nature portfolio

Peer Review File

Cryo-electron microscopy reveals sequential binding and activation of Ryanodine Receptors by statin triplets

Corresponding Author: Professor Filip Van Petegem

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript reports the cryo-EM structures of the ryanodine receptor (RyR) type 1 in complex with atorvastatin, a commonly prescribed medication used to lower blood plasma cholesterol levels. It aims to investigate whether statins directly bind to RyRs, thereby exhibiting their off-target side effects. The authors determined the structure of RyR1 in the presence and absence of atorvastatin, revealing that it binds to a cleft within the transmembrane domain, leading to the opening of the pore. Observing that three molecules are located in the same pocket and directly interact with each other is an unexpected finding that would draw interest from scientists across diverse fields studying protein-ligand interactions. The manuscript also proposes a mechanism for how type 2 RyRs in the heart are spared from the side effects of statins. Overall, the structural data is of high quality, the interpretation of the data is appropriate, and the conclusions are well supported by the data and the available literature. This work is an important contribution to the field and may serve as a foundation for the development of statin analogs that do not interact with RyRs. I only have a few minor concerns.

1) It would be helpful to mention the common therapeutic doses of atorvastatin and how they relate to the concentration used in the cryo-EM experiments. Would atorvastatin occupy these sites at the therapeutic doses in patients?
2) The authors propose that atorvastatin also binds to the ATP binding site. While this may be true, the data presented is not strong enough to reach this conclusion. Could the observed changes be the result of channel opening forced by binding of atorvastatin to the TMD? Unless there is additional support, this section needs to be toned down.
3) The cryo-EM densities shown in Figure 1 are convincing. However, it may be helpful to show the density for the entire binding pocket without removing any density. Panel D probably indicates this, but it is unclear whether the individual densities are segmented or not.
4) It would be helpful to show the relation between panels B and C in Figure 3 directly on the figure, potentially using a rotation arrow. It is not straightforward to understand that they show the same thing, but from a different viewing angle.
5) FSC threshold of 0.5 should be used to define model resolution.
6) In Extended Data Table 1, the map-sharpening B factor is listed as N/A. Is this true? Were the maps shown in the figures not sharpened? This needs to be clarified.

Reviewer #2

(Remarks to the Author)

Please see attachment.

Reviewer #3

(Remarks to the Author)

The manuscript is well written and clearly describes the high resolution cryo-electron microscopy study of atorvastatin binding to the skeletal muscle ryanodine receptor (RyR1) with associated structural changes that explain the molecular interactions between the drug and the ion channel. The results show the molecular interactions between the drug and RyR1 that underlie the significant clinically challenging off-target effects of some statin drugs on skeletal muscle and provide a framework for the design of more specific statin analogues with reduced off target binding to RyR1. The methodology is sound and in my opinion the work meets expected standards in the field. The results generally support the claims and conclusions, although some concerns are outlined below and require addressing in the manuscript.
For the present study the purified RyR1 was bathed in 36μM free Ca2+ (buffered with 2mM EGTA) and 2mM caffeine before flash before flash-freezing. The concerns arise from the use of such a high Ca2+ concentration which could be exerting an inhibitory influence on RyR1 structure, although the channel would be mainly open with the added caffeine. The reason for using such a strongly activating and non-physiological solution should be addressed. The channel would be forced into a conformation that would not be representative of its invitro conformation. It would be useful to examine statin binding to a less strongly activated or even partially closed channel. There are additional concerns with the comparison with RyR2 (Figure 4C). Was the RyR2 structure obtained using the same Ca2+ and caffeine concentrations as those used with RyR1? This should be addressed and a reference for the RyR2 structure should be provided with a comment on the solution conditions and how this might affect structure in the statin binding region.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed my previous comments sufficiently. I have no further comments.

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments and concerns to my full satisfaction; and I feel that they have also done so likewise for the comments of other reviewers.
I therefore support the publication of this paper in Nature Communications as so revised.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concens.
I do not have any additional comments

Responses to the reviewers' comments are shown in blue font.

Reviewer #1:

This manuscript reports the cryo-EM structures of the ryanodine receptor (RyR) type 1 in complex with atorvastatin, a commonly prescribed medication used to lower blood plasma cholesterol levels. It aims to investigate whether statins directly bind to RyRs, thereby exhibiting their off-target side effects. The authors determined the structure of RyR1 in the presence and absence of atorvastatin, revealing that it binds to a cleft within the transmembrane domain, leading to the opening of the pore. Observing that three molecules are located in the same pocket and directly interact with each other is an unexpected finding that would draw interest from scientists across diverse fields studying protein-ligand interactions. The manuscript also proposes a mechanism for how type 2 RyRs in the heart are spared from the side effects of statins. Overall, the structural data is of high quality, the interpretation of the data is appropriate, and the conclusions are well supported by the data and the available literature. This work is an important contribution to the field and may serve as a foundation for the development of statin analogs that do not interact with RyRs. I only have a few minor concerns.

We thank the reviewer for the positive feedback and the constructive remarks below.

1) It would be helpful to mention the common therapeutic doses of atorvastatin and how they relate to the concentration used in the cryo-EM experiments. Would atorvastatin occupy these sites at the therapeutic doses in patients?

The atorvastatin concentrations in blood plasma have been found to range from tens of nanomolar for low dose treatments, to as high as 334nM for high doses (80mg atorvastatin/day), (Stern, R.H. et al.. *J Clin Pharmacol* **40**, 616-23). The concentrations can also rise beyond that in individuals that do not metabolize statins rapidly, or through interference with other drugs (e.g. fimbrozil) or food components (e.g. furanocoumarins in grapefruit) that inhibit CYP3A4, an enzyme involved in statin metabolism. More important is the concentration statins may reach within muscle cells, but this is currently unknown. There is a possibility that they may accumulate, because they can interconvert between lactone and acidic forms, and the latter have reduced membrane permeabilities.

For the cryoEM experiments, we added saturating levels to ensure homogeneity of the particles, as partial occupancies would interfere with identifying the statin densities. The concentration of purified RyR1, which is applied to the cryo-EM grids, is ~5-6μM. With up to 4 molecules binding per subunit (i.e. 16 per full tetramer), this already means we need at least 96μM of statin simply to match the stoichiometry, well above the clinical concentrations.

However, ryanodine binding data from the Sitsapesan lab have established EC50 values of statins for RyRs to range between 300-400nM (Lindsay et al . *Br J Pharmacol* **179**,4941-4957), with increases in ryanodine binding already visible at tens of nanomolar concentrations. This would suggest clinical relevance of RyR1 activation by statins.

We have now added a new 2nd paragraph that outlines this into the Discussion section.

2) The authors propose that atorvastatin also binds to the ATP binding site. While this may be

true, the data presented is not strong enough to reach this conclusion. Could the observed changes be the result of channel opening forced by binding of atorvastatin to the TMD? Unless there is additional support, this section needs to be toned down.

This is a good point with which we agree. We have toned down the section and now write "*It is unclear whether these conformational changes are due to atorvastatin binding to this site, or the three atorvastatin molecules engaging the pVSD domain*"

3) The cryo-EM densities shown in Figure 1 are convincing. However, it may be helpful to show the density for the entire binding pocket without removing any density. Panel D probably indicates this, but it is unclear whether the individual densities are segmented or not.

Indeed, panel D already indicated this, but we now also include additional panels in a new Supplementary Figure 2, which shows the surrounding densities in more detail.

4) It would be helpful to show the relation between panels B and C in Figure 3 directly on the figure, potentially using a rotation arrow. It is not straightforward to understand that they show the same thing, but from a different viewing angle.

We have now altered this figure to show the spatial relationship.

5) FSC threshold of 0.5 should be used to define model resolution.

We have been following the gold-standard guidelines (Scheres and Chen, 2012, Nat Methods 9,853–854). Although this is widely adopted, we realize that not everyone agrees. Therefore, the updated supplementary table 1 now shows the resolution at both FSC=0.143 and FSC=0.5 thresholds.

6) In Extended Data Table 1, the map-sharpening B factor is listed as N/A. Is this true? Were the maps shown in the figures not sharpened? This needs to be clarified.

This is correct. Other than indicated for the density modified maps (updated Supplementary figure 6), the maps shown are the raw maps, which are also the ones uploaded to the EMDB. The methods section has been updated to clarify this.

Reviewer #2:

Van Petegem and coworkers directly demonstrate in this paper that atorvastatin binds to RyR1, the calcium release channel from skeletal muscle; and they show that this binding potentiates the calcium-releasing open state. This is a medically significant finding since statins such as atorvastatin (Lipitor), a drug that is widely administered for the management of cholesterol levels, can have muscle-related symptoms as side effects that may include life-threatening rhabdomyolysis. Susceptibility to such side effects appears to be exacerbated in patients that harbor RyR1 mutations for malignant hypothermia (MH). The findings also provide basic insights into mechanisms of action for ryanodine receptors.

The study is strictly a work in structural biology, without biochemical characterization of atorvastatin binding to RyR1 or electrophysiological study of consequences from such binding. Nevertheless, these results stand on their own as a compelling demonstration of the basis for the effect of atorvastatin binding on RyR1 conformation, which was previously associated with activity. Thus, I recommend the publication of this contribution in *Nature Communications*; however, I do request that the following comments and concerns should be addressed before acceptance:

We thank the reviewer for the positive feedback and constructive comments.

1. From the cited literature, it seems that the biochemistry of atorvastatin binding to RyR1 has not been characterized, and the requirement of DMSO for solubilization would surely frustrate measures of concentration. Indeed, as noted in Methods (lines 344-345), fibers were found on cryo-EM grids suggesting that even in 2% DMSO the atorvastatin concentration was substantially lower than the nominal 10 mM. Nevertheless, the statin affinity at the four sites (Ator1-3 plus the ATP site) is relevant to questions of medical relevance. We are given some insight into the relative affinities from the observation that only the Ator1 site is occupied in the pore-closed structure (36% of the accepted particles by line 91); thus Kd(Ator1) < Kd(Ator2, Ator2, ATP site). Further biochemical analysis of atorvastatin binding to RyR1 would be desirable; but, at a minimum, the issue of statin affinities would seem to merit some discussion.

We have now included additional discussion around the relative affinities of atorvastatin for the different sites, as well as its relationships to clinically relevant concentrations and a previous estimate of the EC50 value for atorvastatin (see also our response to reviewer #1, comment 1). We agree that, for the open state, Ator1 likely has the highest affinity, whereas the atorvastatin bound to the ATP site likely has the weakest (due to the less well-defined density, which may indicate partial occupancy). This is now part of the updated Discussion section.

2. The section on 'Isoform specificity' makes interesting sequence comparisons relevant to the likelihood of statin binding to RyR2 and RyR3 as well as to RyR1 as demonstrated here. It seems to me, however, that the punch line is missing. I would include an added penultimate sentence: "By analogy with the RyR1 structures, RyR2 would not be expected to bind atorvastatin, except perhaps at the ATP site."

As suggested, we now added the following sentence: "*Thus, RyR2 would not be expected to bind atorvastatin in this region or would only do so with much lower affinity*."

We also added a statement in the section on the ATP binding site: *"Since the ATP binding site is conserved among all three isoforms, it would follow that atorvastatin may bind to RyR2 and RyR3 at the same site, but this remains to be confirmed".*

3. The apparent impact of RyR1 disease mutations (notably for MH) on susceptibility to statin side effects is fascinating and particularly relevant to personalized medicine. Thus, the section on binding of atorvastatin to porcine R615C RyR1 is most welcome; however, I am a little puzzled by the findings as described. Moreover, I do not see any discussion of a basis for the impact of this mutation on atorvastatin binding. If I understand correctly, RyR1 is used interchangeably in describing rabbit and pig RyR1; and it appears that this extends to Fig. 7B, which compares RyR1 R615C (presumably pig) with wild-type RyR1 (presumably rabbit). Something needs to be said to justify this comparison since the distinction of 36% closed for wild-type vs. 19% closed for the mutant is taken as evidence for a statin effect. In principle, the distinction could be due to the species difference rather than the mutation. More generally, for clarity, the mutant should be distinguished throughout as 'pig RyR1 R615C' or perhaps 'pRyR1' as in Extended Data Table 2. But how does R615C affect atorvastatin susceptibility, located as it is in the cytosolic shell far away from the S4-S5 linker and sites of atorvastatin binding? This seems unanswerable from present data; but, to my mind, the dilemma should be broached and the discussion could be extended for other MH mutations known to affect susceptibility to statin side effects.

We thank the reviewer for bringing this up and we now have significantly altered the text. We now unambiguously indicate the species (pig RyR1 R615C) throughout, and note the limitation of comparing RyR1 from two different species. "*Although we cannot exclude the possibility that this is due, in part, to species differences in sequence, this observation is in line with the increased contractility of muscle from pigs carrying this mutation*[24]"

A previous clinical study on statin-induced myopathy had identified a patient with the paralogous human RyR1 R614C mutation, motivating us to include this mutation in the study. MH mutations like R614C are known to enhance sensitivity (higher Po) to multiple activating ligands, including caffeine and volatile anesthetics, and cytosolic Ca^{2+}. Just showing one example, we confirmed this for the R615C pig RyR1 mutant using bilayer recordings (Woll et al, 2021, Nat Commun). Thus, an enhanced opening of R615C by atorvastatin is in line with observations for other activators and likely has a common explanation. In regards of the mechanism, one important finding is that R615C has a clear allosteric coupling to the pore: it is located at an interface between two domains (NSol and BSol) that is altered significantly during channel opening. Since the mutation disrupts an ionic and additional hydrophilic interactions, we previously postulated that the R615C mutation destabilizes the closed state of RyR1 more than the open state, thus lowering the energetic barrier for channel opening. This, in turn, allows channel activators, such as atorvastatin, to induce more open-state particles. We include an abbreviated version of this hypothesis in the section describing the R615C mutant.

4. I appreciate that the density attributed to atorvastatin at the ATPsite (lines 199-200) is too weak for model building. Nevertheless, it could be interesting to describe how the two features shown in Fig. 5B relate to the bound ATP, which is not easy to discern in comparing to Fig. 5C. In particular, could one of the two features correspond to the dihydroheptanoic acid group in place of the triphosphate group of ATP and the other blob correspond to the aromatic portion in place of the adenine moiety?

We now include an additional figure (supplementary figure 8 in the updated manuscript) to address this. We have also added our best fit for Atorvastatin into this site, however due to the poor density we did not put this into the deposited structure. It is possibly that this is poor occupancy or that if the atorvastatin binds in multiple binding conformations.

5. The structural studies on pig RyR1 615C are not adequately described. The muscle membrane preparation of this protein is described (line 309) as a variant of the wild-type rabbit protein, which is fine; however, I cannot find any reference to the mutant protein in any of the succeeding sections of Methods. It would suffice to say that purification, cryo-EM sample preparation and data collection procedures were the same as for the rabbit protein, but aspects of data processing are specific and, I believe, model building should be included. It seems to me that the previous structure paper on pig RyR1 (Ref. 37) should be cited in this section and not just in Methods and indirectly in the figure legend.

It appears that the atomic model for pig RyR1 has not been improved from the earlier structure (Ref. 37; PDBid 6X35) and is not being deposited. Since the open-state structure here is at appreciably higher resolution (3.36 Å) than that reported with deposition 6X35 (4.20 Å) and rabbit RyR1 structures have been much improved meanwhile as well, a much improved model for pig RyR1 can be expected – not to mention the benefits from details of interactions with atorvastatin molecules.

We thank the reviewer for this useful suggestion. Indeed, the open-state model of R615C pig RyR1 is substantially better in resolution than what we previously reported, and we therefore completed model building of the open state pig R615C RyR1. As suggested, we also include a more thorough description of the sample handling and unambiguously state in all of the methods sections whether the procedure was the same or different . We also reference the previous study in the main text.

Minor comments:

1. The ‘mechanism of opening’ describes the binding of Ator2 and Ator3 as if force generating (“… these two molecules push the S4 helix and S4S5 linker away …”; lines 159-160), which is implausible. A more plausible mechanism would have these statin molecules stabilizing the open-state conformation from an intrinsic open-closed equilibrium, thereby drawing the equilibrium to the open state. If that description is too awkward, I suggest “As a result, it is as if these two atorvastatin molecules push …”

We agree that this was an akward way to present the mechanism and have altered the text accordingly.

2. Line 218. ‘Po’ is undefined and should be replaced, perhaps by ‘channel open probability’.

done

3. It would be appropriate and desirable to state the resolution levels of the maps in the text and not only in the tables and FSC curves (which are not even legible in my printing of Extended Data Fig. 3).

done

Reviewer #3

The manuscript is well written and clearly describes the high resolution cryo-electron microscopy study of atorvastatin binding to the skeletal muscle ryanodine receptor (RyR1) with associated structural changes that explain the molecular interactions between the drug and the ion channel. The results show the molecular interactions between the drug and RyR1 that underlie the significant clinically challenging off-target effects of some statin drugs on skeletal muscle and provide a framework for the design of more specific statin analogues with reduced off target binding to RyR1. The methodology is sound and in my opinion the work meets expected standards in the field. The results generally support the claims and conclusions, although some concerns are outlined below and require addressing in the manuscript.

We thank the reviewer for the positive feedback and comments.

For the present study the purified RyR1 was bathed in 36μM free Ca2+ (buffered with 2mM EGTA) and 2mM caffeine before flash before flash-freezing. The concerns arise from the use of such a high Ca2+ concentration which could be exerting an inhibitory influence on RyR1 structure, although the channel would be mainly open with the added caffeine. The reason for using such a strongly activating and non-physiological solution should be addressed. The channel would be forced into a conformation that would not be representative of its invitro conformation. It would be useful to examine statin binding to a less strongly activated or even partially closed channel.

We're glad the reviewer is bringing this up. We simply chose to use strong activating conditions to maximize the number of particles that have atorvastatin bound, as this enables a higher resolution to be obtained. This consideration is critical to allow an unambiguous identification of the binding site and of the binding pose, which would not be possible when only a small proportion of the particles has statins bound. Since the statins were already predicted to enhance the open probability, this suggested selective binding to open channels. Indeed, this appeared to be the case for the ator2 and ator3 molecules, where we only see binding to the open state. It's also important to note that Ca^{2+} alone is incapable of producing open RyR1 under cryo-EM conditions, as previously shown (Des Georges *et al*, 2016, *Cell* 67,145-157). Thus, adding several activators is a practical solution to ensure a sufficiently high number of open channels that will enable a high resolution. For the same reason, we wanted to ensure using sufficient Ca^{2+} to produce robust activation but, more importantly, to ensure that all of the activating Ca^{2+} binding sites are fully occupied to avoid heterogeneity that would again negatively impact resolution. We also note that other studies have shown that the peak in ^{3}H-ryanodine binding occurred at Ca^{2+} levels higher than 10μM (e.g. Yuan et al, 2021, *Acta Neuropathologica Communications* 186). The Des Georges *et al* paper also showed that 30μM free Ca^{2+} provided a robust activated conformation and, most importantly, neither in that study, nor in ours, do we observe any additional density for Ca^{2+} bound to an inactivating site.

Of course, we do not wish to claim that caffeine would be needed in a more physiological context. Indeed, previous planar lipid bilayer electrophysiology and ryanodine binding experiments have shown that atorvastatin also enhances the open probability of RyR1 in the absence of caffeine (Lindsay et al, *Br J Pharmacol* **179**,4941-4957). As requested, we now

outline the rationale of using the strong activating conditions in the Methods section and address this point in the main text.

There are additional concerns with the comparison with RyR2 (Figure 4C). Was the RyR2 structure obtained using the same Ca2+ and caffeine concentrations as those used with RyR1? This should be addressed and a reference for the RyR2 structure should be provided with a comment on the solution conditions and how this might affect structure in the statin binding region.

This seems to be a misunderstanding, since Figure 4C does not show a structure of RyR2. We merely show the structure of RyR1 bound to atorvastatin, indicate the side chains involved in interactions, and color the ones that are different between RyR1 and RyR2 in red. Indeed, there currently does not exist a reference structure of RyR2 with only Ca and caffeine bound and hence we did not provide a superposition for this reason. We have altered the figure legend to avoid this confusion.

Comments on Ms. NCOMMS-25-39182 by Molinarolo *et al.*

Van Petegem and coworkers directly demonstrate in this paper that atorvastatin binds to RyR1, the calcium release channel from skeletal muscle; and they show that this binding potentiates the calcium-releasing open state. This is a medically significant finding since statins such as atorvastatin (Lipitor), a drug that is widely administered for the management of cholesterol levels, can have muscle-related symptoms as side effects that may include life-threatening rhabdomyolysis. Susceptibility to such side effects appears to be exacerbated in patients that harbor RyR1 mutations for malignant hypothermia (MH). The findings also provide basic insights into mechanisms of action for ryanodine receptors.

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