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Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle

Péter Szentesi, Beatrix Dienes, Candice Kutchukian, Tamas Czirjak, Ana Buj-Bello, Vincent Jacquemond, and Laszlo Csernoch
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1st Editorial Decision **15-Aug-2022**

Dear Dr Csernoch,

Re: JP-RP-2022-283650 "Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle" by Péter Szentesi, Beatrix Dienes, Candice Kutchukian, Tamas Czirjak, Ana Buj-Bello, Vincent Jacquemond, and Laszlo Csernoch

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EDITOR COMMENTS

Reviewing Editor:

Thank you for submitting your work to the Journal of Physiology. Two reviewers have assessed your manuscript and while both were enthusiastic about the potential impact of the work, they also raised important points that need to be addressed. Reviewer 2 in particular has indicated that the hypothesis of CICR being responsible for the propagation of calcium release in the MTM1 deficient mice is not possible and is not supported by the prevailing mammalian skeletal muscle literature. This reviewer has provided several references to support their stance and offered a potential alternative mechanism for the observed results. Reviewer #1 has also indicated that the modelling should consider potential alternative mechanisms. These concerns need to be addressed before a final decision regarding acceptance can be made.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Your report has been carefully considered by two referees and a review editor, all of which are experts in the field. Both reviewers find your work interesting but each referee has provided numerous suggestions designed to improve your report; some of these key points are summarized in the review editors comments. Please carefully consider reviewer comments when revising your report; we look forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

General comments:

The aim of the study by Szentesi and colleagues was to investigate the cause of the dysfunctional excitation-contraction coupling in skeletal muscle fibres of mice with 3-phosphoinositide phosphatase myotubularin (MTM1) deficiency. By comparing confocal calcium transients recorded from single fibres of MTM1-deficient mice to mathematically modelled simulations using known physiological parameters for excitation-contraction coupling, the authors concluded that t-tubule disruptions are a key determinant of the impaired SR calcium release, and intriguingly that modelling allowed them to discern that calcium-induced calcium release was responsible for the delayed SR calcium release, and which possibly played a role in counteracting the defective SR calcium release. The novelty of this study lies in the modelling of the excitation-contraction coupling processes to explain the defective SR calcium release observed with confocal imaging in the intact fibres of the MTM1-deficient mice, and which were apparently due to the co-existence of both voltage-gated and calcium-gated RyR1 opening. The imaging of the disrupted t-tubular network in fibres from MTM1-deficient mice with Di-8-Anepps would be sufficient evidence to allude to the fact that action potential propagation would be impaired along the sarcolemma, and which leads to the delayed, prolonged and blunted SR calcium release observed in the MTM1-deficient fibres. Nonetheless, validating through modelling that calcium-induced calcium release exists in this diseased model challenges known assumptions that RyR1 functions as a prominently mechanically-activated channel via DHPR interaction in healthy muscle and appears to be an important advance in improving our understanding of RyR1 function. An important next step is confirming that a calcium-activating site exists on RyR1 in the muscles of these MTM1 deficient mice and to explain structurally why this pathological condition is required to enable calcium-activation of RyR1. I am generally satisfied with the authors detailed description in the Discussion of the potential limitations and assumptions of their mathematical

approach. However, does the modelling consider that the prolonged SR calcium release could be explained by a potential slowed closing of the RyR1, a RyR1 leak, or slowed SR calcium reuptake?

Specific comments:

Line 49 - I suggest changing to "disruption of T-tubules results in impaired SR calcium release".

Lines 98-111 - For the lay reader, a description is lacking as to how the MTM1 deficiency leads to t-tubular disruptions and other ECC defects.

Line 115 - Change "myotubularin-deficient" to MTM1.

Line 271 - Citation missing to support the claim that calcium waves are observed in cardiac and skeletal muscle.

Line 540, 550-552 - Consider adding literature support here supporting the assumption that RyR1 has a calcium activating site. Why is this site activated only under diseased when potentially the t-tubular-DHPR-RyR1 interaction is disrupted?

Line 586 - Why were 4-5 week old mice used for the experiments?

Line 602 - Do the experiments being performed at room temperature have any consequences for interpretation of the simulation of the ECC disruption that would be observed at physiological temperature?

Referee #2:

This ms by Szentesi et al explores potential mechanisms of propagation of Ca release in MTM1 deficient skeletal muscle fibres. It is assumed that RyRs in these mutant fibres can propagate Ca release through sensitivity to cytoplasmic Ca. This is contrary to the prevailing view of the field. RyR1 does not support Ca induced Ca release. There is not any evidence presented in the ms that shows CICR in the fibres and I find the referencing selective to support the notion of CICR, as many papers that have shown CICR absence from mammalian muscle fibres are simply ignored. Having said that, the model can be altered to more appropriately search for a mechanism that supports propagation of Ca2+ release into sections of the mutant fibres that do not initially respond to applied voltage.

Major points:

• It has been shown by across many papers that CICR is not operational in mammalian skeletal muscle (This is not an exhaustive list: Shirokova et al 1996, JGP, 1998, J.Physiol, 1999, J.Physiol; Launikonis & Stephenson, 2000, J.Physiol; Cully et al 2014, J.Physiol). For example, the citation of Zhou et al (2005) JGP (line 276, p. 10) says that Ca waves propagate at 0.2 um/ms, used as evidence for potential propagation speed in the mutant fibres, is data from frogs only! (CICR is well established in frog: Shirokova papers, etc). Mouse fibres never show propagation due to CICR in sparks. RyRs open in concert and show no propagation due to the rise in cytoplasmic Ca (Zhou et al 2005).

• The propagation speed of the Ca waves in the study are indeed acknowledged as being fast, about 3.5 mm/s. This is way too fast to be considered as a voltage-independent propagation of Ca release. There are studies that show the propagation of Ca waves (voltage-independent) are never faster than 0.1 mm/s (Figueroa et al 2012, J.Physiol; Cully et al 2014, J.Physiol). Even if the hypothesis that RyR are sensitive to Ca in the mutant fibres is considered, the rate of propagation needs to be clamped below 0.1 mm/s. This excludes CICR as the mechanism causing the propagation. I would like to see the CICR hypothesis excluded, so the authors can move forward to more likely mechanisms to explain the observation.

• What is the mechanism? It has been shown that action potentials propagate within the t-system network of healthy muscle fibres from rats in the range 5-15 mm/s (Posterino et al 2000, J.Physiol; Edwards et al 2011, J.Physiol). This rate fits with the observation presented and is hinted at in the Discussion but not currently considered. I suggest the authors include this possibility in their model. The slightly slower rate of their observation (3.5 mm/s propagation) would seem quite reasonable for a fibre with an altered t-system due to MTM1 deficiency.

• The complexity and connectivity of the t-system network throughout the length of the fibre should be acknowledged. Line-scans (1 dimension of space) tracking Ca release won't see the possible pathways of action potentials and resultant Ca release through the t-system network. Cully et al (2017, Nat Commun) provide the best representation of the human muscle t-system in 3D. Longitudinal connections are shown throughout the long axis of the fibre, providing multiple pathways passed locally depolarized or disrupted tubules.

END OF COMMENTS

Confidential Review **26-Jul-2022**

Texts in blue are the responses to the Reviewing Editor and the Referees. Texts in black are from the Referee comments.

REQUIRED ITEMS

We confirm that Laszlo Csernoch, the last author, is the Vice Rector for Scientific Affairs who responsible for 'Research Governance' at University of Debrecen. Please visit this link: https://unideb.hu/en/leaders-university

Response to the Reviewing Editor

We carefully considered all comments from the Reviewers and we provide detailed answers to all of them.

Regarding the main issue raised by Referee #2, which would reject the possibility that calcium induced calcium release (CICR) is responsible for the propagation of calcium release in the MTM1 deficient mice, we do agree with him/her that functional CICR is not supported by the prevailing mammalian skeletal muscle literature, and we concur with the quoted references. However, this is only true with respect to healthy intact fully differentiated mammalian muscle fibres. Conversely, CICR has been demonstrated to operate in a number of distinct conditions under which integrity of the t-tubule system of differentiated fibres is deficient, a condition that directly relates to the status of the MTM1-deficient fibres. This is now clarified in the revised manuscript and we provide a thorough discussion of the proposed alternative mechanism.

Regarding the alternative mechanisms suggested by Referee #1, we also provide a detailed argument as to why we believe they are not making a major contribution to the detected and modelled phenotype.

Response to Referee #1

General

Nonetheless, validating through modelling that calcium-induced calcium release exists in this diseased model challenges known assumptions that RyR1 functions as a prominently mechanically-activated channel via DHPR interaction in healthy muscle and appears to be an important advance in improving our understanding of RyR1 function. An important next step is confirming that a calcium-activating site exists on RyR1 in the muscles of these MTM1 deficient mice and to explain structurally why this pathological condition is required to enable calcium-activation of RyR1. I am generally satisfied with the authors detailed description in the Discussion of the potential limitations and assumptions of their mathematical approach. However, does the modelling consider that the prolonged SR calcium release could be explained by a potential slowed closing of the RyR1, a RyR1 leak, or slowed SR calcium reuptake?

With respect to the *step of confirming that a calcium-activating site exists on RyR1 in the muscles of these MTM1 deficient mice*, there is heavy evidence in the literature both at the functional and structure level (including the most recently described cryo-EM structure) that this site is present and, under appropriate conditions, activatable, in normal RyR1 channels (*Sárközi S, Szegedi C, Szentesi P, Csernoch L, Kovács L & Jóna I. Regulation of the rat sarcoplasmic reticulum calcium release channel by calcium. J Muscle Res Cell Motil 2000, 21:131-138.; Samsó M. A guide to the 3D structure of the ryanodine receptor type 1 by cryoEM. Protein Science 2017, 26:52-68.*). Since there is no indication whatsoever that the RyR1 encoding gene suffers any specific mutation in MTM1 deficient fibres, we believe that it is quite reasonable to assume that the calcium-activating site is also present and operant in this particular disease condition. In the absence of any reason to suspect the opposite, raising and testing the alternate hypothesis would be somewhat far-etched.

Regarding a structure-related *explanation as to why this pathological condition is required to enable calcium-activation of RyR1*, we believe that the most straightforward explanation is the loss of t-tubule integrity and obligatory concomitant loss of the RyR1 channel control by the DHPR. As also detailed in our response to Referee #2, this is a situation that has been clearly acknowledged to promote Ca^{2+}-sensitive activation of DHPR-free RyR1s in distinct models including saponin permeabilization, osmotic shocks, immaturity of the t-system (in either young undifferentiated or in de-differentiating muscle fibres, *Shirokova N, Shirokov R, Rossi D, Gonzalez A, Kirsch WG, Garcia J, Sorrentino V, Ríos E. Spatially segregated control of Ca^{2+} release in developing skeletal muscle of mice. J Physiol 1999, 521:483-495.*). This is now better acknowledged in the revised manuscript in the Discussion, page 19, line 500-526.

The question of whether the altered Ca^{2+} signals could be explained by an alternative mechanism such as slowed closing of the RyR1, RyR1 leak, or slowed SR calcium reuptake is, we believe unlikely, for the following reasons:

Slowed closing of the RyR1 channels would be a situation analogous to what we previously described in the presence of the scorpion toxin Maurocalcin (*Pouvreau S, Csernoch L, Allard B, Sabatier JM, De Waard M, Ronjat M, Jacquemond V. Transient loss of voltage control of Ca^{2+} release in the presence of maurocalcine in skeletal muscle. Biophys J 2006, 91:2206-2215.; Lukács B, Sztretye M, Almássy J, Sárközi S, Dienes B, Mabrouk K, Simut C, Szabó L, Szentesi P, De Waard M, Ronjat M, Jóna I, Csernoch L. Charged surface area of maurocalcine determines its interaction with the skeletal ryanodine receptor. Biophys J 2008, 95:3497-3509.*). Indeed, under this particular condition, we demonstrated that a substantial fraction of the RyR1 population remain open following the end of voltage-activation of the EC coupling machinery. The functional outcome is very different from everything we have observed (and modelled) in the MTM1-deficient fibres. Indeed, this produces no change in the calcium transient during the voltage pulse, but only after the end of the pulse where there is a prolonged phase of sustained calcium elevation. This is very different from the present situation.

A RyR1 leak is also, we believe an unlikely mechanism, or at least very hard to detect under the present conditions. Indeed, affected RyR1s would be leaking independent of voltage activation of the system and thus before and after the voltage pulses. In contrast, everything we describe here occurs in concurrence with voltage activation.

Slowed SR calcium reuptake is also hard to foresee as a mechanism contributing to the altered calcium transients. For one thing, the present experiments were performed in the presence of a high concentration of intracellular EGTA so that global cytosolic changes in Ca^{2+} are limited in amplitude, which limits activation of the SERCA pump and its contribution to the shaping of the transients. In addition, slowed reuptake would translate in a systematic slower decay of the calcium transients after the end of the pulse, for which we never had any clear evidence (see for instance Fig.3 in *Al-Qusairi L, Weiss N, Toussaint A, Berbey C, Messaddeq N, Kretz C, Sanoudou D, Beggs AH, Allard B, Mandel JL, Laporte J, Jacquemond V, Buj-Bello A. T-tubule disorganization and defective excitation-contraction coupling in muscle fibres lacking myotubularin lipid phosphatase. PNAS 2009, 106:18763–18768.*). Finally, assuming that the reuptake would be involved, attempting to reproduce the spatial inhomogeneities of calcium transients from a slowed reuptake would require assuming that the SERCA pump density or activity is affected in a space-dependent manner, for which there has been absolutely no experimental evidence provided.

Specific comments:

Line 49 - I suggest changing to "disruption of T-tubules results in impaired SR calcium release".

The change has been done.

Lines 98-111 - For the lay reader, a description is lacking as to how the MTM1 deficiency leads to t-tubular disruptions and other ECC defects.

This is a very important but also very tricky question that, we are afraid, nobody can answer yet. The mechanism is likely involving MTM1 interactions with other proteins, specifically BIN1 and DNM2 which are believed involved in processes of membrane curving, membrane tubulation and tubule fission, which would play a role in proper t-

tubule maintenance in the differentiated fibres (see for a recent discussion of these issues in *Gómez-Oca R, Cowling BS, Laporte J. Common pathogenic mechanisms in centronuclear and myotubular myopathies and latest treatment advances. Int J Mol Sci 2021, 22:11377.*). This is now acknowledged in the revised manuscript in the Introduction, page 5 lines 115-120.

In addition, we previously demonstrated that accumulation of the MTM1 phosphoinositide substrates may also play a role in the deficient SR Ca^{2+} release (*Rodríguez EG, Lefebvre R, Bodnár D, Legrand C, Szentesi P, Vincze J, Poulard K, Bertrand-Michel J, Csernoch L, Buj-Bello A, Jacquemond V. Phosphoinositide substrates of myotubularin affect voltage-activated Ca^{2+} release in skeletal muscle. Pflugers Arch 2014, 466:973–985.*) and this is now also specifically mentioned in the revised manuscript in the Introduction, page 5 line 121.

Line 115 - Change "myotubularin-deficient" to MTM1.

The change has been done.

Line 271 - Citation missing to support the claim that calcium waves are observed in cardiac and skeletal muscle.

We inserted appropriate references as suggested.

Line 540, 550-552 - Consider adding literature support here supporting the assumption that RyR1 has a calcium activating site. Why is this site activated only under diseased when potentially the t-tubular-DHPR-RyR1 interaction is disrupted?

Additional literature has been added that demonstrates the ability of type1 Ryanodine receptors (RyR1) to be activated by calcium. These not only include data from isolated and reconstituted channels where the single channel open probability is increased (activation) and then inhibited (inactivation) by calcium (if calcium concentration is raised from the resting level to the µM and then even higher, respectively; *Sárközi S, Szegedi C, Szentesi P, Csernoch L, Kovács L, Jóna I. Regulation of the rat sarcoplasmic reticulum calcium release channel by calcium. J Muscle Res Cell Motil 2000, 21:131–138.*) but – in response to the questions raised by the Referee #2– experiments where calcium does activate RyR1 *in situ* if the T-tubular system is disrupted. The later include measurements on Saponin-treated fibres (*Weisleder N, Zhou J, Ma J. Detection of calcium sparks in intact and permeabilized skeletal muscle fibers. Methods Mol Biol 2012, 798:395-410.; Bodnar D, Geyer N, Ruzsnavszky O, Olah T, Hegyi B, Sztretye M, Fodor J, Dienes B, Balogh A, Papp Z, Szabo L, Muller G, Csernoch L, Szentesi P. Hypermuscular mice with mutation in the myostatin gene display altered calcium signalling. J Physiol 2014, 592:1353–1365.*), fibres exposed to osmotic shock (*Wang X, Weisleder N, Collet C, Zhou J, Chu Y, Hirata Y, Zhao X, Pan Z, Brotto M, Cheng H, Ma J. Uncontrolled calcium sparks act as a dystrophic signal for mammalian skeletal muscle. Nat Cell Biol 2005, 7:525-530.*), developing myotubes (*Shirokova N, Shirokov R, Rossi D, Gonzalez A, Kirsch WG, Garcia J, Sorrentino V, Ríos E. Spatially segregated control of Ca^{2+} release in developing skeletal muscle of mice. J Physiol 1999, 521:483-495.*), and fibres from animals carrying mutations that interfere with proper T-tubular formation (*Kutchukian C, Szentesi P, Buj-Bello A,*

Csernoch L, Allard B, Jacquemond V. Ca^{2+}-induced sarcoplasmic reticulum Ca^{2+} release in myotubularin-deficient muscle fibres. Cell Calc 2019, 80:91–100.).

Line 586 - Why were 4-5 week old mice used for the experiments?

It was shown that MTM1 KO mice are viable, but their lifespan is severely reduced. They develop a generalized and progressive myopathy starting at around 4 weeks of age, with amyotrophy and accumulation of central nuclei in skeletal muscle fibers leading to death at 6–14 weeks (*Buj-Bello A, Laugel V, Messaddeq N, Zahreddine H, Laporte J, Pellissier JF, Mandel JL. The lipid phosphatase myotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice. 2002, PNAS 99:15060–15065.*).

Line 602 - Do the experiments being performed at room temperature have any consequences for interpretation of the simulation of the ECC disruption that would be observed at physiological temperature?

The differing temperature of our experiments (20-22 ^{o}C) as compared to that in the living mouse should minimally affect, if any, the calculated changes in plasma membrane/t- tubule electrical activity. It should though slow down, to some extent, the kinetics of the Ca^{2+} changes but this should not affect any of our conclusions.

Response to Referee #2

General

We understand the concerns of the distinguished Referee as healthy adult mammalian skeletal muscle fibres indeed do not support calcium-induced calcium release (CICR). This has been demonstrated by a number of laboratories and using a several different methods (a list of these are mentioned by the Referee under their first Major point). We, however, must disagree with the Referee in their conclusion(s) drawn from this fact. Most of the concerns are reflected at below (in response to the Major comments), here we would like to point to two aspects of our manuscript.

First, we did not wish to stress in our MS that we think CICR is the sole mechanism responsible for the spatio-temporal pattern (a wave front-like appearance) in the line-scan images. Quite the contrary as we demonstrate in Fig. 14 that the apparent speed of propagation in most cases cannot be explained by CICR. If this was not clear from the text, we do apologize.

Second, we give an alternative solution in Fig. 15 which does not involve CICR at all. In this framework the wave front is due to the delayed activation by voltage within the T-tubular system. We have now rewritten the text to make this point clearer. Why we did not consider further mechanisms is explained below in response to the third Major point raised.

Major

It has been shown by across many papers that CICR is not operational in mammalian skeletal muscle (This is not an exhaustive list: Shirokova et al 1996, JGP, 1998, J.Physiol, 1999, J.Physiol; Launikonis & Stephenson, 2000, J.Physiol; Cully et al 2014, J.Physiol). For example, the citation of Zhou et al (2005) JGP (line 276, p. 10) states that Ca waves propagate at 0.2 um/ms, used as evidence for potential propagation speed in the mutant fibres, is data from frogs only! (CICR is well established in frog: Shirokova papers, etc). Mouse fibres never show propagation due to CICR in sparks. RyRs open in concert and show no propagation due to the rise in cytoplasmic Ca (Zhou et al 2005).

As stated above, we share the view of the Referee that CICR does not operate in a healthy adult mammalian skeletal muscle fibre (see *e.g.* in addition the elegant experiment from the lab of dr. Ríos where calcium was un-caged right outside of a mammalian fibre and no regenerative propagation was seen; *Zhou J, Launikonis BS, Ríos E, Brum G. Regulation of Ca^{2+} sparks by Ca^{2+} and Mg^{2+} in mammalian and amphibian muscle. An RyR isoform-specific role in excitation-contraction coupling? J Gen Physiol 2004, 124:409-428.*). These experiments should, however, be extended to include the similarly extensive literature of mammalian skeletal muscle where the T-tubular system is disrupted or not yet exists. For the former, using Saponin to permeabilize or osmotic shock to disrupt the T-tubules (in otherwise healthy fibres), have demonstrated the existence of calcium sparks in adult mammalian skeletal muscle indicating the ability of type 1 Ryanodine receptors (RyR1) to respond to calcium under such un-healthy conditions (for an incomplete list see *e.g. Weisleder N, Zhou J, Ma J. Detection of calcium sparks in intact and permeabilized skeletal muscle*

fibers. Methods Mol Biol 2012, 798:395-410.; Wang X, Weisleder N, Collet C, Zhou J, Chu Y, Hirata Y, Zhao X, Pan Z, Brotto M, Cheng H, Ma J. Uncontrolled calcium sparks act as a dystrophic signal for mammalian skeletal muscle. Nat Cell Biol 2005, 7:525-530., respectively). For the latter, evidence from developing mammalian skeletal muscle have shown that in areas where the T-tubular system is not yet present calcium sparks are present whereas in areas with existing T-tubules they are not (*Shirokova N, Shirokov R, Rossi D, Gonzalez A, Kirsch WG, Garcia J, Sorrentino V, Ríos E. Spatially segregated control of Ca^{2+} release in developing skeletal muscle of mice. J Physiol 1999, 521:483-495.*). All these experiments have clearly demonstrated that RyR1, if not in its normal, i.e. voltage-sensor controlled condition is capable of being activated by calcium. In addition to the above, evidence from mammalian skeletal muscle fibres isolated from diseased muscle have demonstrated the existence of calcium sparks under such conditions (e.g. *Kutchukian C, Szentesi P, Allard B, Trochet D, Beuvin M, Berthier C, Tourneur Y, Guicheney P, Csernoch L, Bitoun M, Jacquemond V. Impaired excitation-contraction coupling in muscle fibres from the dynamin2R465W mouse model of centronuclear myopathy. J Physiol 2017, 595:7369–7382.*). In particular, our group has previously shown that calcium sparks exist in MTM1-defficent mouse skeletal muscle fibres and spontaneous calcium release can occur in voltage-clamped cells clearly indicating that not voltage fluctuations, rather calcium-induced activation underlies the opening of RyR1 in such conditions (*Kutchukian C, Szentesi P, Buj-Bello A, Csernoch L, Allard B, Jacquemond V. Ca^{2+}-induced sarcoplasmic reticulum Ca^{2+} release in myotubularin-deficient muscle fibres. Cell Calc 2019, 80:91–100.*).

The propagation speed of the Ca waves in the study are indeed acknowledged as being fast, about 3.5 mm/s. This is way too fast to be considered as a voltage-independent propagation of Ca release. There are studies that show the propagation of Ca waves (voltage-independent) are never faster than 0.1 mm/s (Figueroa et al 2012, J.Physiol; Cully et al 2014, J.Physiol). Even if the hypothesis that RyR are sensitive to Ca in the mutant fibres is considered, the rate of propagation needs to be clamped below 0.1 mm/s. This excludes CICR as the mechanism causing the propagation. I would like to see the CICR hypothesis excluded, so the authors can move forward to more likely mechanisms to explain the observation.

What is the mechanism? It has been shown that action potentials propagate within the t-system network of healthy muscle fibres from rats in the range 5-15 mm/s (Posterino et al 2000, J.Physiol; Edwards et al 2011, J.Physiol). This rate fits with the observation presented and is hinted at in the Discussion but not currently considered. I suggest the authors include this possibility in their model. The slightly slower rate of their observation (3.5 mm/s propagation) would seem quite reasonable for a fibre with an altered t-system due to MTM1 deficiency.

Based on the above we felt obliged to explore the possibility of the observed wave front to be due to CICR. We share the view of this Referee that the observed apparent speeds (see Fig. 14) are greater (by far in some cases) than what would be expected from CICR. This has been acknowledged in the MS and an alternative hypothesis was tested. In that framework the degree of disruption in the T-tubular system was assumed not to be homogenous, rather a more severe disruption was assumed to be surrounded by less severe disruptions and not-affected areas giving rise to a spatially changing T-

tubular dis-integrity. This manifests as a spatially changing "ρ" within the framework of the model. This was explored in Fig. 15 and we concluded that this could indeed explain the observations even if CICR is not present. If this conclusion was not clear from the text, we do apologize. In the revised version we now stress this more. However, based on the findings one cannot exclude the possibility that CICR does indeed operate in MTM1-defficient muscle.

As stated above, we gave an alternative explanation for the observed apparent speed of propagation independent of CICR. We do acknowledge that there might be alternative possibilities and *in situ* propagation of an action potential (AP) along longitudinal T-tubules could take place and contribute to the activation of MTM1-defficient fibres in areas which are otherwise in- or poorly accessible by the corresponding "transversal" T-tubules. Under the conditions used in our study – voltage-clamped fibres placed in solutions that prevent the activation of voltage-gated sodium channels (presence of 2 μM tetrodotoxin and 140 mM TEA being the major cation) – this is not an alternative as AP-s cannot develop. This possibility was thus not included into the model.

The complexity and connectivity of the t-system network throughout the length of the fibre should be acknowledged. Line-scans (1 dimension of space) tracking Ca release won't see the possible pathways of action potentials and resultant Ca release through the t-system network. Cully et al (2017, Nat Commun) provide the best representation of the human muscle t-system in 3D. Longitudinal connections are shown throughout the long axis of the fibre, providing multiple pathways passed locally depolarized or disrupted tubules.

We acknowledged in the Discussion of the original MS that longitudinal T-tubules could contribute (not as conducting AP-s) to the activation of the fibre. One should, however, consider the fact that under our conditions propagation of the voltage along the tubular system is purely electrotonic. This means that to initiate activation in a neighbouring area by propagation along the longitudinal T-tubules requires that the propagation along the corresponding "transversal" T-tubule be "slower" (otherwise the activation would be initiated by the corresponding "transversal" T-tubule as the distance is shorter). In this framework spatially distinct (and ever more affected) "transversal" T-tubules should be assumed to get spatio-temporarily distinct and propagating activation. This framework is not different from what we assumed in our model. Nevertheless, this aspect is now discussed in more detail in the revised MS in the Discussion, page 19, line 500-526.

Dear Dr Csernoch,

Re: JP-RP-2022-283650R1 "Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle" by Péter Szentesi, Beatrix Dienes, Candice Kutchukian, Tamas Czirjak, Ana Buj-Bello, Vincent Jacquemond, and Laszlo Csernoch

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.

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- 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:

Thank you for the thorough revisions of your manuscript and for submitting your work to the Journal of Physiology. Reviewers 1 and 3 are satisfied with the revisions and believe the proposed mechanism of CICR to be present in diseased mammalian skeletal muscle to be novel. Reviewer 2, however, remains unconvinced. The authors are encouraged to provide additional experimental evidence that CICR exists in diseased mammalian skeletal muscle, if the data are available. If no additional experimental evidence exists, then the authors are obliged to openly acknowledge throughout the manuscript, including in the abstract, that more experimental evidence is required to support their modelling data.

As accurately noted by Reviewer #1, much of the details of the animal handling and procedures are not in the current manuscript but they cite their previous papers where they performed the original experiments that were used for this modeling dataset.

Senior Editor:

Your revised manuscript has been reviewed by the two original reviewers and an additional referee. The third (and new) reviewer became involved in the review process to arbitrate differing opinions between reviewers 1 and 2. Reviewer 2 remains unconvinced about the author statements about CICR (see reviewer comments for details). However, reviewers 1 and 2 believe that your work has merit. In revising your manuscript please respond to the concerns raised by both reviewer 1 and 3 in your revised report.

REFEREE COMMENTS

Referee #1:

The authors have sufficiently addressed my previous concerns. Thank you.

****

Referee #2:

I am still greatly concerned with the statements of CICR without evidence for a such a mechanism in muscle fibres with only RyR1. The literature cited in the response letter provide no actual evidence of CICR, but only claims of it. A problem here may be that Ca sparks are assumed by some to be evidence for CICR. They are not.

The paper by Wang (2005) with osmotic-shock sparks was clearly explained through a subsequent, careful approach taken by another lab to show these events were due to depolarization (Pickering et al 2009, JGP). There is no evidence of CICR in these experiments of Wang.

Saponin is known to increase the leakiness of the RyR, probably by altering the DHPR-RyR interaction (Launikonis & Stephenson, 1997, J.Physiol). The leakiness of the RyR indicates the lowered inhibition of the RyR to opening, and the opening is likely due to the [Ca2+]SR, not the cytoplasmic Ca2+, which is the source of CICR. There's no evidence of transversal propagation of Ca release/sparks (open RyRs opening the near neighbour RyRs, obviously delayed in time) in any of these mammalian skeletal fibres treated with saponin.

Myotubes without a full t-system haven't employed their full inhibition on the RyRs to prevent Ca sparks (Zhou et al 2005, AJP). Again, this is likely [Ca2+]SR causing the release events, not CICR.

Activation of the RyR1 by luminal Ca2+ is not CICR. Is this what the authors are trying to suggest by saying CICR. Please change the text to exclude CICR and indicate luminal activation by Ca, if so. Regardless, this type of propagation is too slow to match that observed (Cully et al 2014).

Experiments in bilayers of isolated RyR1 without the same ionic conditions as in the fibre are not evidence for how the RyR will operate in the fibre.

If the authors would like to claim CICR is occurring, they are obliged to show it - this would require imaging Ca release/sparks under voltage-independent release where it is observed that Ca release propagates transversally across the fibre. Such as Zhou et al 2005 JGP show CICR in frog but its absence in mouse fibres, as there is only ever a concerted opening of RyRs in mammalian fibres. That is, the conditions shown in frogs in Zhou et al 2005 would have to recapitulated in MTM1-def fibres to make claims of CICR.

An alternative approach would be to apply mM Bapta through the pipette to the MTM1-def fibres and depolarize them. Any CICR will be suppressed. A continuation of the propagating Ca release following voltage activation under these conditions cannot be CICR.

Please show sequential opening of RyRs on the MTM1 deficient mice to claim functional CICR (in the absence of any voltage activation) or suppress it under conditions that stop the diffusion of released Ca. Without this, the suggestions of CICR should be removed from the ms.

****

Referee #3:

The manuscript by Szentesi et al is a continuation of this group's work on the mechanisms of ECC impairment in MTM1 knockout mice, a model for X-linked centronuclear myopathy. Muscle fibers from MTM1 KO mice exhibit numerous

ultrastructural derangements including regions disorganized or missing t-tubules and fewer triads. ECC alterations include inhomogeneous t-tubule charge-gated Ca2+ release and CICR in regions lack charge-mediated Ca2+ release. The goal of the present study was to generate a quantitative model that reproduced the ECC defects seen in MTM1 KO muscle fibers.

Their formulation included standard models of t-tubule depolarization, voltage and time dependence of charge 1 and 2 interconversion, t-tubule charge movement, voltage- and Ca2+ dependent RyR activation and Ca2+-dependent RyR inactivation. Their model reproduces the major spatial and temporal defects seen in MTM1 KO muscle. The major conclusion was that impaired t-tubule conductance leads to the loss of charge-gated Ca2+ release which then allows CICR in these regions. The suggestion that CICR occurs in MTM1 KO mouse muscle is novel and contradicts previous findings in healthy muscle.

Although these findings provide insight into the mechanisms underlying ECC impairment MTM1 KO muscle, they are largely unsurprising given the previously reported ultrastructural defects, thus the work is likely to have moderate impact.

A major limitation is the use of parameters derived from healthy animal in simulations of diseased muscle. The use of a normal value of SR Ca2+ is a concern given the authors observe a wide range of Ca wave propagation speed and a dependence of Ca wave propagation on free SR Ca content. However, the authors acknowledge this limitation, and the model can be refined as these values are obtained from the KO mice.

The manuscript was well written and clear, I found only a few typographical errors.

Line 100: Saponin should not be capitalized.

Line 393: mal-function should be malfunction.

Lines 446-447: "... becomes worth and worth..." do you mean "worse and worse" ?

END OF COMMENTS

1st Confidential Review **09-Sep-2022**

Response to the Reviewers' comments

Reviewer#2

While we understand the criticism of the Reviewer regarding calcium-induced calcium release (CICR) we are puzzled with their concern.

For the former a number of questions need to be addressed.

1. Should we have considered CICR in our simulations at all?

   We completely agree with the Reviewer and have acknowledged in our previous response that there are compelling evidence that CICR does not operate in healthy adult mammalian skeletal muscle fibres. However, in our earlier report on the appearance of calcium sparks in MTM1 deficient fibres (Kutchukian et al., Cell Calcium, 2019, 80:91-100.) we state that "*Indeed, one may simply picture CICR contribution to Ca^{2+} homeostasis as a collateral consequence of RYR1 channels uncoupling from the t-tubule voltage control, with no specific biological relevance to muscle function. However, one may also consider that the capacity of RYR1 to be gated by Ca^{2+} under conditions when it loses its normal control, grants the muscle fibers with an alternative compensatory mechanism to help sustain EC coupling under such distress situations.*" (see page 99, bottom paragraph in left column). We are convinced that if such a claim is stated in a paper based on experimental data, a subsequent report using mathematical modelling should, inevitably, test if that statement could be verified with the simulations. Based on the above we felt obliged to test whether CICR could explain the experimental observations or not.

2. Do experimental evidence indicate the presence of CICR in MTM1 knock-out animals?

   As presented in Fig. 7 of the above referenced report, calcium sparks with extremely large (greater than 4 μm) full-width at half maximum (FWHM) were observed in these fibres with an appreciable frequency. This morphological feature of these calcium release events excludes the possibility that they have arisen from a single cluster of Ryanodine receptors (RyRs) located in the membrane of a single sarcoplasmic reticulum (SR) surrounding an individual myofibril. Rather, RyRs from different SR should have contributed to these events. As there could not be protein-protein interactions (concerted opening) between these calcium release channels two possibilities remain. The activation of the independent but neighbouring clusters of RyRs happened randomly at the same time or these clusters "communicated" with one another *via* a different mechanism. The former seems extremely unlikely as the frequency of spark occurrence was 336.7±53.4 Hz/mm^2 = 0.33x10^{-6} ms^{-1}μm^{-2} in these fibres as reported in the aforementioned paper. This means that the probability of two sparks (at least 2 are needed to give rise to an FWHM of 4 μm) to occur in neighbouring clusters of RyRs (*i.e.* within an area of less than 4 μm^2 and within a time window of less than 5 ms; based on the size of the myofibrils and sarcomere length and the rate of line-scanning, respectively) is less than 10^{-5}. In other words, one in every 10000 would be an event with the given FWHM if they occurred randomly. We have recorded 84 such events (see panel B of Fig. 7 of the given paper). In addition, sparks with FWHM of greater than 6 μm were also seen, indicating the close to

simultaneous activation of at least 3 neighbouring clusters of RyRs, reducing the possibility (based on the above) to less than $2x10^{-10}$. This was the reason that led us to propose the possibility of CICR being the underlying reason for synchronized opening of RyRs seen under this diseased condition. This is mentioned in the Discussion in lines 508-514.
We, nevertheless, accept the criticism of the Reviewer that this is not a direct evidence for the existence of CICR, only a very likely scenario.

3. Why test CICR for global calcium release?

   As presented in Fig. 9B of our MS clear linear wave fronts were detected in the calcium transients in the line-scan images. The linearity of these wave fronts is a clear indication that they could not arise from a simple diffusion of calcium ions into areas where no calcium release occurs. Former studies on calcium transients in cardiac cells have shown that such linear wave fronts are likely to be associated with the presence of CICR (*e.g.* Takamatsu & Wier, FASEB J. 1990, 4(5):1519-1525; Galimberti & Knollmann, J Mol Cell Cardiol. 2011, 51(5):760-768.). Based on the above (point #2) it seemed quite reasonable to investigate whether the wave fronts detected in the skeletal muscle fibres of MTM1 deficient animals could also be attributed to CICR. In our report we, on the one hand, demonstrated that indeed the incorporation of CICR could give rise to wave fronts that resemble those measured. However, on the other hand, we also pointed out – see Figs. 14 and 15 – that CICR cannot explain all, maybe only a smaller portion of the detected transients.

4. Could previous publications be relevant for CICR?

   The distinguished Reviewer argues that the references we quoted in our former response (Wang et al. 2005) are insufficient to prove that CICR operates in skeletal muscle under certain modified conditions. We must agree with the Reviewer that those experiments bear no direct evidence for the presence of CICR under the conditions tested. However, one equally cannot rule out the possibility that CICR indeed operated under those conditions. Similarly, the fact that in healthy mammalian skeletal muscle fibres CICR could not be evoked doesn't prove unequivocally that it could not exist under diseased conditions. Furthermore, as discussed below, the experiments referenced by the Reviewer (Pickering et al. 2009; Launikonis & Stephenson, 1997) do not rule out the possible involvement of CICR.
   We accept the statement of the Reviewer that Pickering and coworkers have demonstrated that the activation of the dihydropyridine receptors (DHPRs) following membrane depolarization are involved in the activation of calcium release events after a sudden volume change of a skeletal muscle fibre. However, other investigations, some from our lab (Csernoch et al., J Physiol 2004, 557:43-58; Csernoch et al., J Membr Biol 2008, 226:43-55) have demonstrated that depolarization in an adult mammalian fibre under voltage-clamp conditions will not initiate a calcium spark. In other words, the direct mechanical coupling between the DHPR and the RyR will not give rise to calcium sparks, rather, to long-lasting small amplitude events (also seen by Pickering et al.). As in their report by Pickering and coworkers used nifedipine to block the DHPR and subsequently calcium sparks due to changes in tonicity, this, at the very least, leaves the possibility open that calcium entering through the DHPRs (as

the depolarization was long enough to allow the channel to open) activated the RyRs via CICR.

In their report Launikonis & Stephenson investigated the effect of saponin on SR calcium loading on skinned skeletal muscle fibres and found that it reduced the ability of the SR to accumulate calcium as a non-treated fibre. However, they applied saponin directly into the "intracellular solution" (as the fibre had its surface membrane removed) and the application lasted for 30 min. In the measurements mentioned in our former response (Bodnar et al., J Physiol 2014, 592:1353–1365) saponin was applied to an enzymatically isolated fibre with intact surface membrane for a very short time (usually less than 1 min). Furthermore, membrane permeability was continuously monitored as a fluorescent dye was present in the external solution and as soon as dye entry was observed the saponin containing solution was wash away. Therefore, it is unlikely that in those experiments saponin had any effect on the SR membrane. This, again, at the very least, leaves the possibility open that CICR was the underlying reason for the activation of calcium sparks under the referenced condition.

Thus, taken together, we believe that the only conclusion that can be drawn from the existing data is that in healthy adult mammalian skeletal muscle fibres CICR doesn't exist, while in an MTM1 deficient fibre CICR can explain some but not all aspects seen at the level of global calcium transients and local calcium release events.

For the latter – *i.e.* the concern regarding the inclusion of CICR in the MS – we must disagree with the Reviewer for two reasons.

1. First, and foremost, we clearly demonstrate and stress in the MS, in connection to Figs. 14 and 15, that CICR cannot explain all wave fronts measured in MTM1 deficient fibres. We, therefore, propose and demonstrate an alternative mechanism for this phenomenon, based solely on the disruption and consequent inappropriate conduction of voltage in the T-tubular system (Fig. 15). In those simulations CICR was not involved. As stated in the MS (lines 214-216 in the revised MS) expect for the conditions where it was explicitly mentioned otherwise R_m was set zero (this is now further strengthened in the 2nd revision; lines 300-301), *i.e.* there are usually no RyRs that could be activated by calcium. This was the case for the simulations in Fig. 14 as well. If this was not clear from the text and/or from our previous response, we do apologize. We have thus modified the text (lines 455-456 and the corresponding figure legend) to further stress that CICR was not included in the simulations for Fig. 15 where we explain how wave fronts with apparently large speeds could be due to the disrupted T-tubular network.
2. Second, based on what we have discussed above, it would have been inappropriate not to test for CICR in the simulations as in our previous report we explicitly suggested its presence. Furthermore, again as detailed above, observations regarding calcium release events in MTM1 deficient fibres can be explained if CICR is assumed to operate under these conditions. While we do accept that the simplest explanation might not be the correct one, to completely neglect the possibility that it exists is unjustified.

We would, finally, like to express our gratitude to the Reviewer for the thorough analysis of our MS and the valuable criticisms made.

Response to the Reviewers' comments

Reviewer #3

We completely agree with the Reviewer that the parameters used in the simulations are from measurements in healthy muscles. However, we would like to point out that almost all parameters are connected to DHPR and RyR. We believe that the MTM1 mutation does not alter these channels. We also think that the diffusion of Ca^{2+} is independent of the mutation. Thus the parameters that were modified (R_m, τ, and ρ) well describe the effects of the mutation. In addition, in our previous report (Al-Qusairi et al., 2009, PNAS 106:18763–18768) we demonstrated that there is no significant difference in SR calcium content between the affected and non-affected regions of MTM1 KO animals. This is now explicitly stated in the Introduction in lines 117-118.

Thank you for pointing out the typographical errors. All errors have been corrected in the revised MS.

Response to the Reviewing Editor

First of all, we would like to thank you for the opportunity to answer the reviewers' questions again. We believe that now all questions will be answered and the manuscript will finally be accepted.

In response to the criticism raised by Reviewer#2 we have detailed our previously published experiments that support the idea of CICR. In particular, the Reviewer mentions: "*Please show sequential opening of RyRs on the MTM1 deficient mice to claim functional CICR (in the absence of any voltage activation)*". In our report published in Cell Calcium (Kutchukian et al., Cell Calcium, 2019, 80:91-100.) on MTM1 deficient fibres we actually did just that (see Fig. 7 of the article). Namely, a calcium release event is presented where sequential opening of neighbouring clusters of Ryanodine receptors (RyRs) is seen. The above mentioned figure (panel B) also presents that calcium sparks with full-width at half maximum (FWHM) of greater than 4 μm were observed in large numbers in these cells. As argued in the response to the Reviewer the likelihood of detecting such events due to random opening of neighbouring RyR clusters is negligible (for certain large events it is less than $2x10^{-9}$) whereas concerted (protein-protein interaction) opening of the channels in distinct clusters is also impossible due to structural reasons. In a different publication from our lab (Al-Qusairi et al., 2009, PNAS 106:18763–18768) we also gave evidence that resting cytosolic [Ca^{2+}] as well as the SR calcium content are not affected significantly in these muscles (see response to Reviewer#3). Taken together, we believe that these experimental data clearly indicate that CICR is present in this diseased condition. Again, we, nevertheless, acknowledge in the Discussion that other mechanisms contribute to the disease-associated alterations of voltage-activated Ca^{2+} release.

We would also like to mention that in our response to the comments of Reviewer#2 – where they argue that the literature quoted in our former response is inappropriate for indications of CICR – we point out why the arguments used (additional reports quoted) by the Reviewer do not rule out/question the presence of CICR. Furthermore, we acknowledge, both in our response to the criticism and in the revised MS that not all features seen can be explained solely by assuming the presence of CICR. Rather, by the presence of improper conduction of the voltage along the affected Transverse-tubular system.

Taken together we are convinced that 1) the inclusion of CICR in the modelling is justified and 2) we have given proper consideration to all possible mechanisms that might underlie the altered calcium homeostasis in this severe muscle pathology.

2nd Revision - Editorial Decision **04-Nov-2022**

Dear Dr Csernoch,

Re: JP-RP-2022-283650R2 "Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle" by Péter Szentesi, Beatrix Dienes, Candice Kutchukian, Tamas Czirjak, Ana Buj-Bello, Vincent Jacquemond, and Laszlo Csernoch

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Yours sincerely,

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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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- '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.

EDITOR COMMENTS

Reviewing Editor:

Thank you for the thorough revisions of your manuscript and for submitting your work to the Journal of Physiology. Reviewers 1 and 3 are completely satisfied with the revisions and believe the proposed mechanism of CICR to be present in diseased mammalian skeletal muscle to be novel. Reviewer 2 has a few remaining concerns that the authors are encouraged to carefully consider and address.

REFEREE COMMENTS

Referee #1:

I have no further comments as the reviewer sufficiently addressed my concerns in their previous revision.

Referee #2:

***

I agree with the authors that there is no problem considering CICR in the model. I agree you have been careful with wording in regards to CICR in the series of papers already published. However, the problem is it is not supported by experimental evidence. If this was provided, this would be an amazing paper. I continue to have problems. I think an acknowledgement that the MTM1 mutant could have lowered RyR stability which may allow propagation of Ca waves due to luminal Ca activation would be a much more likely explanation for the slow waves and wide sparks previously observed.

Introduction.

Citing the work of Osmotic sparks is poor. There is no biophysical analysis in this paper that shows that Ca release in the sparks propagate, to provide evidence of CICR (eg. Zhou et al 2005, JGP do this very well. This should be the standard to make such a claim). The sparks in Wang are shown to be due to depolarization upon osmotic challenges by Steele's group. I don't see your alternative explanation.

p.5. "While the data...there was no evidence that CICR...". You mean the experiments were conducted well, but showed no evidence of CICR?

p.12. di-8-annepps staining needs to be used in conjunction with Ca2+ imaging to justify statements about where EC coupling is function or not (Fig 7). Otherwise, this is an assumption. Please acknowledge this as such.

p.16. Ca waves in mammals are shown NOT to be due to CICR (that is CICR is cyto Ca activation) but are shown to be due to luminal Ca activation of RyR1 (Cully 2014). You are mis-citing the work. It is possible luminal activation due to a lowering of local RyR stability in the mutant allows propagating slow waves and explains the result of wide sparks in Kutchukian et al 2019, Cell Calcium. Additionally, Duke & Steele, 2008, Cell Calcium showed large local Ca release events in mouse fibres and concluded they were due to spontaneous electrical events. This is another explanation that does not include CICR.

p. 20. Line 519 onwards. These examples of sparks are NOT consistent with CICR. Sparks are most likely activated by luminal Ca after the RyR closed state has been made less stable (by saponin, osmotic shock etc). There is no evidence of CICR in mouse fibres in Figueroa (2012). A photoreleased Ca spot would cause immediate Ca release in the fibre if there where CICR in mouse, as it does in the frog. However, it does not.

Line 529 onward: very confusing that you exclude longitudinal propagation of APs due to TEA and TTX in your studies but you include excitability in your explanation for Ca release propagation at higher rates not possible in voltage-independent Ca waves.

***

Referee #3:

The authors have made all the requested revisions.

END OF COMMENTS

2nd Confidential Review **19-Oct-2022**

EDITOR COMMENTS

Reviewing Editor:

Thank you for the thorough revisions of your manuscript and for submitting your work to the Journal of Physiology. Reviewers 1 and 3 are completely satisfied with the revisions and believe the proposed mechanism of CICR to be present in diseased mammalian skeletal muscle to be novel. Reviewer 2 has a few remaining concerns that the authors are encouraged to carefully consider and address.

First of all, we would like to thank you again for the opportunity to answer the questions of Reviewer #2. We believe that finally all questions have been answered and the manuscript will be acceptable for publication in the Journal.

Response to the Reviewers' comments

Referee #2:

The questions and notes raised by the Referee are in Italic.

I agree with the authors that there is no problem considering CICR in the model. I agree you have been careful with wording in regards to CICR in the series of papers already published. However, the problem is it is not supported by experimental evidence. If this was provided, this would be an amazing paper. I continue to have problems. I think an acknowledgement that the MTM1 mutant could have lowered RyR stability which may allow propagation of Ca waves due to luminal Ca activation would be a much more likely explanation for the slow waves and wide sparks previously observed.

We agree with the Referee that modified RyR stability could also be an underlying phenomenon for the observations in MTM1 mutant skeletal muscle. This is now explicitly acknowledged in the Discussion of the revised MS. It was not included in the model as there is evidence that the SR calcium content is not altered in these fibres (Al-Qusairi et al., 2009, PNAS 106:18763–18768) and there is no data about the open probability of RyR from MTM1 deficient muscle that would endorse lowered stability.

At this point we would like to mention that the observed relative number of sparks and embers in MTM1 KO mice was 10:1 (unpublished data). As the latter is considered as an indication of the opening of a single RyR while the former of the opening of multiple channels, this ratio clearly indicates that the simultaneous random opening of neighboring RyR can, essentially, be ruled out to explain the appearance of sparks with such a high frequency. This points to the fact that communication between channels in different RyR clusters does happen. This could in theory, as acknowledged above, be due to changes in intra-SR calcium content, albeit we have no evidence for such a change.

Introduction.

Citing the work of Osmotic sparks is poor. There is no biophysical analysis in this paper that shows that Ca release in the sparks propagate, to provide evidence of CICR (eg. Zhou et al 2005, JGP do this very well. This should be the standard to make such a claim). The sparks in Wang are shown to be due to depolarization upon osmotic challenges by Steele's group. I don't see your alternative explanation.

We acknowledge that our MS does not provide biophysical evidence (e.g. by the photo-release of caged-calcium and the consequent propagation of a calcium wave) for CICR. Nevertheless, calcium was released, in this case from the SR, and calcium waves were observed. This, inevitably, lowers SR calcium content and could be, as discussed, the initiation of altered RyR function.

As for the results of Steele's group, we apologize if our previous answer was not clear. Authors in the referenced paper show that an osmotic challenge is associated with a prolonged (several minutes long) depolarization and the appearance of short (spark-like) and long (ember-like) calcium release events. They also demonstrate that blocking the DHPR interferes with the generation of these events. While a possible interpretation of these results could be

that all these events were due to the physical interaction of DHPRs and RyRs, an alternative interpretation also exists. This is supported by the number of observations on voltage clamped mammalian fibres where depolarizing pulses could never initiate a spark only an ember. The alternative explanation for the observations of the Steele's group would then be that the ember-like events were due to direct interaction between the DHPRs and RyRs, while the spark-like events were due to calcium entering through the DHPR (during the long depolarization).

p.5. "While the data...there was no evidence that CICR...". You mean the experiments were conducted well, but showed no evidence of CICR?

Thank you for pointing out that this sentence is not completely clear. We modified it as follows:

While the data were conclusive, there was no evidence that the spatio-temporal profiles of $[Ca^{2+}]_i$ seen in MTM1 deficient muscle fibres could indeed be generated by CICR during voltage-clamp activation.

p.12. di-8-annepps staining needs to be used in conjunction with Ca^{2+} imaging to justify statements about where EC coupling is function or not (Fig 7). Otherwise, this is an assumption. Please acknowledge this as such.

We agree with the Referee that a spark experiment with simultaneous di-8-annepps staining could prove that sparks occur at places of disrupted T-tubules. Until then this remains an assumption. Thus we modified the text as suggested.

"These observations raise the possibility that in MTM1 deficient fibres areas with both normal and modified ECC could be present."

p.16. Ca waves in mammals are shown NOT to be due to CICR (that is CICR is cyto Ca activation) but are shown to be due to luminal Ca activation of RyR1 (Cully 2014). You are mis-citing the work. It is possible luminal activation due to a lowering of local RyR stability in the mutant allows propagating slow waves and explains the result of wide sparks in Kutchukian et al 2019, Cell Calcium. Additionally, Duke & Steele, 2008, Cell Calcium showed large local Ca release events in mouse fibres and concluded they were due to spontaneous electrical events. This is another explanation that does not include CICR.

We would like to drawn the attention of the Reviewer that we did not say that the calcium waves observed by Cully et al. (2014) were the consequence of CICR. We just stated that calcium waves were detected in mammalian skeletal muscle as well. We have extended this sentence in the revised MS with "under special conditions" to stress that this is not a physiological condition, as well as acknowledge the interpretation suggested by Cully and coworkers.

p. 20. Line 519 onwards. These examples of sparks are NOT consistent with CICR. Sparks are most likely activated by luminal Ca after the RyR closed state has been made less stable (by saponin, osmotic shock etc). There is no evidence of CICR in mouse fibres in Figueroa (2012). A photoreleased Ca spot would cause immediate Ca release in the fibre if there where CICR in mouse, as it does in the frog. However, it does not.

We agree that data from Figueroa et al. (2014) provide clear evidence for absence of CICR in healthy adult mouse skeletal muscle. We used their data on the speed of calcium wave propagation in frog to compare with those observed in MTM1 mouse fibres. Again, we are not claiming that CICR operates in normal mouse fibres, but that it could operate in MTM1-deficient fibres experiencing destruction of the T-tubular network. Our expectation is, therefore, that photorelease of Ca^{2+} in such areas of MTM1-KO fibres would cause immediate Ca^{2+} release.

We modified the text to make our explanation clearer.

Line 529 onward: very confusing that you exclude longitudinal propagation of APs due to TEA and TTX in your studies but you include excitability in your explanation for Ca release propagation at higher rates not possible in voltage-independent Ca waves.

Please consider the first column (V_m) in Figure 4 of the MS. Here it is clearly visible (please follow e.g. the pink traces from bottom to top) that if the distance is increasing from the surface membrane (r/a: from 0.8 to 0.2), the time to reach a given membrane potential is also increasing. In other words, the longer the distance from the surface membrane to a given point in the interior of the fibre, the longer will the time be to reach a given degree of depolarization. This is also true if longitudinal propagation is assumed, thus if the depolarization is arriving through longitudinal tubules to a disrupted T-tubule area (i.e. first transversally in a distant T-tubule and then longitudinally in a "longitudinal" T-tubule, therefore increasing the distance), it would have a longer delay. This will appear as if the excitation would be "traveling".

Thus we modified the text to make it more clear.

Dear Dr Csernoch,

Re: JP-RP-2022-283650R3 "Disrupted T-tubular network accounts for asynchronous calcium release in MTM1 deficient skeletal muscle" by Péter Szentesi, Beatrix Dienes, Candice Kutchukian, Tamas Czirjak, Ana Buj-Bello, Vincent Jacquemond, and Laszlo Csernoch

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

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Thank you for the thorough revisions of your manuscript. This is a well-conducted modelling study that will likely have a sustained impact on the field.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Your revised report is now acceptable for publication. Congratulations on the completion of an excellent study.

3rd Confidential Review **10-Nov-2022**