Protein kinase C (PKC)-mediated phosphorylation of TRPM2 Thr738 counteracts the effect of cytosolic Ca2+ and elevates the temperature threshold

Makiko Kashio, Satoru Masubuchi, and Makoto Tominaga
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Alexander Binshtok (Referee #2)

Review Timeline:

| Event                      | Date       |
|----------------------------|------------|
| Submission Date            | 20-May-2022|
| Editorial Decision         | 04-Jul-2022|
| Revision Received          | 30-Jul-2022|
| Editorial Decision         | 24-Aug-2022|
| Revision Received          | 25-Aug-2022|
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Senior Editor: David Wyllie

Reviewing Editor: Carole Torsney

Transaction Report:

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

Re: JP-RP-2022-283350 "Protein kinase C (PKC)-mediated phosphorylation of TRPM2 Thr738 counteracts the effect of cytosolic Ca2+ and elevates the temperature threshold" by Makiko Kashio, Satoru Masubuchi, and Makoto Tominaga

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 3 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

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

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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I hope you will find the comments helpful and have no difficulty returning your revisions within 4 weeks.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,
REQUIRED ITEMS:

- Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See Information for Authors for further details.

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- Please ensure that any tables are in Word format and are, wherever possible, embedded in the article file itself.

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- A Statistical Summary Document, summarising the statistics presented in the manuscript, is required upon revision. It must be on the Journal's template, which can be downloaded from the link in the Statistical Summary Document section here: https://jp.mssubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

- Papers must comply with the Statistics Policy https://jp.mssubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

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- If n (less than or equal to) 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.
- Exact p values must be stated. Authors must not use ‘greater than’ or ‘less than’. Exact p values must be stated to three significant figures even when ‘no statistical significance’ is claimed.

- Statistics Summary Document completed appropriately upon revision

- Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily ‘readable’ from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type ‘Abstract Figure’. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal’s premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

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EDITOR COMMENTS

Reviewing Editor:

The referees have highlighted that this is a well performed study with clear findings that expands knowledge of temperature detection. However both scientific referees have raised some issues that need to be addressed. Moreover, there is clarification required regarding the statistical analysis employed as outlined by the Statistics Editor.

In addition, the following points should be addressed - in addition to the referee comments:

Report ‘mean (SD)’ not ‘mean {plus minus} SD’

Exact P values should be provided. For a given comparison the exact* p values must be stated to three significant figures, even when ‘no statistical significance’ is being reported. NB This must be provided in main text (if appropriate); figures or tables/figure legends; and the Statistical Summary Document, Asterisks may be used if precise p values are cited in the legend and where writing out p values would distort figure presentation.

N needs to be defined (e.g. x cells from y slices in z animals) in the Methods and statistical summary document.

Statistical summary document to be completed.

Supplemental figures are not allowed. Either incorporate in to manuscript figures of refer to results in manuscript text.

Senior Editor:

You manuscript has been assessed as being potentially acceptable for publication in The Journal of Physiology, however several queries have been raised by referees and our statistics editor - these need to be addressed. Please also note the comments from the Reviewing Editor, of particular note in addition to comments about data analysis and presentation is the fact that we do not allow supplemental figures.

Please ensure any revised submission complies with our statistical reporting requirements

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REFEREE COMMENTS

Referee #1:

I have been asked for an opinion on the statistics described in this paper further to concerns expressed during initial scrutiny. These concerns are:
1. 'Authors employed One-way ANOVA analysis throughout which I do not think is appropriate and think would be helpful to get statistic editor advice. Figure 1C - if data presented in this manner ie both PMA and FK data on same graph should this be 2-way ANOVA? In this instance there appears to be x1 control group and I am uncertain how this influences use of one or two-way ANOVA? Also confirm that Fig 2D,E and 3E,F should be 2-way ANOVA rather than one-way ANOVA While Fig 5B - use of one-way ANOVA appears appropriate - advice required re appropriate analysis for Fig 5D,E especially as a different range of Ca concentrations employed in one group in E.'

I agree that the methods and results do not hang together coherently, and that the presentation is confusing. The authors state in the statistical methods that they performed one-way ANOVA throughout, but Figure 1C, for example, depicts a factorial design with two factors (group and time), as the editor states. The figure legend implies that a between group analysis was conducted at each time point - if so, how was this done? Was it simply independent t-tests at each timepoint, with Bonferroni correction of alpha? Or was it t-tests as a follow-up to a significant interaction in a 2-way ANOVA? The same issue applies to the other examples cited by the editor. Please clarify.

The sample sizes are small, leading to a risk of exaggerated effect sizes versus the true effects for effects found to be statistically significant - Type M (magnitude) error. Caution should be exercised in drawing conclusions from this study.

Referee #2:

In this MS, the authors studied intracellular factors affecting the temperature threshold of TRPM2 channels. These regulations are well described for TRPV1 and TRPM8 channels. Authors previously demonstrated that signaling substantially affects the temperature threshold of the TRPM2 channel. In the current study authors used biochemical and electrophysiological approaches to show that other factors, beyond H2O2, also change the temperature threshold of TRPM2 channels. The authors demonstrated that TRPM2 channels are prone to PKC but not PKA phosphorylation and that this PKC phosphorylation elevated the temperature threshold for TRPM2 activation. Using alanine substitutions, the authors suggested possible candidates for the TRPM2 phosphorylation by PKC. Interestingly, the authors also show the interplay between the effect of phosphorylation and the effect of the activation-mediated elevation of intracellular calcium.

This is a well-performed study, and the results are clear, solid and well presented. The conclusions are well supported by the results. The revealed mechanisms of the temperature threshold regulation of TRPV2 channels add important insight to the physiology of temperature detection and TRPV2 channel biophysics.

I have only one general comment. I think that although the results are clear, the text needs to be improved. Sometimes the terms used are confusing, and some of the statements are too general. Some parts need English polishing. Just a few examples:

Page 3 lines 58-59 " TRP channels have significant effective stimuli" - need to be rewritten.

Page 3 lines 69-70 - thermal pain and hyperalgesia are two different phenomena.

On page 4 line 85 authors wrote, " Despite the clinical significance of TRPV1 in pain sensation, the detailed mechanisms by which its temperature thresholds are modulated have not been fully characterized." This statement misleads the reader as the paper studies TRPM2 and not TRPV1.

Page 11, line 261 - the sentence starting " For TRPM2 below..." needs to be rewritten.

Those are just a few examples. I suggest that the authors proofread the article.

Referee #3:
Kashio et al. present a study based on biochemistry and molecular biology as well as Patch Clamp techniques in which they investigated mechanisms relevant for modulation of the heat threshold in TRPM2. Previously the group published a paper about the effect of H2O2 on the heat threshold of TRPM2. Such findings are important because they allow us to understand how the modulation of the activity of an ion channel works at body temperature.

Here the authors show another mechanism for the regulation of TRPM2: PMA treatment increases the phosphorylation of TRPM2 and leads to an elevated temperature threshold. The experiments were performed with phos-tag SDS PAGE analysis. This effect was independent of changes in the calcium permeability as was shown with experiments with fixed cytosolic calcium concentrations. Thereby the phosphorylation is suggested to counteract the effect of physiological Calcium at the interior which leads to increased current amplitudes and decreased temperature thresholds. The authors then used site-directed mutagenesis to identify the phosphorylation sites relevant for the PMA-mediated phosphorylation and verified T738A as relevant. This site is postulated to decrease the effect of cytosolic Ca2+ on TRPM2 threshold and mediate the PMA effect.

1. Manuscript describes the experiments well, however abbreviations are not sufficiently introduced. They should ideally be introduced in every section when used first. Furthermore, interpunctuation regulations are not appropriately followed and more paragraphs may improve reading experience.

2. The authors describe that cytosolic calcium decreases the temperature threshold for TRPM2 activation, therefore, have they tried to apply repeated heat ramps in short sequence with fixed and non-fixed calcium? Does this desensitize the channels (in contrast to TRPV3 for example, which shows increasing current with repeated stimulation)? This would be an interesting control for the experiments shown in Fig. 2. How often were individual cells stimulated with heat?

Furthermore Fig. 2 should profit from some decent dashed lines indicating the actual thresholds in the sample recordings in A and B panels (same is true for Fig. 3 A). In addition, in panel C (of Fig. 2) the dashed lines were hardly visible. Is this due to a reduced resolution in the online version or does this need to be fixed?

3. It should be considered to explain the color code in a legend. For example, in Fig. 1 there would be space for a legend on the right side to explain the color-based grouping. Furthermore also Fig. 2 and other Figures lacks the color code. It is better to mentioned it in the Figure legend. When was the PMA applied? At the moment where it is written in the Figure - supposedly not.

4. Arrhenius plots may profit from better explanation; in addition, why are two colors used in Fig. 3 panels C and D: what are the blue tints intended to clarify?

5. Fig. .5 Do the lines between box plots in panels D and E signify that there were repeated measures performed? Or were these independent samples?

END OF COMMENTS
Responses to reviewers’ comments

We greatly appreciate the reviewers’ thoughtful review and helpful suggestions, which helped substantially improve our manuscript. We have now revised the manuscript according to the reviewers’ recommendations. In addition to responses to the reviewers’ comments, we have replaced one reference regarding thermo-TRP (Kashio 2021) with a more recent citation (Kashio and Tominaga 2022) that has updated information concerning TRP thermoregulation.

Below, we provide a point-by-point description of the changes we have made in the revised version.

Reviewing Editor:

The referees have highlighted that this is a well performed study with clear findings that expands knowledge of temperature detection. However both scientific referees have raised some issues that need to be addressed. Moreover, there is clarification required regarding the statistical analysis employed as outlined by the Statistics Editor.

We thank the Reviewing Editor for conducting the review process to improve our manuscript.

We have revised our manuscript according to the reviewers’ recommendations as follows.

Report 'mean (SD)' not 'mean {plus minus} SD'

We have made the requested update.

Exact P values should be provided. For a given comparison the exact* p values must be stated to three significant figures, even when 'no statistical significance' is being reported.

NB This must be provided in main text (if appropriate); figures or tables/figure legends; and the Statistical Summary Document, Asterisks may be used if precise p values are cited in the legend and where writing out p values would distort figure presentation.

In the revised version we include exact p values in the figures and figure legends, as well as in the Statistical Summary Document as appropriate.

N needs to be defined (e.g. x cells from y slices in z animals) in the Methods and statistical summary document.

We have defined "N" in the Methods and in the Statistical Summary Document.
Statistical summary document to be completed.

We have completed the Statistical Summary Document.

Supplemental figures are not allowed. Either incorporate into manuscript figures or refer to results in manuscript text.

We now present all figures in the main manuscript.

Senior Editor:

You manuscript has been assessed as being potentially acceptable for publication in The Journal of Physiology, however several queries have been raised by referees and our statistics editor - these need to be addressed. Please also note the comments from the Reviewing Editor, of particular note in addition to comments about data analysis and presentation is the fact that we do not allow supplemental figures.

Please ensure any revised submission complies with our statistical reporting requirements

We thank the Senior Editor for evaluating our manuscript. We have responded to the points raised and made appropriate updates in the revised manuscript.

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REFEREE COMMENTS

Referee #1:

1. 'Authors employed One-way ANOVA analysis throughout which I do not think is appropriate and think would be helpful to get statistic editor advice. Figure 1C - if data presented in this manner ie both PMA and FK data on same graph should this be 2-way ANOVA? In this instance there appears to be x1 control group and I am uncertain how this influences use of one or two-way ANOVA? Also confirm that Fig 2D,E and 3E,F should be 2-way ANOVA rather than one-way ANOVA. While Fig 5B - use of one-way ANOVA appears appropriate - advice required re appropriate analysis for Fig 5D,E especially as a different range of Ca concentrations employed in one group in E.'

We thank the reviewer for their thoughtful review and insightful suggestions.

We agree with the reviewer’s opinion that two-way ANOVA is more suitable for Fig.1C. We
prepared all samples for the PMA/FK-treated group together with one common control group consistent with the method described in our previous manuscript. We have now executed additional control experiments for the FK-treated groups in order to avoid the statistical multiplicity problem. Five control experiments were conducted together with a positive control (6h after PMA pretreatment shown below (left); these experiments were NOT used for analysis in the relevant manuscript figure) to confirm successful execution of the test system. Because no statistical difference was observed between the additional and the previous data in both control and PMA (6h) groups (Figure below, right), we concluded that the additional control data we collected can be used as a control for the FK-treated groups. Moreover, we have adopted two-way ANOVA followed by post hoc Dunnett’s multiple comparison for the PMA/FK-treated groups.

We realized that the whiskers in the previous version of Figure 1C were not correct (i.e., no min and max values within the 1.5x interquartile range, but rather 95% confidence limits) and we have made corrections in the revised version.

We have also updated the analyses for the revised Fig. 2E,F (previous Fig. 2D,E) and Fig. 3F,G (previous Fig. 3E,F) to use two-way ANOVA. As the reviewer noted, the earlier figures showing the results for T738A and T738D (previous Fig. 5D,E) included different Ca²⁺ concentration ranges for the mutants. Therefore, we decided to separate the results for T738A (revised Fig. 5D,E) and T738D (revised Fig. 5F,G) and applied two-way ANOVA and one-way ANOVA, respectively. Moreover, we realized that the data in Fig. 5B (ADPR current density in mutant channels) should be analyzed by Kruskal-Wallis ANOVA followed by a post hoc Dunn’s test due to the non-normal distribution of the data. We have corrected the
analysis of the results shown in this figure. We have provided detailed information in the statistical summary document.

I agree that the methods and results do not hang together coherently, and that the presentation is confusing. The authors state in the statistical methods that they performed one-way ANOVA throughout, but Figure 1C, for example, depicts a factorial design with two factors (group and time), as the editor states. The figure legend implies that a between group analysis was conducted at each time point - if so, how was this done? Was it simply independent t-tests at each timepoint, with Bonferroni correction of alpha? Or was it t-tests as a follow-up to a significant interaction in a 2-way ANOVA? The same issue applies to the other examples cited by the editor. Please clarify.

We have updated the statistical analysis in the revised Fig. 2E,F (previous Fig. 2D,E), Fig. 3F,G (previous Fig. 3E,F) and Fig. 5D,E to two-way ANOVA followed by post hoc multiple comparisons. Post hoc analyses were used only for cases when statistical significance was observed in ANOVA, as appropriate. To clearly describe the procedure, we added statements regarding the statistical analyses in the legend for each figure.

The sample sizes are small, leading to a risk of exaggerated effect sizes versus the true effects for effects found to be statistically significant - Type M (magnitude) error. Caution should be exercised in drawing conclusions from this study.

We thank the reviewer for this advice. Because of technical limitations, the sample size in some experiments, particularly those involving western blotting, was small. However, we believe that the main points we intended to address (i.e., the effect of phosphorylation, Ca^{2+} concentration, and mutation on the temperature threshold for TRPM2 activation) are based on a sufficient number of samples (N>10) and thus the results would support our conclusions.

**Referee #2:**

I have only one general comment. I think that although the results are clear, the text needs to be improved. Sometimes the terms used are confusing, and some of the statements are too general. Some parts need English polishing. Just a few examples:

We thank the reviewer for their interest in our study and for their helpful suggestions. We have thoroughly checked our manuscript and modified the points that the reviewer raised. Moreover, our manuscript has been checked by a native English speaker.
Page 3 lines 58-59 "TRP channels have significant effective stimuli" - need to be rewritten. 
We have changed the sentence to “TRP channels have characteristic effective stimuli” (Line 59 in the revised manuscript).

Page 3 lines 69-70 - thermal pain and hyperalgesia are two different phenomena. 
We have deleted the term “thermal pain” (Line 81 in the revised manuscript).

On page 4 line 85 authors wrote, "Despite the clinical significance of TRPV1 in pain sensation, the detailed mechanisms by which its temperature thresholds are modulated have not been fully characterized." This statement misleads the reader as the paper studies TRPM2 and not TRPV1. 
We have updated the sentence to “Despite the physiological and clinical significance of thermo-TRPs, the detailed mechanisms by which the temperature thresholds of these channels are modulated have not been fully characterized.” (Line 83-85 in the revised manuscript).

Page 11, line 261 - the sentence starting "For TRPM2 below..." needs to be rewritten. 
We have updated the sentence to "At a temperature below the threshold for TRPM2 activation, Q_{10} values were low for both PMA (-) and PMA (+)... which corresponds to Q_{10} values that reflect temperature-dependent ion diffusion (1.2~1.5)." (Line 267-270 in the revised manuscript).

Those are just a few examples. I suggest that the authors proofread the article. 
We thank the reviewer for this comment. To improve our manuscript, it has been checked by a native English speaker.

Referee #3:

1. Manuscript describes the experiments well, however abbreviations are not sufficiently introduced. They should ideally be introduced in every section when used first. Furthermore, interpunctuation regulations are not appropriately followed and more paragraphs may improve reading experience.

We thank the reviewer for this helpful suggestion. We have defined the abbreviations in the results and in the figures when they first appear. Moreover, our manuscript has been
checked by a native English speaker.

2. The authors describe that cytosolic calcium decreases the temperature threshold for TRPM2 activation, therefore, have they tried to apply repeated heat ramps in short sequence with fixed and non-fixed calcium? Does this desensitize the channels (in contrast to TRPV3 for example, which shows increasing current with repeated stimulation)? This would be an interesting control for the experiments shown in Fig. 2. How often were individual cells stimulated with heat?

In all recordings, heat stimulation was applied only once. As we mentioned in the Results (Line 256-259 in the revised manuscript), substantial Ca²⁺ influx in the presence of 2 mM extracellular Ca²⁺ through the activated TRPM2 pore caused sustained current activation even after the temperature decreased to room temperature. Therefore, we applied a TRPM2 inhibitor (flufenamic acid; FFA) to inhibit the residual current. Because of this technical limitation, repeated heat stimulations in the presence of extracellular Ca²⁺ cannot be applied to TRPM2. We carried out additional experiments to define the effect of repeated heat stimulation in the presence of fixed minimal cytosolic Ca²⁺ (100 nM) since TRPM2 cannot be activated in the complete absence of cytosolic Ca²⁺. Repeated heat stimulation caused reproducible TRPM2 activation, although the current amplitude did progressively decline (Figure indicated below). This phenomenon was recapitulated in 4 independent recordings. Therefore, these results indicate that TRPM2 is desensitized by multiple heat stimulations in the presence of cytosolic Ca²⁺ (100 nM). The densitization/desensitization process is very interesting to explore as part of ion channel function, but more thorough investigations are needed to understand how TRPM2 is desensitized (the role of Ca²⁺ ions, etc.). We will carry out these investigations in future studies.
To clearly describe the experimental conditions, we added the statement “All results were obtained from independent cells that were not exposed to repeated heat stimulations.” in the Methods (Line 190-191 in the revised manuscript).

Furthermore Fig. 2 should profit from some decent dashed lines indicating the actual thresholds in the sample recordings in A and B panels (same is true for Fig. 3 A). In addition, in panel C (of Fig. 2) the dashed lines were hardly visible. Is this due to a reduced resolution in the online version or does this need to be fixed? We added lines to Fig. 2A, B and Fig. 3A to indicate the point of the temperature threshold. The dashed lines in all figures, including Fig. 2C, were modified to increase visibility.

3. It should be considered to explain the color code in a legend. For example, in Fig. 1 there would be space for a legend on the right side to explain the color-based grouping. Furthermore also Fig. 2 and other Figures lacks the color code. It is better to mentioned it in the Figure legend. When was the PMA applied? At the moment where it is written in the Figure - supposedly not. We added color coding, which is described in the updated figure legends. We agree that the phrase “in the presence and the absence of PMA treatment” used throughout the original version could cause confusion. As such, we updated the description to PMA (-) and PMA (+) that correspond to without and with PMA “pretreatment”. Moreover, we added the sentence “All results were obtained from independent cells that were not exposed to repeated heat
stimulations." in the Methods, and "PMA treatment (100 nM 20 min) was applied 2~3h before current recordings were collected." to all relevant figure legends to reference the experimental conditions (Line 190-191 and figure legends of Fig. 2A,B, Fig. 3A and Fig. 5C in the revised manuscript).

4. Arrhenius plots may profit from better explanation; in addition, why are two colors used in Fig. 3 panels C and D: what are the blue tints intended to clarify?

We used black and red color coding to indicate PMA (-) and PMA (+) conditions, respectively, throughout manuscript. In the revised Fig. 3D,E (previous Fig. 3C,D), we wanted to represent differences in cytosolic Ca\textsuperscript{2+} concentrations with different color densities and thus adopted a gradient of blue shading to show the temperature thresholds of these traces. We have modified the figure legend accordingly to explain the assignment of colors to cytosolic Ca\textsuperscript{2+} concentrations.

5. Fig. 5 Do the lines between box plots in panels D and E signify that there were repeated measures performed? Or were these independent samples?

All recordings were obtained from independent cells that were not exposed to repeated stimulations. We have clearly mentioned this experimental condition in the Methods (Line 190-191 in the revised manuscript).
Dear Dr Kashio,

Re: JP-RP-2022-283350R1 "Protein kinase C (PKC)-mediated phosphorylation of TRPM2 Thr738 counteracts the effect of cytosolic Ca2+ and elevates the temperature threshold" by Makiko Kashio, Satoru Masubuchi, and Makoto Tominaga

Thank you for submitting your revised Research Article to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

Please advise your co-authors of this decision as soon as possible.

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

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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I hope you will find the comments helpful and have no difficulty returning your revisions within 4 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

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REVISION CHECKLIST:
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To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,
REQUIRED ITEMS:

- Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

- If n [less than or equal to] 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

- Statistics Summary Document completed appropriately upon revision

EDITOR COMMENTS

Reviewing Editor:

If the Statistical Summary Document has errors please describe what is incorrect?:
Thank you for completing the Statistical Summary Document (SSD) - regarding the 'hypothesis'. Suggest reword from:

We aimed to reveal functional regulation of TRPM2 by cytosolic Ca2+ ions and PKC-mediated TRPM2 phosphorylation.

To:

We hypothesised that TRPM2 will be functionally regulated by cytosolic Ca2+ ions and PKC-mediated TRPM2 phosphorylation

SSD - in the column panel A, please also briefly describe specific experimental aim/question

Comments to the Author:
The authors have fully addressed the concerns raised by the reviewers and Statistical Editor. There are a few minor points to attend to in the ms and Statistical Summary Document (SSD):

1. The authors were requested to report 'mean (SD)' not 'mean {plus minus} SD'. Although the rebuttal indicates they have made the requested update there are a number of places where the term mean{plus minus}SD is used (for example Figure 4 legend). Please amend
2. Figure 1 panel C - please report exact p value rather than p<0.001. The only exception is if p is less than 0.0001, in which case '<' is permitted. This may be helpful for subsequent figures also

Senior Editor:

If the statistical summary document has errors please describe what is incorrect.

Reviewing Editor has suggested:

Thank you for completing the Statistical Summary Document (SSD) - regarding the 'hypothesis'. Suggest reword from:

We aimed to reveal functional regulation of TRPM2 by cytosolic Ca2+ ions and PKC-mediated TRPM2 phosphorylation.

To:

We hypothesised that TRPM2 will be functionally regulated by cytosolic Ca2+ ions and PKC-mediated TRPM2 phosphorylation.

SSD - in the column panel A, please also briefly describe specific experimental aim/question

Comments to the Author:

There are a couple of outstanding issues that would be easier if you could correct at this stage, rather than at proof or production stage. If you carry these out I will accept your manuscript once these have been received. Thank-you for submitting this work to The Journal of Physiology

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REFEREE COMMENTS

Referee #1:

I am satisfied by the authors' responses to my original comments.

Referee #2:

The authors adequately addressed my concerns

END OF COMMENTS
Responses to reviewers’ comments

We greatly appreciate the reviewers’ thoughtful review. We have now revised the manuscript according to the reviewers’ recommendations. Below, we provide a point-by-point description of the changes we have made in the re-revised version.

Reviewing Editor:

Statistical Summary Document (SSD) 'hypothesis'.
According to editor’s suggestion, we have changed the hypothesis in SSD to
“We hypothesized that TRPM2 will be functionally regulated by cytosolic Ca^{2+} ions and PKC-mediated TRPM2 phosphorylation.”

SSD - in the column panel A, please also briefly describe specific experimental aim/question
We have filled in experimental aim/question on column panel A.

Comments to the Author:
1. The authors were requested to report 'mean (SD)' not 'mean {plus minus} SD'. Although the rebuttal indicates they have made the requested update there are a number of places where the term mean{plus minus}SD is used (for example Figure 4 legend). Please amend
We have amended the corresponding errors.

2. Figure 1 panel C - please report exact p value rather than p<0.001. The only exception is if p is less than 0.0001, in which case ‘<’ is permitted. This may be helpful for subsequent figures also
We have amended the corresponding part.

Senior Editor:
Statistical Summary Document (SSD) 'hypothesis'.
According to editor’s suggestion, we have changed the hypothesis in SSD to
“We hypothesized that TRPM2 will be functionally regulated by cytosolic Ca^{2+} ions and
PKC-mediated TRPM2 phosphorylation."

SSD - in the column panel A, please also briefly describe specific experimental aim/question
We have filled in experimental aim/question on column panel A.

REFEREE COMMENTS

Referee #1:
I am satisfied by the authors' responses to my original comments.

Referee #2:
The authors adequately addressed my concerns

We greatly appreciate the reviewers' helpful suggestions which improve our manuscript.
Dear Dr Kashio,

Re: JP-RP-2022-283350R2 "Protein kinase C (PKC)-mediated phosphorylation of TRPM2 Thr738 counteracts the effect of cytosolic Ca2+ and elevates the temperature threshold" by Makiko Kashio, Satoru Masubuchi, and Makoto Tominaga

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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Authors should note that it is too late at this point to offer corrections prior to proofing. The accepted version will be published online, ahead of the copy edited and typeset version being made available. Major corrections at proof stage, such as changes to figures, will be referred to the Reviewing Editor for approval before they can be incorporated. Only minor changes, such as to style and consistency, should be made at a proof stage. Changes that need to be made after proof stage will usually require a formal correction notice.

All queries at proof stage should be sent to TJP@wiley.com.

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Yours sincerely,

David Wyllie
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Thank-you for making these additional changes/clarifications; I am happy to accept this work for publication in The Journal of Physiology.

2nd Confidential Review