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Nitrosylation of Cardiac CaMKII at Cys290 Mediates Mechanical Afterload-Induced Increases in Ca2+ Transient and Ca2+ Sparks

Chidera Collette Alim, Don M. Bers, Juliana Mira Hernandez, Christopher Y Ko, Ye Chen-Izu, Julie Bossuyt, Erin Shen, and Sonya Baidar
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Nazha Hamdani (Referee #1)

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Dear Professor Bers,

Re: JP-RP-2022-283427 "Nitrosylation of Cardiac CaMKII at Cys290 Mediates Mechanical Afterload-Induced Increases in Ca2+ Transient and Ca2+ Sparks" by Chidera Collette Alim, Don M. Bers, Juliana Mira Hernandez, Christopher Y Ko, Ye Chen-Izu, Julie Bossuyt, Erin Shen, and Sonya Baidar

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

Yours sincerely,

Bjorn Knollmann
Senior Editor
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- You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

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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.msubmit.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.

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

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- 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.

- A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

EDITOR COMMENTS

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Both reviewers recognized the merits of this study but also suggested significant additional experiments or analysis that will improve the manuscript. Reviewer 1 specifically requests additional experiments while both reviewers highlight deficiencies in the statistical analysis. In particular, hierarchical statistical analysis is essential to avoid bias introduced by data clustering. The issue of the linearity and possible saturation of the Fluo-4 Ca reporter also needs addressing.

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Also, as indicated by Reviewer 2, please include appropriate hierarchical (nested) statistics to detect any bias due to clustering of data.

Senior Editor:

I concur with the reviewing editor. Please note that a responsive resubmission will likely require new data to address the reviewers concerns. Please adhere to the statistical guidelines, as mentioned also by the reviewers. In particular, all data points should be shown for n<30, and data analyzed using a hierarchical clustering analysis. The resubmission will require a complete statistical table and appropriate ethical approvals.

Please also note, regarding statistics: raw data need to be included.

REFEREE COMMENTS

Referee #1:

The present study "CaMKII nitrosylation mediates afterload-enhanced Ca transients" demonstrated that CaMKIIδ activation by S-nitrosylation at the C290 site is essential in mediating the intrinsic afterload-induced enhancement of myocyte SR Ca uptake, release and Ca transient amplitude (Anrep effect). The authors showed also that the NOS1 mechano-chemotransduction pathway is beneficial in allowing the heart to increase contractility to limit the reduction in stroke volume when afterload is elevated. This is a very important area of investigations as it gathers novel insights into understanding of the contribution of CaMKII activity and post-translational modifications to the myocyte modulation. The study is well designed and written, however there still some comments that have to be addressed and gaps that have to be considered.

1. Is S-nitrosylation the only redox modification that may occur on the Cys290 or for example S-glutathionylation could also occur, if so what would be then the effect of this S-glutathionylation on the CaMKII activity and myocyte function?

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3. What is the effect of the point mutation in CaMKIIδ (cys290A) on the CaMKII expression and activity?

4. What are the molecular mechanisms underlying CaMKIIδ activation by cys290 S-nitrosylation mediating the intrinsic afterload-induced enhancement of myocyte SR Ca uptake, release and Ca transient amplitude?

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6. Please state in each correlation the R-squared and the p values.

7. The authors should indicate the precise p-values? Please indicate the P values of *, **, and ***.

8. Unfortunately there is no ethical approval stated.

Referee #2:

This is a careful study of the effects of afterload and NO donors on Ca cycling in the heart., using a knockout approach to define the role of a nitrosylation site on CamKII. The work is novel. I have two major concerns that require attention. (1) It is necessary to use hierarchical statistics to avoid potential problems of pseudoreplication. (2) here is a possibility that the SR CA content measurements are affected by a lack of resolution due to indicator saturation.

Introduction. I feel that this could be written better to get the "message" across better. As it is, I felt that I was getting lost in your hydrogel, NO signalling, pre- and afterload.

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Do you really need to use "MCT" as an abbreviation? I don't think it helps readability.

Fig 2A. It isn't clear to me what this shows. I would prefer to see a time course of fluorescence. I.e. like 2D but on a longer scale including the caffeine. This would allow the reader to assess the stability of the cell over time.

Fig 2B (and elsewhere). I think that you are performing the statistical tests on the cells and ignoring the fact that, on average, three cells come from each animal. This raises the possibility of pseudo replication (Eisner, 2021). I realize that this would probably give spurious increase of significance and you find no significant change. However, I think it better practice to test with nested statistics. (I tis potentially a bigger problem for Figs 4 and 5 - see below).

Fig 2C. If you are plotting these linear regressions, please test to see if there is indeed a significant correlation.

Fig 2E. We need exact p values. Also, given that the data are paired, have you considered joining the dots (spaghetti plot) to emphasize the direction of effect of ISO in each cell?

Figs 2 and 3. It is interesting that GSNO has no effect on the amplitude of decay rate of the Ca transient in WT despite increasing spark rate. This requires comment.

Fig 3. The text does not seem to refer to the SR Ca load measurements. I have several questions here. (1) Nowhere are we shown specimen caffeine-induced records (discounting the green colour image of Fig 2A). This makes it impossible to assess their quality. (2) What is the resolution for measuring changes of SR content? The issue here is that it looks like F/Fo is about 9 at the peak of the caffeine response. The peak fluorescence achievable with the indicator is given by $F_{max} = F_o([K/Ca] +1]$ (Cheng et al., 1993). Assuming that resting Ca is 100 nM, if we take a value for Kd of 400 nM, then $F_{max} = 5F_o$., in other words, it is impossible to obtain a value of 9. A Kd of 800 nM gives F_{max} of $9K_o$. However, this would mean that the indicator is completely saturated and cannot discriminate changes of Ca. A lower affinity indicator is required - what value do the authros think is appropriate for the Kd of fliuo-4? From a practical point of view, what is the highest value of fluorescence that can be recorded? Until we know, this, we cannot decide whether the lack of apparent effect on SR content means anything other than indicator saturation.

Fig 4. The statistical analysis has been performed with unpaired t tests (D-G) or Mann-Whitney (H). These tests require that the samples are independent. However, in each experiment between 4 and 5 cells are taken from each animal. It is important to redo the analysis with hierarchical (nested) statistics. Please also give exact p values so that the reader can assess the significance.

Fig 4. You nicely show effects of afterload on the decay of the Ca transient and attribute these to increased SERCA pumping. How can you exclude a role for altered calcium buffering (Kentish & Wrzosek, 1998)?

Fig 5. Again, hierarchical statistics are required. It may be that the differences will persist, but the appropriate test needs to be performed.

REFERENCES

Cheng H, Lederer WJ & Cannell MB. (1993). Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. Science 262, 740-744.

Eisner DA. (2021). Pseudoreplication in physiology: More means less. J Gen Physiol 153.

Kentish JC & Wrzosek A. (1998). Changes in force and cytosolic Ca2+ concentration after length changes in isolated rat ventricular trabeculae. J Physiol 506 (Pt 2), 431-444.

END OF COMMENTS

Confidential Review **06-Jun-2022**

Editor and Referee Comments and Responses

Reviewing Editor:

Both reviewers recognized the merits of this study but also suggested significant additional experiments or analysis that will improve the manuscript. Reviewer 1 specifically requests additional experiments while both reviewers highlight deficiencies in the statistical analysis. In particular, hierarchical statistical analysis is essential to avoid bias introduced by data clustering. The issue of the linearity and possible saturation of the Fluo-4 Ca reporter also needs addressing.

We appreciate that the merits of our study were recognized, and we have endeavored to address all of the statistical and technical issues raised.

The ethics statement is included but it appears in the Cardiomyocyte Isolation section. It would be better placed in the preceding section where animals are first mentioned (Conventional echocardiography and Doppler imaging).

We have moved the ethics statement to the beginning of Methods (red font).

Please ensure that data are presented as means +/- SD rather than SEM. Please also state the P values for each comparison rather than simply P<0.05 etc.

We have revised the data format, made error bars for ±SD and included specific P values.

Also, as indicated by Reviewer 2, please include appropriate hierarchical (nested) statistics to detect any bias due to clustering of data.

We have revisited our data and applied nested statistics to the datasets where appropriate.

Senior Editor:

I concur with the reviewing editor. Please note that a responsive resubmission will likely require new data to address the reviewers concerns. Please adhere to the statistical guidelines, as mentioned also by the reviewers. In particular, all data points should be shown for n<30, and data analyzed using a hierarchical clustering analysis. The resubmission will require a complete statistical table and appropriate ethical approvals.

All data points are now shown, and the data analysis has been reviewed. We also attached the statistical table and added a separate section for the ethical approvals (red font).

Please also note, regarding statistics: raw data need to be included.

Additional raw data were added as appropriate (as noted below Referee 2, Fig 2B comment).

Referee Comments

Referee #1:

The present study "CaMKII nitrosylation mediates afterload-enhanced Ca transients" demonstrated that CaMKIIδ activation by S-nitrosylation at the C290 site is essential in mediating the intrinsic afterload-induced enhancement of myocyte SR Ca uptake, release and Ca transient amplitude (Anrep effect). The authors showed also that the NOS1 mechano-chemotransduction pathway is beneficial in allowing the heart to increase contractility to limit the reduction in stroke volume when afterload is elevated. This is a very important area of investigations as it gathers novel insights into understanding of the contribution of CaMKII activity and post-translational modifications to the myocyte modulation. The study is well designed and written, however there still some comments that have to be addressed and gaps that have to be considered.

We appreciate your recognition of the novelty and contribution of our study.

1. Is S-nitrosylation the only redox modification that may occur on the Cys290 or for example S-glutathionylation could also occur, if so what would be then the effect of this S-glutathionylation on the CaMKII activity and myocyte function?

We have previously measured S-nitrosylation rather directly in our JBC paper (Erickson et al. 2015; Fig 3D) and showed that compared to WT CaMKIIδ, the C290A and C273S mutants both reduced cysteine nitrosylation, and that the double mutant CaMKIIδ (C290A+C273S) caused further reduction.
Having said that, we cannot rule out other possible post-translational modifications at these S-nitrosylation sites, including glutathionylation and oxidation. In fact, we showed that C290A could also limit autonomous CaMKIIδ activation by H_2O_2 (Fig 5C-D in the same Erickson *et al.* 2015 paper). Multiple post-translational modifications in this key regulatory (autoinhibitory) domain of CaMKIIδ *all* promote autonomous activity (autophosphorylation at T287, *O*-GlcNAcylation at S280, oxidation at M281/M282, and nitrosylation at C290). Thus, we suspect that potential glutathionylation or oxidation at C290 or other post-translational modifications in this domain are likely to promote autonomous CaMKIIδ activation. This point is now mentioned in the revised manuscript (pg 9, red font).

2. Would it be possible to confirm using mass spectrometry the S-nitrosylation of cys290 after treatment with GSNO? -chemo-transduction

While this is possible, it is not practical for us to do this confirmation of our prior nitroso-cysteine antibody results noted above, in a timely fashion. We are planning to use mass spectrometry in future follow-up studies, especially to distinguish relative *S*-nitrosylation at C290 and C273 on CaMKIIδ.

3. What is the effect of the point mutation in CaMKIIδ (cys290A) on the CaMKII expression and activity?

As for CaMKII activity, we showed in the Erickson 2015 paper above that CaMKIIδ-C290A was activated by Ca-CaM comparably to WT CaMKIIδ, but also that it prevented autonomous activation by GSNO exposure. Here we have confirmed that the expression level of CaMKIIδ in the CaMKIIδ-C290A knock-in mouse was comparable to the WT littermates (new Fig. 1D).

4. What are the molecular mechanisms underlying CaMKIIδ activation by cys290 S-nitrosylation mediating the intrinsic afterload-induced enhancement of myocyte SR Ca uptake, release and Ca transient amplitude?

We are indeed interested in identifying the upstream molecular mechanisms that transduce afterload into NOS1 and CaMKII activation to cause the afterload-induced enhancement of Ca sparks and transients. That is currently the focus of another extensive detailed project (led by one of the co-authors) in a major, but separate study.

5. Does the point mutation in CaMKIIδ (cys290A) have any effect of cysteine on the CaMKII structure? or does it affect the binding of CaMKII to any partners (for example calmoduline)?

We do not expect the C290A point mutation (replacing one methyl group H with HS) to significantly alter overall CaMKII structure, but cannot rule out some change. However, we do know that it does not interfere at all with full activation by Ca-CaM (see above). In another project we focus on directly quantifying CaM-CaMKII affinity and off-rates (Simon *et al.* 2021), where phosphomimetic CaMKIIδ S287D enhances CaM affinity and off-rate is further slowed by oxidation (MM281/282). We are now extending that study to include *S*-nitrosylation and *O*-GlcNAcylation site effects and PTM interactions, but that parallel project is still ongoing.

6. Please state in each correlation the R-squared and the p values.

The R-squared and P values have been included.

7. The authors should indicate the precise p-values? Please indicate the P values of *, **, and ***.

P-values have been included throughout the figures

8. Unfortunately there is no ethical approval stated.

The ethical approval has been placed at the beginning of Methods– highlighted in red font.

Referee #2:

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We appreciate that the merits of our study were recognized, and we have endeavored to address your concerns below.

Introduction. I feel that this could be written better to get the "message" across better. As it is, I felt that I was getting lost in your hydrogel, NO signalling, pre- and afterload.

We have revised the Introduction with this in mind.

Statistics. We are shown *, **, and *** but I could not find what levels of significance these correspond to. The methods section simply states that $p<0.05$ was used as a criterion. Please clarify. Also, give exact p values for both significant and insignificant results.

We have replaced the asterisks with specific p values to clarify.

Do you really need to use "MCT" as an abbreviation? I don't think it helps readability.

No, we don't need it. Mechano-chemo-transduction is spelled out in the few places it appears.

Fig 2A. It isn't clear to me what this shows. I would prefer to see a time course of fluorescence. I.e. like 2D but on a longer scale including the caffeine. This would allow the reader to assess the stability of the cell over time.

A time course of fluorescence has been included in the revised (new) Fig 3A.

Fig 2B (and elsewhere). I think that you are performing the statistical tests on the cells and ignoring the fact that, on average, three cells come from each animal. This raises the possibility of pseudo replication (Eisner, 2021). I realize that this would probably give spurious increase of significance and you find no significant change. However, I think it better practice to test with nested statistics. (It is potentially a bigger problem for Figs 4 and 5 - see below).

We revisited our data and applied nested statistics to the datasets where appropriate. New data reanalysis using nested statistics is now included in (new) Figs 3B, 4B, 5D-H, and 6B-F.

Fig 2C. If you are plotting these linear regressions, please test to see if there is indeed a significant correlation.

The R-squared and P values have been included in (new) Fig 3C.

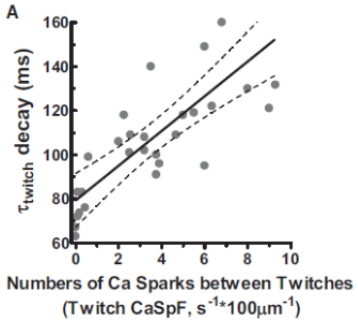
Fig 2E. We need exact p values. Also, given that the data are paired, have you considered joining the dots (spaghetti plot) to emphasize the direction of effect of ISO in each cell?

Thank you. We now indicate specific p values and joined dots for the paired data in (new) Figs. 3 & 4.

Figs 2 and 3. It is interesting that GSNO has no effect on the amplitude of decay rate of the Ca transient in WT despite increasing spark rate. This requires comment.

The $[Ca]_i$ decline during a twitch is dominated by the removal of cytoplasmic Ca by SERCA and NCX (>90% via SERCA in mouse). Although SR Ca release may not turn off abruptly at the peak of the Ca transient, the kinetics of [Ca]i decline, even with 0.5 mM caffeine (a substantial RyR sensitizer) present, are virtually identical to those in the absence of caffeine (Trafford *et al.* 2000; Eisner *et al.* 2000; Bers, 2014, Pg 118). So under normal conditions, even a sensitized RyR is unlikely to appreciably alter the kinetics of twitch [Ca]i decline. Put another way, a

substantial SR Ca leak is 0.5-1 µmol/l cytosol/sec, while SERCA is operating at a rate that is ~100 times higher during most $[Ca]_i$ decline. However, in some extreme conditions, SR Ca release events during $[Ca]_i$ decline can significantly slow twitch [Ca]i decline. An example of this was in mice with very leaky RyR2 ($CaMKII\delta_C$ transgenic; very leaky RyR) crossed with PLB-KO mice that drives $[Ca]_{SR}$ very high (Guo et al. 2012). In that case, we correlated the tau of twitch [Ca]i decline with Ca spark frequency. Thus, late SR Ca release typically does not alter [Ca]i decline but can do so under certain conditions.



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A K_d of 400 nM for Fluo-4 is typical in buffer solutions. However, most Ca indicators have much lower affinity in cardiomyocytes than in solution. Bassani, Bassani & Bers (1995) and Hove-Madsen & Bers (1992 PMID: 1420876) demonstrated 2-3 times lower affinity in cells compared to solutions for Indo-1 (844 nM vs. 250 nM; Fig 2 in PMID 7787031). Harkins…Baylor, 1993 (BiophysJ 65:865-881) also measure K_d for Fluo-3 in skeletal muscle fibers to be 1100 nM (2-3 times that in salt solutions). Ljubojevic *et al.* (2011 PMID: 21575569) also found K_d of Fluo-4 in cardiac myocyte cytosol to be 1200 nM.

For Fluo-4 in cardiomyocytes, we typically use K_d = 1100 nM, which gives a value of F_{max}/F_0 of ~12 F_0 (for resting $[Ca]_i$ of 100 nM), which is consistent with intentional indicator saturation. So, during caffeine-induced Ca transients, we are indeed, nearing saturation, and now acknowledge that this limits our ability to detect modest differences in SR Ca load (pg 6-7, red font).

Fig 4. The statistical analysis has been performed with unpaired t tests (D-G) or Mann-Whitney (H). These tests require that the samples are independent. However, in each experiment between 4 and 5 cells are taken from each animal. It is important to redo the analysis with hierarchical (nested) statistics. Please also give exact p values so that the reader can assess the significance.

Nested statistics analysis was applied to the datasets in (new) Fig 5D-H. The conclusions drawn from the hierarchical (nested) statistics are consistent with the results from our previous analysis.

Fig 4. You nicely show effects of afterload on the decay of the Ca transient and attribute these to increased SERCA pumping. How can you exclude a role for altered calcium buffering (Kentish & Wrzosek, 1998)?

We assume that you are asking about the effect of higher force to increase Ca affinity of TnC. As such, there could be more TnC-bound Ca at the peak of contraction, which would tend to reduce the Ca transient amplitude, but we observed Ca transients to be larger with the afterload. The higher Ca buffering under afterload *could* allow $[Ca]_i$ decline driven by SERCA to be faster initially, but then it should also have snubbed the Ca transient peak. So, while we cannot rule out an effect of altered myofilament Ca buffering (acknowledged now; pg 7, red font), the SR seems to be the predominant driver of the faster $[Ca]_i$ decline. A side note is that

Kentish & Wrzosek changed preload to increase force (which also changes afterload), but in our case preload was fixed.

Fig 5. Again, hierarchical statistics are required. It may be that the differences will persist, but the appropriate test needs to be performed.

Nested statistics are used in (new) Fig 6B-F, but do not alter our conclusions.

References

Bassani JW, Bassani RA, Bers DM. Calibration of indo-1 and resting intracellular [Ca] in intact rabbit cardiac myocytes. Biophys J. 1995;68(4):1453-60. PMID: 7787031; PMCID: PMC1282040.

Bers DM. Cardiac sarcoplasmic reticulum calcium leak: basis and roles in cardiac dysfunction. Annu Rev Physiol. 2014;76:107-27. PMID: 24245942.

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Eisner DA. (2021). Pseudoreplication in physiology: More means less. J Gen Physiol 153.

Eisner DA, Choi HS, Díaz ME, O'Neill SC, Trafford AW. Integrative analysis of calcium cycling in cardiac muscle. Circ Res. 2000;87(12):1087-94. PMID: 11110764

Erickson JR, Nichols CB, Uchinoumi H, Stein ML, Bossuyt J, Bers DM. S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ. J Biol Chem. 2015;290(42):25646-56. PMID: 26316536; PMCID: PMC4646208.

Guo T, Zhang T, Ginsburg KS, Mishra S, Brown JH, Bers DM. CaMKIIδ$_C$ slows $[Ca]_i$ decline in cardiac myocytes by promoting Ca sparks. Biophys J. 2012;102(11):2461-70. PMID: 22713561

Harkins AB, Kurebayashi N, Baylor SM. Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. Biophys J. 1993;65(2):865-81. PMID: 8218910; PMC1225787.

Hove-Madsen L, Bers DM. Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. Biophys J. 1992;63(1):89-97. PMID: 1420876; PMCID: PMC1262127.

Kentish JC & Wrzosek A. (1998). Changes in force and cytosolic Ca2+ concentration after length changes in isolated rat ventricular trabeculae. J Physiol 506 (Pt 2), 431-444.

Ljubojević S, Walther S, Asgarzoei M, Sedej S, Pieske B, Kockskämper J. In situ calibration of nucleoplasmic versus cytoplasmic Ca^{2}+ concentration in adult cardiomyocytes. Biophys J. 2011;100(10):2356-66. PMID: 21575569.

Simon M, Ko CY, Rebbeck RT, Baidar S, Cornea RL, Bers DM. CaMKIIδ post-translational modifications increase affinity for calmodulin inside cardiac ventricular myocytes. *J Mol Cell Cardiol*. 2021;161:53-61. PMID: 34371035

Trafford AW, Díaz ME, Sibbring GC, Eisner DA. Modulation of CICR has no maintained effect on systolic Ca2+: simultaneous measurements of sarcoplasmic reticulum and sarcolemmal Ca2+ fluxes in rat ventricular myocytes. *J Physiol*. 2000;522 Pt 2(Pt 2):259-70. PMID: 1063910

Dear Don,

Re: JP-RP-2022-283427R1 "Nitrosylation of Cardiac CaMKII at Cys290 Mediates Mechanical Afterload-Induced Increases in Ca2+ Transient and Ca2+ Sparks" by Chidera Collette Alim, Don M. Bers, Juliana Mira Hernandez, Christopher Y Ko, Ye Chen-Izu, Julie Bossuyt, Erin Shen, and Sonya Baidar

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory minor revision.

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

Best wishes

Bjorn Knollmann
Senior Editor
The Journal of Physiology

REQUIRED ITEMS:

(1) You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

(2) Please also include your ethics approval reference number in the Methods section.

(3) Your manuscript must include a complete Additional Information section
Currently, the Author Contributions section seems to be missing?

(4) Please provide (in the article file) a legend to accompany your Abstract figure.

EDITOR COMMENTS

The manuscript is almost acceptable, but a statement on food and water is still missing, and details of anesthesia for survival studies (i.e., echos) are still lacking. Also, please either increase the number of cells to show that spark frequency is reduced, or rewrite the sentence on page 7 that currently states "an apparently lower baseline Ca2+ spark frequency (although P=0.18 in the nested compound comparison)". Since there is no statistical significant difference in baseline spark frequency, a difference should not be implied.

Please also attend to the 4 'Required Items' above.

REFEREE COMMENTS

Referee #1:

The authors have satisfactorily addressed most of my concerns.

Referee #2:

A nice study.

_____

END OF COMMENTS

1st Confidential Review **12-Aug-2022**

Editors Comments and Responses

REQUIRED ITEMS:

(1) You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

Thank you for highlighting these items. Additional information and changes for the Methods, Authors Contributions, and abstract figure legend have been included in the manuscript and highlighted in red font.

Changes for animal procedures in Methods as follows:

For echocardiography imaging:

Transthoracic echocardiography was performed in mice anaesthetized by isoflurane inhalation (1.5%), which was later individually adjusted (1-3%) to achieve a stable heart rate of 400-500 beats/minute to avoid fusion of the waves. Core body temperature was carefully monitored and maintained at 37 ºC during the entire procedure.

For cardiomyocyte isolations:

The animals were kept at standard temperature, humidity, and lighting. Food and drinking water were provided ad libitum. Mechanism of death was exsanguination while the mice were unconscious.

(2) Please also include your ethics approval reference number in the Methods section.

The ethics approval reference number has now been included.

All animal handling and laboratory procedures were conducted in compliance with the NIH guidelines for animal research and with approval of the Institutional Animal Care and Use Committee at the University of California, Davis (protocol #: 21572).

(3) Your manuscript must include a complete Additional Information section. Currently, the Author Contributions section seems to be missing?

Authors Contributions have been added to the Additional Information section as follows:

C.C.A, C.Y.K, Y.C, D.M.B, and J.B conceived the conceptual design of the study. C.C.A and J.M.H performed data acquisition and analysis. C.C.A, C.Y.K, J.M.H, E.Y.S, S.B, Y.C, D.M.B, and J.B performed visualization and interpretation of results. C.C.A drafted

the manuscript, C.C.A, C.Y.K, J.M.H, E.Y.S, S.B, Y.C, D.M.B, and J.B edited and revised it critically for important intellectual content. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

(4) Please provide (in the article file) a legend to accompany your Abstract figure.

An abstract figure legend as follows has been added as follows:

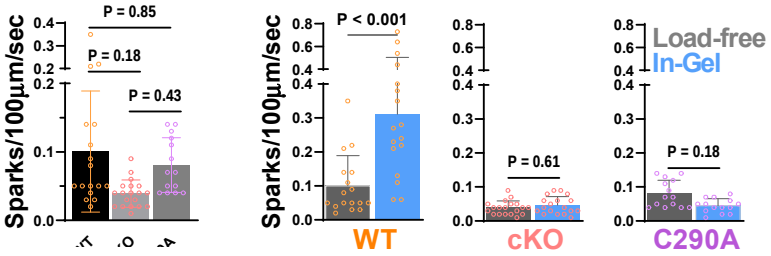
CaMKII S-nitrosylation at Cys-290 mediates mechano-chemo-transduction and afterload-induced increases in Ca^{2+} transient amplitude and Ca^{2+} sparks.

EDITOR COMMENTS

The manuscript is almost acceptable, but a statement on food and water is still missing, and details of anesthesia for survival studies (i.e., echos) are still lacking. Also, please either increase the number of cells to show that spark frequency is reduced, or rewrite the sentence on page 7 that currently states "an apparently lower baseline Ca2+ spark frequency (although P=0.18 in the nested compound comparison)". Since there is no statistical significant difference in baseline spark frequency, a difference should not be implied.

Thank you for specifying these points. We have included the statement on food and water as well as details for anesthesia (shown in section (1) above). The sentence on page 7 has also been re-written (shown below). Edits in the manuscript are indicated in red font color.

Figures 6A-B show that CaMKII-cKO and WT myocytes exhibited mean Ca spark frequencies of 0.03 and 0.1 sparks/100 µm/sec, respectively (P = 0.18, nested one-way ANOVA, multiple comparison test). Given the activating effect of CaMKII on diastolic RyR2, a difference might have been expected (Wehrens *et al.* 2004; Guo *et al*., 2006; but in resting myocytes CaMKII activation is typically low (Erickson *et al.,* 2011).



Dear Don,

Re: JP-RP-2022-283427R2 "Nitrosylation of Cardiac CaMKII at Cys290 Mediates Mechanical Afterload-Induced Increases in Ca2+ Transient and Ca2+ Sparks" by Chidera Collette Alim, Don M. Bers, Juliana Mira Hernandez, Christopher Y Ko, Ye Chen-Izu, Julie Bossuyt, Erin Shen, and Sonya Baidar

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

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EDITOR COMMENTS

The MS is now acceptable. Excellent work!

2nd Confidential Review **28-Sep-2022**