CCAAT-binding complex and HapX display highly variable but evolutionary conserved synergetic promoter-specific DNA recognition. Nucleic Acids Res. *48*, 3567–3590. https://doi.org/10.1093/nar/gkaa109.

Gsaller, F., Hortschansky, P., Beattie, S., Klammer, R.V., Tuppatsch, K., Lechner, B.E., Rietzschel, N., Werner, E.R., Vogan, A.A., Chung, D., et al. (2014). The Janus transcription factor HapX controls fungal adaptation to both iron starvation and iron excess. EMBO J. *33*, 2261–2276. https://doi.org/10.15252/embj.201489468.

Gsaller, F., Hortschansky, P., Furukawa, T., Carr, P.D., Rash, B., Capilla, J., Muller, C., Bracher, F., Bowyer, P., Haas, H., et al. (2016). Sterol Biosynthesis and Azole Tolerance is Governed by the Opposing Actions of SrbA and the CCAAT binding complex. PLoS Pathog. *12*, e1005775. https://doi.org/10.1371/journal.ppat.1005775.

Hortschansky, P., Eisendle, M., Al-Abdallah, Q., Schmidt, A.D., Bergmann, S., Thon, M., Kniemeyer, O., Abt, B., Seeber, B., Werner, E.R., et al. (2007). Interaction of HapX with the CCAAT-binding complex–a novel mechanism of gene regulation by iron. EMBO J. *26*, 3157–3168. https://doi.org/10.1038/sj.emboj.7601752.

Hortschansky, P., Haas, H., Huber, E.M., Groll, M., and Brakhage, A.A. (2017). The CCAAT-binding complex (CBC) in Aspergillus species. Biochim Biophys Acta Gene Regul Mech *1860*, 560–570. https://doi.org/10.1016/j.bbagrm.2016.11.008.

Huber, E.M., Hortschansky, P., Scheven, M.T., Misslinger, M., Haas, H., Brakhage, A.A., and Groll, M. (2022). Structural Insights into Cooperative DNA Recognition by the CCAAT-Binding Complex and its bZIP Transcription Factor HapX (Structure).

Huber, E.M., Scharf, D.H., Hortschansky, P., Groll, M., and Brakhage, A.A. (2012). DNA minor groove sensing and widening by the CCAAT-binding complex. Structure *20*, 1757–1768. https://doi.org/10.1016/j.str.2012.07.012.

It takes two to tango: Rycals and ATP snuggle up to bind ryanodine receptors

Raika Pancaroglu[1] and Filip Van Petegem[1,*]
[1]Department of Biochemistry and Molecular Biology, The Life Sciences Institute, The University of British Columbia, Vancouver, BC, Canada
*Correspondence: petegem@mail.ubc.ca
https://doi.org/10.1016/j.str.2022.05.022

In this issue of *Structure*, Melville and colleagues used cryo-EM to study the binding of ryanodine receptors to Rycals, compounds with the potential to treat skeletal and cardiac muscle disorders. Unexpectedly, they found that Rycal packs against an ATP in a peripheral pocket, which stabilizes the closed channel state.

The largest ion channels known to date, ryanodine receptors (RyRs), play crucial roles in a variety of tissues. They are mostly studied for their role in the contraction of various types of muscle tissue. When triggered to open, they release Ca^{2+} stored in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER). Mammalian genomes encode three isoforms (RyR1–3) that are expressed in different tissues. RyRs form homotetrameric assemblies consisting of ~5000-residue subunits. Due to their size (>2MDa), they have been popular targets for cryo-EM studies. A multitude of cryo-EM structures have captured RyRs in different states and bound to different molecules (des Georges et al., 2016). Overall, they resemble the shape of mushrooms, with the stalk forming the transmembrane region and a large cap extending into the cytosol (Figure 1).

Malfunctioning of RyRs is closely linked to disease (Pancaroglu and Van Petegem, 2018). RyR1 is the predominant isoform found in skeletal muscle and is targeted by hundreds of disease-associated mutations that can lead to myopathies such as central core disease (CCD) or malignant hyperthermia (MH), a condition whereby volatile anesthetics or muscle relaxants can trigger opening of the mutant RyR1. This results in a hypermetabolic response, muscle rigidity, and a dangerous rise in body temperature. Mutations in the cardiac RyR2 have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT), a type of stress-induced arrhythmia that can lead to sudden cardiac death. Although such genetic disorders are rare, an increasing body of data has suggested that RyRs are also major culprits or accomplices in a range of more common conditions including Alzheimer's disease, sarcopenia, atrial fibrillation, and heart failure progression (Kushnir et al., 2018). Consequently, RyRs are very attractive drug targets with the potential to be used in treatment for a wide range of illnesses.

A number of cryo-EM structures have visualized the binding of small molecules to RyRs. These molecules include ATP and Ca^{2+}, physiologically relevant ligands that can activate RyRs (Ca^{2+} can also inhibit RyRs at higher concentrations). Both ATP and Ca^{2+} have been found to bind cooperatively at domain-domain interfaces that involve the C-terminal region of the RyR (des Georges et al., 2016). The same region also binds caffeine, which stimulates channel opening as well. Diamide insecticides, which activate RyRs and cause paralysis in insects, bind to pseudo-voltage-sensing domains in the transmembrane region (Ma et al., 2020). Ryanodine, a plant alkaloid that led to the discovery of RyRs, binds directly in the ion permeation pathway (des Georges et al., 2016) and can block the channel at high concentrations. At lower levels it leads to subconductance states

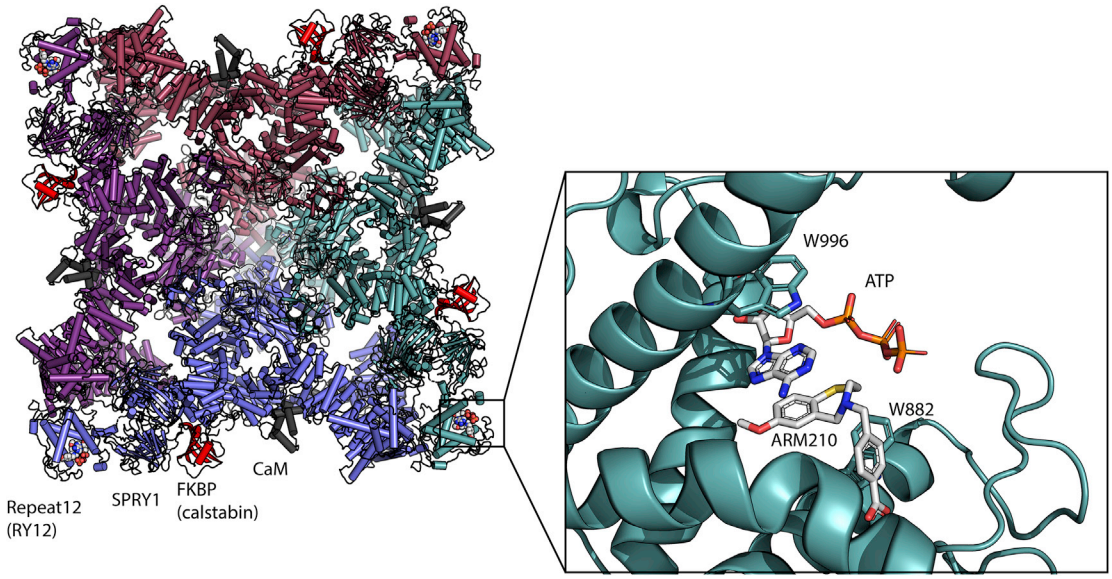

Figure 1. The binding site for ATP and ARM210, the Rycal used in this study, in RyR1

Top view of RyR1, facing from the cytosol toward the SR membrane, showing the four subunits in different colors. FKBP (calstabin) is shown in red and calmodulin (CaM) in dark gray. ATP and ARM210 are shown in spheres, bound to the Repeat12 domain. This domain is separated from FKBP through the SPRY1 domain. The inset shows a detail of the Repeat12 domain, with ATP and ARM210 in sticks. Two Trp residues involved in binding are also shown.

and is considered toxic. Thus far, all of these molecules have been found to bind either close to, or directly within, the RyR transmembrane region, even though there is plenty of binding surface available throughout the large cytosolic region.

Although there are a couple of loss-of-function mutations, a common observation for RyRs in pathological conditions is a gain-of-function phenotype, whereby RyRs have increased sensitivity to cytosolic or luminal ligands. Sometimes referred to as "leaky RyRs," these channels could greatly benefit from inhibitory molecules. Because directly blocking the RyR pore might have adverse effects, the ideal candidate would be an allosteric inhibitor, capitalizing on the long-range allosteric coupling between the massive cytosolic shell and the pore-forming domain. One promising class of molecules is "Rycals," benzothiazepine derivatives that have been shown to prevent the "leak" of Ca^{2+} through overactive RyRs (Kushnir et al., 2018). Rycals have been proposed to stabilize the binding between RyRs and either FKBP12 or FKBP12.6 (FK506 binding proteins of 12 or 12.6 kDa, respectively, also known as "calstabins"). However, the role of FKBPs in the pathophysiology of RyRs has been the topic of a long ongoing debate. In particular, there is disagreement as to whether the pathological conditions truly cause a significant decrease in affinity for the FKBPs. Specific high-affinity binding of Rycals has also been questioned, with an alternative proposal that they instead bind to multiple low-affinity sites (Mei et al., 2013). Identifying the binding site for Rycals in RyRs and observing the mechanism of action would aid in solving this controversy.

In this issue of *Structure*, Melville et al. (2022) employed cryo-EM to study the binding of RyR1 to ARM210, a Rycal that is being tested in clinical trials for patients with RyR1-related myopathy. The study now shows, for the first time, a direct binding of a Rycal to an RyR, but in a location and manner that is completely unexpected. The binding site resides in a small domain located in the extreme corners of the RyR (Figure 1). Known as the "RY12 domain" or "Repeat12," this domain forms a horseshoe-shaped structure consisting of two pseudo-symmetrical halves. ARM210 binds within the cleft of the horseshoe. The specific site is particularly intriguing, as it is >180 Å away from the pore-forming domain. An even greater surprise is a small molecule accomplice: Rycal stacks against an ATP molecule that is also bound to the same domain. Because the Repeat12 domain shows a high flexibility relative to the remainder of the RyR, its local resolution is somewhat lower. Yet, the Rycal can be seen to form π-stacking interactions with the adenine ring of ATP. Both Rycal and ATP also make stacking interactions with nearby tryptophan residues (Figure 1). Melville et al. have also validated the binding site by showing that residues lining the cleft are critical for the binding and functional effect of the Rycal. A thorough analysis has shown that there were no open channels in this cryo-EM dataset, despite the presence of ATP, Ca^{2+}, and caffeine in the sample, which was previously shown to result in open channel structures (des Georges et al., 2016). Thus, the binding of the Rycal appears to prevent open channel conformations.

A major advance, this study now definitively shows that Rycals can bind RyRs directly. More importantly, the binding is clearly dependent on the exact conditions used, as sufficient amounts of ATP are required. This might explain why other reports have failed to show specific high-affinity binding of Rycals to RyRs. The study also posits new questions. One pertains to the promiscuity of the site. Melville et al. showed that the Repeat12 domain can also be occupied by two molecules of ADP. The requirement of ATP for Rycal binding is also reminiscent of dantrolene, a molecule used to treat MH episodes in the operating room. It was previously

found that the inhibition of RyR1 by dantrolene also requires ATP (Diszhazi et al., 2019). Therefore, the Repeat12 domain might potentially be able to accommodate a range of molecules with significantly different structures.

An important unanswered question remains about the exact mechanism of Rycals. Although proposed to stabilize the interaction between FKBP/calstabin and RyRs, the current structure does not show any distortion of the FKBP binding site. However, the structure was solved in the presence of FKBP, and it is possible that structural changes in its binding site are only observed in its absence, which could still explain an altered affinity. The SPRY1 domain is critical for binding of FKBP (Yuchi et al., 2015) and is adjacent to the Repeat12 domain. Small shifts in SPRY1, mediated by conformational changes in the Repeat12 domain, could readily alter the FKBP binding site.

The study has pushed the resolution limits of RyR cryo-EM structures, and multiple regions are now much better resolved compared with previous cryo-EM structures. However, future improvements of the Rycal binding are still welcome, and this might come from crystallographic studies on the isolated Repeat12 domain. Although a previous crystal structure of the RyR1 Repeat12 domain displayed a very distinct conformation that would be incompatible with ligand binding (Yuchi et al., 2015), an unpublished structure of the RyR3 Repeat12 domain has shown binding of ATP (PDB ID: 6UHH). Also of interest will be the interplay between Rycals and disease mutations of RyRs. Cryo-EM structures of disease-mutant RyRs have shown that the mutations can result in distinct pathological or primed conformations (Iyer et al., 2020; Woll et al., 2021). It will be of interest to see whether the Rycals can revert these back to WT conformations.

The path is now fully open for further structure-guided development of a novel generation of therapeutics.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

des Georges, A., Clarke, O.B., Zalk, R., Yuan, Q., Condon, K.J., Grassucci, R.A., Hendrickson, W.A., Marks, A.R., and Frank, J. (2016). Structural basis for gating and activation of RyR1. Cell *167*, 145–157.e17. https://doi.org/10.1016/j.cell.2016.08.075.

Diszhazi, G., Magyar, Z.E., Motyan, J.A., Csernoch, L., Jona, I., Nanasi, P.P., and Almassy, J. (2019). Dantrolene requires Mg(2+) and ATP to inhibit the ryanodine receptor. Mol. Pharmacol. *96*, 401–407. https://doi.org/10.1124/mol.119.116475.

Iyer, K.A., Hu, Y., Nayak, A.R., Kurebayashi, N., Murayama, T., and Samso, M. (2020). Structural mechanism of two gain-of-function cardiac and skeletal RyR mutations at an equivalent site by cryo-EM. Sci. Adv. *6*, eabb2964. https://doi.org/10.1126/sciadv.abb2964.

Kushnir, A., Wajsberg, B., and Marks, A.R. (2018). Ryanodine receptor dysfunction in human disorders. Biochim Biophys Acta Mol. Cell Res. *1865*, 1687–1697. https://doi.org/10.1016/j.bbamcr.2018.07.011.

Ma, R., Haji-Ghassemi, O., Ma, D., Jiang, H., Lin, L., Yao, L., Samurkas, A., Li, Y., Wang, Y., Cao, P., et al. (2020). Structural basis for diamide modulation of ryanodine receptor. Nat. Chem. Biol. *16*, 1246–1254. https://doi.org/10.1038/s41589-020-0627-5.

Mei, Y., Xu, L., Kramer, H.F., Tomberlin, G.H., Townsend, C., and Meissner, G. (2013). Stabilization of the skeletal muscle ryanodine receptor ion channel-FKBP12 complex by the 1,4-benzothiazepine derivative S107. PLoS One *8*, e54208. https://doi.org/10.1371/journal.pone.0054208.

Melville, Z., Dridi, H., Yuan, Q., Reiken, S., Wronska, A., Liu, Y., Clarke, O.B., and Marks, A.R. (2022). A drug and ATP binding site in type 1 ryanodine receptor. Structure *30*, 1–10. https://doi.org/10.1016/j.str.2022.04.010.

Pancaroglu, R., and Van Petegem, F. (2018). Calcium channelopathies: structural insights into disorders of the muscle excitation-contraction complex. Annu Rev. Genet. *52*, 373–396. https://doi.org/10.1146/annurev-genet-120417-031311.

Woll, K.A., Haji-Ghassemi, O., and Van Petegem, F. (2021). Pathological conformations of disease mutant Ryanodine Receptors revealed by cryo-EM. Nat Commun. *12*, 807. https://doi.org/10.1038/s41467-021-21141-3.

Yuchi, Z., Yuen, S.M.W.K., Lau, K., Underhill, A.Q., Cornea, R.L., Fessenden, J.D., and Van Petegem, F. (2015). Crystal structures of ryanodine receptor SPRY1 and tandem-repeat domains reveal a critical FKBP12 binding determinant. Nat Commun. *6*, 7947. https://doi.org/10.1038/ncomms8947.