Conditional mutations in SERCA, the Sarco-endoplasmic reticulum Ca^{2+}-ATPase, alter heart rate and rhythmicity in *Drosophila*

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Abbreviations : CS Canton-Special, CSK Cytoskeleton buffer, CyO Curly of Oster, DHPR Dihydropyridine receptor, FITC Fluorescein isothiocyanate, FR Frequency, HL3 Hemolymph like solution 3, MESA Maximum entropy spectral analysis, PBS Phosphate buffered saline, PIPES 1,4 Piperazine bis (2-ethanosulfonic acid), PMSF phenyl methyl sulfonyl fluoride, RI Rhythmicity index, SERCA Sarco-endoplasmic reticulum calcium ATPase, SR Sarcoplasmic reticulum, UAS Upstream Activator Sequence, WT Wild type

Abstract

To analyze the role of cytosolic calcium in regulating heart beat frequency and rhythm, we studied conditional mutations in *Drosophila* Sarco-endoplasmic reticulum Ca^{2+}-ATPase, believed to be predominantly responsible for sequestering free cytosolic calcium. Abnormalities in the amount or structure of the SERCA protein have been linked to cardiac malfunction in mammals. *Drosophila* SERCA protein (dSERCA) is highly enriched in *Drosophila* larval heart with a distinct membrane distribution of SERCA at cardiac Z-lines, suggesting hitherto unrecognized zones for calcium uptake into the sarcoplasmic reticulum. Heart beat frequency is strikingly reduced in mutant animals following dSERCA inactivation, (achieved by a brief exposure of these conditional mutants to non-permissive temperature). Cardiac contractions also show abnormal rhythmicity and electrophysiological recordings from the heart muscle reveal dramatic alterations in electrical activity. That all effects of the mutation can be phenocopied by expression of mutant SERCA protein in muscle strongly suggests that SERCA normally functions to control general pacemaker activity in insect cardiac tissue. Overall, these studies underscore the utility of the *Drosophila* heart to model SERCA dysfunction dependent cardiac disorders and constitute an initial step towards developing *Drosophila* as a viable genetic model system to study conserved molecular determinants of cardiac physiology.

Key Words: SERCA, Heart, *Drosophila*, Physiology, Genetics

Introduction

Cardiac physiology has been a major area of investigation in both basic and translational research. In addition to its obvious medical significance, cardiac muscles also provide the opportunity of studying a highly specialized organ-system. While maintaining general similarities with skeletal muscle function, muscles in the heart are optimized for periodic and synchronized contraction-relaxation through the entire lifetime of the animal. Mechanistically, this integrates cellular machinery that handles calcium entry through L-type calcium channels and calcium-induced calcium release, with calcium removal via uptake and buffering to produce cycles of contraction (Trafford et. al. 2002).

Studying the molecular determinants of cardiac disorders assumes much importance since a major portion of human ailments comprises cardiac diseases. In particular, disorders of the heart that derive from malfunctioning of calcium homeostasis are well known (Morgan 1991; Chen 1999). For instance, the sarco-endoplasmic reticulum calcium ATPase (SERCA), a membrane protein that pumps free cytosolic calcium into intracellular stores, has been implicated in several forms of cardiac disorders (Qi et. al. 1997; Schwinger et. al. 1999; Minamisawa et. al. 1999). In these cases, it is either pathology that leads to reduced SERCA activity, or mutations in the protein that impair the functioning of this pump, that lead to maladjusted calcium handling in cardiac muscle cells. Although significant information exists regarding this subset of disorders, studies that investigate and interpret the function of SERCA *in vivo*, with special emphasis on its molecular interactions and signaling mechanisms, will greatly promote understanding in this field.

Drosophila has been a highly valuable genetic model system for studying a diverse range of problems in biology. The richly annotated genome sequence makes it possible to focus studies on homologs of interesting genes in vertebrates. Indeed, homologs of most vertebrate genes are found in flies and thus disease modeling has progressed with impressive rapidity over the last five years (Yoshihara et. al. 2001). Several studies have analyzed conserved molecular mechanisms that control heart development and function in *Drosophila* (Dowse et. al. 1995; Gu and Singh 1995; Gajewski et. al. 1997; Johnson et. al. 1998; Curtis et. al. 1999; Johnson et. al. 2002; Dulcis and Levine 2003).

In order to outline specifically the role of SERCA in cardiac function *in vivo*, we have developed a fly model of SERCA-dependent heart dysfunction. Using recently characterized mutations in dSERCA (Sanyal et. al. 2004), we assess the cellular context in which SERCA regulates cardiac function. We demonstrate that SERCA is present in insect heart muscles and shows a membrane-bound enrichment at the muscle Z-line. SERCA dysfunction leads to a severe reduction in heart beat indicating a requirement for SERCA-dependent calcium sequestration in controlling normal heart beat in *Drosophila*. In the temperature sensitive dSERCA mutants (Ca-P60A^{Kum170} and Ca-P60A^{Kum295}), both the strength and regularity of heartbeat is reduced in the most severe mutant strain after a heat pulse and several animals become completely arrhythmic. Further, expression of a mutant SERCA protein in cardiac muscle of an otherwise wild type fly, leads to similar cardiac defects. Direct measurements of extracellular electric fields near the pacemaker indicate profound alterations in electrical activity in SERCA mutant animals, indicating a

role for SERCA dependent processes in maintaining proper pacemaker activity in the invertebrate heart.

Materials and methods

Fly rearing

Flies were reared on standard sucrose-agar fly medium at 25°C. dSERCA mutant flies were maintained balanced over the CyO chromosome. Canton-S, the background wild type strain for the isolation of the dSERCA mutants, was used as the control strain in all experiments.

Larval dorsal vessel immunohistochemistry

Wandering third-instar larvae were dissected mid-ventrally to keep the dorsal vessel intact. Dissected preparations were fixed in 3.5% calcium-free paraformaldehyde and blocked in PBS-containing 0.15% Triton, 2% BSA and 5% normal goat serum for one hour. Antibody dilutions were as follows: affinity-purified anti-dSERCA at 1:500, MAb3 at 1:5, anti-alpha actinin at 1:2. FITC-conjugated phalloidin was used to visualize actin. Appropriate secondary antibodies conjugated to fluorescent Alexa dyes (Molecular Probes, Eugene, OR) were used. Images were acquired using a Nikon LSCM system. Anti-alpha-actinin antibody was from J. Saide (Saide et. al. 1989) and MAb3 was from T. Volk (Wu et. al. 1995; Park et. al. 1996).

For experiments that involved detergent extraction of membrane prior to fixation, animals were dissected in normal saline and then incubated for 5 minutes in CSK buffer (10 mmolar PIPES, 100 mmolar KCl, 300 mmolar Sucrose, 2.5 mmolar MgCl[2], 1 mmolar PMSF, 1% aprotinin, and 1% Triton X-100). This was followed by normal fixation and staining procedures as outlined above.

Measurement of heartbeat parameters in mutant *Drosophila*

Heartbeat was monitored optically during the P1 (white) pupal stage, within an hour after the initial stage of pupariation, when the cases are translucent. Individual pupae were placed on a slide in an Olympus BH2 microscope modified for heartbeat recording by the addition of a phototransistor in the optical path of one of the eyepieces capable of registering changes in brightness caused by the movement of the heart. A drop of distilled water was placed on the pupa to facilitate light and temperature transmission. The microscope stage temperature was controlled by a Sensortek TS2 system. Each pupa was tested at 5 temperatures: 20°C, 25°C, 30°C, 35°C, and 37°C. The animal was allowed to equilibrate at each temperature for 1.5 minutes after which, 30 seconds of data were collected by a microcomputer with a DASH8 A/D conversion system sampling at 100 Hz.

The two major parameters of heartbeat determined here are frequency (FR) and rhythmicity index (RI; a quantitative measure for the regular periodicity of heartbeat). These parameters were determined, as previously described, by data analysis using custom software developed by us (Dowse et. al. 1995; Johnson et. al. 1998; Levine et. al. 2002).

Two experimental protocols were followed to test the effects of the SERCA mutations on heart function. In the first, wild type flies (Canton-S) and each of the mutant strains were tested as above directly from culture without a heat shock. In the second, all flies, including wild-type controls, were heat shocked to 41°C after collection by immersing a glass vial containing the individual pupa in a water bath. The rate of temperature rise was measured with a probe and it took 1.5 minutes to reach the appropriate temperature. Each pupa was thus exposed for 2.5 minutes to ensure a full

minute at the high temperature. The vial was chilled to ambient in water and the pupa then allowed to rest for approximately 15 minutes before testing.

Electrophysiological recordings from *Drosophila* cardiac muscle

Extracellular recordings were made from the contractile chamber of the 3rd instar larval heart as described previously (Papaefthmiou and Theophilidis 2001). For recordings under restrictive conditions, the animals were heat pulsed before recording. All recordings were done in standard HL3 ringers used for physiology (Stewart et. al. 1994) with 1.5 mmolar external calcium.

Results

SERCA is enriched at Z-discs in *Drosophila* cardiac muscle

dSERCA is a highly conserved gene as compared to that from several other species (Fig. 1a). Expectedly, it shows the highest similarity (85% identity) to SERCA genes from other arthropod classes. Significantly, it shows a high degree of identity (about 70%) to the mammalian SERCA1, 2 and 3 genes (Magyar et. al. 1995). It is also interesting to note that the phosphorylation site, Asp[351] (Maruyama and MacLennan 1988), critical sites for binding calcium, Glu[309], Glu[771], Asn[796], Thr[799], Asp[800] and Glu[908] (Clark et. al. 1989) and Thapsigargin (a specific inhibitor of SERCA) binding S3 stalk (Asp[254-Lys[262]) and M3 loop (Val[269]-Gly[277]) (Zhong and Inesi 1998; Asahi et. al. 2000) are all identical. Residues that are known to bind phospholamban in mammals (Lys[397]-Val[402]) are, however, completely different (Toyofuku et. al. 1993).

The larval dorsal vessel, the analog of the vertebrate heart, is a tubular structure that spans the entire length of the animal. Of the two pacemakers, the posterior one pumps hemolymph in an anterograde manner (Dulcis and Levine 2003). To visualize the dorsal vessel and the muscles that comprise this contractile portion of the heart, we employed several methods. The dorsal vessel can be visualized using nomarski optics (that outlines the pericardial cells), by staining with FITC conjugated Phalloidin (that binds actin), with MAb3, (a mouse monoclonal antibody that stains the surface of the pericardial cells (Park et. al. 1996)), or with antibodies against alpha-actinin, (a protein that is a component of the Z-line) (Fig. 1b). This shows that cardiac muscles in the *Drosophila* larva contain components of the fundamental contractile machinery of

muscles. We also observed that cardiac muscles are arranged in a stereotypic net-like fashion, an architecture likely to be optimized for repeated cycles of contraction.

The anti-SERCA antibodies show strong staining in the larval dorsal vessel, and at higher magnification, a unique staining pattern for dSERCA is revealed (Fig. 1c). SERCA in cardiac muscle appears enriched in distinct bands in addition to a more diffuse distribution. Such localization of SERCA may indicate the existence of specialized zones for rapid sequestration of cytosolic calcium into intra-cellular stores in these muscle cells.

To identify the subcellular zone where SERCA is concentrated, we compared its localization with that of alpha-actinin, a well-described structural element of muscle fibers. Alpha-actinin, a relatively rigid component of the contractile machinery of muscles localized to the Z-bands, serves to tether actin filaments and determines intra-filament spacing in contractile bundles. Its presence and role in insect muscle has been described in several studies (Saide et. al. 1989; Vigoreaux et. al. 1991; Fyrberg et. al. 1998; van Straaten et. al. 1999). Figure 1d clearly shows that the SERCA immunopositive bands colocalize strongly with the alpha-actinin bands. This indicates an enrichment of SERCA at the Z-line. In the context of the cellular architecture of myofibers, high levels of SERCA at the Z-line, at least in these muscles, may correspond to specialized zones of calcium influx, such as at the triad junctions (Jensen 1977; Franzini-Armstrong 1999; Franzini-Armstrong et. al. 1999; Felder et. al. 2002). Locally concentrated SERCA pumps may serve to quickly sequester and pump the cytosolic calcium back into intra-cellular stores. By quickly resetting calcium levels in the cytoplasm, this would enable very efficient and rapid feedback control of free calcium in

the cytoplasm, thereby enabling rapid and highly periodic contractions of these muscle fibers.

We addressed the mechanism of SERCA localization by asking whether this required direct, high-affinity, protein-protein interactions with components of the Z-line by using weak detergent solution to extract membrane fractions from larval preparations before visualizing SERCA. Figure 1e shows a single heart muscle fiber double stained for alpha-actinin and dSERCA. After membrane extraction, the entire dSERCA signal is lost while alpha-actinin remains intact. This implies that the localization of SERCA seen in figure 1d arises primarily due to local concentration of membrane-associated SERCA in close apposition to the Z-lines. These areas may correspond to the calcium release units described earlier (Franzini-Armstrong 1999; Felder et. al. 2002).

dSERCA regulates heart beat in *Drosophila*

The structural conservation of *Drosophila* SERCA and its strong expression in the heart suggested important functions in generating or maintaining cardiac contractions and rhythm *in vivo*. We directly examined SERCA contribution to FR and RI in living animals by comparing these parameters in wild-type (WT) heart with those of *dSERCA* mutant hearts either with or without prior heat shock. We chose two temperature sensitive alleles, *CaP60A*$^{\mathrm{Kum170}}$ and *CaP60A*$^{\mathrm{Kum295}}$ for these experiments. Another allele *CaP60A*$^{\mathrm{A11}}$, is a very weak allele and expectedly, did not show significant changes in heart beat parameters (however, see supplementary data and table S1 for a complete statistical analysis across genotypes and temperatures). Thus, the two strong alleles and especially *CaP60A*$^{\mathrm{Kum170}}$ was selected for further analysis. In the temperature sensitive

mutants, recordings from the flight muscle indicate serious defects in muscle action potential generation. This defect is seen both in the unheated animals and also in mutants that have been subjected to a heat pulse at restrictive temperatures. Thus, while failures in action potential generation are observed in unheated dSERCA mutants, the frequency of such failures is highly elevated when these mutants are heat pulsed (Sanyal et. al. 2005). We anticipated that any effects attributable to this perturbation of SERCA should manifest themselves as temperature-dependent defects in relevant aspects of cardiac function. Table 1 displays FR and RI values for wild-type and *dSERCA* mutant hearts recorded at 25°C, before or after brief exposure to 41°C (see materials and methods).

Flies bearing mutations in dSERCA tested without any prior heat shock showed significantly reduced heart beat frequencies (in Hz) compared to wild-type flies ($F = 22.65$, $P = 0.0001$, Table I). There was no meaningful variation in RI among strains. These reduced FR values are statistically significant, but alone do not conclusively demonstrate a role for SERCA in regulation of heartbeat frequency. To conclusively map these phenotypes to mutations in dSERCA, we investigated whether temperature-dependent perturbation of SERCA resulting from prior exposure to 41°C for 1 minute would exacerbate the slow heartbeat phenotype as involvement of SERCA would predict.

The conditioning temperature pulse induced significant effects on heart beat frequency, with the most striking change in strain *CaP60A*^{Kum170}, the more severe of the two alleles (Table 1). Heart beat frequency dropped in both alleles below that of wild-type flies treated in the same manner. WT flies remained essentially unchanged after the heat pulse while *CaP60A*^{Kum170} had its frequency significantly lowered by the pulse (CS: $F \sim 0.0$, $P = 0.98$; *CaP60A*^{Kum170}: $F = 25.38$, $P = 0.0001$). *CaP60A*^{Kum295} had a frequency

already very low before the pulse and remained unchanged by it (table I). Both the interval between beats and the duration of each individual beat (systole) were lengthened in animals with reduced heart rate (Figure 2a). Taken in sum, these data demonstrate an essential function for SERCA in regulating the frequency of heartbeat in *Drosophila*.

SERCA perturbation had no significant effect on the RI of flies that remained rhythmic after being exposed to the heat pulse (Table 1). Only these animals were used to calculate and compare RI values. However, animals that showed no significant rhythmicity based on autocorrelation analysis were not included in these calculations, and there were fewer rhythmic animals after pulsing in the mutant strains compared to wild type. WT showed no reduction in rhythmicity as a result of the pulse, while *CaP60A*^{Kum295} had a 5.3% reduction, and the severe *CaP60A*^{Kum170} strain lost 70%. MESA spectra, representative of rhythmicity of heart beat clearly showed this difference. Figure 2b shows representative spectra from wild type and *CaP60A*^{Kum170} animals with and without a heat pulse. As shown by the arrowheads, multiple peaks in the heated *CaP60A*^{Kum170} animals indicate highly arrhythmic heart beat in these animals. Thus SERCA perturbation has clear effects on both frequency and rhythmicity of heart beats.

The cellular origin of these defects could either be in the heart muscle themselves or potentially in the neurons that innervate these muscles (Dulcis and Levine 2003). To test if impairing SERCA function in cardiac muscle alone leads to similar phenotypes, we constructed and analyzed flies that have muscles mutants for dSERCA in a wild type background. This was accomplished by using a transgenic fly in which a copy of the mutant dSERCA coding region is placed under the control of an upstream UAS element (Brand and Perrimon 1993). (A wild type transgene is not predicted to rescue the

phenotypes as the mutations are strongly dominant, thus we constructed a dominant negative transgene for our experiments). We expressed the mutant protein in muscle using the mef2-GAL4 driver which has been shown to have high expression in all muscle tissue, including cardiac muscle (Bour et. al 1995). Results were as for the mutant *CaP60A*^{Kum170} flies. Flies showed a significantly lower heart rate after being heat pulsed (F = 10.8, P = 0.01, Table 1). As with the *CaP60A*^{Kum170} mutant strain, the number of rhythmic animals was reduced by 50% post pulse. When the transgene was expressed using a pan-neuronal driver (elav-C155-GAL4), no changes in heartbeat were observed (data not shown). These experiments conclusively prove that the effects on heart beat in the dSERCA mutants are caused by mutations at the SERCA locus and they derive from SERCA perturbations in cardiac muscle.

dSERCA function controls electrical activity in cardiac tissue

Although our observations suggest that loss-of-function SERCA mutations affect cardiac properties, it is not clear whether they affect electrical excitability or muscle contraction in response to such electrical changes. In order to directly measure electrical activity from cardiac tissue, we recorded extracellularly from the dorsal vessels of live larvae (Figure 3a). (Larval hearts also show dramatic effects of SERCA perturbation). Typically, the heart of a dissected larva continues to beat for up to 30 minutes in HL3 ringer’s solution. Electrical activity from the heart accurately depicts these contractions, with periodic and rhythmic spikes (Papaefthmiou and Theophilidis 2001).

Upon heating, the wild type animals do not show any significant changes in their electrical activity. *CaP60A*^{Kum170} mutants, on the other hand, showed dramatic reduction in overall activity (Figure 3b). Beats became infrequent and erratic with no discernible

periodicity. Each individual beat is also slowed down. These changes correlate well with observed changes in the physical beating of the heart. Thus, it is clear that dSERCA dependent calcium sequestration directly affects excitability and permits repetitive contractions of the fly heart.

Discussion

There are several reasons for analyzing SERCA functions in the *Drosophila* heart. While the development of the *Drosophila* heart involves cellular mechanisms and genetic pathways remarkably similar to those used in vertebrates, study of the physiological regulation of heart function in *Drosophila* has only recently begun in earnest (Johnson et. al. 2002; Johnson et. al. 1997; Johnson et. al. 2000; Johnson et. al. 2001). SERCA is arguably an excellent entry point for a comparative study of cardiac physiology. SERCA is a highly conserved protein and performs a key role in regulating muscle contractility in all animals. We show here that a) *Drosophila* SERCA is highly enriched in the heart and b) SERCA dependent functions play a physiologically significant role in regulating heartbeat frequency and rhythmicity. This study is not only the first step in developing a genetic model system to study cellular functions of SERCA and associated molecules, but also adds a new line of attack on cardiac physiology in general.

Localization of *Drosophila* SERCA

Although the presence of SERCA in invertebrate muscle has been reported (Zhang et. al. 2000; Zwaal et. al. 2001), no study has described the role of this molecule in the insect heart. Using antisera against two carefully selected 20 amino acid peptides from *Drosophila* SERCA, we demonstrate the protein's selectively high expression in the heart and further its association with specific subcellular zones (Figure 1). Specifically, SERCA shows strong co-localization with alpha-actinin, an integral component of muscle Z-line (Figure 1d). Similar specialized zones of sarcoplasmic reticulum have been previously described. Ryanodine receptor clusters in the sarcoplasmic reticulum are found in close apposition to the DHPR receptors in T-tubules (Franzini-Armstrong 1999;

Franzini-Armstrong et. al. 1999; Felder et. al. 2002). This organization serves to couple tightly calcium entry from outside the muscle cell to calcium-triggered release from intra-cellular stores. The dynamics of contraction in the heart muscle cell are highly specialized for the quick entry and sequestration of calcium. This allows repetitive and rapid contraction-relaxation cycles. It is conceivable, given such dedicated physiology that elevated SERCA concentrations near sites of calcium entry form zones of rapid calcium sequestration into the SR. This would likely enable rapid decline of local calcium to resting levels in these cells following each contraction episode.

The mechanism involved in generating this specific subcellular distribution of SERCA remains unknown. Our data indicate that SERCA is not physically associated with structural components of the Z-line. That SERCA localization depends on association with specialized membrane rather than tight interaction with cytosolic scaffold proteins is evidenced by its sensitivity to mild detergent extraction, a process that leaves alpha-actinin distribution essentially unchanged. While this in itself is not surprising, the fact that membrane specializations exist that place high concentrations of SERCA in close apposition to the Z-line (and the T-tubule calcium entry sites) is novel. This indicates hitherto undetected areas of sarcoplasmic reticulum that allow rapid calcium sequestration. The mechanisms that might restrict SERCA to these regions are currently unknown and we offer speculations for its potential significance.

Functions of SERCA in the insect heart

In order to test the function of dSERCA in the *Drosophila* heart, we analyzed effects of conditional SERCA mutations on cardiac contraction cycles. The most striking

observation is that *CaP60A*^{Kum170} mutants show clearly reduced FR that is further aggravated upon incubation at restrictive temperatures coupled with a reduced number of rhythmic animals. Both the interval between two subsequent beats and the duration of each beat are increased. The strong temperature-dependence of the phenotype is compelling evidence that this defect derives from perturbation of the temperature-sensitive SERCA protein. In addition, targeted expression of mutant SERCA protein in cardiac muscles is sufficient to cause these changes. Electrical activity in the heart is also severely altered in the mutants and taken together with the fact that the insect heart is myogenic, it is likely that SERCA directly controls electrical excitability in cardiac musculature.

There are two possible mechanisms that may contribute to these phenotypes that are not mutually exclusive. First, there is likely a delay in the restoration of normal low resting calcium levels owing to defective calcium sequestration by a mutant SERCA molecule, inhibiting relaxation and prolonging the refractory period between beats. Any delay in calcium uptake into the SR would prevent relaxation and re-initiation of contraction. Second, the *Drosophila* heart pacemaker requires a Ca^{2+} spike (Dowse et. al. 1995; Gu and Singh 1995; Johnson et. al. 1998). Given the widely observed process of calcium dependent inactivation of its own channels (Peterson et. al. 2000), prolonged elevated concentration of this ion would keep the critical pacemaker Ca^{2+} channels inactive longer than normal. The resolution of these issues using detailed genetic, physiological and calcium imaging experiments should inform us about the roles of SERCA.

Our analyses of SERCA in a genetic model organism, is significant because several diseases that arise from defective SERCA function have been described in mammals. Cardiac disorders are known to be associated with reduced SERCA2a function in cardiac muscles of higher vertebrates (MacLennan 2000; Periasamy and Huke 2001). The development of a model system amenable to genetic manipulations as described in this study will help in elucidating the mechanism of SERCA-dependent disease. It will also allow analysis of known and novel mutations, genetic screens to isolate other molecules that play a role in regulating calcium dynamics and molecular screens to identify other binding partners.

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Figure legends

Fig. 1. The SERCA gene in *Drosophila* is highly conserved. (a). dSERCA is the most closely related to arthropod SERCA but also has very high homology to mammalian SERCA genes. (Dm =*Drosophila melanogaster*; Sm = *Schistosoma mansoni*; Hs = *Homo sapiens*; Rn = *Rattus norvegicus*; At = *Arabidopsis thaliana*; Pa = *Paramecium aurelia*; Pf = *Plasmodium falciparum*; Sc = *Saccharomyces cerevisiae*). (b). The larval dorsal vessel (heart) components. The larval dorsal vessel can be visualized using nomarski optics to outline the pericardial cells, staining with Mab3 to label the surface of pericardial tissue, staining with Phalloidin to label muscle actin or staining with antibodies to alpha-actinin, a component of muscle Z-lines. In all images, posterior is to the left and all views are from the ventral side. Scale bar = 100 microns. (c). SERCA is highly enriched in cardiac muscle. dSERCA staining in dorsal vessel musculature shows a striated pattern interspersed with more diffuse staining. Scale bar = 20 microns. (d). dSERCA is enriched at the Z-line and is entirely membrane bound. Double staining of a single cardiac muscle showing SERCA (red) and alpha-actinin (green). The merged image shows the co-localization of the two proteins in these muscle cells. Alpha-actinin marks the muscle Z-line, thereby suggesting a concentration of dSERCA at the Z-line. (E). A single merged image of cardiac muscle preparations from which all membrane has been extracted prior to fixation, stained for SERCA and alpha-actinin. While alpha-actinin staining is unaltered, the SERCA staining is completely removed. This indicates the membrane association of SERCA and thus, zones of SERCA enrichment that correspond to Z-lines. Scale bar = 10 microns.

Fig 2. (a). Comparison of optically recorded heartbeat of the conditional mutant SERCA strain *CaP60A*^{Kum170} with that of wild-type animals. Shown are sample recordings done at 25°C after the pupae were heat shocked to 41°C. Wild-type animals (left trace) display more rapid and regular beats than *CaP60A*^{Kum170} animals (right trace). Frequent "failures" or missed beats are seen in heated *CaP60A*^{Kum170} animals (arrows). The ordinate is to the same scale in both cases and is normalized. The abscissa is time in seconds. Scale bar = 5 seconds. (b). Representative MESA spectra from four animals tested at 25°C both without and with a prepulse to 41°C. Overall, Wild Type was unaffected by the pulse either for FR or RI. The sharp single peaks at both temperatures are one indication of strong, regular rhythmicity reaffirming the RI scores. *CaP60A*^{Kum170} flies suffered a significant decrement in rhythmicity and frequency after a prepulse. The multiple peaks in the spectrum derived from the pulsed animal (arrowheads) show this effect clearly. The ordinate is the spectral power and the abscissa is frequency in Hertz.

Fig 3. SERCA regulates electrical activity in the heart pacemaker. (a). Schematic of recording configuration. Extracellular recordings are made from the posterior contractile elements (pacemaker) of the larval heart. (b). Electrical rhythms of the beating larval heart. Brief inactivation of the conditional SERCA mutation in *CaP60A*^{Kum170} severely perturbs heartbeat, while the effect of heat on wild type is minimal. Scale =5 seconds.

Table 1. FR and RI of various genotypes recorded at 25°C without or with a prepulse at 41°C.

Genotype	Parameter	-heat shock	+heat shock
CS	FR	2.4 ± 0.1	2.4 ± 0.1
	RI	0.48 ± 0.04	0.38 ± 0.12
	n	20	20
CaP60A^{Kum295}	FR	1.7 ± 0.1	1.7 ± 0.1
	RI	0.53 ± 0.05	0.40 ± 0.05
	n	19	18
CaP60A^{Kum170}	FR	1.8 ± 0.1	1.2 ± 0.1
	RI	0.51 ± 0.04	0.40 ± 0.08
	n	20	6
Mef2-GAL4 UAS-*CaP60A*^{Kum170}	FR	2.1 ± 0.2	1.0 ± 0.1
	RI	0.38 ± 0.06	0.20 ± 0.08
	n	4	3

Table of frequency and rhythmicity index values for various genotypes recorded at 25°C without or with a heat pulse. The heat pulse regimen, when applied, is shown schematically above the table. Both mutant SERCA alleles and transgenic animals expressing a dominant negative SERCA protein only in muscles, showed significant differences in frequency as compared to wild type. This reduction in FR was exacerbated with the heat treatment, as expected for temperature sensitive conditional alleles. RI values for these animals are not significantly altered. However, this calculation only involves animals that continued to be rhythmic after a heat pulse. Significantly, several

animals in both mutant and transgenic classes, but not wild type, became severely arrhythmic after the heat pulse.

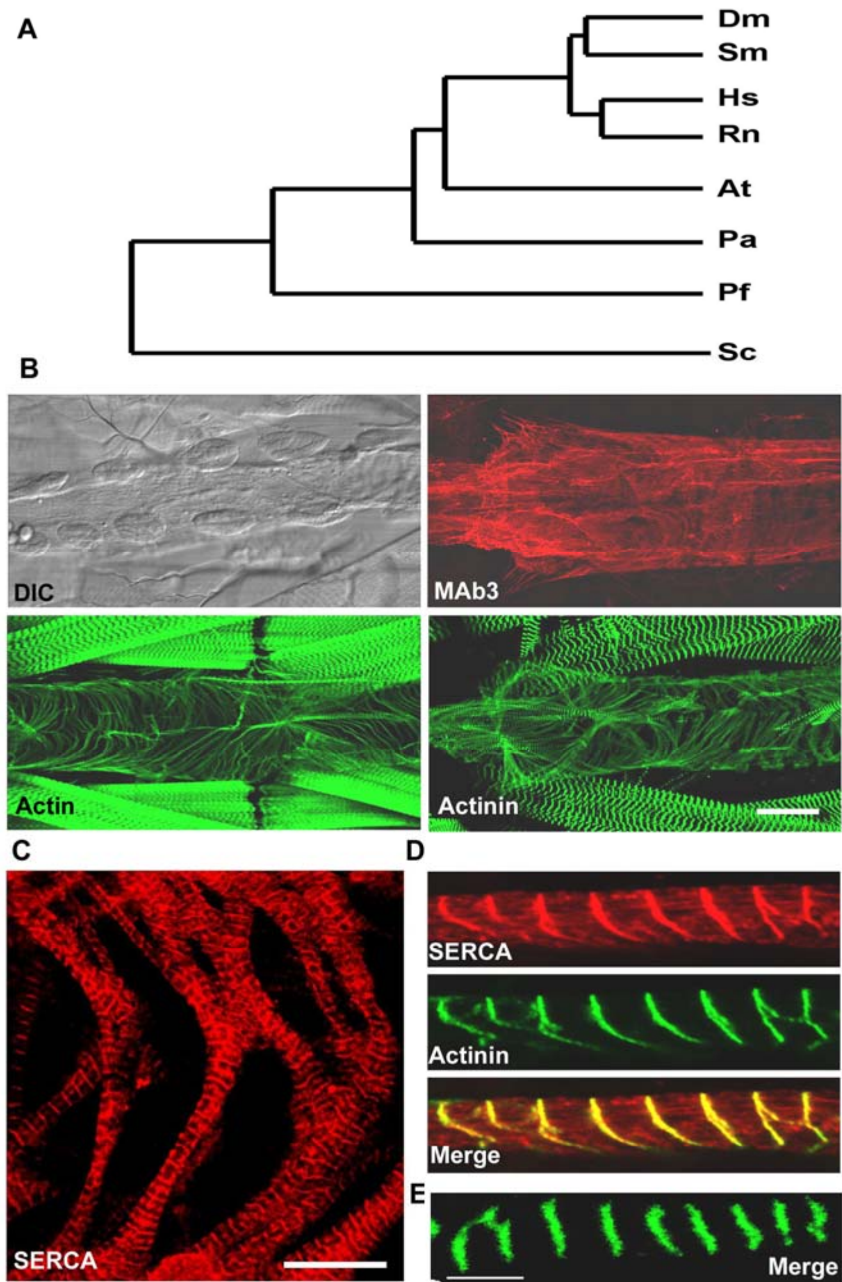


Figure 1.

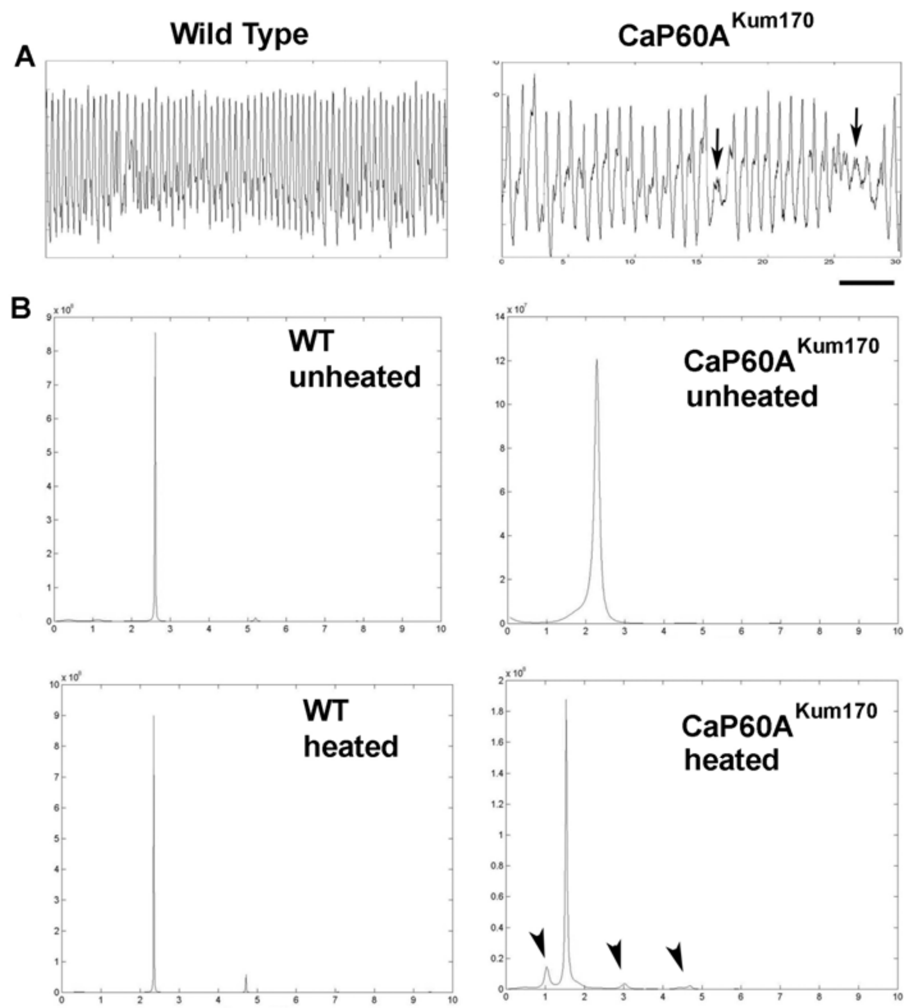
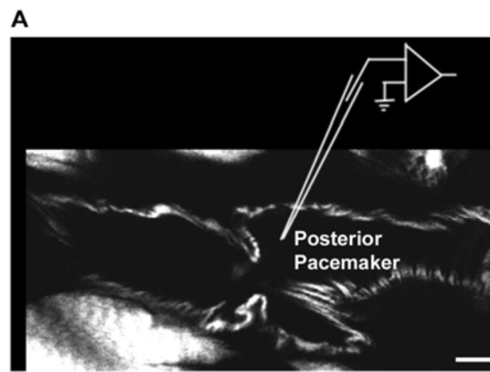


Figure 2.



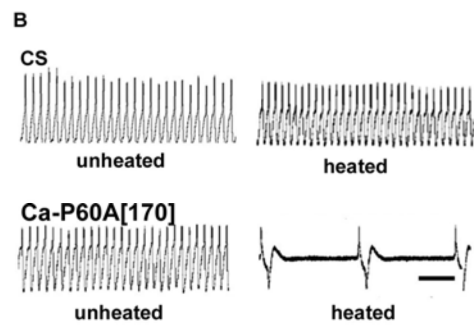


Figure 3.

Supplementary Data

Flies bearing mutations in dSERCA tested without any prior heat shock showed significantly reduced heart beat frequencies (in Hz) across all temperatures compared to wild-type flies, (W-T [CS], 2.8 ± 0.01, n = 97, > CaP60A^{Al1}, 2.4 ± 0.1, n = 100; = CaP60A^{Kum170}, 2.0 ± 0.05, n = 99; = CaP60A^{Kum295}, 1.9 ± 0.05; $F = 52.6$, $P = 0.0001$; SAS GLM procedure, Ryan-Einot-Gabriel-Welsch [REGWF] multiple F test, $\alpha = 0.05$, df = 390). (20 flies were tested in all cases here and for pulsed flies below, however only rhythmic animal by temperature results were used in the calculation). Results for individual temperatures are shown in Table S1. There was no meaningful variation in RI among strains across temperatures, although CaP60A^{Kum295} had a marginally higher RI ($F = 4.4$, $P = 0.005$).

We also investigated whether temperature-dependent perturbation of SERCA resulting from prior exposure to 41°C for 1 minute would exacerbate the slow heartbeat phenotype as involvement of SERCA would predict.

The conditioning temperature pulse induced significant effects on heart beat frequency, with the most striking change in strain CaP60A^{Kum170}, the most severe of the three alleles (Table S1). Pooled across temperatures, heart beat frequency dropped in all three alleles below that of wild-type flies treated in the same manner. W-T flies remained unchanged after the heat pulse ($F = 1.2$, $P = 0.27$). (Here and below, Hz ± S.E.M.: W-T, 2.8 ± 0.1, n = 98; > CaP60A^{Al1}, 2.3 ± 0.1, n = 72; = CaP60A^{Kum295}, 2.1 ± 0.1, n = 92; > CaP60A^{Kum170}, 1.4 ± 0.1, n = 29; $F = 42.8$, $P = 0.0001$; SAS GLM procedure, [REGWF] multiple F test, $\alpha = 0.05$, df = 290). CaP60A^{Kum295} and CaP60A^{Kum170} had their frequencies significantly lowered by the pulse, but CaP60A^{Al1} did not (data not shown).

SERCA perturbation had no significant or clear cut effect on the RI of flies that remained rhythmic after being exposed to the heat pulse (Table S1). However, animal by temperature combinations that showed no significant rhythmicity based on autocorrelation analysis were not included in the calculations, and there were fewer rhythmic animals after pulsing in the mutant strains compared to wild type. W-T showed no reduction in rhythmicity as a result of the pulse, while $CaP60A^{Kum295}$ had a 3% reduction, $CaP60A^{A11}$ showed a 27% drop, and the severe $CaP60A^{Kum170}$ strain lost 71%.

Temperature had a clear effect on FR in all strains tested. Regression of slope on temperature was significant for all strains both with and without a temperature pulse (data not shown). There was an effect of genotype on slope and intercept for non-pulsed ($F = 68.46$, $P < 0.0001$ SAS GLM Procedure, Homogeneity of slopes test) and pulsed ($F = 7.5$, $P < 0.0066$) animals, but no clear pattern emerged. Linear regression is an inadequate representation of the effects of temperature on RI.

We then expressed the mutant protein in muscle using the mef2-GAL4 driver which has been shown to have high expression in all muscle tissue, including cardiac muscle. Results were as for the mutant $CaP60A^{Kum170}$ flies. Grouped across temperatures, flies showed a significantly lower heart rate after being heat pulsed. (Not-pulsed, 2.1 ± 0.2; pulsed = 1.0 ± 0.1; $F = 20.5$, $P = 0.0001$, SAS GLM procedure, REGWF multiple F test, $\alpha = 0.05$, df = 43). As with the $CaP60A^{Kum170}$ mutant strain, the number of rhythmic animal by temperature cells was reduced from 96% to 35%. RI was reduced, but not quite significantly (Not-pulsed, 0.38 ± 0.06; pulsed = 0.20 ± 0.08; $F = 3.5$, $P = 0.076$, SAS GLM procedure, REGWF multiple F test, $\alpha = 0.05$, df = 43.)

Table S1

Not Temperature shocked
Temperature °C

Genotype	20	25	30	35	37
CS	2.0 ± 0.1	2.4 ± 0.1	2.8 ± 0.1	3.2 ± 0.1	3.4 ± 0.1
	0.45 ± 0.06	0.48 ± 0.04	0.43 ± 0.04	0.37 ± 0.05	0.33 ± 0.05
	19	20	19	20	20
CaP60A^{Al1}	1.8 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	2.9 ± 0.1
	0.35 ± 0.05	0.51 ± 0.05	0.53 ± 0.05	0.51 ± 0.05	0.46 ± 0.1
	20	20	20	20	20
CaP60A^{Kum295}	1.4 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
	0.37 ± 0.05	0.53 ± 0.05	0.58 ± 0.04	0.55 ± 0.05	0.54 ± 0.06
	16	19	20	20	20
CaP60A^{Kum170}	1.5 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
	0.44 ± 0.04	0.51 ± 0.04	0.51 ± 0.03	0.40 ± 0.03	0.39 ± 0.03
	19	20	20	20	20

Temperature shocked
Temperature °C

Genotype	20	25	30	35	37
CS	2.0 ± 0.1	2.4 ± 0.1	2.8 ± 0.2	3.3 ± 0.1	3.3 ± 0.1
	0.40± 0.05	0.38 ± 0.12	0.40 ± 0.05	0.33 ± 0.05	0.37 ± 0.05
	19	20	20	19	20
CaP60A^{KumAl1}	1.6 ± 0.2	2.0 ± 0.1	2.53 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
	0.24 ± 0.14	0.37 ± 0.07	0.39 ± 0.08	0.36 ± 0.06	0.40 ± 0.04
	8	12	14	19	19
CaP60A^{Kum295}	1.7 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
	0.29 ± 0.04	0.40 ± 0.05	0.46 ± 0.05	0.40 ± 0.05	0.42 ± 0.05
	14	18	20	20	20
CaP60A^{Kum170}	1.0 ± 0.04	1.2 ± 0.1	1.5 ± 0.2	1.7 ± 0.2	1.5 ± 0.3
	0.32 ± 0.11	0.40 ± 0.08	0.39 ± 0.13	0.35 ± 0.04	0.15 ± 0.04
	5	6	5	6	7

The tables show measurement of heart beat parameters in wild type and Ca-P60A mutant animals both before and after a heat pulse at restrictive temperatures. For each genotype, the first row is FR, the second row is RI and the third the number of animals tested in each category.