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Noncanonical EF-hand motif strategically delays Ca²⁺ buffering to enhance cardiac performance

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Abstract

EF-hand proteins are ubiquitous in cell signaling. Parvalbumin (Parv), the archetypal EF-hand protein, is a high-affinity Ca²⁺ buffer in many biological systems. Given the centrality of Ca²⁺ signaling in health and disease, EF-hand motifs designed to have new biological activities may have widespread utility. Here, an EF-hand motif substitution that had been presumed to destroy EF-hand function, that of glutamine for glutamate at position 12 of the second cation binding loop domain of Parv (ParvE101Q), markedly inverted relative cation affinities: Mg²⁺ affinity increased, whereas Ca²⁺ affinity decreased, forming a new ultra-delayed Ca²⁺ buffer with favorable properties for promoting cardiac relaxation. In therapeutic testing, expression of ParvE101Q fully reversed the severe myocyte intrinsic contractile defect inherent to expression of native Parv and corrected abnormal myocardial relaxation in diastolic dysfunction disease models *in vitro* and *in vivo*. Strategic design of new EF-hand motif domains to modulate intracellular Ca²⁺ signaling could benefit many biological systems with abnormal Ca²⁺ handling, including the diseased heart.

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Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS

J.M.M. was responsible for the original concept and had a key role in study design and data interpretation. W.W. designed and conducted most experiments. M.S.B., F.I.H., M.L.A., J.D. and E.B. aided the *in vivo* heart function tests. E.A. generated the Ad5 and rAAV vectors for the Parv proteins. Q.L. and J.D.P. provided plasmid constructs of modified Parv. J.P.D. contributed to the measurement of Parv Ca²⁺ and Mg²⁺ dissociation rates. J.M.M. and W.W. prepared the manuscript with contributions from J.D.P. and J.P.D.

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Calcium (Ca^{2+}) is a ubiquitous intracellular second messenger that participates in diverse intracellular processes ranging from muscle contraction to gene expression and cell survival¹. In the heart, rapid release and reuptake of intracellular Ca^{2+} is essential for normal contraction (systole) and relaxation (diastole)². Precise control of cardiac intracellular Ca^{2+} relies on a myriad of specialized regulatory proteins, including Ca^{2+} channels, transporters and binding proteins. Impaired intracellular Ca^{2+} handling has an important role in heart disease, the leading cause of morbidity and mortality in developed countries^{3–7}. Notably, slowed cardiac Ca^{2+} sequestration and poor myocardial relaxation performance cause diastolic dysfunction, a common form of heart disease in the elderly⁸. There are currently no clinical treatments to enhance active myocardial relaxation for correcting cardiac diastolic dysfunction.

Consonant with the prominent role of Ca^{2+} in heart disease, much effort has been directed at correcting defective Ca^{2+} handling in diseased cardiac muscle, primarily through interventions targeting the Ca^{2+} ATPase in the sarcoplasmic reticulum^{9–11}. An alternative approach for enhancing cardiac Ca^{2+} handling centers on a non-ATP-requiring Ca^{2+} buffering mechanism by exploiting the cation-binding specificity of EF-hand motif proteins¹². However, native EF-hand Ca^{2+} buffers have the severe negative effects of suppressing contraction as a result of Ca^{2+} buffering occurring too early in the contraction cycle. Thus, a major re-design of the EF-hand motif to achieve optimized physiological Ca^{2+} buffering must be accomplished before application to the diseased heart.

Studies on the archetypal EF-hand protein Parv^{13–16} have revealed the canonical EF-hand motif core sequence to be a 12-residue ‘pocket’ for binding Ca^{2+} and Mg^{2+} in which the glutamate at position 12 defines the metal-binding specificity^{17–21}. Parv has two EF-hand motifs, the CD and EF domains, that bind Ca^{2+} with very high affinity ($\sim 10^8 \text{ M}^{-1}$) and Mg^{2+} with moderate affinity ($\sim 10^4 \text{ M}^{-1}$)^{14,16}. Parv is a cytosolic Ca^{2+} buffer that is crucial for attaining a high speed of muscle relaxation in fast-twitch muscle. In the specialized swim-bladder muscle of the male toadfish, Parv promotes ultra-rapid contractile cycles (~ 200 contractions per second) for aquatic transmission of the ‘boatwhistle’ mating call²². There, the biological tradeoff is lower muscular power for greater contractile cycle speed²². Hence, although Parv is a ‘delayed’ Ca^{2+} buffer, by virtue of Mg^{2+} dissociation before Ca^{2+} binding, substantial amounts of Ca^{2+} are buffered by wild-type (WT) Parv too early in the contractile process and thereby blunt contractile force^{16,23,24}.

Translating EF-hand Ca^{2+} buffers to other biological systems in which Parv is not normally expressed, including the heart, has been challenging. WT Parv corrects slow myocyte relaxation in diastolic heart disease models but does so through a mechanism of premature termination of the Ca^{2+} transient that markedly reduces cardiac contraction^{23,25–27}. Gaining enhanced relaxation speed at the expense of diminished contraction in cardiac muscle is strictly contraindicated by the physiological necessity of sustaining vigorous heart contraction *in vivo*. Thus, WT Parv and other known Ca^{2+} buffers²⁸ are unsuitable for cardiac therapeutic applications. A major unmet challenge for Ca^{2+} buffering-based therapeutics is to strategically re-design the canonical EF-hand motif of Parv to optimally time Ca^{2+} buffering on the physiological scale. In theory, a new ‘ultra-delayed’ Ca^{2+} buffer could enhance relaxation without the side effect of diminished contraction.

RESULTS

EF-hand motif re-design

We reasoned *a priori* that re-designed EF-hand motifs optimized for the heart would require a combination of reversed cation-binding sensitivities: a relative increase in Ca^{2+} dissociation rate and a relative decrease in Mg^{2+} dissociation rate. In theory, if such a motif

could be designed, it would lead to a further temporal delay in Ca^{2+} binding owing to a further slowing of Mg^{2+} dissociation kinetics²⁴. There have been multiple previous attempts to refine EF-hand motif structure and function to gain biological functionality^{29–32}. Substitution of residue 12 to glutamine in the calmodulin EF-hand motif causes fully defective Ca^{2+} binding and function, indicating that glutamine is a biologically ‘forbidden’ substitution at this position²¹. Because the Parv EF-hand motifs bind Ca^{2+} with much greater affinity than those in calmodulin, we speculated that modifications, particularly at residue 12 in the archetypal EF-hand structure of Parv, could be used to develop new biologically useful Ca^{2+} and Mg^{2+} ($\text{Ca}^{2+}/\text{Mg}^{2+}$) binding motifs. The alignment of WT human α and carp β isoforms of Parv (α -Parv and β -Parv, respectively) highlights the conserved homology of this motif and the residues of the CD- and EF-domain Ca^{2+} binding loops that we modified, including the conserved loop residues 1, 3 and 12 (Fig. 1a,b). Our biochemical and cell studies used carp β -Parv complementary DNA as a template. To directly track cation binding, we engineered a tryptophan residue (ParvF102W) for stopped-flow biochemical determination of metal-binding properties using tryptophan fluorescence detection^{33,34}. The F102W substitution did not affect binding affinities or kinetics compared to those of WT Parv (Fig. 1 and Supplementary Fig. 1) and was functionally indistinguishable from WT Parv (data not shown); thus, the F102W substitution served as a control for EF-hand motif substitutions. In most of the constructs, we disabled the CD or EF site to simplify EF-hand motif analysis (Supplementary Fig. 1). As an aid in describing these constructs, we show two- and three-dimensional diagrams that highlight alterations in the geometries of the Ca^{2+} -binding sites of the CD and EF domains (Fig. 1b). We characterized the metal-binding properties of numerous Parv mutants *in vitro* using recombinant proteins (Supplementary Fig. 1). Most of the re-designed Parv EF-hand motifs showed lowered Ca^{2+} and Mg^{2+} binding affinities in parallel (Supplementary Fig. 1). On the basis of computational models²⁴, we theorized that engineered EF-hand motifs with some combination of lowered Ca^{2+} and heightened Mg^{2+} binding affinities would be the most efficacious in optimally delaying Ca^{2+} buffering for application in diseased myocytes.

Among the modified EF-hand motifs examined, ParvE101Q was distinguished by having inverted effects on relative Ca^{2+} and Mg^{2+} binding affinities (Supplementary Fig. 1). ParvE101Q contains the triple substitution of D51A, E101Q and F102W (Fig. 1b), and the D51A substitution has been shown to eliminate physiological Ca^{2+} binding at the CD domain (the D51A substitution also influences cation binding at the EF loop). In this context, the E101Q substitution further substantially lowered Ca^{2+} binding affinity at the EF domain (nearly 100-fold), whereas Mg^{2+} -binding affinity was more than doubled (Supplementary Fig. 1). In stopped-flow kinetic experiments, the ParvE101Q substitution resulted in unique and opposite changes in Ca^{2+} and Mg^{2+} dissociation rates: an increased Ca^{2+} dissociation rate and a decreased Mg^{2+} dissociation rate (Fig. 1d). On the basis of these findings, we took forward ParvE101Q as a lead candidate for an optimally delayed Ca^{2+} buffer to enhance cardiac function.

Cellular function of modified EF-hand motifs

To directly evaluate the physiological role of re-designed EF-hand motifs, we used gene transfer (Fig. 1c) to efficiently transduce (>95%) adult rat cardiac myocytes with ParvE101Q and other Parv variants (Fig. 2a). We detected expression of ParvE101Q in a striated pattern co-aligning with actin-containing thin myofilaments (Supplementary Fig. 2). This labeling pattern was lost after membrane permeabilization (Supplementary Fig. 2a), indicating a weak association of Parv with the myofilaments of intact cells. As determined by quantitative RT-PCR (qRT-PCR) (Supplementary Fig. 2b) and semiquantification of protein expression from western blot analyses (Supplementary Fig. 2c), the expression of ParvE101Q reached a stable level at 48 h and was threefold to fivefold lower than that of

WT Parv. On the basis of our previous estimation of WT Parv expression after gene transfer³⁵, we estimated the expression level of ParvE101Q to be ~0.01 mM.

To determine the effects of re-designed EF-hand motifs on contractility, we electrically stimulated myocytes at 0.2 Hz and monitored their sarcomere length shortening during each twitch. As previously reported^{26,35}, WT Parv severely depressed the amplitude of the sarcomere length shortening while speeding myocyte relaxation (Fig. 2b), highlighting the severe negative side effect of WT Parv proteins. The modified Parv proteins ParvD51A and ParvD92N accelerated relaxation and also significantly decreased the amplitude of sarcomere length shortening ($P < 0.001$; Fig. 2c), indicating insufficient delayed Ca^{2+} buffering in these constructs.

In marked contrast, ectopic expression of ParvE101Q did not cause the contractile depression defect caused by expression of WT Parv or the other Parv variants. Expression of ParvE101Q increased contractile amplitude by 186% over WT Parv and even by 24% over control nontransduced myocytes (Fig. 2c,d). ParvE101Q also significantly ($P < 0.01$) decreased the time from peak to 50% relaxation ($T_{50\%}$), a parameter used to quantify relaxation performance independent of contraction amplitude-dependent effects, indicating increased relaxation speed (Fig. 2d). ParvD51A expression also showed modest inverted changes in $\text{Ca}^{2+}/\text{Mg}^{2+}$ affinities, but this was not sufficient to enhance contractility (Fig. 2c), indicating the specific effect of E101Q on Ca^{2+} buffering.

We next tested the effects of ParvE101Q on the function of rabbit cardiac myocytes, which have intracellular Ca^{2+} handling properties that are highly comparable to those of human myocytes². Consistent with the findings in rat myocytes, ParvE101Q expression markedly enhanced cell contractility and accelerated relaxation in a dose-dependent manner (Fig. 3a–f). Moreover, ParvE101Q conferred enhanced contractility and faster relaxation effects over a range of pacing frequencies (Fig. 3g,h).

To elucidate the mechanism underlying the effect of ParvE101Q on cell contraction and relaxation, we monitored intracellular Ca^{2+} cycling using Fura-2 in rabbit myocytes. ParvE101Q significantly reduced the time course of Ca^{2+} transient decay without altering peak Ca^{2+} transient amplitude ($P < 0.001$; Fig. 4a). We also observed these effects at increased pacing frequencies from 0.2 to 2 Hz (Supplementary Fig. 3a,b). Moreover, assessment of sarcoplasmic reticulum Ca^{2+} storage by caffeine-induced Ca^{2+} release revealed no difference in the amount of releasable sarcoplasmic reticulum Ca^{2+} in ParvE101Q compared to control (nontransduced) myocytes (Fig. 4b). In live-cell confocal imaging studies, the frequency and amplitude of spontaneous sarcoplasmic reticulum Ca^{2+} release events, called Ca^{2+} sparks, were similar in control and ParvE101Q myocytes (Supplementary Fig. 3c–f).

The expression of key Ca^{2+} -handling proteins, including the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), calsequestrin (CSQ), phospholamban (PLN) and cardiac troponin I (cTnI), was unaltered by ParvE101Q (Supplementary Fig. 4a). We also did not detect differences between control and ParvE101Q cells in the phosphorylation status of PLN at Ser16 or of cTnI at Ser23 and Ser24 after β -adrenergic stimulation (Supplementary Fig. 4b,e). We also determined the functional effects of ParvE101Q at two temperatures, which is informative because intracellular Ca^{2+} handling processes are differentially sensitive to temperature³⁶, and we observed the same relative functional effects of ParvE101Q at both temperatures (Supplementary Fig. 5). Taken together, these data indicate that ParvE101Q facilitates Ca^{2+} dissociation from myofilaments and speeds myocyte relaxation without increasing sarcoplasmic reticulum Ca^{2+} release or storage.

As Ca^{2+} amplitude, sarcoplasmic reticulum Ca^{2+} load and the expression and phosphorylation status of Ca^{2+} -handling proteins were all unchanged by ParvE101Q, we next determined whether ParvE101Q affects myofilament proteins and their Ca^{2+} sensitivity. The myofilament Ca^{2+} -sensing protein cTnC is a crucial component of the troponin complex that initiates Ca^{2+} -induced myofilament contraction^{37,38}. The cTnC mutation L29Q, which can cause familial hypertrophic cardiomyopathy³⁹, has altered Ca^{2+} sensitivity⁴⁰ and interacts with the N-terminal extension of cTnI during the structural transition of myofilaments⁴¹. Gene transfer of cTnC L29Q, which stoichiometrically replaces endogenous cTnC, blocked the enhanced contractility caused by ParvE101Q (Fig. 4c), providing evidence that the positive inotropic effect of ParvE101Q is due to effects on myofilament proteins. We next incubated permeabilized myocytes with purified recombinant ParvE101Q protein at a concentration of 0.05 mM (within the concentration range in which WT Parv expression in intact myocytes has cellular effects and above that estimated for ParvE101Q (0.01 mM); Supplementary Fig. 2c)²⁴ to test whether ParvE101Q directly influences myofilament Ca^{2+} sensitivity. In ParvE101Q treated myocytes, steady-state Ca^{2+} -activated isometric force generation showed unaltered myofilament Ca^{2+} sensitivity (pCa_{50} , $-\log[\text{Ca}^{2+}]$ at half-maximal isometric tension), cooperativity (Hill coefficient) and maximum isometric tension (Fig. 4d). These results indicate that ParvE101Q does not directly influence myofilament Ca^{2+} sensitivity; however, we cannot exclude the possibility that potential partner proteins mediating such an effect might have been lost during permeabilization. In addition, we speculate that the increased Mg^{2+} binding affinity of ParvE101Q may buffer local Mg^{2+} , thus facilitating the binding of Ca^{2+} to cTnC to enhance contractility.

β -adrenergic stimulation is one of the central regulatory mechanisms affecting cardiac function and dysfunction^{42,43}. Therefore, we evaluated β -adrenergic responsiveness in ParvE101Q-expressing rabbit myocytes. Both control cells and ParvE101Q-expressing cells showed similar increases in contractility and relaxation speed after isoproterenol treatment (Supplementary Fig. 6). Thus, ParvE101Q-expressing myocytes fully retain signaling pathways that mediate the physiological contractile effects of acute β -adrenergic stimulation. Moreover, ParvE101Q expression has similar effects as isoproterenol in terms of increasing relaxation speed ($T_{50\%}$ values decreased 22% and 29% by ParvE101Q and isoproterenol, respectively).

Therapeutic testing of ParvE101Q

We next tested whether ParvE101Q could correct the pronounced cellular functional deficits in several models of heart disease. In a canine model of coronary microembolization-induced heart failure in which relaxation function is impaired, ParvE101Q gene transfer accelerated the relaxation speed (Fig. 5a,b). In a rabbit ischemic model (chronic ligation of the left circumflex coronary artery (LCX)), the rabbits showed overt symptoms of heart failure and decreased heart performance by echocardiography (Supplementary Table 1). Isolated myocytes from these rabbits showed severe contractile deficits (Supplementary Table 2), which were corrected by *in vitro* ParvE101Q gene transfer (Fig. 5c–f and Supplementary Table 2). We further confirmed that ParvE101Q corrected the slow relaxation of rabbit myocytes in which thapsigargin was used to acutely inhibit SERCA2a function and sarcoplasmic reticulum Ca^{2+} reuptake (Fig. 5g). We also used a cellular ischemia and reperfusion mimetic model, in which ParvE101Q rescued the slowed relaxation in rabbit myocytes (Fig. 5h). In failing myocytes, ParvE101Q increased the Ca^{2+} decay rate (Supplementary Fig. 7), indicating that the Ca^{2+} binding of ParvE101Q underlies the accelerated relaxation caused by ParvE101Q expression in failing myocytes.

We next tested the therapeutic potential of ParvE101Q to correct defective cardiac relaxation and diastolic dysfunction *in vivo*. There is no single best animal model of

diastolic dysfunction, so we used two different models with documented cell-intrinsic slow myocardial relaxation and organ-level diastolic dysfunction. We first used recombinant pseudotyped adeno-associated virus vector (rAAV2/6)-mediated intravascular gene delivery⁴⁴ of ParvE101Q *in vivo* in mice with conditionally ablated SERCA2a in the hearts of adult mice. In this model, cardiac Ca^{2+} -handling dysfunction severely compromises cardiac relaxation due to impaired cytosolic Ca^{2+} removal, a defect that parallels SERCA2a deficiency in human heart diseases⁴⁵ (Fig. 6a and Supplementary Fig. 8). We found that rAAV-mediated ParvE101Q expression enhanced relaxation performance in the SERCA2a-depleted hearts as evaluated by echocardiography (Fig. 6b), real-time hemodynamic catheterization (Fig. 6c) and measurement in Langendorff perfused hearts (Supplementary Fig. 9a,b). These data provide evidence that the beneficial physiological effects of the E101Q EF hand motif might be transferrable to clinical applications.

To further pursue the cardiac therapeutic potential of ParvE101Q *in vivo*, we generated rAAV vectors using human α -Parv and the same optimized EF-motif substitutions as described above for carp β -ParvE101Q. We tested human α -ParvE101Q (containing the triple substitution) in a mouse model of familial cardiomyopathy (restrictive (hypertrophic) cardiomyopathy (RCM))⁴⁶ that has cell-intrinsic diastolic dysfunction and slow myocardial relaxation caused by expression of a mutant cardiac troponin I (R193H), mimicking the functional defects in human patients with cardiomyopathy⁴⁷. This cardiomyopathy model provides an excellent platform to test therapies for the correction of cell-intrinsic slowed active relaxation by myofilament defects. We therapeutically treated adult RCM mice at ~4 months of age, at which time substantial diastolic dysfunction is evident⁴⁸, with human α -ParvE101Q rAAV. Ten weeks after rAAV ParvE101Q gene delivery, we recorded real-time *in vivo* heart hemodynamics using micromanometry pressure-conductance catheterization (Fig. 6d)⁴⁴. Relaxation performance, measured by the peak rate of left ventricular systolic pressure decline ($-\text{dP}/\text{dt}$), was significantly reduced in both untreated RCM mice and in RCM mice treated with α -ParvF102W compared to nontransgenic littermates. RCM mice treated with α -ParvE101Q had improved $-\text{dP}/\text{dt}$ compared to untreated and α -ParvF102W-treated mice, with values not significantly different than those of nontransgenic littermates (Fig. 6e), indicating that ParvE101Q treatment corrected $-\text{dP}/\text{dt}$ deficits in RCM mice. Tau, a load-independent measure of relaxation, was significantly aberrant in saline-treated RCM mice and was corrected to a value nearly identical to that of the nontransgenic littermates by ParvE101Q ($P < 0.05$; Fig. 6f). Other hemodynamic results were not different across treatment groups (Supplementary Table 3).

Thus, in two different animal models of cell-intrinsic diastolic dysfunction, one of which was Ca^{2+} -handling dependent as a result of SERCA2a deficiency and one of which was myofilament dependent, ParvE101Q treatment can correct defective relaxation.

DISCUSSION

Canonical EF-hand $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding motifs were re-designed to elicit new therapeutic functionalities for correcting cell-intrinsic diastolic dysfunction *in vitro* and *in vivo*. Diastolic dysfunction, precipitated by slow cardiac muscle relaxation, is an important and growing human health problem³⁻⁸. Over 500 EF-hand motifs exist in nature, spanning a myriad of Ca^{2+} -dependent biological processes^{1,17,49}. We found that a biologically forbidden, noncanonical EF-hand glutamate-to-glutamine substitution at residue 12, shown previously to fully disable Ca^{2+} binding in the calmodulin EF-hand motif²¹, inverts and physiologically optimizes the $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding properties of Parv to enhance active relaxation in heart muscle (Fig. 6g). ParvE101Q circumvents the canonical EF-hand motif biological conundrum of compromising cellular power for speed by local control of $\text{Ca}^{2+}/\text{Mg}^{2+}$ content²². This new ultra-delayed Ca^{2+} -buffering EF-hand motif may have

applications in a range of biological systems that are dependent on the timely control of Ca^{2+} signaling, including the heart and nervous system.

In cardiac muscle, the timing of Ca^{2+} buffering in the contractile cycle is crucial for performance. Ca^{2+} buffering that occurs too early, as in the case of ectopic expression of WT Parv, blunts contraction, whereas buffering that occurs too late will have no beneficial effects. Parv is an archetypal EF-hand Ca^{2+} buffer^{14,15}. By virtue of a delicate balance between $\text{Ca}^{2+}/\text{Mg}^{2+}$ cytosolic content and native EF-hand $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding affinities, Parv tailors intra-cellular Ca^{2+} signals for specific physiological processes. This effect is pronounced in the toadfish swim-bladder muscle, the fastest contracting and relaxing muscle among vertebrates, in which Parv functions as a temporary Ca^{2+} depot to aid rapid termination of the intracellular Ca^{2+} signal²². Through a process of Mg^{2+} dissociation before Ca^{2+} binding, Parv is a delayed Ca^{2+} buffer. Even so, ectopic expression of WT Parv substantially and prematurely truncates intracellular Ca^{2+} signaling events such that in muscle systems, power output is markedly depressed^{22,26}. The design and implementation of an optimal ultra-delayed EF-hand Ca^{2+} buffer to overcome this shortcoming could be beneficial to systems requiring robust Ca^{2+} signal initiation followed by rapid signal termination.

The canonical 12-residue EF-hand motif is crucial in defining cation binding affinity and specificity^{13,17,49}. In a survey of 567 EF-hand motifs, glutamate occupied position 12 in 92% of the motifs and was shown to be required for bidentate coordination of Ca^{2+} ions^{13,17,50}. In calmodulin, an EF-hand glutamate-to-glutamine substitution at residue 12 caused a bidentate-to-monodentate conversion and fully eliminated Ca^{2+} binding at the site²¹, indicating that this is a biologically forbidden substitution in this protein. The same substitution in Parv, however, is tolerated and lowers Ca^{2+} affinity while paradoxically increasing Mg^{2+} affinity. We propose that the substitution of glutamate to glutamine at residue 12 not only affects the bidentate binding of Ca^{2+} but also substantially alters the structure of the cation-binding pocket, resulting in a tighter association with the smaller ion, Mg^{2+} . Attaining optimized EF-hand buffers for contractile systems requires some combination of reduced Ca^{2+} affinity and increased Mg^{2+} affinity, which would be affected by altering dissociation rates for these cations²⁴. In the case of ParvE101Q, a reversed relative cation affinity would theoretically provide the precise lag time in Ca^{2+} signal termination to aid in cell relaxation. In support of this conclusion, ParvD51A, which has modest cation affinity inversion compared to ParvE101Q, showed slightly less inhibition of contraction than WT Parv. Because ParvD51A fully disables the CD loop, the remaining EF loop must confer the inverted $\text{Ca}^{2+}/\text{Mg}^{2+}$ affinity, which was further enhanced by E101Q. Thus, in terms of the ability to optimize cardiac Ca^{2+} buffering on the physiological time scale, we found the following hierarchy: ParvE10Q (optimally delayed), ParvD51A (moderately delayed) and WT Parv (insufficiently delayed).

ParvE101Q provides a new approach to correct slow relaxation and diastolic dysfunction. Diastolic dysfunction is defined by abnormally slow left ventricular relaxation and filling and is a physiological trigger for diastolic heart failure, a major and growing clinical syndrome in which the active relaxation phase of the cardiac cycle is slowed^{8,51,52}. There is no cure or direct clinical treatment for abnormally slow active heart muscle relaxation. Previous reports of applying WT Ca^{2+} buffers in models of diastolic dysfunction showed correction of relaxation defects but also the characteristic side effect of severe contractile depression²³. ParvE101Q, in comparison with WT Parv, speeds the relaxation of diseased myocytes while sustaining contraction. Collectively, the unique transient Ca^{2+} depot functions of ParvE101Q could provide a new therapeutic approach for the diseased heart. Further unique features of ParvE101Q are that it does not require energy and does not alter sarcoplasmic reticulum Ca^{2+} loading or leak, which are beneficial attributes in the context of

the energy-starved and arrhythmogenic diseased heart^{53–55}. The potential therapeutic use of ParvE101Q in diastolic dysfunction is further justified by its effectiveness in myocytes from large mammals with human-like Ca^{2+} handling, its cell-intrinsic effectiveness at pacing frequencies in the human heart rate range and its full correction of contraction defects inherent in WT Parv when used as a Ca^{2+} buffer.

A biologically forbidden EF-hand motif E101Q substitution yielded optimized $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding properties to serve physiologically relevant ultra-delayed Ca^{2+} buffering and correction of defective relaxation performance in diseased cardiac muscle. Manipulating the EF-hand Ca^{2+} binding motif could also be a new approach for implementing intracellular Ca^{2+} buffers for a range of other biological systems that require refined intracellular Ca^{2+} signal processing.

ONLINE METHODS

Recombinant adenovirus vectors

The sequence of carp WT Parv was derived from the protein sequence of carp β -Parv (Swiss-Prot accession number P02618) using the most favorable codons (that is, those that facilitate expression in mammalian cells). WT and point mutations of Parv were cloned into pDC316 shuttle vectors and then into recombinant adenoviral vectors using the AdMax system (Microbix)⁵⁶. A Flag-tag sequence (DYKDDDDK) was added to the N terminus of Parv to facilitate immunodetection of protein expression. Gene expression of each vector was controlled by the CMV promoter, and the polyadenylation signal was provided by SV40. High-titer ($\sim 10^{11}$ – 10^{12} plaque forming units (PFU)) and plaque-purified adenoviral stocks were produced, and purified and viral aliquots were stored at -80°C .

Biochemical determination of mutant Parv metal-binding properties

To measure the metal-binding affinity of Parv and its mutants (Supplementary Fig. 1), we used nontagged recombinant proteins purified directly from bacteria⁵⁷. The Ca^{2+} binding parameters (association constant and number of binding sites) were measured at 25°C using equilibrium dialysis⁵⁷. This method uses either EDTA or ethylene glycol tetraacetic acid (EGTA) to regulate the free calcium ion concentration and $^{45}\text{Ca}^{2+}$ to determine the amount of metal ion bound as a function of $[\text{Ca}^{2+}]$ after reaching equilibrium. The affinity and stoichiometry are best estimated from an objective fit of the data using a Marquardt-nonlinear least-squares procedure (SSQMIN) to the equation $Y = nK_a[\text{Me}^{2+}] / (1 + K_a[\text{Me}^{2+}])$, where Y is equal to the moles of metal ion (Me^{2+}) bound per mole of protein, n is the number of binding sites, and K_a the affinity constant for these sites³⁴. The direct Mg^{2+} binding affinity (K_{Mg}) was calculated from a competition experiment in the presence of 2 mM Mg^{2+} .

For dissociation rate measurements (Fig. 1d), the recombinant proteins ParvF102W and ParvE101Q were purified by using the GST tag-based protein expression and purification system⁵⁸. Briefly, nontagged Parv mutants were cloned into the pGEX-KG plasmid downstream of the thrombin recognition site and transformed into *E. coli* (BL21(DE3)LysS) for large-scale protein expression. The GST-Parv fusion protein was purified and digested by thrombin to remove the GST tag and yield Parv with two additional amino acids at the N terminus. Milligram amounts of Parv or its mutants were produced with a purity $>95\%$. The biochemical measurement of the Ca^{2+} and Mg^{2+} dissociation rates of the Parv mutants used methods reported previously with modifications⁵⁹. Briefly, metal-dissociation rates (k_{off}) were measured using a stopped-flow instrument (Applied Photophysics Ltd.), and tryptophan emission was monitored through an ultraviolet-transmitting black glass filter. Each k_{off} value represents an average of at least three separate experiments, with each averaging at least five traces fit with a single exponential equation.

Adult cardiac myocyte isolation, culture and gene transfer

The procedures used in this study were in agreement with the guidelines of the Internal Review Board of the University of Minnesota Committee on the Use and Care of Animals. Adult ventricular cardiac myocytes were isolated from female Sprague Dawley rats or male New Zealand white rabbits using previously described methods²⁶. Myocytes were incubated with recombinant adenovirus containing WT Parv or Parv variants at a multiplicity of infection (MOI) of 500 in FBS-free M199 medium for 1 h. This titer has been shown to achieve maximal (95–100%) efficiency of synchronous gene transfer *in vitro* without cytotoxicity by previous studies^{35,56,60} and this study. Cells were cultured in FBS-free M199 medium for up to 96 h after gene transfer, with the medium being changed every 24 h. For functional studies, myocytes from rats were transferred to a stimulating chamber 18 h after gene transfer and cultured under constant field stimulation (0.2 Hz, 7.0 V) using a MyoPacer Field Stimulator (IonOptix), with the medium being changed every 12–16 h.

qRT-PCR, western blotting and immunofluorescence assays

Expression of WT Parv and ParvE101Q in cultured myocytes was detected 24–96 h after cell isolation and gene transfer by qRT-PCR, western blotting and immunofluorescence assays. qRT-PCR was run using the iCycler Real-Time PCR Detection System (Bio-Rad). The reverse transcription reaction was done using reagents from Applied Biosystems (#N808-0234). A total of 200 ng RNA was used per 10 µl reaction volume. Each reaction (20 µl) contained 1× PCR buffer, 5.5 mM MgCl₂, 100 nM of each of the forward and reverse primers, either 100 nM fluorescent probe or 0.1× SYBR Green, 10 nM fluorescein, 200 µM of each dNTP, 0.025 U µl⁻¹ Taq polymerase (Invitrogen) and 2 µl of the reverse transcription product (complementary DNA). The protocol used was: 1 min at 95 °C followed by 40 cycles of 9 s at 95 °C and 1 min at 60 °C. 18s ribosomal RNA was used as an internal control. The primary antibodies used were to Flag tag (1:1,000, Sigma, F3165), CSQ (1:1,000, ABR, PA1-913), PLN (1:1,000, Abcam, Ab2865), PLN phosphorylated at Ser16 (1:1,000, Upstate, 07-052), SERCA2a (1:500, Sigma, S1314), NCX (1:500, Swant, P11-13), cTnI (1:1,000, Chemicon, AB1627), cTnI phosphorylated at Ser23 and Ser24 (1:1,000, Cell Signaling, 4004S) and actin (1:5,000, Sigma, A2103). Secondary antibodies were conjugated to IRDye 800 (Rockland) or Alexa Fluor 680 (Molecular Probes), and signals were visualized and quantified using the Odyssey system (Licor). For immunofluorescence assays, cells were fixed with 3% paraformaldehyde and incubated first with rabbit antibodies to actin (1:1,000) and mouse antibodies to Flag tag (1:1,000) and then with fluorescent probe-conjugated secondary antibodies (antibodies to mouse IgG-FITC or to rabbit IgG-Texas red; 1:1,000, Molecular Probes). Fluorescence images were taken by a confocal microscope (Olympus FluoView 500).

Myocyte contraction, Ca²⁺ handling and myofilament Ca²⁺ sensitivity

Measurement of the sarcomere shortening of unloaded cardiac myocytes used the Myocytes Calcium and Contractility Measurement System (IonOptix) as previously described⁶⁰. Briefly, cover slips containing myocytes were transferred to a stimulation chamber and filled with M199 medium on the stage of a microscope (Nikon, Eclipse TE2000). Myocytes were viewed under a ×40 objective (numerical aperture (NA) 1.3), and images were collected (240 Hz) by a charge-coupled device (CCD) camera (MyoCam, IonOptix). For experiments examining the response to β-adrenergic stimulation, recordings were made before and 10 min after application of isoproterenol (10 nM, Sigma). The myocyte transient analysis software (Ionoptix) was used to determine baseline sarcomere length, peak sarcomere length shortening, percentage sarcomere length shortening, time to maximal peak shortening and time from peak to 25%, 50% and 75% relaxation. The experimental temperature was set at 37 °C or 29 °C (the specific temperatures used are listed in the figure legends) to determine further whether Ca²⁺ buffering underlies the acceleration of relaxation by ParvE101Q.

Intracellular Ca^{2+} transport is known to depend on temperature, especially for SERCA, which has a much higher temperature dependence ($Q_{10} = 3\text{--}5$)^{61–63} than does Parv ($Q_{10} = 1.9$)⁶⁴. By comparing the results at different temperatures, we have previously verified that Ca^{2+} buffering underlies increased relaxation conferred by WT Parv²⁶.

For Ca^{2+} transient measurement, myocytes were loaded with Fura-2AM (a ratiometric Ca^{2+} indicator; 2 μM , Molecular Probes) for 10 min at room temperature after a de-esterification period of 20 min in M199 medium⁶⁵. Fura-2 fluorescence was measured using a spectrophotometer (Stepper Switch, IonOptix). Initially, Fura-2 was excited at 360 nm (the isosbestic point independent of Ca^{2+}) and then continuously at 380 nm (Ca^{2+} -dependent fluorescence). Emission was collected at >510 nm by a photomultiplier tube. Ratiometric data were collected and analyzed online using commercial software (IonOptix). To measure caffeine-induced sarcoplasmic reticulum Ca^{2+} release, Fura-2-loaded myocytes were electrically stimulated (1 Hz) for 1 min and then locally perfused with caffeine (20 mM) using a rapid capillary tube perfusion system. Visualization of spontaneous Ca^{2+} sparks used a confocal laser-scanning microscope (LSM510, Carl Zeiss) following methods described previously⁶⁶. All the functional experiments were performed at 1.8 mM CaCl_2 .

Myofilament Ca^{2+} sensitivity was determined by measuring the steady-state Ca^{2+} -activated isometric force on permeabilized cardiac myocytes, as previously described⁶⁷. The relaxing solution pCa ($-\log [\text{Ca}^{2+}]$) value was 9.0, and the maximal activating solution pCa was 4.0. The sarcomere length was set at 2.1 μm , and the isometric tension-pCa relationship was obtained by measuring isometric tension generation in response to various amounts of Ca^{2+} activation ranging from nominal (pCa = 9.0) to maximal (pCa = 4.0) Ca^{2+} concentrations. The Hill coefficient and pCa50 were determined with a nonlinear least-squares fitting algorithm. The production of recombinant ParvE101Q protein used the same method as described above for the dissociation rate measurements.

***In vitro* disease models**

To obtain myocytes with contractile deficiencies, a LCX ligation was used to produce the rabbit ischemic heart disease model⁶⁸. The rabbit was sedated with subcutaneous ketamine (10 mg per kg body weight) and xylazine (5 mg per kg body weight), intubated orally and ventilated with a pressure-controlled ventilator with 1–3% isoflurane in 100% oxygen at a peak inspiratory pressure of 15.0 cm H_2O and a respiratory rate of 50 breaths per min. The heart was exposed using a left thoracotomy, and a 6-0 prolene suture was tied around the proximal portion of the LCX 1–2 mm distal to the left atrium. A chest tube was inserted through a separate stab incision, and the thoracotomy was closed in layers. Sham-operated rabbits underwent thoracotomy without LCX ligation. Echocardiography was performed using an HP Sonos 5500 ultrasound machine to verify myocardial infarction and measure cardiac function 2–4 weeks after surgery. Systolic and diastolic dimensions and wall thicknesses were measured in M-mode in the parasternal short-axis view at the level of the papillary muscles. The ejection fraction was calculated from the M-mode parasternal short-axis view. Diastolic function was assessed by conventional pulsed-wave spectral Doppler analysis of mitral valve inflow patterns (early (E) and late (A) filling waves).

The cellular ischemia and reperfusion mimetic model was achieved by incubating cells with a modified ischemia mimetic solution (140 mM NaCl, 8 mM KCl, 1 mM MgCl_2 , 1.25 mM CaCl_2 , 6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.02% BSA, pH 6.0) described previously⁶⁹ for 15 min followed by reperfusion with M199 medium (pH 7.4) for 15 min. Cell contraction amplitude was severely blunted during ischemia mimetic solution incubation and partially recovered 15 min after reperfusion. The canine failing cardiac myocytes were kindly provided by H. Sabbah at the Henry Ford Hospital Heart and Vascular Institute.

rAAV production and *in vivo* gene delivery

For the *in vivo* disease model studies, WT Parv from both carp β -Parv and human α -Parv (GenBank accession number NM_002854.2) were generated. In each case, the rAAV2/6 vector constructed used a constitutive promoter (CMV), human α -Parv (or carp β -Parv) and SV40pA flanked by AAV2 ITRs. Transgenes were constructed with point mutations to generate proteins of interest. All constructs were sequence verified. The rAAV2/6 virus was generated using a dual plasmid system and standard calcium phosphate transfection techniques in HEK293 cells. The virus-containing cells and supernatant were harvested, processed through microfluidization to lyse the remaining cells and purified over a heparin sulfate column. Viral titers averaged 2.1×10^{13} vector genomes per ml. Intravascular rAAV gene delivery was performed in adult mice as described before⁴⁴, and each mouse received 1×10^{12} vector genomes in a volume of 300 μ l in the tail vein. Gene expression was verified by immunohistochemistry at least 10 weeks after rAAV-mediated gene transfer.

In vivo cardiomyopathy models of cell-intrinsic compromised Ca^{2+} removal or myofilament defects

Inducible cardiac myocyte-specific excision of the SERCA2a (knockout) mice (kindly provided by K.B. Andersson and G. Christensen) was generated by crossing SERCA floxed mice with MerCreMer transgenic mice⁴⁵. Knockout mice were injected intravascularly with the rAAV β -ParvE101Q vector or saline vehicle. At 3 weeks, mice from all groups (floxed only, knockout and knockout plus vector treatment) were injected with tamoxifen (1 mg intraperitoneally). Six weeks after treatment with tamoxifen, echocardiography was performed, which was followed 1 week later by *in vivo* pressure-conductance catheterization for real-time hemodynamics and Langendorff pressure recordings⁷⁰.

The familial RCM mouse model, which carries a cardiac troponin I mutation (R193H) and develops diastolic dysfunction and slow myocardial relaxation (as in human RCM disease), was described previously⁴⁶. At ~4 months of age, at which time substantial diastolic dysfunction is evident⁴⁸, the mice were therapeutically treated with human α -ParvE101Q rAAV vectors or a control human α -ParvF102W rAAV vector or saline. Ten weeks after rAAV gene delivery, real-time heart hemodynamics were recorded *in vivo* using micromanometry pressure-conductance catheterization as described previously⁷⁰.

Statistics

Data are expressed as the mean $\pm$ s.e.m. Two-way ANOVA, one-way ANOVA and unpaired *t*-test were used as appropriate for determining statistical significance among groups. $P < 0.05$ was deemed significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EF-hand motif design and biochemical kinetic analysis. **(a)** Sequence alignment of the two EF-hand Ca²⁺ binding domains (the CD and EF domains) of human α -Parv (WT), carp β -Parv (WT) and Parv variants. The 12-amino-acid metal-binding sequences are highlighted in boxes, and the substitutions are shown in red. **(b)** Two- and three-dimensional diagrams of the CD and EF domains of ParvE101Q (containing the D51A, E101Q and F102W substitutions). Broken lines in the loop domains (top) indicate interaction between the six coordinating residues and Ca²⁺. **(c)** Schematic of the adenovirus vector-expressing Parv constructs driven by a cytomegalovirus (CMV) promoter and a prokaryotic expression vector containing a GST tag system for Parv protein purification from *Escherichia coli*. The thrombin recognition site between the GST tag and Parv facilitates the removal of the GST tag during protein purification. The resultant Parv protein has two additional residues at its N terminus. ITR, inverted terminal repeat; aa, amino acids. **(d)** Representative traces and summarized data of the Ca²⁺ and Mg²⁺ dissociation rates of ParvF102W and ParvE101Q. Δ Trp fluorescence, change in tryptophan fluorescence. Data are shown as the means $\pm$ s.e.m. $n = 16$ –41 experiments. * $P < 0.01$ compared to ParvF102W by unpaired t test.

Figure 2.

Functional screening of Parv motifs in rat adult cardiac myocytes. **(a)** Immunofluorescence images showing ParvE101Q expression in adult rat cardiac myocytes 72 h after gene transfer. Red, actin-specific antibody; green, Flag-specific antibody; blue, nuclear staining with DAPI; control, untreated. Scale bars, 20 μm . **(b)** Representative sarcomere length (SL) traces, summarized sarcomere length shortening and the cell relaxation parameter ($T_{50\%}$) of untreated (control) and WT Parv-expressing myocytes 72 h after gene transfer. Data are shown as the means $\pm$ s.e.m. $n = 39$ –43 cells from 3–4 rats. [†] $P < 0.001$ compared to controls. **(c)** Representative sarcomere length traces and summarized sarcomere length shortening (fold change compared to WT Parv) comparing the effects of WT Parv and the indicated Parv variants. Data are shown as the means $\pm$ s.e.m. $n = 43$ –51 cells from 3–4 rats. [†] $P < 0.001$ compared to WT Parv. **(d)** Representative sarcomere length traces, summarized sarcomere length shortening and the cell relaxation parameter ($T_{50\%}$) of control and ParvE101Q-expressing myocytes 72 h after gene transfer. The experimental temperature was 29 $^{\circ}\text{C}$. Data are shown as the means $\pm$ s.e.m. $n = 39$ –48 cells from 3–4 rats. ^{*} $P < 0.01$ compared to controls.

Figure 3.

Effects of ParvE101Q on rabbit adult cardiac myocyte contractility and relaxation. **(a,b)** Expression of WT β -Parv (WT Parv) and ParvE101Q in rabbit cardiac myocytes 24–96 h after gene transfer as determined by western blot **(a)** and immunofluorescence assay **(b)**. Red, actin-specific antibody; green, Flag-specific antibody; blue, nuclear staining with DAPI. Scale bars: top, 200 μm ; bottom, 20 μm . **(c,d)** Representative original **(c)** and normalized **(d)** traces of sarcomere length (SL) shortening from an untreated (control) myocyte and a myocyte with ParvE101Q gene transfer for 72 h. The experimental temperature was 29 $^{\circ}\text{C}$. **(e,f)** Summarized data showing the effects of ParvE101Q on sarcomere length shortening **(e)** and relaxation ($T_{50\%}$; **f**) at the indicated times after gene transfer. Data are shown as the means $\pm$ s.e.m. $n = 28-69$ cells from 3–5 rabbits. $^{\dagger}P < 0.001$ compared to controls by two-way analysis of variance (ANOVA). **(g,h)** Summarized data showing the effects of ParvE101Q on sarcomere length shortening **(g)** and relaxation ($T_{50\%}$; **h**) 48 h after gene transfer and at the indicated pacing frequencies. Data are shown as the means $\pm$ s.e.m. $n = 43-63$ cells from 4 rabbits. $^{\dagger}P < 0.001$ compared to controls by two-way ANOVA.

Figure 4.

Effects of ParvE101Q on Ca²⁺ handling and myofilaments. **(a)** Representative Ca²⁺ transient traces (left), summarized data on Ca²⁺ transient amplitude (middle) and time from peak to 25% ($T_{25\%}$) or 75% ($T_{75\%}$) decay (right) of untreated (control) and ParvE101Q-expressing rabbit adult cardiac myocytes. 360/380, the ratio of emitted Fura-2 fluorescence at 360 nm and 380 nm excitations. Data are shown as the means $\pm$ s.e.m. $n = 43$ –46 cells from 3–4 rabbits. [†] $P < 0.001$ compared to controls. **(b)** Representative traces (left) and summarized data showing the effects of ParvE101Q on the amplitude (sarcoplasmic reticulum (SR) Ca²⁺ store; middle) and decay ($T_{75\%}$ caffeine decay; right) of caffeine-induced sarcoplasmic reticulum Ca²⁺ transient 72 h after gene transfer. The experimental temperature was 29 °C. Data are shown as the means $\pm$ s.e.m. $n = 25$ cells from 3 rabbits. **(c)** Representative sarcomere length (SL) traces (left and middle) and summarized data (right) showing the effects of co-transferring cTnI L29Q and ParvE101Q on the contractility of rat adult myocytes 72 h after gene transfer. +ParvE101Q is treatment with ParvE101Q; -ParvE101Q is no treatment. Data are shown as the means $\pm$ s.e.m. $n = 38$ –41 cells from 4 rats. * $P < 0.05$ compared to the -ParvE101Q group by one-way ANOVA. The experimental temperature was 37 °C. **(d)** Summarized data showing myofilament Ca²⁺ sensitivity (pCa₅₀; left), cooperative molecular interactions along the thin filament (Hill coefficient; middle) or the relative maximum tension (right) before (control) and after incubating permeabilized myocytes with recombinant ParvE101Q (0.05 mM for 10 min). $n = 3$ separate experiments. Data are shown as the means $\pm$ s.e.m.

Figure 5.

ParvE101Q rescues depressed contraction and relaxation in failing myocytes and myocytes with induced relaxation defects. **(a)** Immunofluorescence images showing ParvE101Q expression in cardiac myocytes from a canine heart failure model (HF) 48 h after gene transfer. Red, actin-specific antibody; green, Flag-specific antibody; blue, nuclear staining with DAPI. Scale bars, 20 μ m. **(b)** Summarized data of the effects of ParvE101Q on the prolonged relaxation time course ($T_{50\%}$) of canine failing myocytes at 37 $^{\circ}$ C. Data are shown as the means $\pm$ s.e.m. $n = 21$ –45 cells. * $P < 0.05$ compared to normal (untreated) cells, # $P < 0.05$ compared to HF by one-way ANOVA. **(c,d)** Representative original **(c)** and normalized **(d)** sarcomere length (SL) traces of adult cardiac myocytes isolated from heart failure rabbit myocytes (HF; not treated) without or with ParvE101Q gene transfer (HF + ParvE101Q). **(e,f)** Summarized data showing the effects of ParvE101Q on sarcomere length shortening **(e)** and relaxation ($T_{50\%}$; **f**) of cells from HF rabbits 48 h after gene transfer at a pacing frequency of 0.2 Hz. Data are shown as the means $\pm$ s.e.m. $n = 29$ –35 cells from 2 rabbits. $\dagger P < 0.001$ compared to the -ParvE101Q group by unpaired t test. **(g,h)** Representative normalized sarcomere length traces of the effects of ParvE101Q on the slow relaxation induced by thapsigargin (TG, 50 nM; **g**) and ischemia-reperfusion (I-R) mimetic treatment **(h)** in rabbit cardiac myocytes at 29 $^{\circ}$ C. Controls were untreated cells.

Figure 6.

Systemic rAAV delivery of ParvE101Q rescues defective relaxation in cardiomyopathy models of cell-intrinsic diastolic dysfunction *in vivo*. **(a)** Experimental timeline for study of mice with inducible cardiac myocyte-specific SERCA2a deficiency (mice with a floxed (FI) *ATP2A2* allele and a MerCreMer transgene⁴⁵ in which gene deletion was induced by tamoxifen treatment). Echo, echocardiography. **(b)** Summarized echocardiography data showing the effects of ParvE101Q on *in vivo* diastolic (relaxation) dysfunction in SERCA2a-deficient mice (KO) (KO + E101Q) in comparison to control SERCA2a floxed (FI) mice. IVRTc, isovolumic relaxation time corrected by heart rate (square root of the R wave to R wave interval); Tei index, isovolumic contraction time and isovolumic relaxation time divided by the ejection time. Data are shown as the means $\pm$ s.e.m. * P < 0.05 compared to FI, # P < 0.05 compared to KO by one-way ANOVA. n = 2–3 mice. **(c)** Summary of Millar micromanometry catheterization data for contractility and relaxation performance by ParvE101Q in SERCA2a-deficient mice *in vivo*. Data are shown as the means $\pm$ s.e.m. n = 2–3 mice. * P < 0.05 compared to FI, # P < 0.05 compared to KO by one-way ANOVA. **(d)** Experimental protocol using RCM transgenic (Tg) mice with therapeutic α -ParvE101Q gene transfer *in vivo*. Adult mice were treated with rAAV vectors containing human α -ParvE101Q (RCM + α -ParvE101Q), human α -ParvF102W (as WT Parv; RCM + α -ParvF102W) or saline vehicle (RCM + saline). Ten weeks later, *in vivo* real time hemodynamics of mice from the three groups and a nontransgenic (Ntg) control group were recorded by pressure-conductance catheterization. LV, left ventricular; P-V, pressure-volume. **(e,f)** Summary of Millar micromanometry catheterization data for relaxation function, including the peak rate of left ventricular systolic pressure decline ($-dP/dt$; **e**) and Tau, a load-independent measure of relaxation (**f**). Data are shown as the means $\pm$ s.e.m. n = 5–15 mice. * P < 0.05 compared to Ntg. F102W, human α -ParvF102W; E101Q, human α -ParvE101Q. **(g)** Schematic model of the strategic design of the ultra-delayed EF-hand Ca^{2+} buffer ParvE101Q for optimizing Ca^{2+} binding during the diastolic phase to accelerate active relaxation while preserving contractility. WT Parv is defective for cardiac application, as it buffers Ca^{2+} too early in systole to cause blunted Ca^{2+} amplitude and contraction.