The functional effect of CPVT-associated CaM mutants on RyR modulation

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Abstract

- Using [^{3}H]ryanodine binding assays, we determined the effect of CPVT and IVT associated CaM mutants on CaM's modulation of RyR1 and RyR2 from native SR membranes that were isolated from porcine skeletal and cardiac muscle, respectively.

Introduction

Calmodulin (CaM) is a 148 residue protein, with four Ca^{2+} binding domains (EF-hands): two high affinity Ca^{2+} binding sites in the C-lobe and two EF-hand like domains in the N-lobe (shown in Figure 1). Functional studies have shown that both the Apo and Ca-bound states of CaM modulates the activity of the SR Ca^{2+} release channel, known as the ryanodine receptor. This ligand-gated channel is responsible for release of Ca^{2+} from the SR in response to an action potential, a process known as Excitation-Contraction coupling (E-C coupling) in striated muscle. Mammalian tissues express three different RyR isoforms: RyR1, RyR2 and RyR3. Notably, skeletal muscle and cardiac muscle predominately expresses RyR1 and RyR2, respectively. [1]

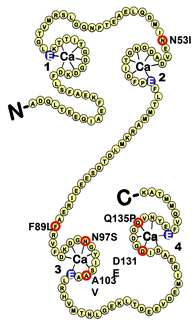


Fig. 1: The structure of CaM with four Ca-binding domains: two on the C-lobe and two on the N-lobe. Mutations being studied can be found located at different places on the protein.

The focus of this project was on CaM's modulation of RyR1 and RyR2 activity. With 0.1 μM Ca^{2+}, CaM (in its Apo state) has been shown to increase RyR1 activity. However, with more than 1 μM Ca^{2+}, CaM (in its Ca-bound state) decreases RyR1 activity. The modulation of RyR2 activity is different from that of RyR1, in that CaM decreases RyR2 activity at both high and low Ca^{2+} concentrations.

Catecholaminergic polymorphic ventricular tachycardia (CPVT) represents a treatable cause of sudden cardiac death in young adults and children. Mutations in CaM have been associated with severe forms of CPVT, with life-threatening arrhythmias occurring in these adolescents.[2] Six mutations (A104V, N54I, F90L, N98S, D132E, and Q136P) are believed to affect the Ca-binding affinity of CaM, therefore affecting the modulation of the RyR2 which is essential in E-C coupling. By studying the functional effects of CPVT-associated CaM mutants on RyR1 and RyR2 modulation, we can begin to determine how these mutants are associated with CPVT and their mechanism of action.

CPVT-Associated Mutants

N54I has is thought to cause CPVT by directly increasing RyR2 activity at diastolic cytoplasmic Ca levels (0.03-0.1 μM). [3]

F90L, associated with Idiopathic Ventricular Fibrillation, is suggested to be important for high-affinity cooperative calcium binding.[4]

N98S, associated with CPVT, has been shown to decrease Ca affinity and abolish the CaM-mediated inhibition of RyR2 activity at diastolic cytoplasmic Ca levels (0.03-0.1 μM). [3]

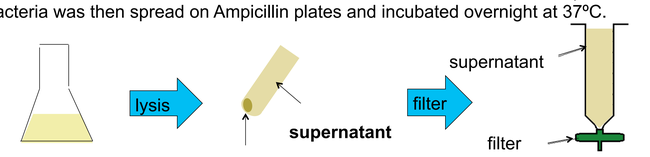
A104V, associated with Ventricular Tachycardia, has also been shown to decrease Ca affinity. However, this mutation did not change the affinity of CaM binding to RyR2.[5]

D132E and Q136P, both CPVT-associated CaM mutants, have been shown to impair Ca sensitivity of the C-lobe.[2]

Experimental Approach

Site-directed Mutagenesis

1uL of each mutated CaM plasmid was added to 45uL of Life technologies BL21(DE3)pLysS competent cells. The cells were then incubated on ice for 30 min. The bacteria was heat shocked by submerging the tubes in a 42ºC water bath for 45 seconds and returning the tube back to ice for 2 minutes. 200uL of SOC was added and the cells were incubated for 60 min at 37ºC and shaken at 225rpm.The bacteria was then spread on Ampicillin plates and incubated overnight at 37ºC.



Bacteria Grow Up and Protein Expression

CaM glycerol stocks were used to inoculate an overnight starter culture of 250mL Luria Broth (100ug/mL Ampicillin). This was grown up for over 12 hours at 30ºC shaking at 225rpm. For each construct, 245mL of starter culture was distributed to a 4L flask containing 0.85L of LB (50ug/mL Ampicillin), starter OD_{600} was 0.1. Bacteria were grown up for 2 hours at 37ºC shaking at 225rpm until OD_{600} was between 0.8-1.0. 440uL of 1M IPTG was added to induce expression, and the cells were further incubated at 37ºC shaking at 225rpm for 4 hours. Cells were spun down at 6000xg for 15 min in a SLA-3000 rotor at 4ºC using the RC-5B centrifuge. Supernatant was discarded and the pellet was resuspended in 20mM TRIS buffer. Cells were frozen overnight at -80ºC.

Protein Purification

The pelleted cell debris was resuspended in 40mLs of homogenization buffer. On ice, the pellet was homogenized using a Bradson Sonicator for five 30s bursts.The cell debris was spun down at 9200 rpm in a SS34 rotor for 30 min at 4ºC. The supernatant was collected and 50uL was set aside for gel electrophoresis. The pelleted cell debris was discarded. 10mM $CaCl_2$ was added to the cell lysate to promote CaM to be in a Ca-bound, open conformation, which exposes hydrophobic residues. The cell lysate was run over a column that contained 20mL of Phenyl Sepharose. CaM's hydrophobic residues bound to the column, and other proteins were filtered through. Wash buffers were used to wash away any unbound non-specifically bound proteins. Finally, CaM was eluted by running buffer which contains a Ca^{2+} chelator, EGTA, over the column. This causes CaM to return to a closed conformation, effectively breaking the hydrophobic interaction with the sepharose. After confirming that the elutes contained CaM by gel electrophoresis (Fig. 2), the elutes were dialyzed overnight in 4L of dialysis buffer II for 8 hours at 4ºC. Protein concentration was determined by absorbance at 280 and by BCA assay methods.

SR Prep and [^{3}H]ryanodine Binding Assay

Pig skeletal SR vesicles were stripped of endogenous CaM by 30 min incubation with myosin light chain CaM binding peptide at 37ºC and sedimented by two centrifugation steps. The SR was incubated with subsaturating (100 nM) and saturating (800 nM) wt CaM or CaM mutants for 3 h at 37ºC with 15nM [^{3}H]ryanodine and either 30nM or 30μM Ca^{2+}. Unbound [^{3}H]ryanodine was removed by filtration. Cardiac SR vesicles were assayed using the same conditions as [^{3}H]ryanodine binding assay detailed above.

References

(1) Tripathy, A., Xu, L., Mann, G., Meissner, G. Calmodulin Activation and Inhibition of Skeletal Muscle Ca2' Release Channel (Ryanodine Receptor). *Biophysical Journal* **69**, 106-119 (1995).
(2) Makita, N. et al. Novel Calmodulin Mutations Associated With Congenital Arrhythmia Susceptibility. *Circ Cardiovasc Genet* **7**, 466-474 (2014).
(3) Hwang, H. S. et al. Divergent Regulation of Ryanodine Receptor 2 Calcium Release Channel by Arrhythmogenic Human Calmodulin Missense Mutants. *Circ Res* **114**, 1114-11124 (2014).
(4) Marsman, R. F. et al. A Mutation in CALM1 Encoding Calmoduling in Familial Idiopathic Ventricular Fibrillation in Childhood and Adolescence. *JACC* **63**, 259-66 (2014).
(5) Gomez-Hurtado, N. et al. NOD1, a new player in cardiac function and calcium handling. *Cardiovascular Research* **106(3)**, 375-386 (2015).

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Results and Figures

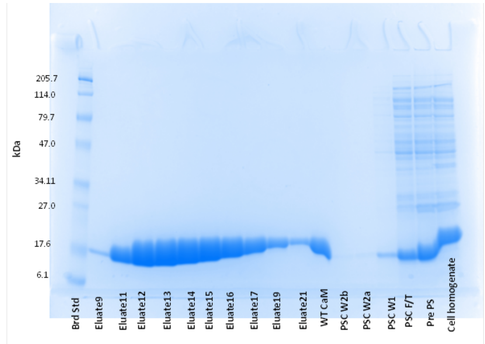

Fig. 2: Gel electrophoresis. Elutes with highest A^{280} were run on the gel to determine fractions that contained protein.

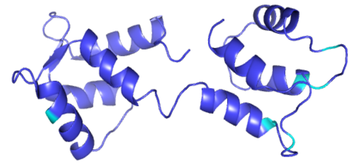

Fig. 3: Calmodulin in its Apo-state (Ca-free state). CPVT and IVT mutations are highlighted in cyan.

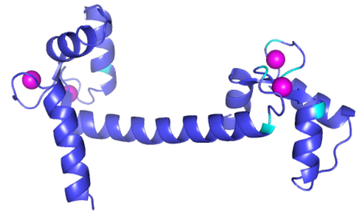

Fig. 4: Calmodulin in its Calcium-bound state. CPVT and IVT mutations are highlighted in cyan.

Functional effect of CPVT-associated mutants on [3H]ryanodine binding to skeletal SR

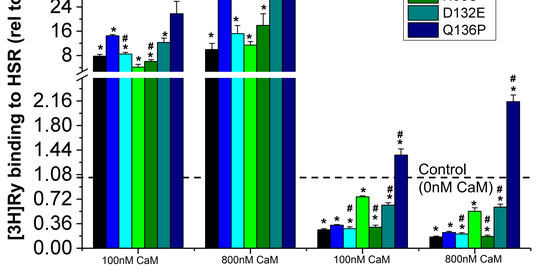

Fig. 5: Data of the functional effect of CPVT-associated CaM mutants on skeletal RyR1 modulation. When bound to low concentrations of Ca^{2+}, wild type CaM (in its apo-state (Fig.4)) will activate the RyR1, activating Ca^{2+} release. Wild type CaM in its Ca^{2+}-bound state (Fig.3) inhibits the RyR1, preventing Ca^{2+} leak. Shown in Figure 3, the mutations D132E and Q136P act quite similar to Apo-CaM, likely due to decreased Ca^{2+} sensitivity (lower Ca^{2+} binding affinity).

Functional effect of CPVT-associated mutants on [3H]ryanodine binding to cardiac SR

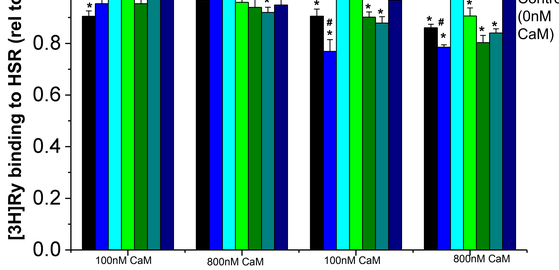

Fig. 6: Data of the functional effect of CPVT-associated CaM mutants on cardiac RyR2 modulation. In both high and low Ca^{2+} concentrations, the wild type CaM will inhibit activity of the RyR2, allowing no Ca^{2+} flow. Shown in Figure 6, the mutations N54I and F90L act quite similar to the control (0nM CaM), and are shown to abolish modulation of the RyR2.

* P<0.05, relative to control # P<0.05, relative to wt CaM

Discussion and Conclusion

The Functional Effect of CPVT-associated mutants on Skeletal RyR1:

- A novel aspect of this project was the study of the CPVT-associated mutants and their functional effect on skeletal RyR1.
- Q136P strongly activated RyR1 at all Ca^{2+} concentrations. D132E acted more like the wild type than Q136P, however, this mutation increases RyR1 activation. As shown in previous studies with RyR2, mutations D132E and Q136P impaired Ca^{2+} sensitivity of the C-lobe, which could be a potential cause of the high activation of the RyR1.
- At only high Ca^{2+} concentrations, N54I, F90L, and N98S caused increased activity of RyR1 compared to wild type. At low Ca^{2+} concentrations, A104V was a strong activator.

The Functional Effect of CPVT-associated mutants on Cardiac RyR2:

- At high and low concentrations of Ca^{2+}, CaM inhibits activity of the RyR2.
- Consistent with previous publications, N54I and F90L abolished the CaM-mediated inhibition of RyR2 activity, which is evidenced by their similarity to the control (0nM CaM) in these experiments.
- At low Ca^{2+} concentrations, A104V and Q136P were strong activators of RyR2; however, at low Ca^{2+} concentrations, A104V was a strong inhibitor of RyR2.
- Q136P was a modest activator of RyR2 at all Ca^{2+} concentrations, and at all Ca^{2+} concentrations, N98S and D132E were similar to the wild type.

Future Directions:

This study adds a great deal to our understanding of these mutants' affect on RyR function, but given the complexity of the results these CPVT and IVT mutants should be studied further to elucidate how single point mutations in CaM contribute to these devastating disorders.