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TRPV1 in arteries enables a rapid myogenic tone

Thieu Phan, Hoai Ton, Hajnalka Gulyás, Robert Porszasz, Attila Toth, Rebekah Russo, Matthew W Kay, Niaz Sahibzada, and Gerard P Ahern
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Corresponding author(s): Gerard Ahern (gpa3@georgetown.edu)

The referees have opted to remain anonymous.

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

Dear Dr Ahern,

Re: JP-RP-2021-281576 "TRPV1 in arteries enables a rapid myogenic tone" by Thieu Phan, Hoai Ton, Hajnalka Gulyás, Robert Porszasz, Attila Toth, Rebekah Russo, Matthew Kay, Niaz Sahibzada, and Gerard P Ahern

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 Referees and the reports are copied below.

Please let your co-authors know of the following editorial decision as quickly as possible.

As you will see, in its current form, the manuscript is not acceptable for publication in The Journal of Physiology. In comments to me, the Reviewing Editor expressed interest in the potential of this study, but much work still needs to be done (and this may include new experiments) in order to satisfactorily address the concerns raised in the reports.

In view of this interest, I would like to offer you the opportunity to carry out all of the changes requested in full, and to resubmit a new manuscript using the "Submit Special Case Resubmission for JP-RP-2021-281576..." on your homepage.

We cannot, of course, guarantee ultimate acceptance at this stage as the revisions required are substantial. However, we encourage you to consider the requested changes and resubmit your work to us if you are able to complete or address all changes.

A new manuscript would be renumbered and redated, but the original referees would be consulted wherever possible. An additional referee's opinion could be sought, if the Reviewing Editor felt it necessary. A full response to each of the reports should be uploaded with a new version.

I hope that the points raised in the reports will be helpful to you.

Yours sincerely,

Professor Don M. Bers
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EDITOR COMMENTS

Reviewing Editor:

Following their impressive mapping of smooth muscle TRPV1 expression in the murine vasculature, this manuscript by Phan et al describes investigations into the role of TRPV1 channels in the regulation of myogenic tone. The authors demonstrate that TRPV1 antagonists decrease vascular tone in a variety of experimental preparations, both ex vivo and in vivo. A specific effect on TRPV1 channels is suggested for most experiments by a absence of an effect of the blockers in TRPV1-null mice. After identifying upregulated TRPM4 channels in their genetic model, the authors also show that this channel also contributes to myogenic vasoregulation. To round off their manuscript, the authors attempt to dissect out the pathway by which mechanical forces activate TRPV1 to regulate vascular tone and tentatively suggest that mechanosensitive GPCRs are responsible.

The general consensus of the referees is that the manuscript presents a well-written, carefully conducted look at the role of TRPV1 in myogenic regulation. However, both referees raise important concerns. Addressing these concerns, which may require additional experimentation, will strengthen the conclusions of the manuscript. In addition to the two expert referees, this reviewing editor has also reviewed the manuscript. In my, this is an impressive description of the involvement of TRPV1 in the regulation of myogenic tone. However, there is some data that is conspicuous in its absence and I give details on this below. Moreover, there are some statistical reporting issues that must be addressed.

1 - In the majority of experiments, the authors confirm the specificity of BCTC by its lack of effect in TRPV1-null mice. However, Figure 5 is missing this important control data. What effect does BCTC have on myogenic tone in their TRPV1-null mice?

2 - It is unclear if the authors have presented technical replicates as biological replicates in some of the Figures. As an example, did the authors repeat their isolated smooth muscle cell experiments (Figure 2A-C) in cells from 47 different wild-type animals, 35 times with BCTC, and in 30 TRPV1-null animals? If so, this seems rather excessive biological replication and may raise ethical concerns. In Figure 2D-F, did the authors investigate myogenic responses in arteries from 51 animals? It seems more likely that the authors have presented technical replicates (multiple cells from a single animal) as biological replicates. Please clarify the unit of assessment in the methods section and in all figure legends. In the case of technical replication, please ensure an appropriate statistical assessment has been used.

3 - Referee 2 highlights the importance of confirming the specificity of BCTC in arteries lacking TRPV1 expression. The authors impressively mapped out TRPV1 expression in their previous work. I believe the authors showed only limited TRPV1 expression in mesenteric or cerebral arteries, and so a lack of effect of BCTC on myogenic tone in these arteries would further strengthen the use of these drugs. Alternatively, the authors might consider looking at the effects of BCTC on calcium signalling evoked by hypo-osmotic solution in cells from larger arteries that have no TRPV1 expression.

4 - I assume inner diameter, but the authors should clarify if they have measured inner or outer blood vessel diameter throughout

5 - Please ensure that the manuscript complies with The Journal of Physiology policies on statistics.

- Please define the unit of assessment, n. It is unclear if n refers to biological replicates (animals) or technical replicates (arteries, or cells from the same animal). If technical replication has been used, the statistical test must account for this.

- Please report the statistical test used in each Figure legend.

Senior Editor:

Please see the Reviewing Editor's comments above. A responsive resubmission with additional data is encouraged.

REFEREE COMMENTS

Referee #1:

This manuscript, by Phan et al, investigates the role of TRPV1 channels in rapid myogenic tone development in skeletal muscle arterioles. They conclude that TRPV1 channels are responsible for the majority of myogenic tone development in skeletal and coronary arteries. While the data are well-presented, I have multiple concerns that should be addressed. Of greatest concern is the lack of consideration given to the biophysical properties of the channel as it relates to the physiological effects seen in vivo, which diminishes the conclusions drawn in the current work. My major and minor comments are as follows:

Major:

• Figure 1 lists the concentration of AMG517 as 10 μM in the text and panel A, but 3 μM in panel D and in the legend. Please clarify, as the IC50 of this drug is sub-nanomolar I believe. Either concentration, in this case, is extremely high. Since nothing happened in the knockout it's probably still specific; I would just like clarification as to the real concentration used and an explanation as to why so high (if there was a reason).

• Figure 3B: 2 data points are missing due to the way the y axis is split. Given the wide variability, the graph should be done in such a way as to include them.

• How is it that resiniferatoxin, given s.c., can cause such a selective sensory nerve effect without substantial vascular effects? IS this to say that the vascular TRPV1 desensitizes differently than the nerve TRPV1 channel? Please include references for the validation of this model, or data to support the loss of sensory nerve function in these mice at the time of infusion. Stating that LacZ expression was absent is insufficient without including the data. Also, functional tests would be ideal to show that you've selectively ablated only pain reflexes and that vascular TRPV1 remains intact. Isolated arterioles would also be effective in showing this is a vascular response.

• Was body temperature measured during the experiments in Figure 4? Could the hyperthermic effects of both drugs also drive peripheral vasodilation independent of vascular TRPV1?

• How do you explain the presence of tone in V1KO mice? Compensation by another TRP channel? What was the effect of BCTC on the time constant for the remaining tone that developed?

• Figure 7: It is extremely difficult to connect TRPV1 and LVGCC function in the way you state. Nifedipine nearly abolishes tone in almost all vessels this size, so the effect of BCTC on top of it is not something you can draw much of a conclusion from in the absence of some complex patch clamp experiments. It is interesting that both V1 and M4 seem to be a source of depolarization to drive CaV11.2 opening, at least in the pharmacological sense. However, these conclusions cannot be properly drawn without measurement of V1, M4 and CaV1.2 currents in these smooth muscle cells.

• If this reflex reactive dilation is physiologically relevant, then the V1KO mice should present an appreciable phenotype due to the lack of such a response. Do they?

• By stating that TRPV1 is rapidly deactivated to promote rebound dilation, the authors imply that TRPV1 is active more often than not. Given the single channel conductance of this channel, this is unlikely - as with most TRP channels, it is most often closed due to the massive amount of ions it can let in when open. How is it that this channel is regulated?

• What about high temperature or proton-evoked calcium signals? Effecting either of these would also be reinforcement of the involvement of V1. The authors present heat as a stimulus in Figure 10's model, but don't test it.

• The PKC/PLC experiments could just as easily be explained by relief of BK channel inhibition, since BK channel feedback is a well-documented mechanism opposing tone development. PKC has such ubiquitous roles in regulating ion channels that the data presented in Fig. 10 do little reinforce the conclusion without the inclusion of KO animals. Also, V1 isn't activated until noxious heat >40{degree sign}C, making it unlikely heat is the driver. The PKC-dependent drop in temperature sensitivity would imply that V1 could be wide-open at body temp - again, given the biophysical properties of the channel, this would be extremely harmful.

• The authors also imply that activation of V1 alters the ion selectivity of the channel. The selectivity of this channel is 9-fold greater for Ca over Na, and more likely to be influenced by the relative concentrations of extracellular Na and Ca than anything else. Without references to changes in selectivity based on stimuli, this is categorically false.

Minor:

• Confirming the specificity of BCTC in V1KO mice would be a nice addition to figure 5, as it would validate the specificity and reinforce your earlier claims.

• Your representative trace in 7B does not match your data in 7D: the summary shows that 50% of the dilation should be due to M4 block and another 50% due to V1 block. The trace in B looks very different. Also that vessel did not fully stabilize in its tone, suggesting there is a slow component to tone as well as the fast one? Perhaps a different trace would be more representative.

Referee #2:

Phan et al study the role of TRPV1 in myogenic tone in heart and skeletal muscle arteries, which they previously identified as expressing this channel in smooth muscle cells. Their data suggest that TRPV1 is activated via a stretch/mechanosensation mechanism that engages PLC and protein kinase C. V1 activity then leads to Cav1.2 activation and SMC constriction.

Overall, the study is well performed and adds to our knowledge of specialization of arteriolar function in different organ systems. I have the following major and minor comments:

Major

1. The study is heavily reliant on TRPV1 pharmacology, specifically BCTC and AMG517. While the TRPV1 null mice they use are convincing and their observations in this model agree with findings in wild type mice, a further comparator is needed. Specifically, these drugs should be shown to have no effect on a wild type artery system that does not express TRPV1, such as mesenteric or cerebral arteries. This would add confidence that these are selective drug effects, and add weight to the argument that the expression of V1 in these arteries supports functional specialization.

2. Figure 10 goes some way to elucidate the mechanistic basis of TRPV1 activation to pressure. Missing here are data from TRPV1 null mice. These experiments should be repeated in TRPV1-/- mice, where the prediction is that the effect of these pharmacological maneuvers should be blunted.

Minor

1. Figure 9D and E, de-endothelialized preps do not constrict to increases in pressure. Why is this? With the SMC contractile machinery still intact one would expect potentially even a more robust constriction due to lack of EC feedback.

2. The abbreviation 'HiK+' is confusing. 'High K+', or '60 mM K+' would be much clearer.

3. Figure 3B should use a normal, rather than divided y-axis.

4. Page 13 "Interestingly, numerous studies have demonstrated a role for TRPM4 in the myogenic tone of

different vascular beds". This statement needs references.

END OF COMMENTS

Confidential Review **23-Mar-2021**

We thank the Editors and Referees for their constructive comments. As suggested, we have performed additional experiments to further support the selectivity of the TRPV1 antagonists. We present new data showing that BCTC does not alter the tone of mesenteric arteries (Fig. 1) or the arterial pressure-diameter relationship in TRPV1-KO mice (Fig. 5). In addition, we have revised the statistical analysis, and substantially changed the text to clarify the points raised by the referees.

Reviewer Editor:

1 - In the majority of experiments, the authors confirm the specificity of BCTC by its lack of effect in TRPV1-null mice. However, Figure 5 is missing this important control data. What effect does BCTC have on myogenic tone in their TRPV1-null mice?

We now show that BCTC has no effect on the myogenic tone (pressure-diameter analysis) of arteries isolated from TRPV1-null mice (Figure 5).

2 - It is unclear if the authors have presented technical replicates as biological replicates in some of the Figures. As an example, did the authors repeat their isolated smooth muscle cell experiments (Figure 2A-C) in cells from 47 different wild-type animals, 35 times with BCTC, and in 30 TRPV1-null animals? If so, this seems rather excessive biological replication and may raise ethical concerns. In Figure 2D-F, did the authors investigate myogenic responses in arteries from 51 animals? It seems more likely that the authors have presented technical replicates (multiple cells from a single animal) as biological replicates. Please clarify the unit of assessment in the methods section and in all figure legends. In the case of technical replication, please ensure an appropriate statistical assessment has been used.

We have clarified the unit of assessment throughout the manuscript and these details are included in Figure legends and the Statistical Summary document. In most case, n refers to the number of mice tested, with the number of arteries/cells also included. Nested-t tests were performed where appropriate.

3 - Referee 2 highlights the importance of confirming the specificity of BCTC in arteries lacking TRPV1 expression. The authors impressively mapped out TRPV1 expression in their previous work. I believe the authors showed only limited TRPV1 expression in mesenteric or cerebral arteries, and so a lack of effect of BCTC on myogenic tone in these arteries would further strengthen the use of these drugs. Alternatively, the authors might consider looking at the effects of BCTC on calcium signalling evoked by hypo-osmotic solution in cells from larger arteries that have no TRPV1 expression.

We now present data showing the effects of the TRPV1 antagonist BCTC on the myogenic tone of non-TRPV1 expressing mesenteric arteries (Figure 1); BCTC fails to

evoke dilation of these vessels. Together with data in TRPV1-null mice, these new data further support the on-target specificity of the TRPV1 antagonists.

4 - I assume inner diameter, but the authors should clarify if they have measured inner or outer blood vessel diameter throughout

Yes, we measured the inner diameter in each case and this is now stated in Methods.

5 - Please ensure that the manuscript complies with The Journal of Physiology policies on statistics.

- Please define the unit of assessment, n. It is unclear if n refers to biological replicates (animals) or technical replicates (arteries, or cells from the same animal). If technical replication has been used, the statistical test must account for this.

- Please report the statistical test used in each Figure legend.

As stated in reply to #2, we have clarified the unit of assessment throughout the manuscript and modified the analysis.

Referee #1

Major:

• Figure 1 lists the concentration of AMG517 as 10 μM in the text and panel A, but 3 μM in panel D and in the legend. Please clarify, as the IC50 of this drug is sub-nanomolar I believe. Either concentration, in this case, is extremely high. Since nothing happened in the knockout it's probably still specific; I would just like clarification as to the real concentration used and an explanation as to why so high (if there was a reason).

The concentration of AMG517 was 3 μM and this is now corrected in the text. The concentration response analysis for BCTC in whole tissue preparations revealed an inhibition of tone in the range 0.3 to 3 μM (**Fig 2C and D**). Therefore, we used concentrations of 1-3 μM in all other experiments. As pointed out by the reviewer these concentrations of antagonists had no effects in arteries from TRPV1-null mice or in mesenteric (non-TRPV1-expressing) arteries.

• Figure 3B: 2 data points are missing due to the way the y axis is split. Given the wide variability, the graph should be done in such a way as to include them.

We have corrected the figure to include all data points.

How is it that resiniferatoxin, given s.c., can cause such a selective sensory nerve effect without substantial vascular effects? IS this to say that the vascular TRPV1 desensitizes differently than the nerve TRPV1 channel? Please include references for the validation of this model, or data to support the loss of sensory nerve function in these mice at the time of infusion. Stating that LacZ expression was absent is insufficient without including the data. Also, functional tests would be ideal to show that you've selectively ablated only pain reflexes and that vascular TRPV1 remains intact. Isolated arterioles would also be effective in showing this is a vascular response.

To clarify, RTX was administered to neonatal mice (P2 and P5) and 8 weeks later BP measurements were performed. High dose capsaicin/RTX treatment permanently deletes almost all TRPV1-expressing sensory neurons (Jancso G, et al. 1977. *Nature* **270**, 741–743; Szallasi A & Blumberg PM, 1992. *Neurosci Lett* **140**, 51–54.) In contrast, our previous studies show that TRPV1 expression in arteries is unaltered by capsaicin/RTX treatment (Czikora A. et al. 2013.; Phan T. et al. 2020). This likely reflects turnover/replacement of TRPV1-expressing arteriolar myocytes. Previously, we showed that the sustained BP increase in response to capsaicin was unaltered in RTX-treated mice (Phan T. et al. 2020), demonstrating a vascular-delimited action. In the current manuscript, we show that the depressor responses to TRPV1 antagonists are unaffected by nerve ablation. We have added these references to the text.

We measured LacZ staining and performed behavioral tests to confirm ablation as previously described (Phan T. et al. 2020). We now clarify in Methods, that we only studied RTX-treated mice that displayed no detectable pain-related behavior (<1s) in response to intraplantar injection of capsaicin. For comparison, WT mice exhibited ~40s of pain-related behavior.

• Was body temperature measured during the experiments in Figure 4? Could the hyperthermic effects of both drugs also drive peripheral vasodilation independent of vascular TRPV1?

This is a very interesting question as TRPV1 agonism/antagonism is well-known to affect body temperature. In our BP experiments with anesthetized mice, the body temperature was maintained at 36°C and BCTC had no hyperthermic effect, presumably due to the effects of anesthesia. In our studies with conscious mice, we used animals that had been treated with RTX as neonates to ablate sensory neurons. We have found (manuscript, in preparation) that sensory neurons are essential for the hyperthermic effects of TRPV1 antagonists. Additionally, the time course of hyperthermia (peaking in >10-20 minutes) does not match the instantaneous and transient depressor effect of antagonists. Therefore, for all these reasons, we conclude that changes in skin blood flow are unlikely to contribute to the hemodynamic effects of TRPV1 antagonism that we report, at least under these experimental conditions.

• How do you explain the presence of tone in V1KO mice? Compensation by another TRP channel? What was the effect of BCTC on the time constant for the remaining tone that developed?

Yes, we propose that tone is restored by compensatory upregulation of other TRP channels. Indeed, TRPM4 and TRPP1 mRNA expression increased in arteries from TRPV1-KO mice. Further, TRPM4 antagonism is greater in KO mice supporting increased functional expression of TRPM4. BCTC had no effect on the development of tone in arteries from KO mice (see new data, Fig. 5B and D).

• Figure 7: It is extremely difficult to connect TRPV1 and LVGCC function in the way you state. Nifedipine nearly abolishes tone in almost all vessels this size, so the effect of BCTC on top of it is not something you can draw much of a conclusion from in the absence of some complex patch clamp experiments. It is interesting that both V1 and M4 seem to be a source of depolarization to drive CaV11.2 opening, at least in the pharmacological sense. However, these conclusions cannot be properly drawn without measurement of V1, M4 and CaV1.2 currents in these smooth muscle cells.

In small resistance arterioles, stretch of myocytes activates a TRP channels/L-type Ca channel pathway to trigger tone (Knot & Nelson, 1998; Earley & Brayden, 2015). We show in skeletal muscle arterioles, using multiple approaches, that TRPV1 participates in this stretch-evoked signaling. In isolated arteriolar myocytes, hypoosmotic stretch activated TRPV1-dependent Ca^{2+} signals. In arteries (both intact and denuded of endothelium) raised intravascular pressure triggered a Ca^{2+} rise and vessel constriction that was sensitive to TRPV1 antagonists. Similarly, a TRPM4 antagonist partly inhibited tone, and when combined with BCTC inhibited myogenic tone to the same extent as nifidepine. Therefore, these data support our hypothesis that TRPV1, and to a lesser extent TRPM4, act as transduction channels downstream of pressure signaling, to depolarize the membrane and activate Cav1.2. This hypothesis agrees with the prevailing theory of myogenic tone whereby several TRP channels (TRPC6, TRPM4 and TRPP1) expressed in cerebral or large primary arteries are proposed to activate Cav1.2 via depolarization. The novel aspect of our study is the participation of TRPV1 that enables rapid changes in myogenic tone.

We agree with the reviewer that measuring stretch activation of TRP currents would be another useful line of exploration. These studies, of course, would require pharmacological validation just as we have employed in the current study; using TRPV1 and TRPV4 antagonists to dissect the individual components of the total current. We plan to perform patch experiments in future studies investigating the molecular aspects of tone. Indeed, there are a number of molecular steps involved in skeletal muscle myogenic tone that are unknown and warrant exploration including the identity of the

GPCR, details of the PLC/temperature regulation and the potential interplay between TRPV1 and TRPM4 channels.

• If this reflex reactive dilation is physiologically relevant, then the V1KO mice should present an appreciable phenotype due to the lack of such a response. Do they?

The precise physiological roles of rapid reactive dilation are unknown, however many studies over the past 30 years have suggested that this phenomenon may aid in the transition from rest to exercise (see Clifford lab papers). To the best of our knowledge this type of analysis has not been performed comparing WT versus TRPV1-null mice.

• By stating that TRPV1 is rapidly deactivated to promote rebound dilation, the authors imply that TRPV1 is active more often than not. Given the single channel conductance of this channel, this is unlikely - as with most TRP channels, it is most often closed due to the massive amount of ions it can let in when open. How is it that this channel is regulated?

The whole cell TRPV1 conductance is governed by several parameters: the no. of channels, the single channel conductance, and the open probability (Po).
TRPV1 expression in arteriolar myocytes is relatively low; both mRNA and the current density evoked by a maximal concentration of capsaicin is ~10% of sensory neurons (Phan T. et al. 2020). Furthermore, heat and PKC are submaximal stimuli of TRPV1. We propose that tonic stretch of myocytes partly opens TRPV1 (low open probability) to generate a standing, small inward current sufficient to depolarize the membrane and activate Cav1.2.

Tonic activation is common in TRP channels. Indeed, the very first TRP channels, characterized in drosophila, are tonically activated by ambient light. Furthermore, TRPV1 is tonically active in thermosensory visceral neurons; hence TRPV1 antagonists produce a robust hyperthermia when administered to humans and most other mammals (doi:10.1523/jneurosci.4833-06.2007; doi: 10.1080/23328940.2015.1040604).

• What about high temperature or proton-evoked calcium signals? Effecting either of these would also be reinforcement of the involvement of V1. The authors present heat as a stimulus in Figure 10's model, but don't test it.

In our recent paper (Phan T. et al. 2020), we extensively characterized TRPV1 function in arteries using agonists (capsaicin, LPA) and antagonists. In the current study, we tested the effects of temperature (22°C versus 32°C, see Fig. 9). These data show that stretch-evoked calcium signaling is strictly dependent on raised temperature. Further, BCTC inhibits the Ca2+ response at 32°C supporting the hypothesis that stretch activates TRPV1 via an increase in temperature sensitivity.

• The PKC/PLC experiments could just as easily be explained by relief of BK channel inhibition, since BK channel feedback is a well-documented mechanism opposing tone development. PKC has such ubiquitous roles in regulating ion channels that the data presented in Fig. 10 do little reinforce the conclusion without the inclusion of KO animals. Also, V1 isn't activated until noxious heat >40{degree sign}C, making it unlikely heat is the driver. The PKC-dependent drop in temperature sensitivity would imply that V1 could be wide-open at body temp - again, given the biophysical properties of the channel, this would be extremely harmful.

We agree that PKC can affect many channels. We state in Discussion: "**It should be noted, however, that our experiments with pharmacologic PKC inhibitors cannot exclude the participation of other PKC-regulated proteins**".

Nonetheless, PKC phosphorylation is a fundamental mechanism for regulating/sensitizing TRPV1 (Premkumar LS & Ahern GP, 2000; Vellani V, et al., 2001) and our data support PKC acting via TRPV1. We show that stretch activation of arteriolar myocytes is dependent on both TRPV1, PKC and temperature in the physiologic range of >32°C (note that resting skeletal muscle temperature is 32°C-35°C). These results are consistent with a mechanism whereby PKC sensitizes TRPV1 to temperature. Furthermore, the activity of BK channels is increased by raised temperature and therefore the temperature dependence of myogenic tone does not support a primary role for BK channels in stretch/PKC signaling.

We repeated the inhibitor experiments with arteries from KO mice. These data are similar to WT mice, suggesting that PLC/PKC signaling is an absolutely conserved signaling pathway for myogenic tone. These data are not surprising, since PLC (Osol *et al.*, 1993; Matsumoto *et al.*, 1995; Gonzales *et al.*, 2014) and PKC (Hill *et al.*, 1990; Korzick *et al.*, 2004) has been shown to be essential for tone in different vessel beds, including cerebral arteries that do not express TRPV1 (Earley *et al*. 2007). Furthermore, TRPM4, which we show is upregulated in arteriolar myocytes of TRPV1-KO mice, is activated by PLC and PKC-signaling pathways (Guinamard *et al.*, 2002; Nilius *et al.*, 2005; Earley *et al*. 2007).

TRPV1 is activated by noxious heat >42°C. However, physical and chemical stimuli of TRPV1 are allosterically coupled to channel gating, reducing the temperature threshold. Capsaicin substantially reduces the temperature threshold to below room temperature, while PKC reduces the threshold to 30-32°C (Sugiura *et al.* 2002).

Tonic partial activation of TRPV1 (low Po) is not predicted to be harmful as demonstrated by TRPV1 function in thermosensory neurons. Moreover, Cav1.2 channels in vascular smooth muscle are tonically open and admitting Ca2+ without apparent toxicity.

• The authors also imply that activation of V1 alters the ion selectivity of the channel. The selectivity of this channel is 9-fold greater for Ca over Na, and more likely to be influenced by the relative concentrations of extracellular Na and Ca than anything else. Without references to changes in selectivity based on stimuli, this is categorically false.

We don't suggest that TRPV1 ion selectivity is altered. Instead, we suggest that stretch activates small TRPV1 currents (due to low channel density and low Po), that is insufficient alone to trigger constriction. That is, TRPV1 provides a small depolarizing current to activate Cav1.2.

Minor:

• Confirming the specificity of BCTC in V1KO mice would be a nice addition to figure 5, as it would validate the specificity and reinforce your earlier claims.

Agreed. We have performed these experiments and the data are added to Figure 5B and D.

• Your representative trace in 7B does not match your data in 7D: the summary shows that 50% of the dilation should be due to M4 block and another 50% due to V1 block. The trace in B looks very different. Also that vessel did not fully stabilize in its tone, suggesting there is a slow component to tone as well as the fast one? Perhaps a different trace would be more representative.

We now present a more representative trace.

Referee #2

Phan et al study the role of TRPV1 in myogenic tone in heart and skeletal muscle arteries, which they previously identified as expressing this channel in smooth muscle cells. Their data suggest that TRPV1 is activated via a stretch/mechanosensation mechanism that engages PLC and protein kinase C. V1 activity then leads to Cav1.2 activation and SMC constriction.

Overall, the study is well performed and adds to our knowledge of specialization of arteriolar function in different organ systems. I have the following major and minor comments:

Major

1. The study is heavily reliant on TRPV1 pharmacology, specifically BCTC and AMG517. While the TRPV1 null mice they use are convincing and their observations in this model agree with findings in wild type mice, a further comparator is needed. Specifically, these drugs should be shown to have no effect on a wild type artery system that does not express TRPV1, such as mesenteric or cerebral arteries. This would add confidence that these are selective drug effects, and add weight to the argument that the expression of V1 in these arteries supports functional specialization.

Agreed. We now show that BCTC fails to evoke dilation of mesenteric arteries that do not express TRPV1 (Figure 1). These new data further support the on-target specificity of the TRPV1 antagonists.

2. Figure 10 goes some way to elucidate the mechanistic basis of TRPV1 activation to pressure. Missing here are data from TRPV1-null mice. These experiments should be repeated in TRPV1-/- mice, where the prediction is that the effect of these pharmacological maneuvers should be blunted.

We performed these experiments and found that the PLC and PKC inhibitors similarly blocked tone in WT and TRPV1-deficient arteries. These data suggest that PLC/PKC signaling is an absolutely conserved signaling pathway for myogenic tone. As we responded to Reviewer 1, these data are not necessarily surprising, since PLC has been shown to be essential for tone in other tissues, including cerebral arteries. Further, TRPM4, which is upregulated in arteriolar myocytes of TRPV1-KO mice, is also activated by PLC and PKC-signaling pathways.

Minor

1. Figure 9D and E, de-endothelialized preps do not constrict to increases in pressure. Why is this? With the SMC contractile machinery still intact one would expect potentially even a more robust constriction due to lack of EC feedback.

The de-endothelialized arterioles do indeed contract substantially to pressure. Fig. 9D and E shows the Ca2+ signal. As shown in Figure 9F, the diameter in control arteries versus BCTC treatment, becomes significantly smaller as pressure is raised to 60 and 100 mM Hg.

2. The abbreviation 'HiK+' is confusing. 'High K+', or '60 mM K+' would be much clearer.

We have changed to “KC”l throughout.

3. Figure 3B should use a normal, rather than divided y-axis.

We have adjusted the axis to show all the data points.

4. Page 13 "Interestingly, numerous studies have demonstrated a role for TRPM4 in the myogenic tone of

different vascular beds". This statement needs references.

Our mistake; TRPM4 has only really been characterized in cerebral arterioles. We have amended the statement: “Interestingly, previous studies have demonstrated a role for TRPM4 in the myogenic tone of cerebral arterioles (Earley *et al.*, 2004; Reading & Brayden, 2007), while a recent study showed that TRPP1 contributes to the modest tone found in primary arteries (Bulley *et al.*, 2018).

Dear Dr Ahern,

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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 revision.

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EDITOR COMMENTS

Reviewing Editor:

Phan et al. have performed new control experiments and, in doing so, have strengthened their conclusions that TRPV1 is a key regulator of myogenic tone.

1 - As highlighted by referee 2, the description of the myogenic tone data and the way it is presented (as Δ diameter [%], Figure 10) leaves room for interpretation. To this reviewing editor, the way the data is presented graphically in summary data, suggests that that the authors have calculated the percentage increase in diameter caused by the various pharmacological inhibitors at 80 mmHg (e.g. the drug was added at 80 mmHg). However, judging by the traces (e.g. Figure 10A), the authors seem to have actually performed a full myogenic response experiment before and after the inclusion of each inhibitor. I believe the authors have then compared the diameter at 80 mmHg in the presence of the drug to that in its absence. If this is the case, these drugs are not really causing a change in diameter, as suggested by the summary figures. Instead, the inhibitors are preventing myogenic tone from developing. If this is the case, might it be more intuitive if the authors calculate myogenic tone in the absence and presence of each of the inhibitors? This obviously assumes that the experiments were also performed in Ca2+-free PSS.

If this is not possible, perhaps the authors could simply show diameter measurements at 80 mmHg in the absence and presence of each of the drugs, and show the data as paired dot plots.

If, instead, the authors did add the drugs at 80 mmHg and measured a change in diameter, then please make sure the example raw data reflects this. An accurate description of delta diameter in the figure legend would also help our readers.

Please note that Referee 2 is interpreting this data differently (as the change in diameter from 20 mmHg to 80 mmHg), and I strongly encourage the authors to focus their attention on ensuring that there is no ambiguity in the way the data is presented.

2 - P-value format. Apologies for not noticing this previously. Please use a single threshold (e.g., $p = 0.05$) for statistical significance, and refrain from using different numbers of asterisks to indicate lower thresholds. This practice falsely suggests some results are perhaps more significant than others. This applies throughout the manuscript.

REFEREE COMMENTS

Referee #1:

All my initial questions were adequately answered, thank you. I only have two further comments:

Figure 6B: Please clarify exactly what was done with KCl and the concentration used in the intravital experiment. Neither the figure nor the text make it clear is meant by "brief vasodilation". Is this the same as the stuff in Figure 8 (meaning dilation after constriction)? Or did you use a hyperpolarizing concentration of KCl (~10 mM) to drive Kir-mediated dilation?

Were the statistics for mRNA expression done on normalized data vs control? This is a statistically valid approach (see PMID 21278720). N=3 is also underpowered given the variability.

Referee #2:

Thank you for addressing my comments. The manuscript is substantially improved by these alterations.

I have a remaining concern over the mechanistic data in Fig 10, which are difficult to interpret. My understanding is that in Fig 10B the authors are comparing diameter of the arteriole at 20 and 80 mmHg and calculating change in tone. Does the TRPV1-null mouse then generate 'normal' tone under these conditions (i.e. you see essentially no change going from 20 to 80 mmHg due to the constriction to pressure?) As currently presented, this is the most natural interpretation, and it appears that under these conditions, PLC inhibition, PKC block and VGCC block all are as effective as in WT arteries with intact TRPV1. From this, the natural conclusion seems to be that TRPV1 is not involved in these signaling pathways influence on tone.

A further panel showing traces to pressure in TRPV1-null mice under control conditions, in the presence of U73122, in the presence of nifedipine, and in the presence of GX109 would help the reader to interpret these experiments.

END OF COMMENTS

1st Confidential Review **18-Oct-2021**

We thank the Editors and Referees once again for their constructive comments. As suggested, we have clarified the description of Fig. 10 and added representative traces of arterial diameter (Fig. 10A). In addition, we have increased the sample number (6 mice) for mRNA analysis (Fig. 7A). We hope the manuscript is now suitable for publication.

Reviewer Editor:

1 - As highlighted by referee 2, the description of the myogenic tone data and the way it is presented (as Δ diameter [%], Figure 10) leaves room for interpretation. To this reviewing editor, the way the data is presented graphically in summary data, suggests that that the authors have calculated the percentage increase in diameter caused by the various pharmacological inhibitors at 80 mmHg (e.g. the drug was added at 80 mmHg). However, judging by the traces (e.g. Figure 10A), the authors seem to have actually performed a full myogenic response experiment before and after the inclusion of each inhibitor. I believe the authors have then compared the diameter at 80 mmHg in the presence of the drug to that in its absence. If this is the case, these drugs are not really causing a change in diameter, as suggested by the summary figures. Instead, the inhibitors are preventing myogenic tone from developing. If this is the case, might it be more intuitive if the authors calculate myogenic tone in the absence and presence of each of the inhibitors? This obviously assumes that the experiments were also performed in Ca2+-free PSS.

If this is not possible, perhaps the authors could simply show diameter measurements at 80 mmHg in the absence and presence of each of the drugs, and show the data as paired dot plots.

If, instead, the authors did add the drugs at 80 mmHg and measured a change in diameter, then please make sure the example raw data reflects this. An accurate description of delta diameter in the figure legend would also help our readers.

Please note that Referee 2 is interpreting this data differently (as the change in diameter from 20 mmHg to 80 mmHg), and I strongly encourage the authors to focus their attention on ensuring that there is no ambiguity in the way the data is presented.

Indeed, in the summary data presented in Fig. 10B, the drugs were added to pressurized vessels and the y-axis represents the change in diameter. We have revised the Fig. 10A to show representative traces of the dilatory response.

2 - P-value format. Apologies for not noticing this previously. Please use a single threshold (e.g., p = 0.05) for statistical significance, and refrain from using different numbers of asterisks to indicate lower thresholds. This practice falsely suggests some results are perhaps more significant than others. This applies throughout the manuscript.

We have revised all figures; removing the asterisks and now show exact p values for relevant data points.

Referee #1:

All my initial questions were adequately answered, thank you. I only have two further comments:

Figure 6B: Please clarify exactly what was done with KCl and the concentration used in the intravital experiment. Neither the figure nor the text make it clear is meant by "brief vasodilation". Is this the same as the stuff in Figure 8 (meaning dilation after constriction)? Or did you use a hyperpolarizing concentration of KCl (~10 mM) to drive Kir-mediated dilation?

We used 40 mM KCl to constrict arterioles and to evoke reactive vasodilation and we now state this in Methods, Results and Figure legends.

Were the statistics for mRNA expression done on normalized data vs control? This is a statistically valid approach (see PMID 21278720). N=3 is also underpowered given the variability.

Thanks for alerting us to that study. For comparative analysis of mRNA in WT versus TRPV1-null mice, data were initially normalized to GAPDH and subsequently each data point (WT and KO mice) was normalized to the mean of the WT values. We also increased the sample size to 6 mice.

Referee #2:

Thank you for addressing my comments. The manuscript is substantially improved by these alterations.

I have a remaining concern over the mechanistic data in Fig 10, which are difficult to interpret. My understanding is that in Fig 10B the authors are comparing diameter of the arteriole at 20 and 80 mmHg and calculating change in tone. Does the TRPV1-null mouse then generate 'normal' tone under these conditions (i.e. you see essentially no change going from 20 to 80 mmHg due to the constriction to pressure?) As currently presented, this is the most natural interpretation, and it appears that under these conditions, PLC inhibition, PKC block and VGCC block all are as effective as in WT arteries with intact TRPV1. From this, the natural conclusion seems to be that TRPV1 is not involved in these signaling pathways influence on tone.

A further panel showing traces to pressure in TRPV1-null mice under control conditions, in the presence of U73122, in the presence of nifedipine, and in the presence of GX109

would help the reader to interpret these experiments.

We apologize for presenting a confusing figure. In Fig. 10B, the drugs were added to vessels pressurized to 80 mm Hg and the y-axis indeed represents the change in diameter. We have revised Fig. 10A to show representative traces of the dilatory response to U73122, U73343, nifedipine and GF 109203X.

We conclude that PLC, PKC and Cav1.2 signaling pathways are critical for myogenic tone in both WT and TRPV1-KO mice. Pharmacological experiments also support a critical role for TRPV1. Although the magnitude of tone is unaffected by disrupting TRPV1 expression, we propose that increased expression of other TRP channels (TRPM4 and TRPP1, see Fig 7A) compensates for the loss of TRPV1, restoring the magnitude but not the kinetics of tone.

2nd Revision - Editorial Decision **11-Jan-2022**

Dear Dr Ahern,

Re: JP-RP-2021-281873XR1 "TRPV1 in arteries enables a rapid myogenic tone" by Thieu Phan, Hoai Ton, Hajnalka Gulyás, Robert Porszasz, Attila Toth, Rebekah Russo, Matthew W Kay, Niaz Sahibzada, and Gerard P Ahern

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology, subject to any modifications to the text and/or satisfactory clarification of the Methods section that may be required by the Journal Office to conform to House rules.

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

The authors have clarified the methods used to assess the actions of various pharmacological agents on the diameter of pressurized arteries. In all, the manuscript provides a convincing demonstration of the potential for TRPV1 channels to modulate artery function.

END OF COMMENTS

2nd Confidential Review **21-Dec-2021**