The Journal of Physiology JP

Blebbistatin reduces calcium buffering in cardiomyoctes: Consequences for cellular electrophysiology

Izzatullo Sobitov, Katharina Ritzenhoff, Marie Gaulrapp, Lea Becker, Aiste Liutkute, Fitzwilliam Seibertz, Funsho E. Fakuade, Fleur E. Mason, and Niels Voigt
DOI: 10.1113/JP287545

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The referees have opted to remain anonymous.

Review Timeline:

Submission Date:	31-Jan-2025
Editorial Decision:	04-Mar-2025
Revision Received:	02-Jun-2025
Accepted:	01-Jul-2025

Senior Editor: Bjorn Knollmann

Reviewing Editor: Brian Delisle

Transaction Report:

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Dear Dr Voigt,

Re: JP-RP-2025-287545 "Blebbistatin reduces calcium buffering in cardiomyoctes: Consequences for cellular electrophysiology" by Izzatullo Sobitov, Katharina Ritzenhoff, Marie Gaulrapp, Lea Becker, Aiste Liutkute, Fitzwilliam Seibertz, Fleur E. Mason, Funsho E. Fakuade, and Niels Voigt

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If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Bjorn Knollmann
Senior Editor
The Journal of Physiology

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

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

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Comments to the Author (Required):
Two expert referees in the field evaluated the manuscript. While the referees thought the work was sound, they expressed minor and major concerns that the manuscript is primarily confirmatory, could benefit from additional experimental studies, and does not significantly impact the field in its current form. Several clarifications and experiments are suggested to improve the significance of the study.

Please also see 'Required Items' above.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy (Required):
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Comments to the Author:
The work is interesting, but a responsive revision would require addressing the concerns raised in the review, which will require new experiments. The authors also need to better explain the novelty of the work, since the effect of blebbistatin on

buffering has been reported before, and address why pro-arrhythmic effects are postulated whereas antiarrhythmic effects of blebbistatin have been reported in hypertrophic cardiomyopathy models (as pointed out by reviewer 1).

REFEREE COMMENTS

Referee #1:

GENERAL COMMENTS

Sobitov and colleagues present a manuscript examining the impact of blebbistatin on Ca2+ buffering in cardiomyocytes. Blebbistatin is used as an uncoupling agent for excitation-contraction coupling by inhibiting myosinII ATPase often in experimental studies performing optical mapping. The authors simultaneously measured whole-cell NCX currents and intracellular Ca2+ to make this assessment following rapid application of caffeine. They also measure a number of ionic currents. Their findings suggest that blebbistatin reduces Ca2+ buffering myocytes by shifting the buffer dissociation constant consistent with a decrease in affinity for Ca2+. In addition, they observed accelerated inactivation of ICa,L and a reduction in IK1 in blebbistatin treated cells. Overall, they conclude that blebbistatin reduces Ca2+ buffering which impacts the cell physiology in a number of ways which can be proarrhythmic, and this needs to be consider in studies using these agents. Overall, the work has several strengths: the detailed single cell studies using a range of techniques and combining patch clamp measures with intracellular Ca2+ measurements, larger sample sizes are provides for the relatively noisy iPSC-aCM model, and the results are clearly present. However, there are some limitations to the work as currently presented:

1. The finding that blebbistatin reduces calcium sensitivity of the myofilaments and hence calcium buffering in cardiomyocytes has been demonstrated and known for some time with earlier papers by Dou et al., 2006 (PMID: 17615158) and Baudenbacher et al., 2008 (PMID: 19033660). Thus, the findings in the present study seem largely confirmatory. The authors need to better clarify any novel or contradictory findings in this study relative to the literature.

2. Why do the authors use Fluo-3 AM rather than the salt form since they are using a patch pipette. Otherwise, the Flou-3 in cell will be diluted during experiment. Also the salt form may overcome some limitations in compartmentalization of the dye allowing focus on cytosolic compartment.

3. Line 90 states that for whole-cell voltage clamp experiments, all measurements were conducted at room temperature which is a limitation for understanding the impact at physiological temperatures given that many of the proteins and binding show remarkable and differential temperature sensitivity. However, it is confusing as line 65 suggests ICa,L measurements were carried out at 37 C.

4. What is the impact of blebbistatin on the decay of ICa,L shown in Figure 1A? This is a consistency check for the ICa,L measures by automated patch clamp.

5. The authors ICa,L measurements in Figures 5 and 6 examining the impact of blebbistatin in the presence and absence of 10 mm EGTA buffering are more complex to interpret than suggested. This amount of EGTA (10 mM) in the pipette buffers cellular Ca2+ to a level that SR stores are largely depleted and Ca2+ transients do not occur in response to ICa,L currents (no contractions). In contrast, the minimal Ca2+ buffering used in Figure 5 will potentially allow Ca2+-induced Ca2+ transients. Thus, it may be that the impact observed is due to the presence or absence of Ca2+ transients rather than a simple change in buffering of intracellular Ca2+ to a different level.

6. The authors suggest that blebbistatin is pro-arrhythmic, but this contrasts the conclusion from the Knollmann group suggesting blebbistatin in the presence of hypertrophic cardiomyopathy mutations is antiarrhythmic (Baudenbacher et al., 2008, PMID: 19033660). How do the authors reconcile these apparently different results. Is this an atrial vs. ventricular difference?

MINOR COMMENTS

1. The authors state that the biexponential decay of ICa,L can be simply separated into CDI (fast tau) and VDI (slow tau). Unfortunately, while those process tend to segregate in that manner, detail biophysical studies suggest there is clear overlap. Perhaps the authors should be more circumspect in this description.

Referee #2:

The study by Sobitov et al. investigates the excitation-contraction uncoupling effects of blebbistatin on Ca2+ signaling properties of human induced pluripotent stem-cell derived atrial cardiomyocytes. The study provides evidence that reduced intracellular Ca2+ buffering by blebbistatin increases the diastolic Ca2+ transients, SR Ca2+ leak and spark frequency, increases ICaL inactivation, without affecting the SR Ca2+ content and ICaL amplitude. These changes the cellular electrophysiological dynamics, authors suggest, are responsible for reported proarrhythmic effects of blebbistatin. Although the manuscript is well-written, the authors fail to provide evidence for some of their assertions or explain the contradictions in their results. The MS will be greatly improved by providing more details on methods and results sections.

Major concerns:

1. How did the authors confirm that their derived cardiomyocytes indeed were atrial of nature? Did the Authors perform any action potential measurements, or did they test the expression of subtype-specific cardiac markers like MLC2a? Did the author test the effects of acute application of Retinoic acid on the calcium signaling parameters? The MS would be greatly strengthened by establishing the atrial nature of these cells.

2. There is no indication on how SR Ca2+ leak was measured. Was it measured by the application of tetracaine in zero calcium zero sodium solutions? Was the leak then quantified as the fraction of caffeine-induced release? Please clarify and include the description in the Methods section. Why was 10mM caffeine used? Generally 3-5 mM caffeine produce the maximum calcium release from SR in cardiomyocytes. Use of the higher concentrations may activate calcium entry on hemi-channels, reported by some investigators.

3. Fig.1 shows ICaL current around 20 pA/pF, while Figs.5-6 show only ICaL amplitudes of 2-5 pA/pF. How can the authors explain these large difference in their results? Are the cells with larger calcium current autophosphrylated? The larger ICa density cells are more consistent with those reported for hiPSC-CMs in the literature! Did the measured Ca-transients reflect this large difference in Ica?

Figure 2 shows greater dispersion of INCX in blebistatine treated cells consistent with larger Ca-transients. Why would increasing the calcium buffering result in larger Ca-transients? Does the dispersion arise from variance in Ica in different cells?

4. Small-conductance Ca2+ activated potassium channels were shown to be present in both ventricles and atria, however functional activation of SK channels is significantly more prominent in atria. Increasing number of studies show the inhibition of SK channels as a potential antiarrhythmic treatment option in AF and other atrial arrhythmias. Did the Authors examine the effect of reduced Ca2+ buffering on the Ca2+ dependent K+ current in their atrial specific CMs. I would recommend doing such experiments.

5. The Authors have used blebbistatin in the concentration of 10 μM. Did the Authors test its dose-dependent effects on the intracellular Ca2+ signaling properties of hiPSC derived atrial CMs?

6. iPSC derived cardiomyocytes have the advantage that they show spontaneous automaticity. Were these cells able to maintain spontaneous beating? If so, did the faster calcium dependent inactivation and the reduced IK1 result in faster beating rates?

7. Authors claim that the intracellular Ca2+ changes due to blebbistatin results in pro-arrhythmic changes. Did the Authors measure any indicators of arrhythmia for instance, EADs or DADs in these cells?

8. Did the authors check on calcium channel current kinetics and amplitude when Ba2+ was the charge carrier through the channel. This may provide some insight on the buffering issue, because acute application of Ba2+ containg solution lasting only a few seconds that fails to activate CICR and rise of cytosolic calcium transients may shed some light on the authors' proposal of compartmented calcium pools.

Minor:

Starting almost every sentence with mean {plus minus} SEM in the figure legends is somewhat odd. I would recommend saying for example Quantification of CaT amplitude instead of Mean {plus minus} SEM CaT amplitude etc.

END OF COMMENTS

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Response to Editor's comments

We greatly appreciate the thoughtful efforts of the Editors and Reviewers on our manuscript and are thankful for the constructive feedback, which has significantly enhanced our work.

In the revised version, we have updated the format of our submitted documents as requested by the Editorial Office. In particular, we have revised the presentation of statistical analysis.

In addition, in the revised version we highlight the novelty of our work and discuss that both increased or decreased buffering can be arrhythmogenic. We also refer to an excellent editorial by Knollmann and Bers (Knollmann & Bers, 2024) which discusses this and accompanies our previous study (Fakuade *et al.*, 2024).

Page 4, line 35: "Considering that about 99% of intracellular Ca^{2+} is bound to buffers, even minor perturbations in the amount of Ca^{2+} buffered may have a major impact on free Ca^{2+}(Smith & Eisner, 2019), thereby influencing EC coupling. Both increased and reduced buffering capacity can increase arrhythmia susceptibility, as discussed recently in an editorial by Knollmann and Bers (Knollmann & Bers, 2024)"

Page 4, line 40: "In this study, we examine the direct effects of blebbistatin (10 µmol/L). We investigate whether blebbistatin selectively disrupts intracellular Ca^{2+} buffering, thereby inducing pro-arrhythmic changes in Ca^{2+} handling and cellular electrophysiology."

Below, we have responded to the specific comments from the Reviewers.

Response to comments of Reviewer 1

Sobitov and colleagues present a manuscript examining the impact of blebbistatin on Ca^{2+} buffering in cardiomyocytes. Blebbistatin is used as an uncoupling agent for excitation-contraction coupling by inhibiting myosinII ATPase often in experimental studies performing optical mapping. The authors simultaneously measured whole-cell NCX currents and intracellular Ca^{2+} to make this assessment following rapid application of caffeine. They also measure a number of ionic currents. Their findings suggest that blebbistatin reduces Ca^{2+} buffering myocytes by shifting the buffer dissociation constant consistent with a decrease in affinity for Ca^{2+}. In addition, they observed accelerated inactivation of $I_{Ca,L}$ and a reduction in I_{K1} in blebbistatin treated cells. Overall, they conclude that blebbistatin reduces Ca^{2+} buffering which impacts the cell physiology in a number of ways which can be proarrhythmic, and this needs to be consider in studies using these agents. Overall, the work has several strengths: the detailed single cell studies using a range of techniques and combining patch clamp measures with intracellular Ca^{2+} measurements, larger sample sizes are provides for the relatively noisy iPSC-aCM model, and the results are clearly present. However, there are some limitations to the work as currently presented:

We greatly appreciate the Reviewer's positive feedback and their constructive comments, which have led us to make improvements to the manuscript.

1. The finding that blebbistatin reduces calcium sensitivity of the myofilaments and hence calcium buffering in cardiomyocytes has been demonstrated and known for some time with earlier papers by Dou et al., 2006 (PMID: 17615158) and Baudenbacher et al., 2008 (PMID: 19033660). Thus, the findings in the present study seem largely confirmatory. The authors need to better clarify any novel or contradictory findings in this study relative to the literature.

We acknowledge that the literature cited by the Reviewer indeed present important findings, using readouts such as force of contraction to show the effects of blebbistatin. However, those studies did not *directly* measure effects of blebbistatin on cytosolic Ca^{2+} buffering, including parameters such as B_{max} and K_d. Furthermore, it has been questioned whether force of contraction studies can be used as conclusive evidence of altered Ca^{2+} buffering, as it is not the sole factor governing the relationship between $[Ca^{2+}]_i$ and force, as discussed by Smith and Eisner (Smith & Eisner, 2019). To our knowledge, therefore, our study is the first to directly measure the effects of blebbistatin on cytosolic Ca^{2+} buffering in cardiomyocytes (iPSC-CM).

In order to better clarify this important point, we have cited the two studies highlighted by this reviewer and added the following text in the discussion:

Page 12, line 263: "Similarly, Ca^{2+} sensitivity reduction was achieved in a concentration-dependent manner in mouse cardiac muscle preparations (Dou *et al.*, 2007; Baudenbacher *et al.*, 2008). However, such studies are mainly based on quantification of blebbistatin effects on contractile force and do not directly quantify effects on cytosolic Ca^{2+} buffering and Ca^{2+} homeostasis."

2. Why do the authors use Fluo-3 AM rather than the salt form since they are using a patch pipette. Otherwise, the Fluo-3 in cell will be diluted during experiment. Also the salt form may overcome some limitations in compartmentalization of the dye allowing focus on cytosolic compartment.

We apologise for any confusion. The experiments involving simultaneous patch-clamp and epifluorescent measurements were performed with fluo-3 loaded cells as well as pipette solution containing fluo-3 pentapotassium salt, originally described in the Supplements. We have now added this information to the Methods section of the revised manuscript for clarity:

Page 6, line 89: "The pipette solution (in mmol/L: EGTA 0.02; GTP-Tris 0.1; HEPES 10; K-aspartate 92; KCl 48; MgATP 1; Na_2ATP 4; pH = 7.2 adjusted with KOH) contained fluo-3 pentapotassium salt, 0.1 mmol/L (Thermo Scientific)."

3. Line 90 states that for whole-cell voltage clamp experiments, all measurements were conducted at room temperature which is a limitation for understanding the impact at physiological temperatures given that many of the proteins and binding show remarkable and differential temperature sensitivity. However, it is confusing as line 65 suggests ICa,L measurements were carried out at 37 C.

We thank the Reviewer for this comment. Whereas manual patch-clamp experiments were performed at 37 °C, all experiments performed by automated patch-clamp (APC) were carried out at room temperature in order to enable calcium- and fluoride-assisted seal formation (Milligan *et al.*, 2009; Seibertz *et al.*, 2022), which is less efficient at 37 °C.

We have now extended the following sentence in the Methods section about APC:

Page 8, line 165: "All measurements were conducted at room temperature, allowing calcium- and fluoride-assisted seal formation (Milligan *et al.*, 2009; Seibertz *et al.*, 2022)."

Analysis of ion channel activity in the present study, specifically $I_{Ca,L}$ peak as well as fast and slow inactivation kinetics, showed similar effects of blebbistatin at both room and physiological temperatures (Figure 1). Furthermore, there is evidence that I_{K1} is not temperature-dependent (Voigt *et al.*, 2010).

4. What is the impact of blebbistatin on the decay of ICa,L shown in Figure 1A? This is a consistency check for the ICa,L measures by automated patch clamp.

We appreciate the Reviewer's suggestion and we have performed the analysis accordingly. Please refer to Figure 1. The observed changes in automated patch-clamp experiments were consistent with manual patch-clamp data.

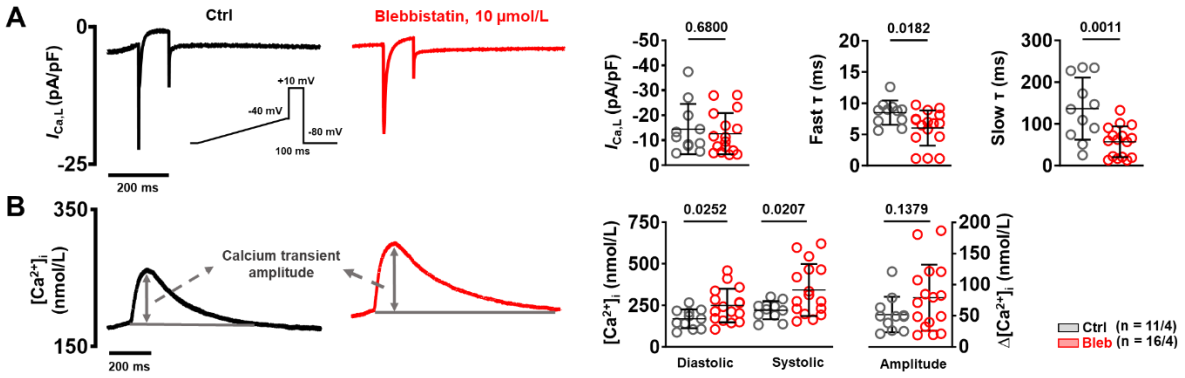

Figure 1. $I_{Ca,L}$ and Ca^{2+} transients (CaT) in iPSC-aCM. **A,** Representative traces of $I_{Ca,L}$ with voltage-clamp protocol (inset) and quantification (mean ± SD) of peak $I_{Ca,L}$ amplitude and inactivation (tau of inactivation) kinetics. **B,** Representative traces of CaT (Fluo-3 AM) and quantification (mean ± SD) of diastolic and systolic $[Ca^{2+}]_i$ and CaT amplitude. Cells were pretreated with blebbistatin (10 µmol/L). Comparisons made using unpaired Student's t-test, Mann Whitney's U-test. p values vs Ctrl. n/N = iPSC-aCMs/batches.

5. The authors $I_{Ca,L}$ measurements in Figures 5 and 6 examining the impact of blebbistatin in the presence and absence of 10 mm EGTA buffering are more complex to interpret than suggested. This amount of EGTA (10 mM) in the pipette buffers cellular Ca2+ to a level that SR stores are largely depleted and Ca2+ transients do not occur in response to ICa,L currents (no contractions). In contrast, the minimal Ca2+ buffering used in Figure 5 will potentially allow Ca2+-induced Ca2+ transients. Thus, it may be that the impact observed is due to the presence or absence of Ca2+ transients rather than a simple change in buffering of intracellular Ca2+ to a different level.

We apologise if there was any confusion concerning the aim of this experiment. We wanted to use EGTA to abolish Ca^{2+}-induced Ca^{2+} release from the SR and therefore cytosolic Ca^{2+} transients, thus allowing us to exclude any direct effect of blebbistatin on Ca^{2+} channel inactivation.

To clarify this, we extended/added the following sentences in the Results section:

Page 11, line 232: "To determine whether Ca^{2+} chelation could mitigate blebbistatin effects, as well as to address the possibility of direct effects of blebbistatin on $I_{Ca,L}$, ethylene glycol tetraacetic acid (EGTA, 10 mmol/L) was added to the internal solution in APC experiments."

and

Page 11, line 241: "Furthermore, direct effects of blebbistatin on $I_{Ca,L}$ can also be excluded, based on these results."

6. The authors suggest that blebbistatin is pro-arrhythmic, but this contrasts the conclusion from the Knollmann group suggesting blebbistatin in the presence of hypertrophic cardiomyopathy mutations is antiarrhythmic (Baudenbacher et al., 2008, PMID: 19033660). How do the authors reconcile these apparently different results. Is this an atrial vs. ventricular difference?

We refer the Reviewer to an excellent editorial by Knollmann and Bers (Knollmann & Bers, 2024), describing the biphasic relationship between Ca^{2+} buffering and arrhythmogenic risk (both too much and too little Ca^{2+} buffering power can increase risk for arrhythmia). Hypertrophic cardiomyopathy has been associated with increased Ca^{2+} buffering (Schober *et al.*, 2012)**.** Therefore, we suggest that normalisation of buffering by a Ca^{2+} desensitiser in this scenario would protect against arrhythmia. On the other hand, reducing buffering to below normal levels, as in the current study, is also thought to be pro-arrhythmic. Indeed, we have recently shown in an *ex vivo* mouse heart model that blebbistatin increases arrhythmia inducibility (Fakuade *et al.*, 2024).

MINOR COMMENT: The authors state that the biexponential decay of $I_{Ca,L}$ can be simply separated into CDI (fast tau) and VDI (slow tau). Unfortunately, while those process tend to segregate in that manner, detail biophysical studies suggest there is clear overlap. Perhaps the authors should be more circumspect in this description.

We thank the Reviewer for highlighting an important nuance in interpreting inactivation kinetics of $I_{Ca,L}$. Although $I_{Ca,L}$ inactivation has broadly been described as involving Ca^{2+}-dependent and voltage-dependent processes with distinct time constants (τ) that can be quantified from bi-exponential current decay, there is evidence showing that these processes are interdependent and that the underlying mechanisms influence each other (Findlay, 2004; Mahajan *et al.*, 2008; Grandi *et al.*, 2010).

Consequently, we have toned-down the wording in the Results section:

Page 10, line 220: “Interestingly, when biphasic inactivation kinetics of $I_{Ca,L}$ at +10 mV were estimated, the fast phase (thought to be dominated by Ca^{2+}- dependent inactivation, CDI), as well as the slow phase (during which VDI is important) were both found to be significantly quicker in the blebbistatin-treated group (Figure 5B).”

We have also changed the legend of figures 5B and 6B, where we no longer specify CDI and VDI.

Further, due to the interdependence of both processes, we suggest that alterations in CDI may drive changes in VDI. The EGTA data are also in line with this hypothesis, as both fast and slow inactivation of $I_{Ca,L}$ showed no difference upon blebbistatin treatment. We altered the text in the Discussion as follows:

Page 13, line: 296: “Additionally, we observed that increased diastolic Ca^{2+} led to enhanced inactivation kinetics of $I_{Ca,L}$, which is in line with increased free cytosolic Ca^{2+}. The processes of CDI and VDI are inter-dependent and therefore we hypothesise that alterations in CDI occurring upon blebbistatin treatment may drive changes in VDI.”

Response to comments of Reviewer 2

The study by Sobitov et al. investigates the excitation-contraction uncoupling effects of blebbistatin on Ca^{2+} signaling properties of human induced pluripotent stem-cell derived atrial cardiomyocytes. The study provides evidence that reduced intracellular Ca^{2+} buffering by blebbistatin increases the diastolic Ca^{2+} transients, SR Ca^{2+} leak and spark frequency, increases I_{CaL} inactivation, without affecting the SR Ca^{2+} content and I_{CaL} amplitude. These changes the cellular electrophysiological dynamics, authors suggest, are responsible for reported proarrhythmic effects of blebbistatin. Although the manuscript is well-written, the authors fail to provide evidence for some of their assertions or explain the contradictions in their results. The MS will be greatly improved by providing more details on methods and results sections.

We thank the Reviewer for their feedback and we have made revisions to the manuscript which we believe address the Reviewer's comments and concerns.

1. How did the authors confirm that their derived cardiomyocytes indeed were atrial of nature? Did the Authors perform any action potential measurements, or did they test the expression of subtype-specific cardiac markers like MLC2a? Did the author test the effects of acute application of Retinoic acid on the calcium signaling parameters? The MS would be greatly strengthened by establishing the atrial nature of these cells.

We thank the Reviewer for this comment. While this study focuses on blebbistatin's effect on Ca^{2+} buffering and ion channels using iPSC-derived atrial cardiomyocytes, our group has previously demonstrated and characterised the atrial phenotype of this cell line (Seibertz *et al.*, 2023*a*). It has been shown that the atrial-subtype directed differentiation protocol produces cardiomyocytes that display distinct atrial-specific properties, notably atrial-like electrophysiology. Although, we did not repeat subtype validation experiments in this study, the same established differentiation protocol and cell line were used (Cyganek *et al.*, 2018).

We did not test acute application of retinoic acid on calcium handling as suggested by this reviewer. It is important to note that retinoic acid is applied on day 3 and 5 post-initiation of differentiation, and it is not present in later stages of myocyte maturation. Electrophysiological and calcium handling experiments were performed after day 35-40 in the absence of retinoic acid (Methods section of the revised manuscript). We therefore feel that investigating acute effects of retinoic acid on cellular electrophysiology is beyond the scope of our study.

Furthermore, we hypothesise that the findings in this study likely apply to iPSC-derived ventricular cardiomyocytes as well and future studies will explicitly test this. We have now added text in the Potential Limitations section:

Page 14, line: 233: "We assume that our findings in atrial iPSC-CM are likely to apply also to ventricular iPSC-CM, although this has to be tested in future studies."

2. There is no indication on how SR Ca2+ leak was measured. Was it measured by the application of tetracaine in zero calcium zero sodium solutions? Was the leak then quantified as the fraction of caffeine-induced release? Please clarify and include the description in the Methods section. Why was 10mM caffeine used? Generally 3-5 mM caffeine produce the maximum calcium release from SR in cardiomyocytes. Use of the higher concentrations may activate calcium entry on hemi-channels, reported by some investigators.

We apologise for any confusion. SR Ca^{2+} leak was measured by quantifying Ca^{2+} sparks via confocal line scan. SR Ca^{2+} leak was calculated from Ca^{2+} spark frequency and Ca^{2+} spark size (Figure 4B). We have clarified this in the Methods section:

Page 7, line 122: "Images were acquired using Zen 2009 acquisition software and analysed using the ImageJ SparkMaster plug-in (Picht *et al.*, 2007). SR Ca^{2+} leak was calculated from Ca^{2+} spark frequency and Ca^{2+} spark size, as previously described (Fischer *et al.*, 2015)."

We agree with the Reviewer that caffeine can affect hemi-channels and indeed we have observed this behaviour in mouse cardiomyocytes (Fakuade *et al.*, 2021*b*). Interestingly, and in contrast, we found that this activity is rare in human atrial cardiomyocytes (Fakuade *et al.*, 2021*b*)and therefore we conclude that this does not play a significant role in the present study.

The protocol for the quantification of SR Ca^{2+} content by application of 10 mM caffeine is an established method published by Varro *et al.* (Varro *et al.*, 1993) and has been extensively and successfully used by us and others (Maier *et al.*, 2003; Stokke *et al.*, 2011; Voigt *et al.*, 2012; Fakuade *et al.*, 2021*a*). Therefore, we feel confident that our use of the method has provided accurate results.

3. Fig.1 shows ICaL current around 20 pA/pF, while Figs.5-6 show only ICaL amplitudes of 2-5 pA/pF. How can the authors explain these large difference in their results? Are the cells with larger calcium current autophosphrylated? The larger ICa density cells are more consistent with those reported for hiPSC-CMs in the literature! Did the measured Ca-transients reflect this large difference in ICa?

We appreciate the Reviewer's attention regarding the differences in $I_{Ca,L}$ current densities displayed in Figure 1 and Figures 5-6. We suggest that this discrepancy arises due to utilisation of two different techniques, rather than biological variability. It is important to note that in manual patch-clamp experiments, cardiomyocytes adhere to coated coverslips, enabling stable "attached" configuration (see Methods section), preserving membrane integrity and yielding higher current densities. In contrast, automated patch-clamp requires cellular suspension, leading to different "spatial confirmation" of cardiomyocytes. While absolute current density values differ, we observe that relative changes in response to blebbistatin are consistent between manual and automated patch-clamp experiments. We have noted this in the Potential Limitations section of the revised manuscript:

Page 14, line: 341: "We acknowledge there are differences in the absolute values of $I_{Ca,L}$ densities measured in manual and automated patch-clamp experiments. We attribute this to variation in the spatial configuration of the cell in the given technique, rather than intrinsic physiological variability. In manual patch-clamp, cells adhere to glass coverslips whereas

automated patch-clamp requires cellular suspension. Importantly, pharmacological response in both of the techniques was similar, supporting the validity of the findings."

4. Figure 2 shows greater dispersion of INCX in blebistatine treated cells consistent with larger Ca-transients. Why would increasing the calcium buffering result in larger Ca-transients? Does the dispersion arise from variance in Ica in different cells?

It appears there may be a misunderstanding about the impact of blebbistatin on calcium buffering. Blebbistatin, as demonstrated in our study, does not increase calcium buffering capacity but rather decreases the affinity of intracellular Ca^{2+} buffers (increased K_d). This reduction in buffer affinity leads to higher free Ca^{2+} levels during both diastole and systole, as observed in blebbistatin-treated cells (Figure 1B).

Regarding the greater dispersion of I_{NCX} observed in blebbistatin-treated cells, this could reflect the heterogeneity in the response of individual cardiomyocytes to changes in intracellular Ca^{2+} buffering upon blebbistatin treatment. Furthermore, our findings of increased SR Ca^{2+} leak and Ca^{2+} spark frequency with blebbistatin (Figure 4) indicate a more dynamic and variable intracellular Ca^{2+} environment.

5. Small-conductance Ca2+ activated potassium channels were shown to be present in both ventricles and atria, however functional activation of SK channels is significantly more prominent in atria. Increasing number of studies show the inhibition of SK channels as a potential antiarrhythmic treatment option in AF and other atrial arrhythmias. Did the Authors examine the effect of reduced Ca2+ buffering on the Ca2+ dependent K+ current in their atrial specific CMs. I would recommend doing such experiments.

We thank the Reviewer for this comment. SK channels have recently gained much attention in the literature. We would like to highlight the finding that trafficking of SK channels to the membrane is upregulated during atrial fibrillation (Heijman *et al.*, 2023). In contrast, the cardiomyocytes used in our study were healthy and were not subjected to tachypacing. Therefore we do not believe that SK channels play a large role in the findings of our current investigation and a detailed investigation into this would be beyond the scope of the study. However, we have added text to the Potential Limitations section, in order to address this point:

Page 14, line 347: "A further potential limitation in the current study concerns the contribution of SK channels, which have gained much attention in the literature recently. These channels, however, are thought to be more important in the setting of AF (Heijman *et al.*, 2023), whereas our study utilises healthy iPSC-CM."

5. The Authors have used blebbistatin in the concentration of 10 µM. Did the Authors test its dose-dependent effects on the intracellular Ca2+ signaling properties of hiPSC derived atrial CMs?

The main aim of this study was to draw awareness of potentially "unwanted" effects of blebbistatin when used as an experimental tool, for example in optical mapping studies to block contraction, thereby allowing efficient imaging. Blebbistatin is usually used in such studies at a concentration of 5-10 µM and there is evidence that 10 µM is optimal (Swift *et al.*,

2021) so we therefore chose this concentration for the present study. We feel that a full investigation of dose-dependent effects of blebbistatin is beyond the scope of our study.

6. iPSC derived cardiomyocytes have the advantage that they show spontaneous automaticity. Were these cells able to maintain spontaneous beating? If so, did the faster calcium dependent inactivation and the reduced IK1 result in faster beating rates?

This is a particularly interesting question in the field of iPSC-CMs. Indeed, spontaneous automaticity is a hallmark of iPSC-CMs, particularly, when cultured in a monolayer (Kim *et al.*, 2015; Casini *et al.*, 2017). In our study, iPSC-CMs initially exhibited spontaneous beating activity. However, over time, spontaneous beating frequency declined, consistent with previously reported observations linked to time-dependent maturation properties of iPSC-CMs (Lundy *et al.*, 2013; Seibertz *et al.*, 2023*b*). This unique property is primarily driven by increased function of I_{K1} and "improved/mature" electrophysiological profile (Kim *et al.*, 2015; Cyganek *et al.*, 2018; Seibertz *et al.*, 2023*b*). Interestingly, our experiments demonstrated reduced I_{K1} upon blebbistatin incubation, pointing to the fact that the observed change in the current density is indeed the effect of the drug.

Furthermore, it is important to note that the patch-clamp technique at the time of experiment may suppress spontaneous activity. Once seal is formed, cells are kept at a holding potential (- 80 mV) prior to the test pulse. This property can effectively interrupt any spontaneous depolarisations the cells may exhibit under baseline conditions.

7. Authors claim that the intracellular Ca2+ changes due to blebbistatin results in pro-arrhythmic changes. Did the Authors measure any indicators of arrhythmia for instance, EADs or DADs in these cells?

The readout for potential pro-arrhythmic activity in our study was SR Ca^{2+} leak in the form of Ca^{2+} sparks, as measured by confocal line scan. The main aim of the study as a whole was to raise awareness about effects of blebbistatin application such as during use as an experimental tool. Blebbistatin has no clinical use as a drug and so further and substantial experiments into EADs and DADs were not performed. Nevertheless, our recent publication demonstrated that using blebbistatin to reduce Ca^{2+} buffering increases the inducibility of arrhythmic behavior in an *ex vivo* mouse heart model (Fakuade *et al.*, 2024).

8. Did the authors check on calcium channel current kinetics and amplitude when Ba2+ was the charge carrier through the channel. This may provide some insight on the buffering issue, because acute application of Ba2+ containg solution lasting only a few seconds that fails to activate CICR and rise of cytosolic calcium transients may shed some light on the authors' proposal of compartmented calcium pools.

This is a very good idea, however, using Ba^{2+} to abolish SR Ca^{2+} release is not possible with syncropatch becauses Ca^{2+} is required for successful seal formation (Milligan *et al.*, 2009). Therefore, we used EGTA, a slow Ca^{2+} buffer, to prevent cytosolic Ca^{2+} transients and we believe this experiment reflects the Reviewer's intention. We found that EGTA does not abolish CDI completely. This might be due to the fact that Ca^{2+} influx through $I_{Ca,L}$ remains largely unchanged (due to the slow buffering kinetics of EGTA) and is therefore able to bring about CDI of $I_{Ca,L}$. Since under these conditions blebbistatin had no effect on CDI, we conclude

that blebbistatin does not effect the CDI caused by the Ca^{2+} entering via $I_{Ca,L}$. We have now added text to the Discussion to convey this:

Page 13, line 300: "The fact that EGTA, a slow buffer, did not completely abolish CDI in the presence or absence of blebbistatin, suggests that Ca^{2+} entering via $I_{Ca,L}$ can exert CDI and this is not affected by blebbistain, pointing towards compartmentalisation of Ca^{2+} signalling"

Minor: Starting almost every sentence with mean {plus minus} SEM in the figure legends is somewhat odd. I would recommend saying for example Quantification of CaT amplitude instead of Mean {plus minus} SEM CaT amplitude etc.

The figure legends have been revised accordingly.

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Dear Professor Voigt,

Re: JP-RP-2025-287545R1 "Blebbistatin reduces calcium buffering in cardiomyoctes: Consequences for cellular electrophysiology" by Izzatullo Sobitov, Katharina Ritzenhoff, Marie Gaulrapp, Lea Becker, Aiste Liutkute, Fitzwilliam Seibertz, Funsho E. Fakuade, Fleur E. Mason, and Niels Voigt

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EDITOR COMMENTS

Reviewing Editor:

The referees agree that the revised manuscript is expected to have a significant impact on the field.

Senior Editor:

The manuscript is now acceptable. Thank you for your excellent contribution!

REFEREE COMMENTS

Referee #1:

The authors present a responsive revised manuscript. The concerns in my original review have been directly and thoughtfully addressed. This is a meaningful study convincingly demonstrating that blebbistatin alters Ca buffering in iPSC-aCMs.

Referee #2:

I have no other comments.