of PLB^{R9C} on SR Ca^{2+} kinetics, we recorded Ca^{2+} transients in electrically paced adult cardiomyocytes overexpressing CFP-PLB^{R9C} or CFP-PLB^{WT}. PLB^{R9C} overexpressing cardiomyocytes exhibited faster SR Ca^{2+} uptake as compared to PLB^{WT}, and a blunted Ca^{2+} uptake/pacing frequency response. We propose that R9C mutation in PLB increases the stability of the PLB pentameric assembly by crosslinking of adjacent cysteine thiols via disulfide bonds, and these effects are enhanced during oxidative stress. The resultant decrease in availability of PLB monomeric species leads to decrease in inhibition of SERCA, inability to respond to stress, and eventual heart failure.

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Myofilament Calcium Sensitization Causes Pause-Dependent Ventricular Ectopy in Mouse Hearts with Acute and Chronic Myocardial Infarction

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Background. Increased myofilament Ca^{2+} sensitivity has been associated with cardiac arrhythmias in several cardiomyopathies. During the course of myocardial infarction (MI), myofilament Ca^{2+} sensitivity is reduced during acute ischemia but increases in the chronic stage. Thus, we aimed to compare the arrhythmogenic patterns during an acute MI or after chronic MI in mice.

Methods. In theacute MI group, isolated hearts were investigated 15 min after left anterior descending (LAD) coronary artery ligation. In the chronic MI group, hearts were harvested 4 weeks after LAD ligation. To measure arrhythmia susceptibility, isolated hearts from both groups underwent a ventricular pacing challenge consisting of pacing trains of increasing frequency, followed by a pause and an extra stimulus, in the presence or in the absence of the Ca^{2+} sensitizer EMD 57033 (3 μM) or the Ca^{2+} de-sensitizer and contractile uncoupler Blebbistatin (3 μM).

Results. Acute MI caused frequent ventricular ectopyduring the steady-state pacing, but not after a pause. Acute MI hearts became susceptible to post-pause ectopy only after Ca^{2+} sensitization with EMD. In contrast, chronic MI hearts exhibited only very modest ventricular ectopy during the pacing trains (33.3%, $p<0.01$ vs acute MI), but consistently exhibited ectopy after a pause (91.6%), which was prevented by Ca^{2+} de-sensitization with blebbistatin (10%, $p<0.05$). Compared to acute MI, the incidence of ventricular tachycardia (VT) was significantly increased in chronic MI, which could be prevented by blebbistatin (MI: 70%, MI+Bleb: 10%, $p<0.05$, n=10 per group).

Conclusion. Different patterns of arrhythmia induction are involved in acute and chronic MI hearts. Our data suggest that increased myofilament Ca^{2+} sensitivity strongly promotes ventricular ectopy and VT's after a pause, but does not affect ectopy during regular pacing.

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Crucial Role for Ca^{2+}/Calmodulin-Dependent Protein Kinase-II Delta in Regulating Diastolic Stress of Normal and Failing Hearts via Titin Phosphorylation

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Rationale: Myocardial diastolic stiffness and cardiomyocyte passive force ($F_{passive}$) depend in part on titin isoform composition and phosphorylation. Ca^{2+}/calmodulin-dependent protein kinase-IIδ (CaMKIIδ) phosphorylates ion channels, Ca^{2+}-handling and myofilament proteins in the heart, but has not been known to target titin.

Objective: To elucidate whether CaMKIIδ phosphorylates titin and regulates $F_{passive}$ in normal and failing myocardium.

Methods and Results: Titin phosphorylation was assessed in CaMKIIδ/γ double-knockout (DKO) and transgenic CaMKIIδC-overexpressing (TG) mouse hearts, as well as human hearts, by Pro-Q-Diamond/Sypro-Ruby staining, autoradiography, and immunoblotting using phosphospecific titin antibodies. CaMKII-dependent site-specific titin phosphorylation was quantified *in vivo* by mass spectrometry using SILAC mouse heart tissue mixed with wildtype (WT) or DKO heart. $F_{passive}$ of single permeabilized cardiomyocytes was recorded before and after CaMKII administration. We detected hypophosphorylation of all-titin in DKO and hyperphosphorylation in TG compared to WT hearts. Conserved CaMKII-dependent phosphosites were identified within titin's PEVK-domain by quantitative mass spectrometry and confirmed in recombinant human PEVK-fragments. CaMKII also phosphorylated the cardiac titin N2B-unique sequence (N2Bus). Phosphorylation at specific PEVK/N2Bus sites was decreased in DKO and amplified in TG *versus* WT hearts. $F_{passive}$ was elevated in DKO and reduced in TG compared to WT cardiomyocytes. CaMKII-administration lowered $F_{passive}$ of WT and DKO cardiomyocytes, an effect blunted by titin-phosphoantibody pretreatment. Human failing hypertrophic hearts revealed higher CaMKII expression/activity and phosphorylation at PEVK/N2Bus sites than nonfailing donor hearts.

Conclusions: CaMKIIδ phosphorylates the titin springs at conserved serines/threonines, thereby lowering $F_{passive}$. Deranged CaMKIIδ-dependent titin phosphorylation occurs in heart failure and contributes to altered diastolic stress.

Platform: Protein Conformation

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Identification of Noninterfacial Amino Acids Important for Molecular Recognition in Calmodulin

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Calmodulin (CaM) contains two structurally homologous domains that cooperatively bind to a range of different target proteins, such that upon binding the opposing N- and C-domains wrap around the CaM-binding sequence. The N-domain has a binding affinity (K_d = 24 μM) that is approximately 3-orders of magnitude weaker than the C-domain (K_d = 11 nM) to the plasma membrane Ca-ATPase (PMCA). These large differences in binding affinities facilitate ordered binding, which is necessary for the productive activation of many target proteins. To better understand design principles that facilitate molecular recognition, we have used directed evolution combined with yeast surface display to identify mutations that enhance binding between the N-domain of CaM and the PMCA, permitting the identification of combinatorial families of mutate N-domain proteins with nanomolar binding affinities (similar to that of the C-domain). All observed mutations occur at noninterfacial sites that are naturally variable in Nature, suggesting that hypervariable sites between different species may fine tune binding affinities. Mutations in the N-domain that selectively destabilize the unbound state commonly result in enhanced binding affinities with other CaM-binding sequences (i.e., skeletal myosin light chain kinase and ryanodine receptor). In contrast, mutations that structurally couple with residues in the binding interface result in selective high-affinity binding to the PMCA; these mutations commonly result in decreased binding to other target proteins. In total, these results indicate the value of using directed evolution approaches to identify underlying principles that determine binding affinities.

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Rational Design of a Ligand-Controlled Protein Conformational Switch

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Achieving precise temporal control over signaling proteins is essential to the understanding and reprogramming of cellular pathways. Direct regulation of enzyme activity at the protein level can be gained by genetically-encoded protein switches that offer the advantages of precision, specificity and minimal invasiveness. The main challenge in engineering these switches lies in the design of an insertable, universal regulatory domain that can render any signaling protein switchable. However, constructing such a domain is one of the most challenging problems in protein design because of the necessity of engineering and controlling multiple protein states. Here we present a rationally designed domain, uniRapR, with a novel structure, that can be used as a universal, insertable switch. UniRapR is natively disordered but robustly adapts a specific conformation upon binding to the small molecule rapamycin. Insertion of uniRapR into diverse kinases including Src, Lyn, FAK, and p38 enabled their specific and temporally controlled activation in living cells. Switchable