on the significance of the paper, but is important information. Given that the triple mutation also appears to reduce gamma affinity (perhaps), it would not be surprising if gamma1 does not coassemble with BK-TM.

Response: We added the experiment of co-IP between BKα-TM-only construct and LRRC26 subunit. The result (Fig. 1C) showed that the BKα-TM-only construct was able to form complexes with LRRC26, as expected from our previous observation that the TM domain of LRRC26 dominates its BK channel-modulatory function and association with the BK alpha subunit. As such, we feel there is no need to perform co-IP between the Ca^{2+}-binding mutants and the gamma1 subunit. We added a little discussion about this observation in the result part (lines 169-171).

4. Also pertinent to Fig. 1, the reviewers had concerns about the apparent Ca-dependence of the gamma1 effects, given that 10 micromolar is only a little above the effect Kd for activation. The data presented leave open the question of the basis for this gamma effect. Does gamma reduce the maximal Ca-dependent gating shift, or is gamma simply shifting the apparent Kd? Using something like 100 micromolar Ca (or a series of Ca concentrations) would better address this. Is this necessary for the points your paper are trying to make? We do think it is important in terms of developing an understanding of gamma and BK-Ca dependence, and certainly impacts on interpretation of your results with the 5D5N mutation. Because of coupling of the VSD and CTD, one might imagine that gamma acting on the TM might influence Ca-dependence and vice versa mutation of 5D5N might also impact on the behavior of the TM. Pertinent to the GV shifts, as you know, zFVh is generally considered a better indication of the energetics of a given shift. The Reviewers discussed this and feel that in this case Vh is probably the most appropriate "Data Descriptor Parameter" (as coined by one reviewer). However, it may be worth mentioning the limitations of Vh in the Methods. Although, in general, most of your GVs seem to involve fairly parallel shifts along the voltage axis, that doesn't apply to all panels (and it may become an issue particularly with more elevated Ca). For any additional experiments you do, it would only be necessary to focus on gamma1.

Response: We agree that it remains unclear how Ca^{2+} and Ca^{2+}-bowl site neutralization affects BK channel modulation by the gamma subunits. Given that the 5D5N mutation on the Ca^{2+}-bowl also reduced the modulatory effect of the gamma subunits, we speculate that there is a mechanistic connection between these two observations, which led us to focus on the study of the interactions between the negatively charged Ca^{2+}-bowl and the gamma subunits' C-terminal positively charged peptides in this manuscript. We would like to leave the questions (how Ca^{2+} and Ca^{2+}-bowl site neutralization affects BK channel modulation by the gamma subunits) for future study as it is challenging to study because: 1) given the large shift in $V_{1/2}$ caused by the gamma1 subunit already, it is difficult to reliably measure the G-V in the presence of very high Ca^{2+} (the expected $V_{1/2}$ can be around -140 mV at 100 μM Ca^{2+}); 2) the impact of Ca^{2+} and 5D5N mutation on the BK channel modulation by the gamma subunit is significant but not large (limited to 30 to 50 mV), which can be difficult to accurately determine the underlying changes in Horrigan-Aldrich allosteric gating parameters; 3) without a 3D structure of the BKalpha/gamma complex, further study will unlikely produce a conclusive answer to the question.

Given the parallel shift in G-V curves observed in most experiments, we think that $V_{1/2}$ is probably the best indicator of the peptide's apparent effects, although $V_{1/2}$ alone doesn't tell the underlying mechanism. High calcium itself can result in an increase in z value compared to zero calcium recording conditions. As we are comparing WT and mutants at the same calcium conditions, we don't feel it is necessary to use $zFV_{1/2}$ for the difference. We are reluctant to use the $zFV_{1/2}$ (unless it is really

necessary) because the z value for BK channel is relatively small compared to many other voltage-gated K^+ channels and thus more prone to be affected by data quality and artifacts. We observed that the fitted z value can often vary significantly from patch to patch, even the currents are optimal and appear to be good in quality and we presume that it may be affected by how the excised membrane patch shapes on the recording pipette tip. Thus, the use of $zFV_{1/2}$ in free energy calculation can produce significant error if n number is not large enough to alleviate the variation.

We agree and had discussed on the possibility of indirect interactions of Ca^{2+} binding at the Ca^{2+}-bowl site and the gamma subunits via allosteric long-range protein conformational changes. However, it is hard to explain the 5D5N mutation in a similar manner. We have expanded the discussion about potential mechanisms (lines 354-379).

5. In Figure 3, a much more extensive evaluation of tail currents needs to be done including display of tail currents on a time base that readers can readily see and a sample GV derived from currents before and after peptide. Is there an unblocking hook in the tails, and where is the tail amplitude being measured? Also, In the example of 3A, the control outward currents at +140 mV appear to be almost twice the amplitude of the inward current at -120 mV. Such extreme rectification seems problematic and is not a feature of WT BK currents. Is there a bandwidth or sampling problem?

Also, the issue was raised that work from the Aldrich lab (Li et al) and also other labs has shown that fast blocking mechanisms can lead to a slowing of deactivation. If the gamma peptides and TAT were acting similarly, that would lead to an increase in apparent tail amplitude, and an apparent gating shift, depending on how the tails are being measured. We think the 5D5N mutation argues that there really is a shift effect of the peptides, but Fig. 3 needs to more rigorously address the separation of block and activation. Also it may be worth considering that the panels in Fig. 3B-E may be compromised by the possibility that the outward current may reflect the simultaneous occurrence of both an activating effect and the blocking effect, given that you are not at saturating activation at +140 mV and 0 Ca2+. As such, the time course difference may not be the optimal way of asserting they are two separate phenomena.

Also, is there a reason for the somewhat unusual concentration scale on panels 3B,C, and G?

Response: As suggested, we added an enlarged view of the tail currents with time scale bar. We appreciate pointing out of the apparent lower amplitude of the tail currents than expected. Given the very fast deactivation at -120 mV in the absence of Ca^{2+}, the low tail current amplitude was caused in part by the 2K low pass filter, which smoothed out the peak of the full tail currents. We looked at the raw data and found that the traces in Fig. 3A are not a good representative of the data as well because of some capacitance-induced spike that also canceled out some tail current. We repeated these experiments with 10K filtering and the amplitude of tail currents appear to be normal now (new Fig. 3A). We also updated Fig. 3B and C to include this new data. The frequency of the low pass filter should not affect the G-V determination from tail currents in this study as it affects all tail currents in the same manner. Therefore, new data with 10K filter have been done only for Fig. 3A-C and Fig. 5 (comparison of the tail currents between WT and 5D5N).

We agree that the difference in dose response and the time courses of outward currents blockade and tail current increase are suggestive and not conclusive for the independence of the blockade and activation. We added data in Fig 5 to compare the tail currents from the same patches before and after application of peptides for both WT and 5D5N mutant channels. The data showed that the 5D5N mutant's tail currents at –120 mV, unlike those of WT, were only slightly affected by peptide in both amplitude and kinetics although the outward currents of WT and 5D5N mutant were similarly blocked, suggesting that the blockade and activation (tail current changes) are not related. Although pore-blocking peptide could potentially cause slowing of deactivation and thus shift in G-V towards the negative voltage direction, such effects were not obvious or major for the used peptides in this study. We added this part of discussion in this revision (lines 335-342).

For experiments in Fig 3B, C, and G, we tested 0, 0.2, 1, 5, and 50 μM of the peptide in the beginning, and we found that the maximal effect was between 5 and 50 μM, then we tested two more concentrations (10 and 20 μM). Our purpose was to identify the optimal concentration of peptides to use for the study.

6. The peptide results including the test of electrostatic interactions, are certainly provocative, but have suffered from the attempt to examine all three gamma subunits, rather than focusing more in depth on gamma1. Do gating shifts depend exclusively on net basic charge? The one mutation of the gamma1 peptide is suggestive but doesn't allow a conclusion. Would a completely scrambled gamma1 peptide have essentially the same effect? Does sequence matter at all? Although the Reviewers and myself do not doubt that the peptide effects are likely to be mediated by electrostatics, it is typical in the pages of JGP that, in order to assert such a conclusion, control experiments assessing the effect of osmotic strength or low ion strength would be included.

Response: We think that the peptide sequence (order of charged residues) likely plays only a minor role because the peptide we used in this study are all different but showed similar effects. The γ2 and γ3 peptides have similar length and positively charges but difference sequences. Similarly, the γ1 and Tat peptides also have similar length and positively charges, but with very difference sequences. The effect of osmotic strength and low ionic strength are added in Figure 6A and C. We added discussion about this (lines 328-333).

7. Finally, there was a sense among reviewers that you have missed an opportunity to put together a more compelling discussion of your work. It is difficult to know exactly what to suggest. However, this might include a more measured assessment of the arguments for and against whether the peptides and gamma subunits are acting by the same mechanism and at the same site. Although you have touched upon that to some extent, the current presentation seems limited. My sense was that it could have been summarized more strongly what the similarities and differences between the peptide vs gamma subunit effects are. My take is that perhaps about 25-30% of the gamma1 effect involves a potential mechanism similar to that of the peptide, based on your observations that both 1 M K and 5D5N each only partially affects the gamma1 effect while completely precluding the peptide effect. Yet, it seems problematic that, in the presence of a full gamma1 gating shift, the gamma1 peptide can still produce essentially its full gating shift. Although there are probably many potential explanations of that observation, to me it suggests that the peptide shift effect is entirely independent of gamma1.

I think it also remains an open question whether the effects of the 5D5N mutations really reflect a disruption of binding to that site, or some other indirect effect. In the Reviewer Consultation session, the topic was also raised that the TAT peptide of course intercalates into membranes and perhaps the gamma peptides might do the same, somehow altering coupling between the CTD and TM domains. Overall, here, I am simply pointing out that we feel the manuscript would benefit from some time spent on developing a more balanced Discussion of the complex set of phenomenology in your paper.

At this point, our sense is that your results do not necessarily lend themselves to definitive conclusions, but are suggestive. You are certainly entitled to put forward your preferred explanations, but balanced consideration of other possibilities is welcome. It might also be useful for readers if you discussed whether, based on the BK channel structures, any of the charged arginines are likely to reach the calcium bowl, assuming a partially extended gamma1 C-terminus.

Response: We agree that the discussion can be improved, and the data are more suggestive than conclusive. We have expanded the discussion in terms of similarity and difference between gamma subunits and the peptides and the potential mechanisms (lines 343-379). We added more thoughts on the potential mechanisms including a possibility that electrostatic interactions might be involved in proper co-assembly of the alpha/gamma subunits during protein folding and maturation rather than a direct impact on channel gating, which can explain the observation.

Although I have tried to highlight and elaborate on what we felt were the most substantive concerns, there are other points raised in the Reviewer comments below that need to be addressed. Overall, I would like to reiterate that there was considerable interest in the phenomenology in your paper.

Christopher Lingle, Ph.D.
On behalf of Journal of General Physiology

Reviewer #1 (Comments to the Authors):

In the paper "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+ bowl site", Yan and colleagues explore the electrophysiological effects of calcium regulatory (RCK) domains of the BK channel on its subunit dependent modulation. The authors find that calcium binding to the 2 RCK domains of the channel differentially affect the subunit dependent shifts of the conductance voltage (GV) relationships, with the Ca-bowl (RCK2) site exhibiting a more pronounced effect. Synthetic peptides derived from positively charged C-terminal fragments of the subunits appear to block and modulate BK channel gating and the channel-peptide interaction is proposed to be at least partially electrostatic in nature. However, the modulation by peptides tethered to the subunit appears to involve non-electrostatic interactions as well. The experimental observations

are important and the data is nicely illustrated. However, there are several aspects of the paper which could be improved. Below are some suggestions to strengthen the work.

The authors should consider performing (semi-)quantitative IP (or comparable) experiments to explore the biochemical association of γ subunit and BKα-TM - it would help discern if the loss of modulatory effects of the γ subunit is due to lack of association or lack of modulation. The same suggestion would apply to BK□(D362A/D367A/5D5N) mutant and γ3 association.

Response: As suggested, we added Co-IP experiments to explore the physical association of the γ1 subunit and BKα-TM truncation. The result (Fig. 1C) showed that the BKα-TM-only construct remained capable in forming a complex with LRRC26, which is expected from our previous observation that the TM domain of LRRC26 dominates its BK channel-modulatory function and association with the BK alpha subunit. As such, we feel there is no need to perform co-IP between the Ca^{2+}-binding mutants and the gamma1 subunit.

As it stands, much of the data (in Figs.1 and 2) and their descriptions are presented in a very phenomenological way. It would be very useful to interpret the data in the context of established gating models of the BK channel. From channel biophysics standpoint, it is not entirely surprising that effects of the γ subunits are Ca dependent. The interdependence of the calcium and γ subunit effects on the BK channel could arise (at least partly) from their convergent allosteric effects on the channel pore. The authors should discuss this and explain why they seem to favor the possibility of a physical interaction between the C-terminal tail of γ and the RCK domains. The authors might also want to compare/contrast their observations with γ subunit regulation of pH dependence of the related Slo3 channel (Leonetti, et al. PNAS 2012). The authors also might consider exploring the effect of γ subunit on Cd2+ modulation (on intact WT BK channels) which appears to act via the RCK1 site- based on the data/hypothesis presented here, one would predict that the effect of γ subunit remains similar with and without Cd2+ ions.

Response: We agree and had speculated the possibility of indirect interactions of Ca^{2+} binding at the Ca^{2+}-bowl site and the gamma subunits via allosteric long-range protein conformational changes. However, it is hard to explain the 5D5N mutation in a similar manner. We have expanded the discussion about potential mechanisms (lines 354-379). We added a sentence for divalent ions that are directly related to the Ca^{2+}-bowl site (lines 333-335). Because our discussion is limited to the Ca^{2+}-bowl site, we could not find a good way to include the pH dependence of Slo3 channels.

The peptides used in this study seem to have 2 effects on BKα - gating modification and blocking. That mutations in the RCK2 site seem to selectively abolish modulation and not blocking (Fig. 5), suggest that the 2 effects of the peptides might be mechanistically different. Nevertheless, it would be very helpful if the authors very clearly described in the main text how the GV curves were obtained particularly in the presence of peptides (steady state vs tail currents). A voltage-dependent block of the peptides (arising due to charge of the peptides or an open-state preference) will affect the shifts of macroscopic GV curves because the normalizing conductances (Gmax¬) are different. Similarly, the I_tails and their kinetics will also be affected because of concurrent unblocking and channel closure. The authors should discuss these possibilities without which it is not really clear, how large the gating shifts caused by the peptides truly are. It would also help if the authors showed the deactivation kinetics of the

channel in the presence of the peptides on an expanded time scale (particularly for Fig. 3F and G and also for Fig. 5E).

Response: Given that the effects of peptides (20 μM) on the voltage-dependence of the BK channel activation reached steady state within ~30s, the data obtained after peptide application for ≥ 1 min were used for plotting the channels' G-V relationship. We added this sentence in the method (lines 130-131). We added enlarged views of the tail currents for the WT and 5D5N mutants before and after peptide application. As it is shown, the peptide's blocking effect on tail currents was not significant. Although pore-blocking peptide could potentially cause slowing of deactivation and thus shift in G-V towards the negative voltage direction, such effects were not obvious or major for the used peptides in this study. We added this part of discussion in this revision (lines 335-342).

In experiments comparing the functional effects of 1M and 140mM KMeSO3, there is also a substantial difference in osmolarity of the 2 solutions. It might be useful to perform experiments with solutions of comparable osmolarity to support the claims.

Response: We have completed the requested control experiments where the 140 mM and 1M KMeSO3 solutions had the same high (1650 mOsm/kg) osmolarity and as shown in Figure 6C, osmolarity alone has little effect on the G-V relationship.

The authors should cite Gonzales-Perez V, et al PNAS 2018 (Regulatory γ1 subunits defy symmetry in functional modulation of BK channels) particularly in context of Fig. 2A while describing the effect of γ1 subunits on the D362A/D367A/5D5N mutant.

Response: Citation to this paper is added (line 184).

In Table 1, while reporting the number of experimental replicates (n), I would request to clearly distinguish number of patches vs number of cells. This is an important point to consider because current data seem to indicate that the association between BK□ and γ subunits might be weak and contribute to cell-cell variabilities, despite the 1:1 ratio of the cDNAs maintained through the transfection strategy used in this study.

Response: To be more accurate, we changed the sentence of definition of n number to "The number of recorded excised inside-out patches from different HEK293 cells" because only one patch was done for each cell.

Reviewer #2 (Comments to the Authors):

In this work, Chen et al. propose that the intracellular C-terminal region of BK is required for BK channel modulation by the γ1 subunit. They discovered positively charged peptides to be BK channel

modulators and proposed a role for the high-affinity Ca2+-bowl site in modulation by positively charged peptides and auxiliary subunits. The modulation by positively charged peptides is an interesting topic by itself. The work is of interest to BK channel researchers and the ion channel's research community in general. However, I have some major concerns that I believe must be addressed before accepting for publication in JGP.

Main concerns

1. My primary concern is the Ca2+ concentration used. The authors refer to the Ca2+ concentration used as 'high' throughout the text, but the concentration used was just 10 µM. I agree that concentration can cause a significant shift in the G-V curve, but it is actually about half the maximum shift caused by Ca2+ because the kD for the high affinity Ca2+ binding sites is in the same range as the concentration used. Here are some kD values found in the bibliography:

Horrigan and Aldrich (2002, JGP) estimated kD to be in the 7.4-11.2 µM range.

Cox et al. (1997, JGP) estimated kD as 10 µM for the closed state.

Carrasquel et al. (2015, JGP) estimated the apparent kD to be 9.9 µM.

Lorenzo et al. (2019, eLife) estimated the apparent kD to be 6 µM.

When the kD for the individual high-affinity Ca2+ binding sites was estimated, the reported values are roughly in the same range (kD(RCK1) = 13 - 24 M and kD(RCK2) = 3 - 5 M) (Sweet and Cox, 2008, JGP; Bao et al., 2002, JGP; Xia et al., 2002, Nature), with the lowest values reported by Lorenzo et al. In none of these cases can 10 M saturate either site.

Given that there is strong evidence in all of these studies that kD is allosterically changed by the voltage sensor domain or pore states, and that the presence of gamma could definitely change the state of the pore, I wonder if the different shift is due to a change in the kD of the different sites or the apparent kD. If the Ca2+ concentration used is at least saturating to the two high affinity Ca2+ binding sites, the article's conclusions may be more robust.

Response: For Figure 1, we showed that Ca^{2+} reduced the efficacy of the modulatory function of the BKγ1-3 subunits ($\Delta\Delta V_{1/2}$), and we speculated that there might be functional relationships between BKα and γ subunits in the intracellular side. We agree that using higher Ca^{2+} concentration can be more informative. However, it is challenging to measure the BK channel currents in the presence of both gamma1 subunits and very high Ca^{2+} because of the large shift caused by both. We need to hold at -200 mV to avoid basal leak currents at 10 µM Ca^{2+}. For the peptide, we added data of 1mM Ca^{2+} and the result showed that the effect of gamma1 peptide was almost abolished by 1mM Ca^{2+} (Figure 5C).

2. I missed the representative records in all the figures but Figures 4 and 5. They should be added to improve clarity.

Response: As suggested, additional examples of raw current traces were included in the figures, see Figures 1D, 2A, 4E, 5A, and 6A.

3. In figure 4, is the scale below the BKα representative record common to the rest of the representative records? What is the value of the vertical line?

Response: Yes. For Figure 4A, the scale below the BKα representative record is common to the rest of the representative records. The scale for the vertical line was added.

4. In Figure 5, a zoom-in of the tails may aid in visualization.

Response: Enlarged view of the tail currents are added (Figure 5B).

5. The authors discovered that the positively charged C-terminal of gamma1-3 or the HIV TAT peptide cause a G-V shift to the left. Do the authors speculate that these peptides interact with the Ca2+ bowl? If this is the case, does the shift disappear when the peptides are tested on the Ca2+ bowl mutant? Can the authors rule out the possibility that these peptides interact with the membrane, altering the electric field profile? It is a reasonable hypothesis given that HIV TAT and other positively charged peptides are known to interact and even cross membranes.

Response: Yes, we speculated that these peptides interact with the Ca^{2+} bowl, and our results (Figure 5C-E) showed that the shifts were greatly reduced or abolished when the peptides were tested on the Ca^{2+} bowl mutant. To largely rule out the possibility that HIV TAT interacts with BK channels in the membrane (effective from both intra- and extracellular sides), we added experiments by application of the HIV-1 TAT peptide from the extracellular side in both inside-out and whole cell recordings. We did not see the activating effect of the TAT peptide when it was applied extracellularly, suggesting that it didn't mediate its effects via an intramembrane action (Fig. 4F and G; lines 248-254).

6. It can be confusing for non-specialists what the authors mean by "shifts in the BK channel's voltage-gating toward hyperpolarizing direction". Perhaps more typical wording regarding the half activation voltage would be clearer.

Response: To facilitate the understanding, we tried to include changes in $V_{1/2}$ values whenever it is needed and minimized the use of "shifts in the BK channel's voltage-gating toward hyperpolarizing direction"

7. In the first paragraph of the result, the V1/2 of BKα alone is mentioned, but it is not shown in Fig. 1A.

Response: The G-V curve of BKα alone is added in Figure 1B.

Minor concerns
1. Line 3 of Introduction: Should be written 'neuron' instead 'neutron'?

Response: Spelling is corrected

2. Some details should be explained in Methods: '1% penicillin and streptomycin' refers to 1% w/v or 1% dilution from a stock solution? If the latter, what was the stock concentration?

Response: The description is changed to "100 I.U./mL penicillin and 100 µg/mL streptomycin" in the Methods parts.

3. Also in Methods: Some details about the electrophysiology rig should be stated. What amplifier (200B?), filters, and AD converters were used? What were the sampling rate and the filter?

Response: We have added details, including instruments, sampling rate, filtering, leak subtraction, tail current measurements, and peptide concentrations and application methods in the Methods and related Figure Legend parts.

Reviewer #3 (Comments to the Authors):

General comments

Chen et al. set out to study how Slo1 gamma subunits and select positively charged peptides alter electrophysiological properties of heterologously expressed Slo1 BK channels. The experiments appear straightforward and the results are of good quality. I don't see anything wrong with the conclusions but I don't find the conclusions presented in the discussion section of this version particularly satisfying either.

• The study utilizes the macroscopic GV V1/2 value as the primary data description parameter, which could be influenced by multiple gating characteristics. One must wonder the exclusive reliance of this data description parameter provides enough insights about the underlying mechanisms. There are so many ways to alter those V1/2 values.

Response: Please see our detailed responses (4^{th}) to the editor's listed concerns.

• The results presented do raise multiple follow-up questions. Some readers many wonder what may happen in WT/mutant heteromultimers. Others may want to whether/how the gamma C terminus approaches the Ca2+ bow sensor sites.

Response: We agree. As the gamma subunits have "all-or-none" effect, it is interesting to know how the WT/mutant heteromultimers behave in terms of BK channel modulation by gamma subunits. However, we considered this to be beyond the focus of this manuscript. We suspect that the peptides might not possess the all-or-none effect of gamma subunits as they likely interact more directly with the Ca^{2+} bowl site. Unfortunately, without a 3D structure of the BKalpha/gamma complex, we are not confident that speculation on the spatial relationship between the Ca^{2+}-bowl and the gamma subunits' C-termini would be helpful.

• The manuscript does show clearly that some short peptides containing positively charged residues noticeably alter functional characteristics of the Slo1 BK channel. The authors failed to say why the

findings are important - I am sure that some readers would want to know. Is it possible that other divalent cation binding proteins are altered by short positively charged peptides?

Response: We think our findings, even limited to BK channels, are important. The findings might be limited to the Ca^{2+}-bowl site, which is unique given that it possesses multiple negatively charged residues in a short sequence. We would prefer not to speculate if our findings are applicable to other calcium binding proteins.

Specific comments

• Page 3-4. "... "all-or-none"..." It is probably appropriate to cite Gonzalez-Perez et al. 2014 also.

Response: The citation of "Gonzalez-Perez et al. 2014" is added.

• Fig. 1.
Others may disagree but I do think it is important to show some raw/primary data sweeps somewhere. After all, this the results in Fig. 1A are fundamental to the whole study? Further, it is unclear how long the pulses were, how frequently the pulses were applied, leak/capacititive current corrections, etc. Something is wrong with the BKalpha alone fit curves (green dashed curves) shown in B, C, an D (also in many other figures)? The curves appear to saturate at >1. Hopefully, this potential issues does not affect the mean V1/2 value presented.

Response: Additional examples of raw current traces were included in the figures, see Figures 1D, 2A, 4E, 5A, and 6A. We have added details, including instruments, sampling rate, filtering, leak subtraction, tail current measurements, and peptide concentrations and application methods in the Methods and related Figure Legend parts. We appreciate your pointing out the issue that curves appear to saturate at >1. We rechecked the data and realized that some data was not properly normalized to the maximal current and this resulted in a Gmax>1. We apologise for this error and we have re-plotted the correctly normalized data to resolve this issue. This didn't alter the $V_{1/2}$ of the data presented.

• Fig. 2
There must be a better way of organizing all the GV curves (I hope). In Fig. 1B-D, the most pertinent comparison was between the two dashed curves and the two solid curves. Here I don't see any obvious logic/rationale for the colors and it took me a long time to go through each graph. Maybe filled symbols vs. open symbols, etc?

Response: As suggested, we improved clarity of the figures by using filled symbols vs. open symbols to show the effects of BKα mutants with/without γ subunits and different colors for different mutants. The reference G-V curves already shown in previous figures are presented with dashed lines.

The authors should clearly define "Triple mutant".

Response: The "Triple mutant" is now defined in the legend.

(Also in other figures) I would like to see all the data points plotted whenever possible (instead of just the mean and SEM values).

Response: We added individual data points.

I don't see much (or any) value in doing null hypothesis testing in this study. But if the authors really want to show the P-values for some reason, please show the exact values instead of showing asterisks.

Response: We agree that null hypothesis testing is not needed. We removed p-values.

• P6 "...about 43 and 75 mV..."
It may be good to state explicitly that the gamma 1 number is from the two component fitting procedure here.

Response: We added "(fitted with a double Boltzmann)" for 43 mV.

• P7 "... indicates in increase in the channel's open probability ..."
This may be true. But the authors should discuss the possibility that the single-channel current size may be affected. If you screen some of the negative charges near the channel mouth, it is conceivable that the unitary current size may change?

Response: As the tail currents were recorded at the same negative voltages, the single channel conductance should be affected by blockade in the same manner in terms of driving force. Therefore, this is a concern (above) only if: 1) there is significant blockade of tail currents by the peptide and together 2) the pre-depolarization to different voltages has different blockade effects on tail currents. However, as shown in Fig 5B, the blockade of the tail currents even after a pre-depolarization to +240 mV (most favorable blockade of outward currents) was not significant, suggesting that a change in unitary current was unlikely,

• Fig. 3

The authors should show the tail currents on a faster time scale so that the readers could verify that the tail currents kinetics do slow down.

Response: Enlarged view of the tail currents is added in Figure 3A.

How reversible are the peptide effects? Also see below.
Ideally different concentrations of the peptides could/should be investigated so that some inferences about the concentration dependence of the "on" and "off" time courses can be made; more mechanistic inferences, such as the number of peptides required for the effects observed, can be made. I realize that this may be difficult because the peptides appear to have multiple effects.

• P8 "... concentration of 20 μM ..."
See above about the concentration dependence.

Response: We added the data of the γ1 peptide wash-off (Fig. 3H-J). The channel-activating effect was reversible as the effect can be washed away in 5 min while the channel-blockade effect largely remained after 5 min washing. This result provides additional support that the peptides' two effects are largely independent and involves different mechanism.

I am not sure exactly how the GV curve data are constructed. Are the data with peptides normalized to the control/no peptide data or are they normalized within themselves? What is "Gmax"? I am assuming that all the data are presented are paired data sets - before peptide and after peptides.

Response: For G-V plot from tail currents, the normalized conductance (G) was obtained by normalization of the tail current peak amplitudes to the maximal amplitude within the same set of tail currents. If not saturated to the maximum even at very positive voltages, further normalization was achieved by fitting the data with Boltzmann function. The data presented was obtained from paired (before and after peptide application on the same patch) and unpaired (added peptide in bath solution; with/without peptides were from different patches) experiments, but we did not see difference in the averaged results between these, so we pooled the data.

• Fig. 4, comparison of V1/2 values, P8
As the authors describe (based on the results in Fig. 3), the gamma1 peptide has multiple and complicated effects on the channel. What do the V1/2 values with the peptide tell us? I don't think it is clear. Changes in V1/2 from the tail current measurements by the peptide may or may not reflect the "voltage dependence of the channel activation" (P8). Either the authors should somehow tease out the activation effect somehow or put a clear disclaimer/qualifier?

Response: We added the peptide wash-off data (Fig. 3H-J). It provides additional evidence that the channel-activating and blockade effects were well separated. We added data in Fig 5 to compare the tail currents from the same patches before and after application of peptides for both WT and 5D5N mutant channels. The data showed that the amplitude and kinetics of the 5D5N mutant's tail currents at –120

mV, were only slightly affected by the peptide, in contrast to its effects on the WT tails. However, the outward currents of both WT and 5D5N mutant were similarly blocked, suggesting that the blockade and activation effects (tail current changes) were not related. Although pore-blocking by the peptide could potentially slow deactivation and thus shift the G-V negatively, such effects were not obvious or major for the peptides used in this study. We have included these points as part of the revised discussion (lines 335-342).

About $V_{1/2}$, please see our detailed responses (4^{th}) to the editor's listed concerns.

The TAT peptide has more presumably positively charged residues. It should have produced a bigger shift?

Response: According to the sequences, the TAT peptide has two more Lysine than the γ1 peptide, but we didn't see any greater effect of these additional charges on $V_{1/2}$.

• P8 "...to be related to the positive charges..."
Or simply the peptide size/length?
It does not look like the effects depend on the peptide sequence that much then?

Response: We think that the order of the charged residues in the peptide only plays a minor role because irrespective of their sequence, the γ1- γ3 peptides all showed similar effects. In addition, the sequence of charged residues in γ1 and Tat peptides are very different, but they have similar lengths and positive charges and yet have similar effects on the V1/2. We have added these points to the discussion (lines 328-333).

• Fig. 5
Perhaps show the original sweeps in a supplementary figure or something?
Somehow, the gamma2 peptide effect is less affected by the 5D5N mutation? Any speculation?

Response: Examples of current traces were added in the figures. We added a speculation "The residual responses of the 5D5N mutant channels to the γ peptides could be due to the remaining 3 negatively charged residues (D892, D900 and E902) of the Ca^{2+}-bowl region." (line 285-286).

• Fig. 6
Some illustrative traces are in order here.
Are the effects other than the "gating/activation" effects diminished with the 1 M KMeSO3 solution?
I expect that many readers would like to see results with a lower ionic strength solution also. The decrease in the Debye-Hückel length from the normal solution to the 1 M solution used is not expected

to be that much. A much bigger change is expected if a (very) low ionic-strength solution is used
Similar ionic-strength experiments with 5D5N are clearly in order.
Perhaps a few osmolarity control experiments?

Response: We have added current traces. We added data (Figure 6C) to test the effects of high osmolarity (same ionic strength) and lower ionic strength (50 mM). Our results showed that neither high osmolarity nor lower ion strength on the intracellular side significantly affected the peptide-induced shift of the $V_{1/2}$. They both decreased outward currents as expected. We didn't see the need of high ionic strength for 5D5N mutant as the peptide doesn't have much effect on the mutant. The high ionic strength itself did induce some shift in $V_{1/2}$. However, it is beyond the scope of this work to study the underlying mechanism and thus not discussed.

• P9 "... in different manners..."
Clearly identical. They could still be mediated by electrostatic interactions - different ranges/scales?
Manipulations of bulk ionic strengths may or may not alter the phenomena you want to study.

Response: We added more thoughts (lines 349-374) on the potential mechanisms including a possibility that electrostatic interactions might be involved in assembly of the alpha/gamma subunits during protein folding and maturation rather than a direct impact on channel gating, which can explain the observation (lines 369-378).

• P10 "... may affect BK channels' voltage dependence of channel..."
Would this really happen? When the peptide is applied from the extracellular side, the intracellular concentration may not be high enough to do anything. The authors could easily check this using whole-cell measurements.

Response: We performed whole cell recording as suggested. Although application of 20 μM Tat peptide on the extracellular side had little effect, some effect was observed when a much higher (100 μM) peptide concentration was used (Figure 4G).

February 13, 2023

Dr. Jiusheng Yan
The University of Texas MD Anderson Cancer Center
Anesthesiology & Perioperative Medicine
1515 Holcombe Blvd.
Houston, Texas 77030

Re: 202213237R1

Dear Jiusheng,

Thank you for submitting your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" to JGP. Your manuscript has now been seen by the 3 original reviewers, whose comments are appended below. You will see that the reviewers feel that the new additions to figures have addressed many of their earlier concerns. However, you will note in their comments that several remaining issues remain. Most importantly, as clarified in a Reviewer Consultation session, a major concern is that many statements in the manuscript, that imply that gating shifts produced by isolated peptides and intact gamma subunits share similar mechanistic underpinnings, are excessively speculative and are not supported by the preponderance of the data (these concerns are summarized more explicitly below). You are certainly entitled to share your preferred explanations for phenomenology, but it needs to be constrained by rigorous consideration of what the data actually allow. You will note that each reviewer provided a list of issues or small fixes that require attention. Below I highlight some of the major points that remain to be addressed.

1. There is concern that the reduction in the gamma1 gating shift at 10 uM Ca is not being rigorously evaluated. I took the liberty of using the HA allosteric gating constants from Yan and Aldrich, 2010, along with C and K constants from Horrigan and Aldrich, 2002. For no LRRC26 (D=21), Vh at 0 Ca2+ was 164 mV and at 10 uM was 32 mV, for delta-Vh of -132 mV. With LRRC26 (D=412), Vh at 0 Ca2+ was 6 mV, and at 10 uM was -88 mV for a delta-Vh=-94. Therefore, 10 uM appears to cause a -38 reduction in the Vh shift. Given that there is uncertainty about such constants, it seems not implausible that most of the effects of elevated Ca are a simple consequence of the predictions of the HA model, and not reflecting screening by Ca of any site of gamma subunit action. These considerations also may pertain to the modest reduction of the gamma subunit gating shifts by mutation of the Ca bowl, although the difference that is reported between Ca bowl mutation and RCK1 mutation implies an interesting difference in the role of RCK1 and RCK2 on gamma subunit action. In short, this calls into question some of the assertions made in the paper and a rigorous evaluation of your results would take this into consideration.

2. The reviewers remain uncomfortable with the co-IP results. First, the BK(TM) lane shows two FLAG-tagged bands, but those are not explained. Second, Rev. #3 points out the necessity of providing more information about the specifics of the solubilization procedures during the IP. Finally, Rev. #2 is
not happy with the assumption that the result with BK(TM) would be similar to what would happen with the Ca binding mutants. At a minimum, the first two concerns need to be addressed.

3. The new version of Figure 3 and the inclusion of examples of currents greatly improves the manuscript and provides better validation of the peptide-induced gating shifts. However, Rev. #3 notes some concerning issues. Either the axis in Fig. 3G is labelled incorrectly, or the measured time constants do not match with the data in Fig. 3A or 3H. In addition, the amplitude of the tail currents following the steps to +140 mV normalized to those following step to +240 mV in Figure 5B imply a substantial gating shift in 5D5N relative to BK WT, that is not reflected in Fig. 5F where the fractional activation for each should be about the same.

4. The section in the Discussion concerned with Cell Penetrating Peptides (CPPs) contains unsubstantiated claims (around line 320). To my knowledge, the FDA has not approved any CPPs for therapeutic use. No citations are given to back up the claims. To date, CPPs appear to be potential tools and no more. It also may be difficult to ignore the chaotropic effects on membranes that such peptides can have (as suggested by Rev. #2), when considering their effects in animal models. Front. Pharmacol., 20 May 2020
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5. Based on the Consultation Session, all reviewers and myself are in agreement with the following summary of your work. "The work identifies a new pharmacological way of activating BK channels that is produced by high concentrations of free peptides with net positive charge. The effect can be abolished by Ca-bowl mutation, elevated Ca, and elevated ionic strength, supporting the idea that it may arise from electrostatic effects involving interactions of a given peptide with the RCK2 region or perhaps Ca-bowl residues. Given the lack of shared sequence among effective peptides, it is unlikely to involve specific binding. Tests of

whether the gating shift effects mediated by full-length gamma subunits involve similar effects on RCK2 suggest that the major effect of full-length gamma subunits is unlikely to involve a similar mechanism. Any apparent Ca-dependence of the gamma1-induced gating shift (at 10 uM) is likely explained by simple HA considerations. The peptide-induced gating shift (-45 mV) gating shift is not occluded by the presence of the gamma1-gating shift. High ionic strength does not affect the gamma1-induced gating shift. A perhaps 33% reduction of the gamma1 gating shift is produced by Ca-bowl mutation, but whether this shift might also arise because of expectations of changes in HA parameters or something related to the peptide actions is not evaluated. At most, one might suggest that up to 33% of the gamma1 gating shift might involve an effect similar to that mediated by the peptides.

In sum, we would suggest that the results identify a new pharmacological means of activating BK channels by high concentrations of basic peptides, but it unlikely to be related to the major mechanism underlying gamma subunit induced gating shifts."

Although the current version of your manuscript does make many of these points regarding differences between peptide effects and intact gamma subunit effects, there remain many statements that would tend to imply to readers that the mechanism underlying the peptide effects accounts for a major component of the gamma subunit effects. We do not find any arguments for how that might occur to be well-grounded, which tends to run counter to the normal expectations for JGP. Having said that, we do feel the results will be of interest to readers.

We would be pleased to receive a suitably revised manuscript that addresses these concerns, which will be re-reviewed, most likely by some or all of the original referees. Based on the scope of the requested changes, we typically anticipate that the revision process will take no longer than 6 months, however, we understand you may need additional time to work on your resubmission to JGP. We therefore ask that you simply keep us informed as to a realistic submission timeline that is appropriate for your particular circumstances. In addition, please do not hesitate to contact me (via the editorial office) if you feel that a discussion of the reviewers' and editors' comments would be helpful.

Please submit your revised manuscript via the link below along with a point-by-point letter that details your responses to the editors' and reviewers' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). If the article is eventually accepted, it would include a 'revised date' as well as submitted and accepted dates. If we do not receive the revised manuscript within one year, we will regard the article as having been withdrawn. We would be willing to receive a revision of the manuscript at a later time, but the manuscript will then be treated as a new submission, with a new manuscript number.

Please pay particular attention to recent changes to our instructions to authors in sections: Data presentation, Blinding and randomization and Statistical analysis, under Materials and Methods, as shown here: https://rupress.org/jgp/pages/submission-guidelines#prepare. Re-review will be contingent on inclusion of the required information (including for data added during revision) and demonstration of the experimental reproducibility of the results (i.e., all experimental data verified in at least 2 independent experiments).

Please note, JGP now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts (when applicable). This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. If your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JGP, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jgp/pages/submission-guidelines#revised

When revising your manuscript, please be sure it is a double-spaced MS Word file and that it includes editable tables, if appropriate.

Please submit your revised manuscript via this link:
Link Not Available

Thank you for the opportunity to consider your manuscript.

Sincerely,

Christopher Lingle, Ph.D.

On behalf of Journal of General Physiology

Journal of General Physiology's mission is to publish mechanistic and quantitative molecular and cellular physiology of the highest quality; to provide a best-in-class author experience; and to nurture future generations of independent researchers.

Reviewer #1 (Comments to the Authors):

In their revised manuscript Yan and colleagues have improved upon the clarity of the manuscript, performed IP experiments to support their interesting observations and significantly improved figures by providing example raw data. I would like to maintain though that the discussion of the mechanism underlying peptide action still remains a little mundane. It would be more interesting to tie in the observations with what is known about BK gating, particularly with respect to widely used and accepted gating models. Instead the authors have chosen to go into territory which appear to be complete tangents.
• In relating tat peptide effect to some physiological relevance (effect of drug delivery mechanisms or consequences of HIV infection) - a lot would depend on concentration of the "free peptide". This point of discussion seemed particularly jarring! Tat peptides, at the concentrations use (20-200uM) would very likely interact with lipids and that might be potentially more relevant topic of discussion here (although still speculative).
• The comment: "electrostatic interaction is shielded from ionic strength changes in solution and strengthened if it occurs mainly within the low-dielectric protein medium, i.e., buried inside the protein complex." - I don't understand this, particularly in the context of BK where charge screening effect have been postulated to be a mechanism distinguishing IbTx and ChTx action on BK. Also, since BKα alone undergoes a large shift in high salt, but the effect of the γ1 peptide is essentially eliminated in high salt (Figs. 5C and 6B) it seems that high ionic strength is indeed inhibiting the γ1 peptide effect on the GV curve. I'm sorry for the trouble in understanding but I'm left a little confused here.
• I would also like point out that in the methods, using the phrase "co-translational assembly" is possibly a stretch. Simply connecting the 2 genes into a single mRNA simply ensures identical mRNA levels of the 2 genes but does nothing to ensure "co-translational assembly". It is unnecessary here.

Reviewer #2 (Comments to the Authors):

Chen et al. propose that "high" Ca2+ and charge neutralization of the Ca2+-bowl site reduce γ subunit efficacy in BK channel modulation. Intriguingly, they also report that positively charged peptides from the C-terminal positively charged regions of the γ subunits caused significant shifts in the G-V of the BK channel toward the hyperpolarization direction. Their findings also suggest that BK channel modulation by the γ1 subunit requires the intracellular C-terminal region of BK. The work has significantly improved since the initial version, and they have addressed the majority of my concerns, though I believe their claims are somewhat speculative and they have overlooked some concerns raised by other reviewers.
1. I disagree with the assertion that co-IP between Ca2+-binding mutants and gamma subunits is unnecessary.
2. The authors also failed to address some of the concerns raised by other reviewers, such as the need to investigate the effects of gamma1 on Cd2+ modulation of the wild-type channel.
3. I agree that experiments with saturating Ca2+ pose an experimental challenge that could not be completed in a reasonable amount of time. If the authors decide not to conduct those experiments, the statement "The efficacy of the γ subunits in BK channel modulation was reduced by high Ca2+" should be softened. I recommend simply avoiding the word "high" and being specific about the concentration used. Using a subjective term such as "high" can lead to misunderstandings. I would classify 10 μM as "intermediate" rather than "high," because it is nowhere near saturating. According to the authors' responses, "it remains unclear how Ca2+ and Ca2+-bowl site neutralization affects BK channel modulation by the gamma subunits," and I believe these limitations should be clearly stated in the manuscript.
4. The citation (Gonzalez-Pereza, 2018) (L189) appears to be incorrect.
5. While I agree that some of the concerns raised in the first review may take a long time to address experimentally, some statements in the manuscript are overly speculative and could be tested in a straightforward manner. For example, while the statement in L285-6 that "the residual responses of the 5D5N mutant channels to the γ peptides could be due to the remaining 3 negatively charged residues (D892, D900, and E902) of the Ca2+-bowl region" is speculative, as the authors stated in their responses to the reviewers. I consider that testing that possibility (that those three negatively charged residues are responsible for the residual response) is a straightforward experiment.

Reviewer #3 (Comments to the Authors):

General comments

The authors have revised the manuscript according to the suggestions and comments made earlier incorporating many sets of results.

Some issues need to be addressed properly or clarified. The most important item may be related to the protein solubilization protocol.

Specific comments
• L44 "... has particularly large..." Perhaps need an article here "a"?
• L58 "... over an exceptionally large". "range" (or something like that) is missing here?
• L66 "LRR" This has not been formally defined yet.
• L68 "voltage-dependent" may be better?
• L68 "intracellular juxta-membrane positively charged residue cluster regions". This mouthful (but I cannot offer any suggestion - sorry)
• L95 "co-transfection of the cDNA constructs". Maybe provide the weight ratio?
• L103 (and other places) "a whole cell configuration". Need a hyphen between "whole" and "cell"?
• L106 "Ca2+ free solution" A hyphen between "Ca2+" and "free"?
• L118-119 "the amplitudes of the tail currents...". Peak amplitudes?
• "Steady-state activation was expressed as ..." This means that each data set (e.g., with a peptide) was normalized to its own largest tail current size, correct? This should be explicitly stated. This issue is relevant for the results like those in Fig. 4A-D.
• L125 "the known amino acid sequence". "known" seems a bit odd to me. "amino-acid sequence"?
• L131 "30s". One space.
• L135 "After centrifugation". This needs to be expanded. Please see below.
• L145-146 "Unpaired...". I don't think null hypothesis testing is needed for the study described.
• L159 "Co-expression of ...". This was done using the fusion construct? It may be better to state this here (or at least in the figure legend).
• L160 "...voltage-dependence ...". Unclear whether this hyphen is required.
• L167-173 / Fig. 1C. To properly evaluate protein-protein interactions, the protein complexes must be fully solubilized, without membrane fragments. Full solubilization requires high-speed (e.g., 100,000 g) centrifugation runs. If the solubilization step is not complete, the proteins may appear complexed via residual membrane fragments. One extreme (trivial) example would be like this; two proteins are present in the same cell - without proper solubilization, they would appear complexed. The immunoprecipitation results may be suggestive but they do not "demonstrate" (L171). The authors need to provide more information about the centrifugation/solubilization step(s).
• Fig. 1C There seem to be two bands close together in each of the first two lanes. Why?
• Fig. 1H The ordinate label could use "mV"?
• Fig. 2E It is better to indicate that the gamma1 results are from the two-component fits in the legend.
• L205 / Fig. 3 The results in Fig. 3 are those without added Ca2+, right? It will easier for the readers to read this in the legend.
• Fig. 3 In E, the ordinate should start at 0 nA like in J. Are the results shown in Fig. 3E really "representative"? I see about a 40% increase in the peak inward tail current size. But the results in A and C suggest that there should be a 100% (or bigger) increase.
• Fig. 3F Do you really need null hypothesis testing here? I am not sure you do.
• Fig. 3G I think something is wrong the results or the ordinate label of the graph. Without Ca2+, Slo1 ionic current deactivation kinetics at -120 mV should not have a time constant value of 4 ms - this is way too slow. This should be closer to 0.1 - 0.5 ms?
• L239 "... to the abundance of positive charges". This is probably true but the possibility that it is all about the # residues/size is not totally excluded from the results presented alone.
• Fig. 5B and 5F I am confused about these two graphs or I am missing something. Fig. 5B BKalpha (5D5N) 140 mV sweeps. Black = without the peptide and orange is with the peptide. I see about a 50% increase in size. In Fig. 6D, at 140 mV, one is expected to see a bigger increase? Is the tail current shown a near outlier experiment?
• L281 "...increasing ionic strength to 1 M..." "... strength with 1 M..." may be better?
• L358 - 360. I am sorry but I read this sentence a few times, but I failed to understand what it is meant.
• "Although ... by an indirect allosteric mechanism". Why? Is it possible that the allosteric communication required is electrostatic in nature?
• Some format clean-up may be required in the References section.

Response to the reviewers' comments

Re: 202213237R1

Editor's comments
Thank you for submitting your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" to JGP. Your manuscript has now been seen by the 3 original reviewers whose comments are appended below. You will see that the reviewers feel that the new additions to figures have addressed many of their earlier concerns. However, you will note in their comments that several remaining issues remain. Most importantly, as clarified in a Reviewer Consultation session, a major concern is that many statements in the manuscript, that imply that gating shifts produced by isolated peptides and intact gamma subunits share similar mechanistic underpinnings, are excessively speculative and are not supported by the preponderance of the data (these concerns are summarized more explicitly below). You are certainly entitled to share your preferred explanations for phenomenology, but it needs to be constrained by rigorous consideration of what the data actually allow. You will note that each reviewer provided a list of issues or small fixes that require attention. Below I highlight some of the major points that remain to be addressed.

Response: Again, we greatly appreciate the editor and reviewers' thorough and constructive review of the revised manuscript. We have revised the manuscript accordingly.

1. There is concern that the reduction in the gamma1 gating shift at 10 uM Ca is not being rigorously evaluated. I took the liberty of using the HA allosteric gating constants from Yan and Aldrich, 2010, along with C and K constants from Horrigan and Aldrich, 2002. For no LRRC26 (D=21), Vh at 0 Ca2+ was 164 mV and at 10 uM was 32 mV, for delta-Vh of -132 mV. With LRRC26 (D=412), Vh at 0 Ca2+ was 6 mV, and at 10 uM was -88 mV for a delta-Vh=-94. Therefore, 10 uM appears to cause a -38 reduction in the Vh shift. Given that there is uncertainty about such constants, it seems not implausible that most of the effects of elevated Ca are a simple consequence of the predictions of the HA model, and not reflecting screening by Ca of any site of gamma subunit action. These considerations also may pertain to the modest reduction of the gamma subunit gating shifts by mutation of the Ca bowl, although the difference that is reported between Ca bowl mutation and RCK1 mutation implies an interesting difference in the role of RCK1 and RCK2 on gamma subunit action. In short, this calls into question some of the assertions made in the paper and a rigorous evaluation of your results would take this into consideration.

Response: We appreciate the suggestion that the Ca^{2+} effect on BK channel modulation by the γ subunits could be intrinsic property of the BK channel allosteric gating mechanisms. We didn't think about this before and we fully agree on this after HA modeling analysis. We added a plot of the simulated G-V curves (Fig. 7F). Consequently, we downplay the importance of this part of data by reorganizing the order of figures. However, this doesn't apply to the Ca^{2+}-bowl mutant because no Ca^{2+} (0 Ca^{2+}) is used in the experiment, and importantly, the mutation itself, unlike the γ1 subunit's impact on gating (potentially a drastic change in the D factor), had little effect on BK channel voltage-gating (i.e., no major effect on voltage-dependent gating parameters).

2. The reviewers remain uncomfortable with the co-IP results. First, the BK(TM) lane shows two FLAG-tagged bands, but those are not explained. Second, Rev. #3 points out the necessity of providing more information about the specifics of the solubilization procedures during the IP. Finally, Rev. #2 is not happy with the assumption that the result with BK(TM) would be similar to what would happen with the Ca binding mutants. At a minimum, the first two concerns need to be addressed.

Response: The upper band (between 50 and 75 kDa) of the FLAG-tagged BKα (TM-only) construct should be dimeric aggregation form, which can be common for membrane protein on SDS-PAGE. The dimeric band can also be observed in BKα wild type samples. We added the centrifugation speed to the method. We added new data to show the co-IP between the γ1 subunit and the BKα 5D5N mutant. As expected, we didn't see major reduction in their association.

3. The new version of Figure 3 and the inclusion of examples of currents greatly improves the manuscript and provides better validation of the peptide-induced gating shifts. However, Rev. #3 notes some concerning issues. Either the axis in Fig. 3G is labelled incorrectly, or the measured time constants do not match with the data in Fig. 3A or 3H. In addition, the amplitude of the tail currents following the steps to +140 mV normalized to those following step to +240 mV in Figure 5B imply a substantial gating shift in 5D5N relative to BK WT, that is not reflected in Fig. 5F where the fractional activation for each should be about the same.

Response: We are very much thankful for the reviewer's notice of the error in Fig. 3G. The Tau-related figures were incorrectly plotted and sorrily unnoticed by us. The plots were corrected now (now Fig. 2G and 2F). In Fig. 5B, the 140 mV tail currents (middle) were not displayed properly (enlarged view without scale bar). The issue was corrected now (now Fig. 4B).

4. The section in the Discussion concerned with Cell Penetrating Peptides (CPPs) contains unsubstantiated claims (around line 320). To my knowledge, the FDA has not approved any CPPs for therapeutic use. No citations are given to back up the claims. To date, CPPs appear to be potential tools and no more. It also may be difficult to ignore the chaotropic effects on membranes that such peptides can have (as suggested by Rev. #2), when considering their effects in animal models. Front. Pharmacol., 20 May 2020
Sec. Experimental Pharmacology and Drug Discovery
Volume 11 - 2020 | https://doi.org/10.3389/fphar.2020.00697

Response: We appreciate the factcheck and pointing this out! We agree the use of cationic cell–penetrating peptides is mostly a research tool so far and we have removed the word "therapeutic".

5. Based on the Consultation Session, all reviewers and myself are in agreement with the following summary of your work. "The work identifies a new pharmacological way of activating BK channels that

is produced by high concentrations of free peptides with net positive charge. The effect can be abolished by Ca-bowl mutation, elevated Ca, and elevated ionic strength, supporting the idea that it may arise from electrostatic effects involving interactions of a given peptide with the RCK2 region or perhaps Ca-bowl residues. Given the lack of shared sequence among effective peptides, it is unlikely to involve specific binding. Tests of whether the gating shift effects mediated by full-length gamma subunits involve similar effects on RCK2 suggest that the major effect of full-length gamma subunits is unlikely to involve a similar mechanism. Any apparent Ca-dependence of the gamma1-induced gating shift (at 10 uM) is likely explained by simple HA considerations. The peptide-induced gating shift (-45 mV) gating shift is not occluded by the presence of the gamma1-gating shift. High ionic strength does not affect the gamma1-induced gating shift. A perhaps 33% reduction of the gamma1 gating shift is produced by Ca-bowl mutation, but whether this shift might also arise because of expectations of changes in HA parameters or something related to the peptide actions is not evaluated. At most, one might suggest that up to 33% of the gamma1 gating shift might involve an effect similar to that mediated by the peptides.

In sum, we would suggest that the results identify a new pharmacological means of activating BK channels by high concentrations of basic peptides, but it unlikely to be related to the major mechanism underlying gamma subunit induced gating shifts."

Although the current version of your manuscript does make many of these points regarding differences between peptide effects and intact gamma subunit effects, there remain many statements that would tend to imply to readers that the mechanism underlying the peptide effects accounts for a major component of the gamma subunit effects. We do not find any arguments for how that might occur to be well-grounded, which tends to run counter to the normal expectations for JGP. Having said that, we do feel the results will be of interest to readers.

Response: We appreciate the summary and agreed findings of this work. We have now been clearer in language to indicate the difference in mechanisms of BK channel modulation by the positively charged peptides and the C-terminal positively charged regions of the γ subunits. It is not our intention to compare the mechanism of the synthetic peptides' actions with the whole mechanisms of BK channel modulation by γ subunits. To be clear, we now added phrases to define that the comparison is limited to the C-terminal positively charged region(s) of the γ subunit(s), as they contribute ~ 40 mV shift in $V_{1/2}$ that is similar to those produced by the synthetic peptides.

Reviewer #1 (Comments to the Authors):

In their revised manuscript Yan and colleagues have improved upon the clarity of the manuscript, performed IP experiments to support their interesting observations and significantly improved figures by providing example raw data. I would like to maintain though that the discussion of the mechanism underlying peptide action still remains a little mundane. It would be more interesting to tie in the observations with what is known about BK gating, particularly with respect to widely used and accepted gating models. Instead the authors have chosen to go into territory which appear to be complete tangents.

Response: For the mechanism underlying the activating effects of the synthetic peptides on BK channels, we think our data is clear and our discussion is concise. As the peptide interacts with the Ca^{2+}-bowl site, they can act similarly as Ca^{2+} in BK channel modulation. This is straightforward and within the known gating model of BK channels. Not sure more discussion of gating models is necessary. Thus, we didn't discuss further about the mechanism of the synthetic peptide. The rest of the discussion focuses on the potential mechanism of the involvement of the Ca^{2+}-bowl site in BK channel modulation by intact γ subunits, which appears to be more complicated in mechanisms and our discussion is more speculative as the data doesn't allow us to draw a conclusion.

• In relating tat peptide effect to some physiological relevance (effect of drug delivery mechanisms or consequences of HIV infection) - a lot would depend on concentration of the "free peptide". This point of discussion seemed particularly jarring! Tat peptides, at the concentrations use (20-200uM) would very likely interact with lipids and that might be potentially more relevant topic of discussion here (although still speculative).

Response: We feel it is necessary to mention the potential pharmacological or physiological effects of TAT-like peptides on BK channels when they are used as vehicles for delivery. We think this cannot be fully ignored as high concentration of TAT-like peptide can be used experimentally and the dose-dependence data showed that the synthetic peptide started to have effect at lower concentration although 20 μM produced the maximal effect. In addition to its sensitivity to mutation of the Ca^{2+}-bowl site, we presented new data in last revision to show that the effect of TAT peptide is clearly dependent on side of application, ruling out the possibility of a major lipid phase action on BK channels. Our guess is that the positively charged peptides should not stay long in the lipid bilayer when crossing the membrane as it is highly energetically unfavorable. We have removed the HIV Tat protein part in the discussion.

• The comment: "electrostatic interaction is shielded from ionic strength changes in solution and strengthened if it occurs mainly within the low-dielectric protein medium, i.e., buried inside the protein complex." - I don't understand this, particularly in the context of BK where charge screening effect have been postulated to be a mechanism distinguishing IbTx and ChTx action on BK. Also, since BKα alone undergoes a large shift in high salt, but the effect of the γ1 peptide is essentially eliminated in high salt (Figs. 5C and 6B) it seems that high ionic strength is indeed inhibiting the γ1 peptide effect on the GV curve. I'm sorry for the trouble in understanding but I'm left a little confused here.

Response: This part of discussion is about intact γ subunits (not synthetic peptides). This ionic strength-insensitive scenario of electrostatic interactions is largely theoretical situation in that both positive and negative charges are buried deep inside protein (or protein complex). It doesn't apply to the electrostatic interactions between BK channels and the synthesis positively charged peptides or toxin blockers, which mainly occur in solution and are readily interfered by ions in solution. Given that we think this scenario is unlikely to happen (stated in last revision), we have removed discussion of this possibility in this revision.

• I would also like point out that in the methods, using the phrase "co-translational assembly" is possibly

a stretch. Simply connecting the 2 genes into a single mRNA simply ensures identical mRNA levels of the 2 genes but does nothing to ensure "co-translational assembly". It is unnecessary here.

Response: They are indeed translated into a single (fused) peptide. During the peptide maturation they can co-assemble efficiently, presumably due to enriched presence of both at the same location. From the very beginning of our studies on the γ subunits, we found that this is an efficient strategy to ensure full modulation of BK channel by the γ1, γ2 and γ3 subunits in HEK293 cells, which is much more effective than the simply co-transfection, even with more γ subunit in molecular DNA ratio. Thus, we feel the term “co-translational assembly” is appropriate although there could be a better term to use.

Reviewer #2 (Comments to the Authors):

Chen et al. propose that "high" Ca2+ and charge neutralization of the Ca2+-bowl site reduce γ subunit efficacy in BK channel modulation. Intriguingly, they also report that positively charged peptides from the C-terminal positively charged regions of the γ subunits caused significant shifts in the G-V of the BK channel toward the hyperpolarization direction. Their findings also suggest that BK channel modulation by the γ1 subunit requires the intracellular C-terminal region of BK. The work has significantly improved since the initial version, and they have addressed the majority of my concerns, though I believe their claims are somewhat speculative and they have overlooked some concerns raised by other reviewers.

1. I disagree with the assertion that co-IP between Ca2+-binding mutants and gamma subunits is unnecessary.

Response: The co-IP between the BKα 5D5N mutant and the γ1 subunit was added (Fig. 6F).

2. The authors also failed to address some of the concerns raised by other reviewers, such as the need to investigate the effects of gamma1 on Cd^{2+} modulation of the wild-type channel.

Response: In the first round of review, the reviewer had suggested “The authors also might consider exploring the effect of γ subunit on Cd2+ modulation (on intact WT BK channels) which appears to act via the RCK1 site- based on the data/hypothesis presented here, one would predict that the effect of γ subunit remains similar with and without Cd2+ ions.”. We are sorry to forget to include a response to this comment in last revision. We had used RCK1 mutant to rule out a major involvement of the RCK1 site. We agree that the use of Cd^{2+} could be helpful to consolidate this. But we feel this additional experiment is not essential and thus not added in this work.

3. I agree that experiments with saturating Ca^{2+} pose an experimental challenge that could not be completed in a reasonable amount of time. If the authors decide not to conduct those experiments, the statement "The efficacy of the γ subunits in BK channel modulation was reduced by high Ca2+" should be softened. I recommend simply avoiding the word "high" and being specific about the concentration

used. Using a subjective term such as "high" can lead to misunderstandings. I would classify 10 μM as "intermediate" rather than "high," because it is nowhere near saturating. According to the authors' responses, "it remains unclear how Ca2+ and Ca2+-bowl site neutralization affects BK channel modulation by the gamma subunits," and I believe these limitations should be clearly stated in the manuscript.

Response: We agree that the use of "high" for Ca^{2+} concentration in terms of BK channel activation is arbitrary. We generally consider 10 μM to be a high concentration because: 1) it produces ~ 150 mV shift in $V_{1/2}$ and 100 μM only further shifted to a limited degree (~ 60 mV more shift) and mM Ca^{2+} is rarely used; 2) physiologically 10 μM is a high concentration in terms of Ca^{2+} inside the cell. Given the raised concern, we have removed all "high" in front of "Ca^{2+}".

4. The citation (Gonzalez-Pereza, 2018) (L189) appears to be incorrect.

Response: An earlier work is now cited instead.

5. While I agree that some of the concerns raised in the first review may take a long time to address experimentally, some statements in the manuscript are overly speculative and could be tested in a straightforward manner. For example, while the statement in L285-6 that "the residual responses of the 5D5N mutant channels to the γ peptides could be due to the remaining 3 negatively charged residues (D892, D900, and E902) of the Ca2+-bowl region" is speculative, as the authors stated in their responses to the reviewers. I consider that testing that possibility (that those three negatively charged residues are responsible for the residual response) is a straightforward experiment.

Response: We were suggested for providing some speculation in last revision. We are unwilling to do more experiments to test the speculation because it won't add much the current results and conclusion. Furthermore, our speculation is not comprehensive. Now, we also added the possibility of other negatively charged regions of the channels in the speculation.

Reviewer #3 (Comments to the Authors):

General comments

The authors have revised the manuscript according to the suggestions and comments made earlier incorporating many sets of results.

Some issues need to be addressed properly or clarified. The most important item may be related to the protein solubilization protocol.

Specific comments
• L44 "... has particularly large..." Perhaps need an article here "a"?

Response: "a" was added.

• L58 "... over an exceptionally large". "range" (or something like that) is missing here?

Response: "range" was added.

• L66 "LRR" This has not been formally defined yet.

Response: “LRR" was defined now.

• L68 "voltage-dependent" may be better?

Response: We changed “voltage” to "voltage-dependence".

• L68 "intracellular juxta-membrane positively charged residue cluster regions". This mouthful (but I cannot offer any suggestion - sorry)

Response: We made the phrase shorted to “C-terminal positively charged residue clusters”

• L95 "co-transfection of the cDNA constructs". Maybe provide the weight ratio?

Response: The weight ratio was added.

• L103 (and other places) "a whole cell configuration". Need a hyphen between "whole" and "cell"?

Response: We added hyphen between "whole" and "cell".

• L106 "Ca2+ free solution" A hyphen between "Ca2+" and "free"?

Response: We added hyphen between "Ca2+" and "free".

• L118-119 "the amplitudes of the tail currents...". Peak amplitudes?

Response: We changed “amplitudes” to “peak amplitudes”.

• "Steady-state activation was expressed as ..." This means that each data set (e.g., with a peptide) was normalized to its own largest tail current size, correct? This should be explicitly stated. This issue is relevant for the results like those in Fig. 4A-D.

Response: To be clear we rephrased the sentence to be “Steady-state activation was expressed as normalized conductance (G/Gmax) obtained by calculation from the peak amplitudes of the tail currents (deactivation at -120 or -150 mV) and subsequent fitting with Boltzmann function.” In most cases, the

fitting and normalized currents (normalized to the maximal currents) gave the same G/Gmax if the plateau is reached in channel activation at the applied voltages. In the absence of Ca^{2+} without an activator, the applied maximal voltages (e.g., 240 mV) may not be able to produce the maximal activation. In this case, the best fitting with a Boltzmann function can provide a factor to normalize to get the approximate G/Gmax. Hope this clarifies better.

• L125 "the known amino acid sequence". "known" seems a bit odd to me. "amino-acid sequence"?

Response: “known” is removed and the sentence is reorganized to be clearer.

• L131 "30s". One space.

Response: The space was added.

• L135 "After centrifugation". This needs to be expanded. Please see below.

Response: The centrifugation speed we used in this study was 17,000 ×g, and this was added to the method.

• L145-146 "Unpaired...". I don't think null hypothesis testing is needed for the study described.

Response: The t-test only applies to the Fig 2F, in which we intended to show the significant difference in the time-course (kinetics) of the peptide-induced outward current blockade and inward tail current increase in amplitude. We agree that the difference is obvious, and t-test is unnecessary. We have removed t-test in both method and result (Fig. 2F).

• L159 "Co-expression of ...". This was done using the fusion construct? It may be better to state this here (or at least in the figure legend).

Response: No, it was not done with a fusion construct as we don’t have one for the TM-only construct and we are also not sure whether it will work because the plasma membrane expression of the TM-only BK channels is sensitive to its C-terminal tag. We did co-overexpression of γ1 subunit with the TM-only BK for this experiment. We phrased the sentence as “Co-expression of the γ1 subunit with the TM domain-only BKα was achieved by co-transfection of their cDNA constructs (equal amount of plasmid DNA in weight)” in the method.

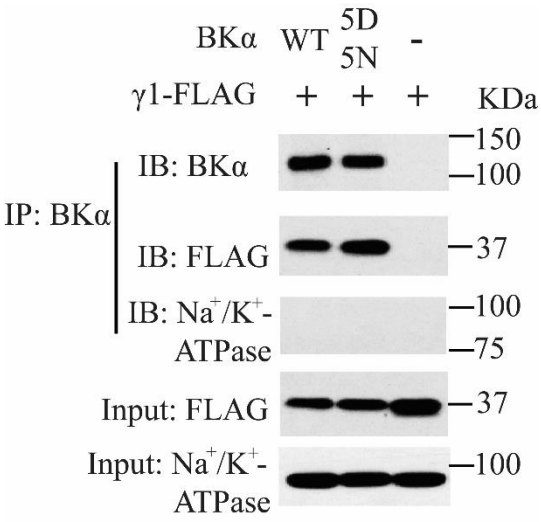
• L160 "...voltage-dependence ...". Unclear whether this hyphen is required.

Response: The hyphen is removed.

• L167-173 / Fig. 1C. To properly evaluate protein-protein interactions, the protein complexes must be fully solubilized, without membrane fragments. Full solubilization requires high-speed (e.g., 100,000 g)

centrifugation runs. If the solubilization step is not complete, the proteins may appear complexed via residual membrane fragments. One extreme (trivial) example would be like this; two proteins are present in the same cell - without proper solubilization, they would appear complexed. The immunoprecipitation results may be suggestive but they do not "demonstrate" (L171). The authors need to provide more information about the centrifugation/solubilization step(s).

Response: We do think this is a reasonable concern. As most immunoprecipitation (IP) experiments are done in regular labs with a benchtop centrifuge suitable for microtubes, we simply follow the common protocol. The centrifugation speed we used to remove the insoluble fractions is 17,000 × g in our study. However, we do not feel that we have the issue you mentioned so far. Firstly, we use a high concentration of detergent (2% DDM) to dissolve the protein. Secondly, before eluting the pull-down proteins, we use 2% DDM to wash the beads three times, and we believe that the proteins in the membrane fragments will be fully solubilized in these steps. Sometimes, we did see some insoluble floats (presumably lipid rafts etc) with some cultured cells after centrifugation, in which cases we used to pass the supernatant through a filter to remove the insoluble floats. Finally, to confirm that there are no insoluble plasma membrane fragments in the IP product, we immunoblotted the IP samples using an Na^+/K^+-ATPase antibody. As showed below, we did not see a band of the plasma membrane mark protein Na^+/K^+-ATPase in the IP products pulled down by a BKα antibody.



• Fig. 1C There seem to be two bands close together in each of the first two lanes. Why?

Response: Usually, we can see a single band for recombinant BK expressed in HEK293 cells immunoblotted in our lab. But if the gels were running for a longer time, a lower weaker band could appear, perhaps because of degradation.

• Fig. 1H The ordinate label could use "mV"?

Response: Yes. "mV" was missed and now added to the labels (now Fig. 7E).

• Fig. 2E It is better to indicate that the gamma1 results are from the two-component fits in the legend.

Response: As suggested, we included a statement “The data obtained with the channel complex of BKα (triple mutant) and γ1 were fitted with a double Boltzmann function” in the legend.

• L205 / Fig. 3 The results in Fig. 3 are those without added Ca2+, right? It will easier for the readers to read this in the legend.

Response: As suggested, a statement “All BK channel currents were recorded in the virtual absence of $[Ca^{2+}]_i$.” was added in the legend.

• Fig. 3 In E, the ordinate should start at 0 nA like in J. Are the results shown in Fig. 3E really "representative"? I see about a 40% increase in the peak inward tail current size. But the results in A and C suggest that there should be a 100% (or bigger) increase.

Response: We agree it is not a good one to be representative. We replaced the Fig. 3E by a more representative one (now Fig. 2E).

• Fig. 3F Do you really need null hypothesis testing here? I am not sure you do.

Response: They are obviously very different. We have removed the t-test in both method and here.

• Fig. 3G I think something is wrong the results or the ordinate label of the graph. Without Ca2+, Slo1 ionic current deactivation kinetics at -120 mV should not have a time constant value of 4 ms - this is way too slow. This should be closer to 0.1 - 0.5 ms?

Response: Thank you very much for pointing out the errors! The Tau values were incorrect here. The Fig. 3G was replotted (now Fig. 2G).

• L239 "... to the abundance of positive charges". This is probably true but the possibility that it is all about the # residues/size is not totally excluded from the results presented alone.

Response: We agree there is other possibility. We changed “appears to” to “is likely” here.

• Fig. 5B and 5F I am confused about these two graphs or I am missing something. Fig. 5B BKalpha (5D5N) 140 mV sweeps. Black = without the peptide and orange is with the peptide. I see about a 50% increase in size. In Fig. 6D, at 140 mV, one is expected to see a bigger increase? Is the tail current shown a near outlier experiment?

Response: In Fig. 5B, the 140 mV tail currents (middle) were not displayed properly (enlarged view without scale bar). The issue was corrected (now Fig. 4B).

• L281 "...increasing ionic strength to 1 M..." "... strength with 1 M..." may be better?

Response: Changed as suggested.

• L358 - 360. I am sorry but I read this sentence a few times, but I failed to understand what it is meant.

Response: Sorry for the confusion. The sentence is no longer needed in this revision and has been removed.

• "Although ... by an indirect allosteric mechanism". Why? Is it possible that the allosteric communication required is electrostatic in nature?

Response: Sorry for the confusion. This sentence is no longer needed in this revision and has been removed.

• Some format clean-up may be required in the References section.

Response: We updated the references with some removal and addition. We formatted the references to JGP style.

Additional major changes made:

Given that the Ca^{2+} effects on BK channel modulation by the γ1 subunit could be at least partially explained by the HA allosteric gating nature of the channel. We downplay its significance and relevance to other parts of this manuscript by moving this part to the near end of the results section and shortened discussion about it. We shortened the discussion (the part related to the effect of 5D5N mutation on BK channel modulation by the γ subunits) to make it to be concise and have less speculation.

Changes of figures (order, number and addition):

Fig. 1D-H is changed to be Fig. 7A-E

Fig. 3 is changed to be Fig. 2

Fig. 4 is changed to be Fig. 3

Fig. 5 is changed to be Fig. 4

Fig. 6A is split into Fig. 5A and Fig. 8A

Fig. 6B,C is changed to be Fig. 5B,C

Fig. 6D,E is changed to be Fig. 8B,C

Fig. 6F is added.

Fig. 7F is added.

April 5, 2023

Dr. Jiusheng Yan
The University of Texas MD Anderson Cancer Center
Anesthesiology & Perioperative Medicine
1515 Holcombe Blvd.
Houston, Texas 77030

Re: 202213237R2

Dear Dr. Yan,

Thank you for submitting your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" to JGP. I have looked over your revised manuscript and feel you have addressed most of the concerns what were raised in the last review cycle. I think the comparison of the peptide effects vs intact gamma effects is more balanced and improves your manuscript.

Before a final decision, I note that there is a issue that needs to be addressed in regards to reporting of statistics. Although I agree with the suggestion made by one of the reviewers in response to the previous version, that a statistical test was not necessary in one case, there are some comparisons that are made where JGP does expect a reporting of a P-Value. Fig. 2E shows an asterisk indicating significance, but the legend needs an exact P value along with the specific test and N's. Might it also be worthwhile to compare, e.g., the gating shift for intact gamma1 vs gating shift via gamma1 peptide? Again, that would require exact P value, the test used, and N's. I am not suggesting that you do that for all of your data, but there may be other cases you should consider for cases in which the text makes an assertion regarding a difference between two different sets of data.

Also related to data presentation, in some cases the manuscript reports n's for particular experiments, but in others there's no indication of how many repeats when into the generation of a given GV. Please check to see that this information is provided in the legends for all parts of your figures. Finally, I note that the horizontal axis label in Fig 4C has a lower case "mV". The same also applies for Fig. 8 and C. Also please be consistent in figure presentation in regards to providing a space between "Voltage" and "mV".

We hope that you will be able to submit a revised manuscript that addresses these points, which we believe will pose no problems, and which may be re-reviewed. Based on the scope of the requested changes, we typically anticipate that the revision process will take no longer than 2 months, however, we understand you may need additional time to work on your resubmission to JGP. We therefore ask that you simply keep us informed as to a realistic submission timeline that is appropriate for your particular circumstances. In addition, please do not hesitate to contact me (via the editorial office) if you feel that a discussion of the reviewers' and editors' comments would be helpful.

Please submit your revised manuscript via the link below, along with a point-by-point letter that details your response to the reviewers' and editors' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). If the article is eventually accepted, it would include a 'revised date' as well as submitted and accepted dates. If we do not receive the revised manuscript within one year, we will regard the article as having been withdrawn. We would be willing to receive a revision of the manuscript at a later time, but the manuscript will then be treated as a new submission, with a new manuscript number.

Please pay particular attention to recent changes to our instructions to authors in the following sections: Data presentation, Blinding and randomization and Statistical analysis, under Materials and Methods, as shown here: https://rupress.org/jgp/pages/submission-guidelines#prepare. Re-review will be contingent on inclusion of the required information (including for data added during revision) and demonstration of the experimental reproducibility of the results (i.e., all experimental data verified in at least 2 independent experiments).

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Sincerely,

Christopher Lingle, Ph.D.
On behalf of Journal of General Physiology

Journal of General Physiology's mission is to publish mechanistic and quantitative molecular and cellular physiology of the highest quality; to provide a best-in-class author experience; and to nurture future generations of independent researchers.

Responses to the editor's comments

Re: 202213237R2

Before a final decision, I note that there is a issue that needs to be addressed in regards to reporting of statistics. Although I agree with the suggestion made by one of the reviewers in response to the previous version, that a statistical test was not necessary in one case, there are some comparisons that are made where JGP does expect a reporting of a P-Value. Fig. 2E shows an asterisk indicating significance, but the legend needs an exact P value along with the specific test and N's. Might it also be worthwhile to compare, e.g., the gating shift for intact gamma1 vs gating shift via gamma1 peptide? Again, that would require exact P value, the test used, and N's. I am not suggesting that you do that for all of your data, but there may be other cases you should consider for cases in which the text makes an assertion regarding a difference between two different sets of data.

Response: We highly appreciate the editor's prompt review and decision. We assume it means Fig. 2F (not 2E). The "asterisk" is actually one of the 3 data points. We agree when comparison is made it is better to have some statistical analysis done. We added back the t-test p-value in Fig. 2F, and the description of the t-test in method and Fig. 2 figure legend. Given that it has been mentioned that the synthetic peptide effects are similar to those contributed by the C-terminal regions of the intact γ subunits, we feel a statistical comparison of the gating shift for intact gamma1 vs gating shift via gamma1 peptide might be not needed as they are very different ~140 vs ~40 mV. We checked the manuscript and found that Fig. 2F is the only one with clearly intended data comparison in Results.

Also related to data presentation, in some cases the manuscript reports n's for particular experiments, but in others there's no indication of how many repeats when into the generation of a given GV. Please check to see that this information is provided in the legends for all parts of your figures. Finally, I note that the horizontal axis label in Fig 4C has a lower case "mV". The same also applies for Fig. 8 and C. Also please be consistent in figure presentation in regards to providing a space between "Voltage" and "mV".

Response: We only indicated N when a bar graph is presented as the N is already listed in Table 1 for all G-V related data and plots. To be clear, we now added a sentence "For all plots of the G/Gmax – voltage (G-V) relationships here and in other figures, the number of repeats (n) used in plotting the individual G-V curve is the same as that of the corresponding data listed in Table 1" in the legend of Fig. 1. The Fig. 4 and 8 have been updated with corrections in case and space for axis label.

April 7, 2023

Dr. Jiusheng Yan
The University of Texas MD Anderson Cancer Center
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Houston, Texas 77030

Re: 202213237R3

Dear Dr. Yan,

I am pleased to let you know that your manuscript, entitled "BK channel modulation by positively charged peptides and auxiliary γ subunits mediated by the Ca2+-bowl site" is scientifically acceptable for publication in Journal of General Physiology. Formal acceptance will follow when it is modified in accordance with any requirements identified by the editorial office in regards to our editorial policies.

Please note items that need attention are listed at the bottom of this email and on the attached marked-up pdf file. Please also be sure to have a copy of the text of your manuscript available as a double-spaced MS Word file and include editable tables, if appropriate.

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Sincerely,

Christopher Lingle, Ph.D.
On behalf of Journal of General Physiology

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- MS Word document of supplemental text needed, if applicable (including figure legends and editable tables)
- Brief Statement describing supplementary information needed, if applicable (in subsection at end of Materials & Methods)
- Please include a data availability statement preceding the Acknowledgments section. Please see https://rupress.org/jgp/pages/editorial-policies#data-availability-statement
- Figures created at sufficient resolution and in acceptable format (including supplemental if applicable). If working in Illustrator, we prefer .ai or .eps file format. If working in Photoshop please use 600dpi/1000dpi .tiff or .psd file format. Minimum resolution at estimated print size: Minimum resolution for all figures is 600 dpi. For figures that contain both photographs and line art or text, 600 dpi is highly recommended. Figures containing only black and white elements (line art, no color, and no gray) should be 1,000 dpi. Maximum figure size is 7 in wide x 9 in high (17.5 x 22.8 cm) at the correct resolution. https://jgp.rupress.org/fig-vid-guidelines
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