Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels

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December 5, 2023

Prof. Peter C Ruben
Simon Fraser University
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Re: 202313505

Dear Professor Ruben,

Thank you for submitting your manuscript, titled "Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels" to JGP. Your manuscript has now been seen by three reviewers, whose comments are appended below. As you will see, while recognizing the potential impact of your work, the reviewers have also identified several issues that the editors agree should be addressed prior to further consideration of the manuscript at JGP. In particular, the reviewers expressed concerns about the conclusion that CBD does not alter the open probability of HCN4 channels and about the docking experiments described. The editors concur, and request that you provide additional information about how the docking was performed. It would also be helpful to complete experiments to test the predictions of the docking results, if possible/feasible.

We would be pleased to receive a suitably revised manuscript that addresses the noted concerns, which will be re-reviewed, preferably by the original referees, pending their availability. Based on the scope of the expected changes, we would anticipate that the revision process will take no longer than 3 months. If you need additional time, however, please let us know, providing what you think will be a realistic submission timeline. 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 reviewers' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). 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. Also, to improve the reproducibility of published content, we have partnered with SciScore. Authors are prompted in eJP to copy and paste the Materials and Methods section of their manuscript for a SciScore assessment when submitting their revised manuscript. Authors are encouraged (not required) to further revise their Materials and Methods if the SciScore is below 4. More information can be found here: https://rupress.org/jgp/pages/submission-guidelines#sciscore

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Thank you for the opportunity to consider your manuscript.

Sincerely,

Jeanne Nerbonne, 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 this paper, the authors describe the regulatory effect of Cannabidiol (CBD) on the cardiac pacemaker channel HCN4. Using whole-cell patch clamp on HEK293T cells expressing HCN4, they showed that CBD potentiate HCN4 by depolarizing the V1/2 of activation and by speeding the kinetics of activation. They report that the potentiation by CBD occurs in a state-dependent mode

with CBD binding to the channel closed state. HCN4 is regulated by cyclic nucleotides, such as cAMP as well as PIP2. The authors provide experimental evidence that the potentiation mechanism of CBD is different from those of cAMP and PIP2. Lastly, authors used computational modeling to underpin the binding site of CBD. They found that CBD binds at the C-terminus of the channel near the S4 voltage sensor. Most of my critique to the paper require further explanation about the state dependence of CDB potentiation and some experimental suggestions. Overall, I believe this paper is a great addition to the scientific literature in this area and requires minor revisions to be ready for publication.

Comments
1. Page 5. Analysis and Statistics. Both equations (I and dV0.5) need to be fixed. What is the meaning of Rate in one of the equations?
2. Page 6. Computational Docking. Can you elaborate further on the criteria used to define the nine highest affinity binding site?
3. Page 7. Instead of showing that the shift is statistically different from 0 mV or not at different doses, it is better to show that the max shift is significantly different than 0 mV (from dose dependence curve fitted). If the effect is different than 0 mV, then one could in principle get a significant result at any concentration as long as one did enough experiments. Maybe better to state what would be a physiologically relevant shift and state from the fitted curve at what concentration that would happen. In addition, please state in the text what concentration would be therapeutically relevant.
4. Page 10. "This suggests that CBD stabilizes the transition to the activated state in addition to its effect on voltage dependence". A shift in voltage dependence is a result of changing the open and/or closing rate. So, I don't really see the necessity to treat these as separate issues. In addition, the effect could be asymmetric so that opening rate is affected more than the closing rate. Maybe this is what the authors are arguing about?
5. Page 10. How fast does CBD bind and unbind to say anything about state dependence? Other studies on lipid like molecules that affect ion channels usually have a very fast on and off rate, but a slow rate of incorporating into the bilayer (e.g. Yazdi et al., JGP 2021). Since the channels undergo much slower open/close transitions than the binding/unbinding I don't think one can draw any conclusions about state dependence from these experiments. In addition, since CBD increases opening, it should bind better to open channels than closed channels.
7. Page 15. Could you please elaborate further on the lipid binding pocket in subunit D from your reference? It does not seem too relevant for the message of the paragraph. Grammar: voltage sensor in line 10.
8. Figure 4. Could you make some mutations of e.g. R397 to test binding mode? Further along you claim: "Mutagenesis to confirm the binding site of CBD would be difficult since the predicted binding site is at the voltage sensor and mutagenesis here tends to drastically change the voltage dependence of channels (Chen et al., 2001)." However, even if the voltage dependence of the mutations is shifted you can still see the effects of your compound?

9. Page 18. "However, since CBD depolarizes the V1/2, these values are expected to be closer to true steady state than the V1/2 calculated for the vehicle". This might not be the best way to describe this. I think it is because the channels open faster....
10. Page 18. "While our results suggest that CBD binds to the closed-state, we have not excluded the idea that CBD doesn't have state dependence, binding to closed- or open-state with no change in affinity. Further, it has been suggested that the pore of HCN4 channels is open at rest (Saponaro et al., 2021). If this is true, the voltage sensor (i.e. CBD's binding site) would still be in the outward or inactive conformation at rest, so our results support CBD binding to the outward conformation of S4." Not sure I understand the logic here? What are you trying to say? How would binding to the outward state of S4 shift the opening to more depolarized voltages? Binding to the outward state of S4 would stabilize the closed state. You might want to change your conclusions.
11. Pag. 22. "CBD's potentiation of HCN4 channels could account for this, counteracting the inhibition of other cardiac potassium channels like IKr (hERG) and IKs (Orvos et al., 2020; Topal et al., 2021)." Not sure I understand this either? Do you suggest that HCN channels are open during the plateau of the action potential and let potassium out of the cell? Maybe better to spell out exactly what you think is going on.

Reviewer #2 (Comments to the Authors):

The authors present electrophysiological evidence that indicates HCN4 are potentiated by cannabidiol (CBD). Further evidence suggests that these effects are independent of the regulation of the channel by other modulators including cAMP and phosphoinositides. The electrophysiological experiments are generally well performed. However, some of the language of interpretation is inaccurate and the computational experiments are limited in their quality and value.

Specific Points to Address:
Title and throughout the manuscript: The generally used full name for HCN channels are "Hyperpolarization-activated cyclic nucleotide-gated" channels rather than "Hyperpolarization and cyclic-nucleotide gated" as listed.

Insufficient and incorrect details of the computational docking methods are provided. Firstly, in the methods and figure legends, PDB: 7NP4 is described as the structure from the human homolog when it is in fact from rabbit HCN4. Since there are sequence differences between human/mouse and rabbit HCN4 it is important to identify the sequence similarity between the species in the potential binding site(s). Furthermore, details of the total number of docking attempts, the number of energy evaluations per docking attempt, size of the evaluation grid, inclusion/exclusion criteria of accepted poses, etc. should be presented in detail.

It is not clear that the computational docking is sufficiently well performed to draw any conclusions, and has limited value to the manuscript. Firstly, there appears to be a limited number of docking attempts (9?) that were used. Based on the statement "Of particular interest was a binding pocket in the center of the VSDs (Fig. 4A,B), where four of the nine highest affinity conformations were localized, one in each monomer" suggests the tetramer of the protein was used, which would oversample any particular binding pocket with so few docking attempts. Secondly, the standard error of docking calculations in Autodock Vina is ~2.85 kcal/mol (J Comput Chem. 2010 Jan 30; 31(2): 455-461). meaning that energetically, all 9 of the docking poses identified in the manuscript are energetically similar and cannot truly be ranked. Thirdly, the cholesterol docking in Supp. Fig. S3 suggests that the hydrophobic alkyl tail of cholesterol would be pointed downward. Given the location of this pocket (at the end of the S4, which is in the area of the polar lipid headgroups based on the Orientations of Proteins in Membranes database, as well as molecular dynamics simulations (Elife. 2023 Jun 21;12:e80303)), this seems like an unlikely favourable conformation due to the high energy of solvating the alkyl tail. Thus, there are no reliable secondary or confirmatory experiments (computational or electrophysiological) performed to provide validation of one particular proposed pocket over another or for the basis of excluding any of the pockets.

The conclusion that CBD did not alter open probability of HCN4 is confusing since there was an obvious large effect on steady-state activation which was fit by a Boltzmann function. The Boltzmann function in this form specifically assesses the difference in probability of the channels being in the open state vs the total available channels (ie. open probability). Moreover, Mayar et al. do not specifically state that CBD alters mHCN1 open probability, but simply HCN1 current density (since they did not observe significant shifts in steady-state activation under their high K+ conditions). While Fig. 1E in your manuscript suggests this is not the case in HCN4, raw traces presented in Fig. 2A and Fig. 2C are consistent with the repetitive pulse data presented in Mayar et al. It is this reviewer's opinion, based on the statement in the discussion "We found that this shift in V1/2 did not result in a significant increase in the open probability of HCN4 channels. In contrast, Mayer et al. found that in intact oocytes CBD increased the open probability of HCN1 channels without shifting the voltage dependence of activation." That the authors may be confounding "current density" with "open probability". These terms are not interchangeable.

Pg. 10 "The protocol was 20 sweeps, for a total of 60 seconds using 120 mV as the activation voltage." Should say -120 mV instead.

Discussion: The mechanism of action proposed in the discussion is somewhat contradictory to the data. The author's state that "when CBD is added it stabilizes the S4 in the activated state, allowing HCN4 channels to open at weak driving forces." but their electrophysiology data suggests binding to the closed state and docking data indicates that CBD binds in the deactivated voltage-sensor configuration. Therefore, it is unclear what leads the authors to infer stabilization of the activated voltage-sensor domain over say destabilization of the closed state. The statement at the end of page 17 "We propose that CBD binds to HCN4 channels (Fig. 5) to stabilize the open state of the channel, by both depolarizing the V1/2 of activation and speeding activation kinetics" conflates mechanism with read-out in the use of the word "by". Depolarization of the voltage-dependence and speeding of activation kinetics is a potential consequence of (and thus the observation) of a stabilized open state (or destabilized closed state), not the mechanism of doing so.

Reviewer #3 (Comments to the Authors):

In their study "Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels", Page and Ruben use whole-cell patch clamp to measure the effects of the phytocannabinoid, cannabidiol, on HCN4 channels. The authors find that CBD is an HCN4 potentiator and shifts the channel activation independent of cAMP and PIP2. The authors also propose that a structurally resolved lipid binding pocket is the most likely site by which CBD exerts its effects. While this is certainly and interesting possibility as compared to a non-specific effect due to changes in membrane properties, the authors should attempt to validate this model experimentally. Given the clinical need for HCN4 potentiators, this would better validate this pocket and CBD as platforms for future pharmaceutical development as the authors suggest.
Strengths: CBD is already in use by many as an FDA-approved and home remedy for a variety of conditions and therefore understanding it's mechanisms of action is of interest. The authors show that the effects of CBD are distinct from PIP2 and cAMP, and identify a potential function for a previously described lipid binding pocket. That CBD potentiates HCN4 without acting at the cAMP-binding pocket is unique and could indeed make it an ideal platform to potentiate HCN4 activation. This in turn has clear relevance for pharmaceuticals to increase heart rate, for example in genetic bradycardias or aging.
Major Concerns:
1) CBD seems to alter the activity of every channel [IC50s are also similar 1-5uM range (Ghovanloo et al., 2018; Orvos et al., 2020; Isaev et al., 2022)] suggesting an indirect mechanism related to the membrane. The direct interaction with the channel posited in Fig. 4 is particularly interesting as many other CBD studies seem to suggest some change in membrane fluidity is responsible. The problem is that the proposed interaction is not tested experimentally. The authors mention the difficulty in working with voltage-sensor mutants and their possible effects on channel gating [mutations of R397 are not functional (Chen et al., 2000)]. However, in the cited Chen et al. paper, the analogous Q401A (Q322A) mutant is functional (Chen et al., 2001). Because the authors suggest that the preferential binding of the CBD benzene to R397 over Q401 could be responsible for these effects, one option is Q401R [likely tolerated as this is analogous to the corresponding residue in hERG (Cowgill et al., 2019)] or Q401K to remove/reverse this preferential binding. Alternatively, given the potential binding of cholesterol at the same site, the authors could look at whether changes in cholesterol compete with CBD (Fürst and D'Avanzo, 2015).

2) Measurements of HCN time constants are complicated and there is some concern with the authors use of a single exponential fit. The authors should highlight in the text that the control HCN4 Tact values at -100mV in Holo are likely not accurate as the activation time ideally needs to be 2-3 time constants for accuracy. More concerning is that the time constants for activation in the cAMP unbound state appear faster than the cAMP bound state. The time constant in the Apo-state would be roughly 1200 ms at -120 mV compared to 1360 and 1270 in the holo-state control and vehicle, respectively. This may be due to fitting a single exponential equation to a multi-step process. I suggest fitting a subset of the data (eg. Apo and Holo control and 5uM CBD data) with the time to 50% activation to validate the data presented.

3) In Fig. 1D the presented IC50 curve does not have any data-points in the middle (going from a non-significant effect at 1 uM to a 100% effect at 5 uM). The authors could consider a 3uM concentration point for this one measurement.

4) More description of the experimental design and statistical analysis would be useful. Based on Ns in Table 1, the data appears to be conducted as a series of matched pairs with control followed by perfusion of a single CBD concentration. If so this should be explicitly stated. The statistical analysis of this data should also account for this as each cell is effectively a single block of the experimental design and can have a matched pair effect. Finally, please provide exact P-values for comparisons of CBD vs control in the Tables 1 and 2.

Minor Concerns:
1) The rationale of the study is given in the discussion, but a single sentence about cannabinoids, heart rate, and potential therapeutic consequences would be useful in the introduction.
2) Comparisons to the therapeutic concentration of CBD occur multiple times but the therapeutic range for CBD is never given.
3) Were midpoints corrected for the liquid-junction potential?
4) Page 7: "Previous studies found that CBD increased the open probability of mHCN1 channels in intact Xenopus laevis oocytes". Is this correct? Is it more accurate to say CBD increases maximal current since it could also reflect an increase in single-channel conductance?
5) In Table 1, the same number of significant figures should be used for each value.
6) The light green color is slightly hard to see. Note that Fig. 3 will be difficult for people with red-green colorblindness.
7) In Figure 2, the zero current level should be indicated on current traces for clarity. Also, it would be beneficial to indicate the -100mV trace on the representative currents to allow comparison between conditions.
8) The diC8-PIP2 current trace shown in Fig. 3C appears to be tiny amplitude (50pA?) is the scale bar correct?
9) PIP2 appears to increase the instantaneous current substantially which warrants a mention. This also makes the measurement of activation in the tail current nearly invisible. For these currents it might be advisable to also measure the GV curve from the activating currents during the hyperpolarizing step. The GV could then be calculated using the driving force (To get the equilibrium potential you could easily fit a line to the three currents measured during the most negative voltage step followed by the corresponding current at -100mV and 0mV)
10) Page 18: "However, since CBD depolarizes the V1/2, these values are expected to be closer to true steady state than the V1/2 calculated for the vehicle, thus the ΔV1/2 may be even larger than reported here" - I would expect the opposite to be true where the isochronal activation in the non-CBD state is artificially hyperpolarized compared to steady-state?
11) The difference with HCN1 should be highlighted in the discussion. While it could be as simple as the difference between oocytes and HEK, it could also hint at the inherent differences in gating that allow HCN1 and HCN2 to activate at more depolarized potentials compared to HCN4.
12) Because CBD and THC have similar structures both containing a benzene ring that the authors think is crucial for cation-pi interactions with the S4 Arg residue, do the authors think the effects on HCN4 gating would be similar?

Dear Professor Ruben,

Thank you for submitting your manuscript, titled "Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels" to JGP. Your manuscript has now been seen by three reviewers, whose comments are appended below. As you will see, while recognizing the potential impact of your work, the reviewers have also identified several issues that the editors agree should be addressed prior to further consideration of the manuscript at JGP. In particular, the reviewers expressed concerns about the conclusion that CBD does not alter the open probability of HCN4 channels and about the docking experiments described. The editors concur, and request that you provide additional information about how the docking was performed. It would also be helpful to complete experiments to test the predictions of the docking results, if possible/feasible.

We would be pleased to receive a suitably revised manuscript that addresses the noted concerns, which will be re-reviewed, preferably by the original referees, pending their availability. Based on the scope of the expected changes, we would anticipate that the revision process will take no longer than 3 months. If you need additional time, however, please let us know, providing what you think will be a realistic submission timeline. 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 reviewers' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). 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. Also, to improve the reproducibility of published content, we have partnered with SciScore. Authors are prompted in eJP to copy and paste the Materials and Methods section of their manuscript for a SciScore assessment when submitting their revised manuscript. Authors are encouraged (not required) to further revise their Materials and Methods if the SciScore is below 4. More information can be found here: https://rupress.org/jgp/pages/submission-guidelines#sciscore

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

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Thank you for the opportunity to consider your manuscript.

Sincerely,

Jeanne Nerbonne, Ph.D.
On behalf of Journal of General Physiology

We thank the editor and reviewers for the opportunity to improve and resubmit our paper. We especially thank the reviewers for their insights and suggestions, which we have largely endeavoured to incorporate into our 'new and improved' version of the manuscript.

Reviewer #1 (Comments to the Authors):

In this paper, the authors describe the regulatory effect of Cannabidiol (CBD) on the cardiac pacemaker channel HCN4. Using whole-cell patch clamp on HEK293T cells expressing HCN4, they showed that CBD potentiate HCN4 by depolarizing the V1/2 of activation and by speeding the kinetics of activation. They report that the potentiation by CBD occurs in a state-dependent mode with CBD binding to the channel closed state. HCN4 is regulated by cyclic nucleotides, such as cAMP as well as PIP2. The authors provide experimental evidence that the potentiation mechanism of CBD is different from those of cAMP and PIP2. Lastly, authors used computational modeling to underpin the binding site of CBD. They found that CBD binds at the C-terminus of the channel near the S4 voltage sensor. Most of my critique to the paper require further explanation about the state dependence of CDB potentiation and some experimental suggestions. Overall, I believe this paper is a great addition to the scientific literature in this area and requires minor revisions to be ready for publication.

Comments
1. Page 5. Analysis and Statistics. Both equations (I and dV0.5) need to be fixed. What is the meaning of Rate in one of the equations?

We are unsure what the reviewer would like changed in these equations. That said, we changed equation 2 to V1/2 instead of deltaV1/2. "Rate" has been replaced with "H" for the Hill-coefficient.

2. Page 6. Computational Docking. Can you elaborate further on the criteria used to define the nine highest affinity binding site?

We attempted to address this comment by elaborating on the docking procedure and changing the text on Page 6 to the following:
"Computational docking was performed using AutoDock Vina (Trott and Olson, 2010; Eberhardt et al., 2021) with the published cryoEM structure of HCN4 (PDB ID: 7NP4) and CBD (DrugBank access: DB09061). Two different search space volumes were used, both greater than 27000 $Å^3$, large enough to include the entirety of the transmembrane region of either a single monomer or the entire tetramer. The entire tetramer was used to examine potential binding sites at interfaces between the monomers. To account for the large search space volume the exhaustiveness parameter was increased from the default of eight to 100 for the monomer and 1000 for the tetramer. The output was the nine highest affinity binding sites calculated, with multiple general locations having multiple predicted binding conformations. In the tetramer sites were also repeated across the different monomers of the channel (Fig. S2)."

To clarify, the nine highest affinity binding sites are the output for AutoDock Vina program. As far as we are aware there is no way to increase or change the output. These conformations were not chosen by us, but were optimized by the program itself. We have since increased the exhaustiveness of the program, which increases the parallel runs the program does before clustering and refining the results into the output (the nine binding conformations or "nine highest affinity binding sites").

3. Page 7. Instead of showing that the shift is statistically different from 0 mV or not at different doses, it is better to show that the max shift is significantly different than 0 mV (from dose dependence curve fitted). If the effect is different than 0 mV, then one could in principle get a significant result at any concentration as long as one did enough experiments. Maybe better to state what would be a physiologically relevant shift and state from the fitted curve at what concentration that would happen. In addition, please state in the text what concentration would be therapeutically relevant.

The shift in V1/2 is not compared to 0 mV. It is compared to the shift observed in the vehicle (DMSO), in order to calculate all statistical significances. The Hill equation was calculated with a base-line for V1/2 shift of zero, the reviewer may be thinking of this.

4. Page 10. "This suggests that CBD stabilizes the transition to the activated state in addition to its effect on voltage dependence". A shift in voltage dependence is a result of changing the open and/or closing rate. So, I don't really see the necessity to treat these as separate issues. In addition, the effect could be asymmetric so that opening rate is affected more than the closing rate. Maybe this is what the authors are arguing about?

Although we understand this comment, we think the reviewer might be mistaken in their assumptions. The voltage dependence (V1/2) of the channel represents the thermodynamic Closed-Open transition, a transition between two conformations with inherent free energies or ΔG. The activation kinetics of the channel represents the transition state energy, or activation energy, of the rate limiting step of activation. What the reviewer may be thinking of is that experimentally kinetics often mirrors changes in the ΔG. Hammond's postulate attempts to explain this by saying that the transition state will resemble the species nearest to it in free energy. Thus, in one-step reactions, changes in energy of the ground state will also change the energy of the transition state. However, in more complex multi-step reactions, such as ion channel gating, there may be effects on the ground state energies without affecting the transition state energy. In line with this, HCN channel activation is a multi-step process. There are examples of HCN channels specifically having kinetics effects that are not mirrored in $V_{1/2}$ (e.g. Magee, Madden, Young 2015). In fact, in this work we show that the effect of CBD on kinetics has a different IC50 than that of the effect on V1/2, suggesting that the two are independent from one another. As such, we feel that it is important to separate these two concepts.

5. Page 10. How fast does CBD bind and unbind to say anything about state dependence? Other studies on lipid like molecules that affect ion channels usually have a very fast on and off rate, but a slow rate of incorporating into the bilayer (e.g. Yazdi et al., JGP 2021). Since the channels undergo much slower open/close transitions than the binding/unbinding I don't think one can draw any conclusions about state dependence from these experiments. In addition, since CBD increases opening, it should bind better to open channels than closed channels.

Our experimental system (bath perfusion) can't accurately resolve on and off binding rates. Nevertheless, we attempted to address this comment in the discussion (Page 18-19). "While our results from the repetitive pulse protocol suggest that CBD binds to the closed-state, we have not excluded the idea that CBD doesn't have state dependence, binding to closed- or open-state with no change in affinity, or that the CBD on-rate is too fast to be accurately measured by this analysis. Future work could be done

to determine whether the effects of CBD are caused by binding to and destabilization of the HCN4 closed state."

We disagree that CBD should bind better to open channels than closed channels, because destabilization of the closed state would have the same effect we observed.

7. Page 15. Could you please elaborate further on the lipid binding pocket in subunit D from your reference? It does not seem too relevant for the message of the paragraph. Grammar: voltage sensor in line 10.

There is further information on this lipid binding pocket in the Discussion (Page 19).

8. Figure 4. Could you make some mutations of e.g. R397 to test binding mode? Further along you claim: "Mutagenesis to confirm the binding site of CBD would be difficult since the predicted binding site is at the voltage sensor and mutagenesis here tends to drastically change the voltage dependence of channels (Chen et al., 2001)." However, even if the voltage dependence of the mutations is shifted you can still see the effects of your compound?

Prompted by the reviewer, we made a mutation to test the binding site: HCN4 Q401R. Although the mutation altered gating such that we were unable to accurately measure steady-state voltage dependence, the mutation also removed any sign of CBD-induced kinetic effects, suggesting we targeted the binding site. See Figure 4E and Figure S4.

9. Page 18. "However, since CBD depolarizes the V1/2, these values are expected to be closer to true steady state than the V1/2 calculated for the vehicle". This might not be the best way to describe this. I think it is because the channels open faster....

This statement was changed to "Steady state $V_{1/2}$ values may be more depolarized than the isochronal $V_{1/2}$ values found here. However, the effect of CBD on $V_{1/2}$ is expected to be preserved at true steady state as well as its effect on activation kinetics."

10. Page 18. "While our results suggest that CBD binds to the closed-state, we have not excluded the idea that CBD doesn't have state dependence, binding to closed- or open-state with no change in affinity. Further, it has been suggested that the pore of HCN4 channels is open at rest (Saponaro et al., 2021). If this is true, the voltage sensor (i.e. CBD's binding site) would still be in the outward or inactive conformation at rest, so our results support CBD binding to the outward conformation of S4." Not sure I understand the logic here? What are you trying to say? How would binding to the outward state of S4 shift the opening to more depolarized voltages? Binding to the outward state of S4 would stabilize the closed state. You might want to change your conclusions.

We agree with the reviewer's comments that these statements are not clear. To remedy this, the text on Page 18-19 was changed to: "While our results from the repetitive pulse protocol suggest that CBD binds to the closed-state, we have not excluded the idea that CBD doesn't have state dependence, binding to closed- or open-state with no change in affinity, or that the CBD on-rate is too fast to be accurately measured by this analysis. Future work could be done to determine whether the effects of CBD are caused by binding to and destabilizing the HCN4 closed state."

11. Pag. 22. "CBD's potentiation of HCN4 channels could account for this, counteracting the inhibition of other cardiac potassium channels like IKr (hERG) and IKs (Orvos et al., 2020; Topal et al., 2021)." Not sure I understand this either? Do you suggest that HCN channels are open during the plateau of the action potential and let potassium out of the cell? Maybe better to spell out exactly what you think is going on.

In an attempt to address this comment we added the following text to Page 22: “However, no increase in QT intervals were reported in clinical trials of CBD in humans (Stanley et al., 2013; Iffland and Grotenhermen, 2017). CBD’s potentiation of HCN4 channels could play a role in this as HCN isoforms including HCN4 are expressed in ventricular cardiomyocytes (Shi et al., 1999; Zhang et al., 2009). Potentiation of HCN4 would result in outward current during the plateau phase of the ventricular action potential counteracting the inhibition of other cardiac potassium channels like I_{Kr} (hERG) and I_{Ks} (Ueda et al., 2009; Orvos et al., 2020; Topal et al., 2021).”

Reviewer #2 (Comments to the Authors):

The authors present electrophysiological evidence that indicates HCN4 are potentiated by cannabidiol (CBD). Further evidence suggests that these effects are independent of the regulation of the channel by other modulators including cAMP and phosphoinositides. The electrophysiological experiments are generally well performed. However, some of the language of interpretation is inaccurate and the computational experiments are limited in their quality and value.

Specific Points to Address:
Title and throughout the manuscript: The generally used full name for HCN channels are "Hyperpolarization-activated cyclic nucleotide-gated" channels rather than "Hyperpolarization and cyclic-nucleotide gated" as listed.

We changed the full name of HCN channels in this paper to “Hyperpolarization-activated cyclic nucleotide-gated channels”

Insufficient and incorrect details of the computational docking methods are provided. Firstly, in the methods and figure legends, PDB: 7NP4 is described as the structure from the human homolog when it is in fact from rabbit HCN4. Since there are sequence differences between human/mouse and rabbit HCN4 it is important to identify the sequence similarity between the species in the potential binding site(s). Furthermore, details of the total number of docking attempts, the number of energy evaluations per docking attempt, size of the evaluation grid, inclusion/exclusion criteria of accepted poses, etc. should be presented in detail.
It is not clear that the computational docking is sufficiently well performed to draw any conclusions, and has limited value to the manuscript. Firstly, there appears to be a limited number of docking attempts (9?) that were used. Based on the statement "Of particular interest was a binding pocket in the center of the VSDs (Fig. 4A,B), where four of the nine highest affinity conformations were localized, one in each monomer" suggests the tetramer of the protein was used, which would oversample any particular

binding pocket with so few docking attempts. Secondly, the standard error of docking calculations in Autodock Vina is ~2.85 kcal/mol (J Comput Chem. 2010 Jan 30; 31(2): 455-461). meaning that energetically, all 9 of the docking poses identified in the manuscript are energetically similar and cannot truly be ranked.

We thank the reviewer for noting the error in labelling of 7NP4 as human instead of rabbit, the text has been changed. This area of the binding pocket, including the S2-S3 linker and S4 is 100% conserved among HCN4 isoforms found in rabbit, mouse and human so we do not predict this to cause any errors in these calculations.

The size of the evaluation grid was large enough that the only size described by the program was > 27000 $Å^3$, as noted in methods section. The number of docking attempts and energy evaluations/docking attempt are standardized by the program, and as such they were not described here.

The methods sections have been adjusted to say ""Computational docking was performed using AutoDock Vina (Trott and Olson, 2010; Eberhardt et al., 2021) with the published cryoEM structure of HCN4 (PDB ID: 7NP4) and CBD (DrugBank access: DB09061). Two different search space volumes were used, both greater than 27000 $Å^3$, large enough to include the entirety of the transmembrane region of either a single monomer or the entire tetramer. The entire tetramer was used to examine potential binding sites at interfaces between the monomers. To account for the large search space volume the exhaustiveness parameter was increased from the default of eight to 100 for the monomer and 1000 for the tetramer. The output was the nine highest affinity binding sites calculated, with multiple general locations having multiple predicted binding conformations. In the tetramer sites were also repeated across the different monomers of the channel (Fig. S2)."

We think that the reviewer may be confusing the output of the Autodock Vina program (the nine conformations of CBD with the highest binding energies of all the clustered and refined binding sites sampled over the course of the calculation) with the exhaustiveness of the sampling process. From Autodock Vina "FAQ":

> "the docking calculation consists of a number of independent runs, starting from random conformations. Each of these runs consists of a number of sequential steps. Each step involves a random perturbation of the conformation followed by a local optimization (using the Broyden-Fletcher-Goldfarb-Shanno algorithm) and a selection in which the step is either accepted or not. Each local optimization involves many evaluations of the scoring function as well as its derivatives in the position-orientation-torsions coordinates. The number of evaluations in a local optimization is guided by convergence and other criteria. The number of steps in a run is determined heuristically, depending on the size and flexibility of the ligand and the flexible side chains. However, the number of runs is set by the exhaustiveness parameter. Since the individual runs are executed in parallel, where appropriate, exhaustiveness also limits the parallelism. Unlike in AutoDock 4, in AutoDock Vina, each run can produce several results: promising intermediate results are remembered. These are merged, refined, clustered and sorted automatically to produce the final result."
> The final result being the nine highest binding energy conformations.

The original docking calculations were done with the default exhaustiveness of 8. To account for the large docking search space as mentioned by this reviewer (i.e. the entire tetramer) we have since run calculations where we increased the exhaustiveness to 100, well beyond the highest recommended by the software (32) for both a single subunit and the full tetramer, and ran a calculation of the full tetramer with an exhaustiveness of 1000. We think that a calculation on the full tetramer was important to potentially identify binding conformations at the interfaces of the monomers.

The increased exhaustiveness searches proposed did not change the two most common general locations for CBD binding. The site/lipid binding pocket, and the site on the C-terminus of the S1 helix were identified repeatedly, in the tetramer across multiple monomers. Less common, a site packed between S4 and S5 helix near the cytoplasmic portion of the membrane was identified in the calculation for a single subunit as well as at the S4-S5 linker.

Thirdly, the cholesterol docking in Supp. Fig. S3 suggests that the hydrophobic alkyl tail of cholesterol would be pointed downward. Given the location of this pocket (at the end of the S4, which is in the area of the polar lipid headgroups based on the Orientations of Proteins in Membranes database, as well as molecular dynamics simulations (Elife. 2023 Jun 21;12:e80303)), this seems like an unlikely favourable conformation due to the high energy of solvating the alkyl tail. Thus, there are no reliable secondary or confirmatory experiments (computational or electrophysiological) performed to provide validation of one particular proposed pocket over another or for the basis of excluding any of the pockets.

This lipid binding pocket was first identified in the CryoEM structure of HCN4 as it was bound to an unknown lipid electron density (Saponaro 2021), most likely a detergent molecule. This is the primary validation of this pocket. It was originally suggested by Saponaro et al. that this site could bind membrane lipids, and thus other lipophilic compounds. We acknowledge that the error of docking calculations is higher than the differences between the calculated binding energies of CBD. However, we originally chose to highlight one potential binding site based on our functional data. The effect of CBD on HCN4 activation suggests a binding site near the S4 voltage sensor or other locations previously identified as important for HCN4 activation. As the C-terminus of S1 has not been previously implicated in HCN4 activation we suggest that this may not be a physiologically relevant binding site, leaving the lipid-binding pocket as the most likely binding site computed.

We attempted to provide secondary evidence for CBD binding to this location by making a mutation at this site: HCN4 Q401R. We performed whole cell patch clamp experiments on this mutant, before and after application of 5 uM CBD. While this channel was so slow and hyperpolarized-shifted that it was unable to quantify V1/2 or tau for activation, there was no qualitative difference before and after application with CBD, suggesting that this mutation has disrupted CBD binding. We think that this provides evidence for CBD binding to this “lipid-binding pocket”

The conclusion that CBD did not alter open probability of HCN4 is confusing since there was an obvious large effect on steady-state activation which was fit by a Boltzmann function. The Boltzmann function in this form specifically assesses the difference in probability of the channels being in the open state vs the total available channels (i.e.. open probability). Moreover, Mayar et al. do not specifically state that CBD alters mHCN1 open probability, but simply HCN1 current density (since they did not observe significant shifts in steady-state activation under their high K+ conditions). While Fig. 1E in your manuscript

suggests this is not the case in HCN4, raw traces presented in Fig. 2A and Fig. 2C are consistent with the repetitive pulse data presented in Mayar et al. It is this reviewer's opinion, based on the statement in the discussion "We found that this shift in V1/2 did not result in a significant increase in the open probability of HCN4 channels. In contrast, Mayer et al. found that in intact oocytes CBD increased the open probability of HCN1 channels without shifting the voltage dependence of activation." That the authors may be confounding "current density" with "open probability". These terms are not interchangeable.

We changed all instances to current density/current instead of open probability. The reviewer is correct, since unitary conductance was not tested in Mayer et al. it was incorrect to state that CBD increased the open probability of HCN1 channels. We thank them for bringing this to our attention.

Pg. 10 "The protocol was 20 sweeps, for a total of 60 seconds using 120 mV as the activation voltage." Should say -120 mV instead.

We changed this statement to say -120 mV.

Discussion: The mechanism of action proposed in the discussion is somewhat contradictory to the data. The author's state that "when CBD is added it stabilizes the S4 in the activated state, allowing HCN4 channels to open at weak driving forces." but their electrophysiology data suggests binding to the closed state and docking data indicates that CBD binds in the deactivated voltage-sensor configuration. Therefore, it is unclear what leads the authors to infer stabilization of the activated voltage-sensor domain over say destabilization of the closed state. The statement at the end of page 17 "We propose that CBD binds to HCN4 channels (Fig. 5) to stabilize the open state of the channel, by both depolarizing the V1/2 of activation and speeding activation kinetics" conflates mechanism with read-out in the use of the word "by". Depolarization of the voltage-dependence and speeding of activation kinetics is a potential consequence of (and thus the observation) of a stabilized open state (or destabilized closed state), not the mechanism of doing so.

We agree with the reviewer that determining the stabilization of activated vs destabilizing of closed is difficult if not impossible. To address this we changed the statement to, “We propose that CBD binds to HCN4 channels (Fig. 5) resulting in both depolarization of the $V_{1/2}$ of activation and speeding of activation kinetics. Thus, CBD allows HCN4 channels to open at weak driving forces.”

Additionally, we changed the second statement to, “We propose that CBD binds to HCN4 channels (Fig. 5) to stabilize the open state of the channel, resulting in both depolarization of the $V_{1/2}$ of activation and speeding of activation kinetics.”

Reviewer #3 (Comments to the Authors):

In their study "Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels", Page and Ruben use whole-cell patch clamp to measure the effects of the phytocannabinoid, cannabidiol, on HCN4 channels. The authors find that CBD is an HCN4 potentiator and shifts the channel activation independent of cAMP and PIP2. The authors also propose that a structurally resolved lipid

binding pocket is the most likely site by which CBD exerts its effects. While this is certainly and interesting possibility as compared to a non-specific effect due to changes in membrane properties, the authors should attempt to validate this model experimentally. Given the clinical need for HCN4 potentiators, this would better validate this pocket and CBD as platforms for future pharmaceutical development as the authors suggest.

Strengths: CBD is already in use by many as an FDA-approved and home remedy for a variety of conditions and therefore understanding it's mechanisms of action is of interest. The authors show that the effects of CBD are distinct from PIP2 and cAMP, and identify a potential function for a previously described lipid binding pocket. That CBD potentiates HCN4 without acting at the cAMP-binding pocket is unique and could indeed make it an ideal platform to potentiate HCN4 activation. This in turn has clear relevance for pharmaceuticals to increase heart rate, for example in genetic bradycardias or aging.

Major Concerns:

1) CBD seems to alter the activity of every channel [IC50s are also similar 1-5uM range (Ghovanloo et al., 2018; Orvos et al., 2020; Isaev et al., 2022)] suggesting an indirect mechanism related to the membrane. The direct interaction with the channel posited in Fig. 4 is particularly interesting as many other CBD studies seem to suggest some change in membrane fluidity is responsible. The problem is that the proposed interaction is not tested experimentally. The authors mention the difficulty in working with voltage-sensor mutants and their possible effects on channel gating [mutations of R397 are not functional (Chen et al., 2000)]. However, in the cited Chen et al. paper, the analogous Q401A (Q322A) mutant is functional (Chen et al., 2001). Because the authors suggest that the preferential binding of the CBD benzene to R397 over Q401 could be responsible for these effects, one option is Q401R [likely tolerated as this is analogous to the corresponding residue in hERG (Cowgill et al., 2019)] or Q401K to remove/reverse this preferential binding. Alternatively, given the potential binding of cholesterol at the same site, the authors could look at whether changes in cholesterol compete with CBD (Fürst and D'Avanzo, 2015).

We agree that CBD is a very promiscuous molecule with documented evidence of membrane fluidity contributing to its effects on ion channels in addition to direct binding. We thank the reviewer for these thoughtful ideas and proposed mutants, and we subsequently made the HCN4 Q401R mutation (Figure 4E, Figure S4). While this mutation was very slow to open, resulting in a linear trace even upon 8 s activation epochs, application of 5 uM CBD had no effect on channel kinetics. We think that this provides functional evidence for CBD binding to HCN4 at the proposed location in the VSD. Notably, the Q401R mutation did not act as predicted by this reviewer. A reason for this could be that the change in polarity in the binding site caused by addition of the arginine was enough to disrupt CBD binding since CBD is so lipophilic (cLogP = 6.33, Chemaxon).

2) Measurements of HCN time constants are complicated and there is some concern with the authors use of a single exponential fit.

We agree with the reviewer that the HCN time constants can be complex. To respond to this concern, we now show the example residuals for our exponential fits (Supplemental Figure 2). As shown, moving to a double fit did not decrease residuals significantly, if at all.

The authors should highlight in the text that the control HCN4 Tact values at -100mV in Holo are likely not accurate as the activation time ideally needs to be 2-3 time constants for accuracy.

We agree with the reviewer and added the following statement to the discussion "As such, τ_{Act} values described in this work from -100 mV may be faster than the true speed."

More concerning is that the time constants for activation in the cAMP unbound state appear faster than the cAMP bound state. The time constant in the Apo-state would be roughly 1200 ms at -120 mV compared to 1360 and 1270 in the holo-state control and vehicle, respectively. This may be due to fitting a single exponential equation to a multi-step process. I suggest fitting a subset of the data (eg. Apo and Holo control and 5uM CBD data) with the time to 50% activation to validate the data presented.

We understand this comment but would like to highlight that direct comparison of the apo and holo conditions is difficult because they use different voltages (Table 1). Similarly, the pre-condition holo state (1360 ms) being compared to the post-condition apo channel is difficult due to the increase in variables: both time and solution perfusion.

To assuage this concern, we can include a small dataset (n = 3) of apo-HCN4 with the holo-HCN4 activation protocol (-100 to -140 mV) which was not included in the paper originally due to the inaccuracy of $V_{1/2}$ measurements made from it with the Apo channel. With this dataset $\tau_{Act\ -120\ mV}$ = 1830 ± 100 ms.

3) In Fig. 1D the presented IC50 curve does not have any data-points in the middle (going from a non-significant effect at 1 uM to a 100% effect at 5 uM). The authors could consider a 3uM concentration point for this one measurement.

To address this comment, we now include a 2.5uM concentration data set in Figure S1 for the calculation of IC50. Inclusion of 2.5uM did not significantly change the IC50 or Hill coefficient.

4) More description of the experimental design and statistical analysis would be useful. Based on Ns in Table 1, the data appears to be conducted as a series of matched pairs with control followed by perfusion of a single CBD concentration. If so this should be explicitly stated. The statistical analysis of this data should also account for this as each cell is effectively a single block of the experimental design and can have a matched pair effect.

To address this comment, we added the following statement to page 5: "Data was collected as matched pairs, with control or "pre-condition" data collection first, followed by perfusion and data collection for a single CBD concentration or vehicle. Due to a small volume of diC8 $PI(4,5)P_2$ stock solution, pre-conditions and perfusion was not performed, opting instead for a standard bath solution of extracellular solution as described above with both 10 μM diC8 $PI(4,5)P_2$ and 5 μM CBD." We also added the following statement to page 6 "Statistical analysis of matched pair conditions was done with a paired Students t-test. Statistical analysis across different conditions consisted of either one-way ANOVA followed by post-hoc Tukey test or unpaired Student's t-tests."

Finally, please provide exact P-values for comparisons of CBD vs control in the Tables 1 and 2.

We added all pre-conditions and subsequent paired student t-test values to table 1. For Table 2, there are multiple instances where the “paired” experiments were not able to be fit to an exponential curve in both conditions. For example, at -100 mV the pre-condition was sometimes linear, but after CBD application, the speed change allowed the current to be fit nicely to the exponential curve. Because of these discrepancies in n’s, performing paired t-tests will be difficult. As such, we suggest leaving Table 2 as originally written without paired t-tests.

Minor Concerns:
1) The rationale of the study is given in the discussion, but a single sentence about cannabinoids, heart rate, and potential therapeutic consequences would be useful in the introduction.

We added the following statement to page 4: “Cannabidiol has been anecdotally reported to alter heart rate (HR) and, in other cases, have no effect on HR. In an effort to determine the molecular basis for potential effects of CBD on HR, we measured the biophysical properties of HCN, a voltage-gated ion channel known to play a major role in controlling HR, in the presence of CBD.”

2) Comparisons to the therapeutic concentration of CBD occur multiple times but the therapeutic range for CBD is never given.

There is a lack of knowledge of the exact bio-availability or the desired therapeutic plasma concentrations to elicit minimum effective doses for CBD (Millar et al. (2019) BJCP). Instead, CBD administration is normally expressed as a dosage. Reviews of current literature have found an average dose of 15mg/kg with larger dosages generally having better therapeutic outcomes. (Millar et al (2019), Arnold et al. (2022). Based on the IC50 of CBD for other therapeutic targets, like Nav channels (1-5 µM), we think it is reasonable to predict that CBD would be effective targeting HCN4 channels at similar dosages to other channels.

3) Were midpoints corrected for the liquid-junction potential?

Yes, all recordings were corrected for the liquid-junction potential.

4) Page 7: "Previous studies found that CBD increased the open probability of mHCN1 channels in intact Xenopus laevis oocytes". Is this correct? Is it more accurate to say CBD increases maximal current since it could also reflect an increase in single-channel conductance?

We changed all instances to current density/current instead of open probability. The reviewer is correct, since unitary conductance was not tested in Mayer et al. it was incorrect to state that CBD increased the open probability of HCN1 channels. We thank them for bringing this to our attention.

5) In Table 1, the same number of significant figures should be used for each value.

To address this comment, we changed the S-value averages in Table 1. We hope this is what the reviewer was referring to.

6) The light green color is slightly hard to see. Note that Fig. 3 will be difficult for people with red-green colorblindness.

We thank the reviewer for bringing this to our attention. Figure 3 conveys information also using labels and different marker styles to ensure it is still accessible to those with Red-green color blindness. However, we can modify 10uM PIP2 condition to more of a magenta shade (#F3919C) to increase contrast with the green if needed.

7) In Figure 2, the zero current level should be indicated on current traces for clarity. Also, it would be beneficial to indicate the -100mV trace on the representative currents to allow comparison between conditions.

Zero current levels were added to Figure 2A. We are unsure which figure panel the reviewer is referring to in the second half of this comment as there are no representative traces in Figure 2 except for in Fig. 2A, which is a superimposition at -120 mV. Are they suggesting a second representative superimposition for -100 mV?

8) The diC8-PIP2 current trace shown in Fig. 3C appears to be tiny amplitude (50pA?) is the scale bar correct?

This scale bar was correct, however, to address this reviewer's concerns in the next point (#9) this representative trace was replaced, as such the scale bars may now be different.

9) PIP2 appears to increase the instantaneous current substantially which warrants a mention. This also makes the measurement of activation in the tail current nearly invisible. For these currents it might be advisable to also measure the GV curve from the activating currents during the hyperpolarizing step. The GV could then be calculated using the driving force (To get the equilibrium potential you could easily fit a line to the three currents measured during the most negative voltage step followed by the corresponding current at -100mV and 0mV)

We understand this comment based on the figure presented, but we think that this is not instantaneous current, but leak and instability in the presented representative trace. We compared the other three cells and recorded an additional cell to test this (n = 5 now) and found no instantaneous current. We chose to remove this representative trace so as not to confuse the reader and have replaced it with the new trace (Figure 3C bottom; Figure 3D).

10) Page 18: "However, since CBD depolarizes the V1/2, these values are expected to be closer to true steady state than the V1/2 calculated for the vehicle, thus the ΔV1/2 may be even larger than reported here" - I would expect the opposite to be true where the isochronal activation in the non-CBD state is artificially hyperpolarized compared to steady-state?

We thank the reviewer for noting this error. We changed the discussion section on page 18: "Steady state $V_{1/2}$ values may be more depolarized than the isochronal $V_{1/2}$ values found here. However, the effect of CBD on $V_{1/2}$ is expected to be preserved at true steady state."

11) The difference with HCN1 should be highlighted in the discussion. While it could be as simple as the difference between oocytes and HEK, it could also hint at the inherent differences in gating that allow HCN1 and HCN2 to activate at more depolarized potentials compared to HCN4.

We attempted to expand on this, as suggested by the reviewer, on page 18: “This difference could be due to the use of intact oocytes versus the HEK 293T cells used in this study. Alternatively, the distinct responses to CBD could be due to isoform specific differences in HCN1 and HCN4 channels. HCN1 and HCN4 differ in both activation kinetics, thought to be due to sequence differences in the S2 helix, as well as the response to cAMP (Moosmang et al., 2001; Chen et al., 2001; Wang et al., 2001; Lolicato et al., 2011). Combined the result is HCN1 channels activating at more depolarized potentials than HCN4 channels. Thus, the distinct responses to CBD could be due to these inherent differences in HCN isoforms.”

12) Because CBD and THC have similar structures both containing a benzene ring that the authors think is crucial for cation-pi interactions with the S4 Arg residue, do the authors think the effects on HCN4 gating would be similar?

Yes, we think that THC may have similar effects as CBD, but we chose to keep this manuscript focused on CBD.

April 3, 2024

Professor Peter C Ruben
Simon Fraser University
Dept. of Biomedical Physiology & Kinesiology
8888 University Drive
Burnaby, BC V5A 1S6
Canada

Re: 202313505R1

Dear Professor Ruben,

I am pleased to let you know that we have received the reviews of your revised manuscript, titled "Cannabidiol potentiates Hyperpolarization and Cyclic-Nucleotide gated (HCN4) channels". The reviewers were very positive about the manuscript and have made only minor comments that we believe you can address readily. The editors agree with the reviewers and consider the manuscript scientifically acceptable for publication in the Journal of General Physiology (JGP). Formal acceptance will follow when it is modified per the referees' remarks and our editorial policies.

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Reviewer #1 (Comments to the Authors):

Authors have responded well to my comments. Maybe a pair of brackets in the denominator in Eq 1 would be good.

Reviewer #2 (Comments to the Authors):

The manuscript has appropriately addressed previous comments/concerns.

Reviewer #3 (Comments to the Authors):

The authors have done a good job revising the manuscript and the addition of experimental data to support the docking simulations validates the authors' conclusions. My comments are all minor details or typos.

My prior minor comment 7 as written was not clear. I was suggesting a horizontal dashed line at 0 current for the traces in Figure 2A and Figure 2C. The second part was an independent recommendation that for the representative currents families throughout the paper (eg. Figure 1A), the authors could indicate which current trace is measured at -100mV in each of the top (control) and bottom (5uM CBD) current families to highlight the shifts in activation.

20 uM CBD in Table 1: ">0.0001" should probably be <0.0001

Figure S1 could simply be used in place of Figure 1D. That the 2.5 uM standard error is larger is probably just because it is in the middle of the Hill curve.

Reviewer #1 (Comments to the Authors):

Authors have responded well to my comments. Maybe a pair of brackets in the denominator in Eq 1 would be good.

We thank the reviewer for their time editing our manuscript. We have added brackets to the denominator in Eq1.

Reviewer #2 (Comments to the Authors):

The manuscript has appropriately addressed previous comments/concerns.

We thank the reviewer for their time editing our manuscript.

Reviewer #3 (Comments to the Authors):

The authors have done a good job revising the manuscript and the addition of experimental data to support the docking simulations validates the authors' conclusions. My comments are all minor details or typos.

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We thank the reviewer for clarifying their previous comment. We have added a horizontal dashed line for Figure 2A and 2C as suggested.

We have also “highlighted” the -100mV traces of the representative currents (Figure 1A, 3A, 3C) by increasing line thickness, as suggested.

20 uM CBD in Table 1: ">0.0001" should probably be <0.0001

We have changed this value to be < 0.0001

Figure S1 could simply be used in place of Figure 1D. That the 2.5 uM standard error is larger is probably just because it is in the middle of the Hill curve.

We have replaced Fig 1D with Fig S1 as suggested.

We thank the reviewer for their time editing our manuscript.