Effect of Experimental Drug S107 on Accessory Protein Binding to the RyR

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Introduction

The physiological basis of muscle-generated motor activity revolves around the precise regulation of intracellular Ca^{2+} ions. Central to this process is a special organelle found in muscle cells called the sarcoplasmic reticulum (SR), which is responsible for the storage, release, and reuptake of the Ca^{2+}. When appropriately signaled by a motor neuron, high-conductance channels in the SR, known as ryanodine receptors (RyRs), release a great volume of Ca^{+2} ions into the muscle cell lumen, to produce muscle contraction; conversely, SERCA ATPase pumps in the SR return the free Ca^{2+} ions from the muscle cell lumen back into the SR, to produce muscle relaxation (Bers, 2002; Carafoli, 2002; Fill et al., 2002).

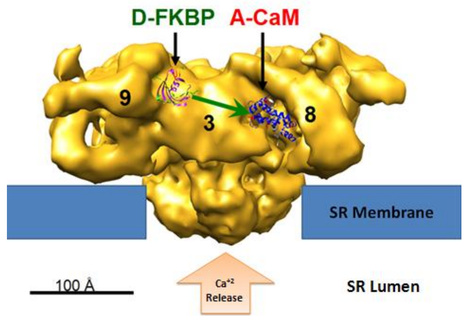


Figure 1. The RyR Ca^{2+} release channel embedded in the SR membrane. The RyR releases Ca^{2+} out of the SR (as part of the excitation-contraction coupling mechanism in muscle). RyR channel activity is in turn regulated by various accessory proteins such as FKBP and CaM which bind to alter its function. I used fluorescently-labeled FKBP (D-FKBP) and CaM (A-CaM) to study the effects of S107 on their binding interaction with RyR in isolated cardiac SR membranes.

Controversy & Goals

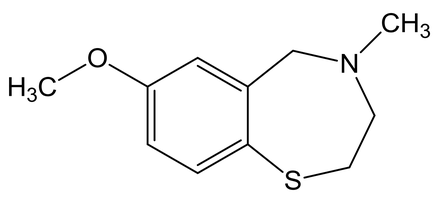


Figure 2. Chemical structure of drug candidate S107:
7-methoxy-4-methyl-2,3,4,5-tetrahydrobenzo[f][1,4]thiazepine
Molecular Weight = 209.31

The new experimental heart drug S107 has been associated with improved muscle performance, though it is still unclear if it directly affects the binding activity of either FKBP or calmodulin (CaM) RyR accessory proteins. The purpose of my research was to investigate whether S107 does in fact directly increase the binding affinity of FKBP12.6, as originally proposed by Dr. Andrew Marks of Columbia University. In addition, I also investigated if S107 affects the binding affinity of CaM as a possible supplementary mechanism of action, or in the case of a negative result for FKBP, as a primary mechanism of action.

Results

FKBP12.0 Binding to RyR2 – Using simplified-saturation binding experiments that detect the bound fluorescence of F-FKBP12.0, we measured changes in the binding affinity of FKBP12.0 for RyR2 caused by S107. For these runs, the glutathione (GSH) antioxidant normally used to control for oxidation during the experiments was substituted for its oxidized form, glutathione disulfide (GSSG). It was found that S107 produced no significant increase in FKBP12.0 binding to the RyR2 (figure 3). However, the standard deviation in these measurements was substantial, possibly because of the over-reduced affinity of FKBP12.0. Therefore, this assay will have to be further optimized.

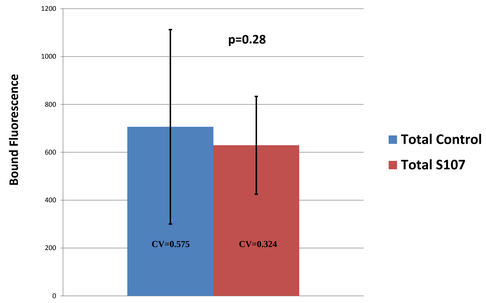


Figure 3. Effect of S107 on FKBP12.0 binding. Simplified-saturation binding assays were performed using a 1 hour preincubation (25˚C) of SR with S107, followed by a 1.5 hour incubation (37˚C) with Binding Media. Data is represented as means SEM (n=5).

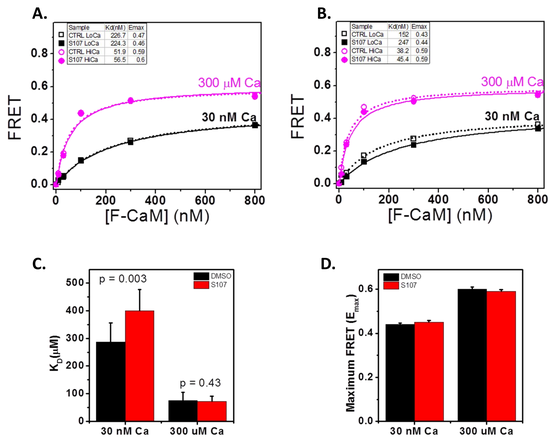
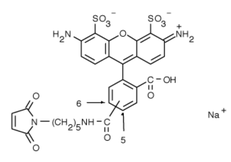


Figure 4. [A] FRET detection of CaM binding to RyR2; 1 hour incubation (25˚C) of SR with S107 and GSH. [B] FRET detection of CaM binding to RyR2; 1 hour incubation (25˚C) of SR with S107 and GSSG. [C] Effect of S107 on the binding affinity of CaM for RyR2; same conditions as B (n=3). [D] Effect of S107 on the maximum FRET efficiency; same conditions as B (n=3).

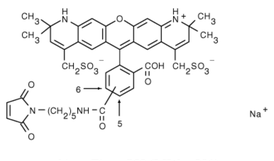
FRET Measurement of CaM Binding to RyR2 – FRET measurements, taken between 520 and 530nm, were used to assess changes in the binding affinity of CaM for RyR2 caused by S107. Initial measurements using regular redox conditions (GSH) detected no significant changes in CaM binding activity at either high or low Ca^{2+} concentrations (figure 4A). Measurements using redox conditions that favored the glutathionylation of RyR2 were also taken (figure 4B), by adding F-CaM after the sample was incubated with S107 and GSSG for 1 hour (Aracena-Parks *et al.*, 2006; Moore *et al.*, 1999). Under these conditions, S107 produced a slight decrease in the binding affinity of CaM (figure 4C), which was significant at low Ca^{2+} concentrations despite no change in E_{max} (figure 4D). Additionally, a dose response of S107 detected an EC_{50} concentration of around just 10nM, in low Ca^{2+} concentrations (figure not shown).

Methods

1. **Preparation of SR Membranes From Pig Heart –** source of functional RyR2 channels
2. **Site-Directed Mutagenesis of FKBP and CaM –** introduced cysteine mutations at specific sites
3. **Expression of FKBP and CaM –** IPTG-induced bacterial expression of mutants and subsequent growth to obtain proteins of interest at lab-scale
4. **Purification of FKBP and CaM –** different specific protocols used to isolate proteins of interest
5. **Site-Directed Labeling of FKBP and CaM –** fluorescently-labeled proteins of interest at mutated cysteine sites with Alexa Fluor
6. **Binding Measurements of FKBP –** measured changes in the binding affinity of FKBP for RyR2 caused by S107 (Cornea *et al.*, 2010)
7. **Fluorescence Resonance Energy Transfer (FRET) Measurements of CaM –** measured changes in the binding affinity of CaM for RyR2 caused by S107 (Guo *et al.*, 2011)



Alexa Fluor 488 (MW = 721)



Alexa Fluor 568 (MW = 881)

Conclusion

Effect of S107 on FKBP – We found that S107 induced no significant increase (or any other change) in the binding affinity of FKBP12.6 or 12.0 for RyR2. This suggests that any increase in the binding of FKBPs to RyR that correlates with S107 treatment under *in vivo* conditions is a product of an indirect, and yet uncharacterized, mechanism. This idea also lends support to the work of other researchers who have instead proposed that FKBP binding to RyR insignificantly reduces Ca^{2+} leak through the channel, and that S107 must therefore elicit its therapeutic mechanism of action through an indirect manner that completely circumvents FKBP (Blayney *et al.*, 2010; Cornea *et al.*, 2010).

Effect of S107 on CaM – Our results show that S107 produced a slight decrease in the binding affinity of CaM for RyR2 (but only in the presence of GSSG). This decrease in binding affinity is significant, but seen only in nM Ca^{2+} concentrations (as in diastole), suggesting that RyR2 channels that have undergone pathological oxidation due to disease, age, or stress bind CaM with reduced affinity if treated with S107. As a result, Ca^{2+} leak through the channel would increase, because S107 would partially negate CaM binding that would otherwise help stabilize the RyR2 and inhibit leak. Based on these results, increased binding affinity of CaM for RyR2 due to S107 is an unsupported explanation for the drug's therapeutic mechanism of action used to improve muscle function.

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Acknowledgments

Minnesota Muscle Laboratory

National Institutes of Health

Grant HL92097