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Slaying a giant: Structures of calmodulin and protein kinase a bound to the cardiac ryanodine receptor

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ABSTRACT

Ryanodine Receptors are Ca^{2+} release channels expressed in the Endoplasmic and Sarcoplasmic Reticulum membranes. Gong et al [1] reported cryo-EM structures of the cardiac RyR2 complexed to Calmodulin, which can downregulate channel opening. Haji-Ghassemi et al [2] report crystal structures of an RyR2 domain with PKA, a kinase promoting channel opening.

Ryanodine Receptors (RyRs) represent the largest and most complex members of the ion channel family. They owe their name to the compound *ryanodine*, an alkaloid found in the South American plant *Ryania speciosa*. RyRs control the release of Ca^{2+} from the Sarcoplasmic (SR) and Endoplasmic Reticulum (ER) [3]. They are expressed in many cell types, and the mammalian genomes encode three different isoforms (RyR1-3). RyRs owe their fame to their key role in muscle excitation-contraction coupling, the process whereby an electrical depolarization of the T-tubular membrane is transmitted into the release of SR Ca^{2+}, triggering contraction in skeletal (RyR1) and cardiac muscle (RyR2). RyRs are targets for hundreds of disease-associated mutations that can result in devastating and often life-threatening conditions [4]. This mostly includes central core disease and malignant hyperthermia for RyR1 variants, and stress-induced arrhythmia (catecholaminergic polymorphic ventricular tachycardia - CPVT) for mutations in RyR2.

Due to their large size, RyRs have been attractive targets for study via electron microscopy methods, which have revealed an overall mushroom shape, with a stalk traversing the membrane and a large cap, which represents the bulk of the protein, extended into the cytosol [3]. Several reconstructions are now available at resolutions better than 4Å [1,3,5,6] (Fig. 1a,b). The stalk resembles a voltage-gated ion channel, containing a pore-forming domain and 4-helix bundles like voltage-sensing domains. The cytosolic cap consists of large α-solenoid regions, decorated with several globular domains. Previous cryo-EM studies have revealed binding sites for activating Ca^{2+}, caffeine, ATP and ryanodine [6]. However, except for FKBP12 and FKBP12.6, most protein binding partners had not been visualized in complex with RyRs at high resolution.

Among the many regulators of RyRs is Calmodulin (CaM), a ubiquitous Ca^{2+} sensing protein that can fine-tune the channel's open probability dependent on the specific isoform and Ca^{2+} concentration. At high Ca^{2+}, CaM has been shown to inhibit both RyR1 and RyR2, whereas at low Ca^{2+} it inhibits RyR2 but activates RyR1 [7]. In a recent report, Gong et al [1] solved cryo-EM structures of porcine RyR2 in complex with CaM in the presence and absence of various ligands, investigating the effects of Ca^{2+}-occupied (Ca^{2+}/CaM) and Ca^{2+}-free CaM (apoCaM) on the conformation of RyR2. One of the complexes with apoCaM reached an overall resolution of 3.6 Å, the highest reported so far for any RyR2 cryo-EM structure. Both apoCaM and Ca^{2+}/CaM bind at the periphery of the cytosolic cap, in a cleft formed by the handle, helical and central domains of RyR2. The exact location differs for apoCaM and Ca^{2+}/CaM, with apoCaM being closer to the cytosolic surface (Fig. 1a,b). For apoCaM, a major interaction is made between the apoC-lobe and a helix previously proposed to be important in CaM binding. The apoN-lobe engages in multiple contacts with RyR2, but not via a typical helical segment found for many apoCaM complexes with other proteins. For Ca^{2+}/CaM, the situation is different: both lobes wrap around an α-helix, resembling a previous crystal structure of Ca^{2+}/CaM bound to the equivalent RyR1 peptide [8] (Fig. 1c). The structures were validated via mutagenesis, showing that interface mutations, whether in CaM or in RyR2, affect the ability of CaM to reduce the channel's open probability and to increase Ca^{2+} release termination.

How does CaM binding modulate channel opening? Several small molecules have the ability to promote opening of RyR2. Depending on the exact cocktail used, the effect of Ca^{2+}/CaM on the RyR2 conformation differs. For example, a mixture of Ca^{2+}, ATP, and caffeine is able to yield cryo-EM structures of open RyRs, but Ca^{2+}/CaM cannot

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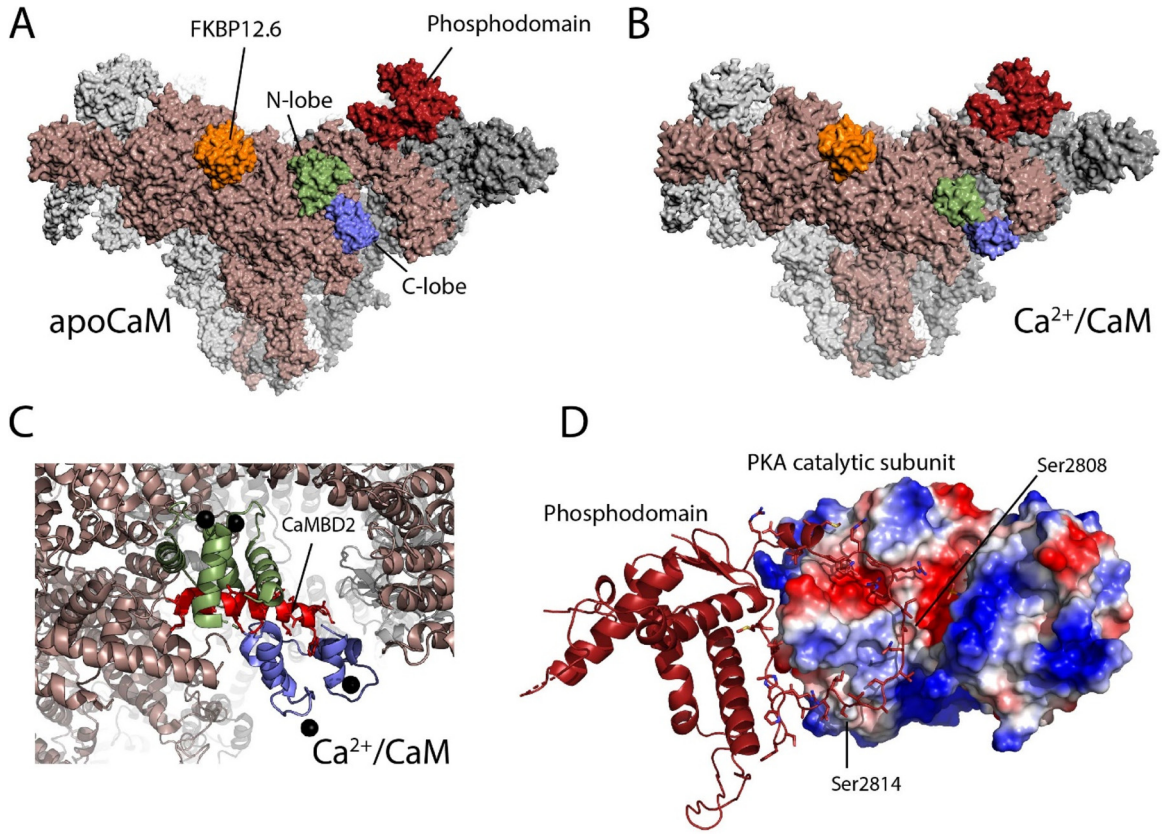

Fig. 1. A. Cryo-EM structure of porcine RyR2 bound to FKBP12.6 (orange) and apoCaM (N-lobe: green; C-lobe: blue) (PDB ID 6JI8). The Repeat34 domain, also known as the phosphorylation domain, is highlighted in red. **B.** Cryo-EM structure of porcine RyR2 bound to FKBP12.6, Ca^{2+}/CaM, ATP, and caffeine (PDB ID 6JIY). **C.** Detail of the Ca^{2+}/CaM binding site in panel B. The previously identified CaM binding peptide, known as CaMBD2, is highlighted in red. **D.** Crystal structure of the RyR2 phosphorylation domain (red cartoon) bound to the catalytic subunit of PKA (shown in electrostatic potential; red: negative; blue: positive potential). (PDB ID 6MM6). The equivalent positions of the human Ser2808 and Ser2814 residues are labeled.

counteract this, only resulting in rotations of the central domain, leaving the transmembrane region unperturbed. In contrast, RyR2 opening by Ca^{2+} and PCB95 can be reversed with Ca^{2+}/CaM, as there are now additional shifts in regions critical in channel gating, resulting in a closed RyR2 structure. One caveat is that these structures represent channels in the presence of detergent, which may affect the channel gating behaviour. But the results highlight the role of the RyR as a signal integrator, with the open probability depending on the exact mixtures of channel activators and inhibitors. Most likely, the precise balance of binding energies associated with each ligand and protein are a critical factor in determining the state of the channel.

RyRs are also under the influence of kinases, including cAMP-dependent protein kinase (PKA) and CaM-dependent kinase II (CaMKII). Excessive phosphorylation of RyRs has been linked to wide range of disorders, including atrial fibrillation, Alzheimer's disease, and much more. RyR phosphorylation is a contentious topic. The exact sites involved in disease, as well as the basal phosphorylation state of RyRs in non-diseased conditions have been under heavy debate. Regardless, a major consensus is that several sites are clear phosphorylation targets, and that phosphorylation seems to facilitate channel opening. In RyR2, the best studied sites include Ser2808 and Ser2814, both located in a flexible loop of the Repeat34 domain [9]. Also termed the phosphorylation hot spot domain, this domain is located on top of the cytosolic surface, where it adopts a very flexible location relative to the rest of the cytosolic cap (Fig. 1a,b). In addition to Ser2808 and Ser2814, there are several other sites in the same loop that can become phosphorylated, but possibly not to the same extent. Ser2030, located at the periphery of the cytosolic cap, has also been proposed as a major site recognized by PKA [10]. In a recent study from our group, we solved crystal structures of the PKA catalytic subunit in complex with the RyR2 phosphorylation domain and an ATP analogue, showing the Ser2808 side chain in the active site [2]. This represents the first description of a PKA complex with a fully folded substrate domain, and shows an extensive embrace between the two proteins, with the phosphorylation domain forming a lasso around a subdomain of PKA (Fig. 1d). This shows that the ability of PKA to recognize its substrates can lie far outside of the canonical PKA recognition motif.

The PKA-RyR2 interface contains targets for multiple disease mutations linked to CPVT, as well as corresponding sites for reported RyR1 variants. Although the complex shows Ser2808 in the active site, the nearby Ser2814 is part of the interface with PKA (Fig. 1d). Primarily regarded as a CaMKII target, Ser2814 phosphorylation has been studied extensively, and, surprisingly, addition of a phosphomimetic at this site (S2814D) increases the affinity for PKA [2]. A structure of the RyR2 S2814D phosphorylation domain in complex with PKA reveals a substantial structural change, with the region including D2814 undergoing a transition to an α-helix. The ability of the S2814D phosphomimetic to change the local secondary structure represents a conformational switch that may couple to the pore-forming region, but the precise allosteric changes remain to be elucidated.

Interestingly, the conformational switch results in additional stabilizing interactions with PKA, explaining the enhanced affinity of the S2814D mutant. Thus, a phosphomimetic at Ser2814, and possibly also an actual phosphorylation, can affect the activity of PKA acting on Ser2808, showing how nearby phosphorylation sites can influence one another. It will be interesting to find out whether prior phosphorylation of Ser2808 similarly impacts the ability of CaMKII to phosphorylate Ser2814.

RyRs remain active topics for investigation via structural biology methods, as many additional kinases, phosphatases, luminal and cytosolic protein partners remain to be mapped. Among these is the voltage-gated calcium channel $Ca_V1.1$, which couples mechanically to RyR1 in skeletal muscle. A lot more structural work is thus on the horizon.

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