

Subhabrata Sanyal · Tricia Jennings · Harold Dowse
Mani Ramaswami

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

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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 evolutionarily conserved 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. 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.

Keywords SERCA · Heart · *Drosophila* · Physiology · Genetics

Abbreviations CS: Canton-special wild-type · CSK: Cytoskeleton buffer · *CyO*: Curly of Oster, multiply inverted second chromosome balancer · 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-ethanesulfonic 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

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; Chien 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

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S. Sanyal (✉) · M. Ramaswami
Molecular and Cellular Biology Department and ARL Division
of Neurobiology, University of Arizona, 1007 E. Lowell Street,
Life Sciences South, AZ, Tucson, USA
E-mail: sanyal@u.arizona.edu
Tel.: +1-520-6216239
Fax: +1-520-6213709

T. Jennings · H. Dowse
Department of Biological Sciences, University of Maine, ME,
Orono, USA

H. Dowse
Department of Mathematics and Statistics, University of Maine,
ME, Orono, USA

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 5 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, 2002; Curtis et al. 1999; 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. For this, we used recently characterized mutations in dSERCA (Sanyal et al. 2004), where we assess the cellular context in which SERCA regulates cardiac function. These EMS induced mutants show temperature sensitive behavioral paralysis followed by unusually prolonged periods of recovery. Since SERCA is likely to be present in all cells, these mutations display multiple phenotypes. Some of these include failures of evoked junction potentials in flight muscles, and reduced evoked junctional currents in larval body wall muscles. The two mutations identified in our screen (alleles 170 and 295) as also two independently isolated mutations in dSERCA are completely dominant with respect to their paralytic phenotype and also recessively lethal. These mutations are not likely to be gain of function mutations because a mutant allele in a SERCA deficiency background, instead of rescuing the mutant phenotypes, leads to lethality, indicating an antimorphic effect.

Here, 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}; Ca-P60A refers to the P type Calcium ATPases at chromosomal position 60A on the right arm of the second chromosome in *Drosophila*), 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

Drosophila strains, rearing and crosses

Flies were reared on standard sucrose-agar fly medium at 25°C. dSERCA mutant flies, which have been "cantonized" by extensive repeated backcrossing to the Canton-S wild-type strain, were maintained balanced over the multiply inverted second chromosome balancer In(2LR)O, Cy chromosome. Canton-S, the background wild type strain for the isolation of the dSERCA mutants, was used as the control strain in all experiments. The UAS-CaP60A^{Kum170} strain was constructed by cloning the mutant cDNA from Ca-P60A^{Kum170} animals in a pUAST vector followed by construction of transgenic flies by routine microinjection procedures. Details of this construct have been reported elsewhere (Sanyal et al. 2005). For our experiments a strain containing a homozygous copy of the transgene on both the second and third chromosomes was used such that the progeny had two copies of the UAS containing transgene. *Mef2-GAL4* lines were obtained from Leo Pallanck at the University of Washington, Seattle and have been described extensively (Ranganayakulu et al. 1996). All other GAL4 lines used in this report are from the *Drosophila* Stock Center at Bloomington.

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 1 h. 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, USA) were used. Images were acquired using a Nikon LSCM system. Anti-alpha-actinin antibody was from Judith Saide (Saide et al. 1989) and MAb3 was from Talila 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 min in CSK buffer (10 mmolar PIPES, 100 mmolar KCl, 300 mmolar Sucrose, 2.5 mmolar MgCl₂, 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 (Ashburner 1989). 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 five temperatures: 20, 25, 30, 35, and 37°C. The animal was allowed to equilibrate at each temperature for 1.5 min after which, 30 s of data were collected by a micro-computer with a DAS8 A/D conversion system sampling at 100 Hz.

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 either with or without a heat shock ($N=20$ for each strain by treatment group). Flies to be heat shocked to 41°C were treated by placing individual pupae of the correct stage in a foam-stoppered glass vial and then immersing the vial in a water bath. The rate of temperature rise was measured with a probe and it took 1.5 min to reach the appropriate temperature. Each pupa was thus exposed for 2.5 min 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 min before testing.

After this initial screen, the most severe of the alleles, Ca-P60A^{Kum170}, and the UAS-CaP60A^{Kum170}/Mef2-GAL4 double transgenic strain (described above) were compared to wild-type in a more detailed protocol designed to probe the phenotypes at higher recording temperatures. In this protocol, P1 pupae ($N=10$ for each strain) were initially tested as above at the same temperatures from 20 to 37°, but the range was extended so that readings were taken for each pupa at five further temperatures from 38 to 42° in 1° increments. The same step protocol of rest and data recording was followed across the full range. After each pupa was tested, it was rapidly cooled on the stage to ambient and heart beating was confirmed both by looking at the output of the optical recording device in the oscilloscope monitor and directly by observation of the heart in a dissecting microscope. This was to ensure that any cessation of heart function was not trivially owing to death of the subject.

Data analysis and statistics

The two major parameters of heartbeat determined here are frequency (FR) and rhythmicity index (RI). FR was determined by Maximum Entropy Spectral Analysis (MESA) adapted to biological time series (Dowse and Ringo 1989; Levine et al. 2002). RI is a quantitative measure of the regularity of heartbeat derived from the autocorrelation function (Dowse et al. 1995; Johnson et al. 1998). Briefly, this function is the correlation of a time series with itself as it is sequentially slipped or “lagged” out of register one sampling interval point at a time (Chatfield 1980). In rhythmic data, recurring maxima of autocorrelation occur each time peaks come into register. The decay in this function is a measure of regularity, here “rhythmicity”, in the signal (Chatfield 1980). We use the height of the third peak (counting the peak at lag zero as one) as a measure of the regularity of the beat (Levine et al. 2002).

Frequency and RI data acquired in this manner were analyzed for effects of genotype and treatment by analysis of variance (ANOVA) [statistical analysis system (SAS)]. Means were compared by the Ryan–Einot–Gabriel–Welsch (REGWF) multiple F test (SAS) ($\alpha=0.05$) which controls for experiment wise error (Einot and Gabriel 1975). Applications of these tests are detailed individually in results. If the heart was known to be functional based on perusal of the record, the FR and RI were included in the data no matter how erratic the beating was. If no heart beat at all was seen in the raw data record, that animal \times temperature cell was treated as a “missing data point” (SAS). The N s in the table reflect the number of pupae (out of the total tested) that had heartbeat for which FR and RI could be calculated.

Electrophysiological recordings from *Drosophila* cardiac muscle

Extracellular recordings were made from the contractile chamber of the third 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

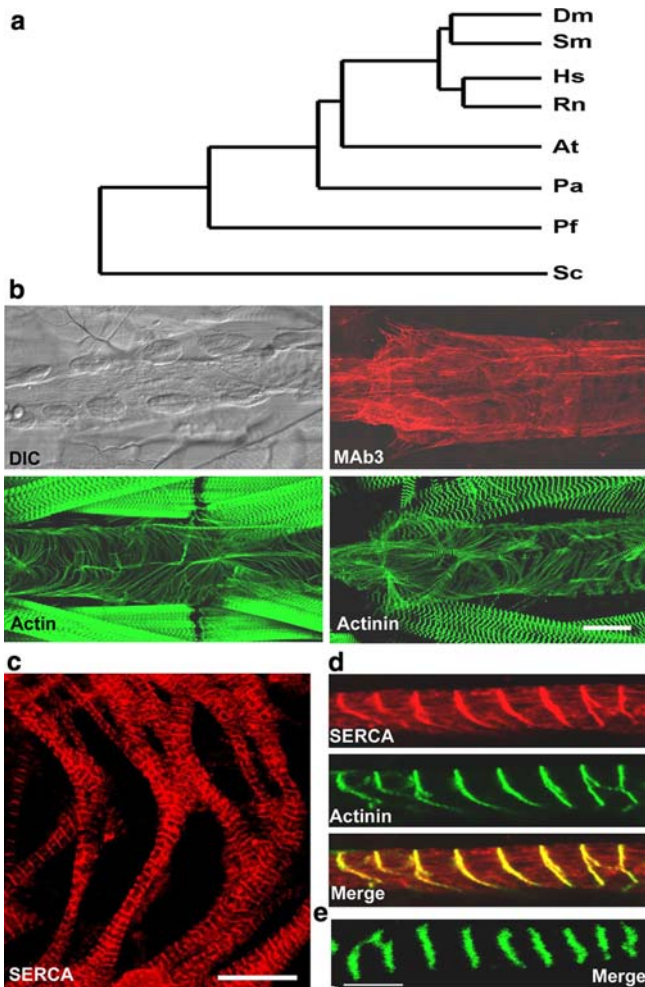


Fig. 1 The SERCA gene in *Drosophila* is highly conserved. **a** dSERCA is the most closely related to arthropod SERCA but also has very high homology to mammalian SERCA genes. (*Dm Drosophila melanogaster*; *Sm Schistosoma mansoni*; *Hs Homo sapiens*; *Rn Rattus norvegicus*; *At Arabidopsis thaliana*; *Pa Paramoecium 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 μ m **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 μ m. **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 μ m

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] (Clarke 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.

To investigate the cellular distribution of dSERCA in the dorsal vessel we used anti-SERCA antibodies that we generated for an earlier study (Sanyal et al. 2005). We believe that these affinity purified antibodies, raised against carefully selected unique peptides, specifically recognize SERCA protein in *Drosophila*. Firstly, the antibodies recognize a band of the correct size in western blots, second, this immunoreactivity is abolished by pre-incubation with competing peptide, third, the muscle staining displays an expected pattern and finally, colocalization experiments show extensive costaining with endoplasmic reticulum markers (anti-KDEL). 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. It is important to note that similar cellular localization has also been observed in vertebrate myocytes (Drago et al. 1998). This points to a general conservation of structure and function of SERCA proteins during evolution and further validates the use of a fly model to study fundamental aspects of SERCA related cardiac disorders in vertebrates.

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 Fig. 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 heartbeat 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 three temperature sensitive alleles, *CaP60A*^{Kum170} (170), *CaP60A*^{Al1} (AL1), and *CaP60A*^{Kum295} (295) for these experiments. 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 temperatures ranging from 20 to 37°C, before or after brief exposure to 41°C according to the first protocol described above.

Flies bearing mutations in *dSERCA* tested without any prior heat shock showed a range of significantly reduced FR compared to wild-type flies when analyzed across all five temperatures ($F=52.6$, $P=0.001$; $W-T > All > 170=295$; Table 1). There was no meaningful variation in RI among strains. These reduced FR values are highly statistically significant, but alone do not conclusively demonstrate a role for SERCA in regulation of heartbeat frequency. To map these phenotypes to mutations in *dSERCA*, we investigated whether temperature-dependent perturbation of SERCA resulting from prior exposure to 41°C for 1 min would exacerbate the slow heartbeat phenotype as involvement of SERCA would predict.

The conditioning temperature pulse induced significant effects on FR, with the most striking change in strain 170, the most severe of the three alleles (Table 1). Heart beat frequency dropped in all alleles below that of wild-type flies treated in the same manner ($WT > all = 295 > 170$). WT flies remained essentially unchanged after the heat pulse ($F=0.03$, $P=0.86$) while 170 had FR significantly lowered ($F=130$, $P=0.0001$). FR in 295 was also lowered significantly ($F=4.5$, $P=0.035$). AL1 remained unchanged ($F=2.01$, $P=0.16$). Both the interval between beats and the duration of each individual beat (systole) were lengthened in animals with reduced heart rate (Fig. 2a). Taken in sum, these data demonstrate an essential function for SERCA in regulating the frequency of heartbeat in *Drosophila*.

Regularity of beat was also reduced in all three mutant strains, with the most severe reduction in 170, while WT was unaffected (WT: $F=1.2$, $P=0.27$; AL1: $F=8.87$, $P=0.0033$; 295: $F=16.34$, $P=0.0001$; 170: $F=75.2$, $P=0.0001$) (Table 1). However, animals that showed no recognizable heartbeat in their raw data records were not included in these calculations, and, importantly, there were fewer animals with recognizable heartbeat after pulsing in the mutant strains compared to wild type. WT showed no reduction in animals with beating hearts as a result of the pulse, while 295 had a 3% reduction, AL1 lost 27%, and the severe 170 strain lost 41%. In this regard, MESA spectra are not only useful for reporting FR, but are generally representative of rhythmicity of heartbeat, and clearly emphasize this difference. Figure 2b shows representative spectra from WT and 170 animals with and without a heat pulse. As shown by the arrowheads, multiple peaks in spectra from the heated 170 animals indicate highly arrhythmic heart beat which is quantified by the RI statistic. Thus SERCA perturbation has clear effects on both FR and RI of heartbeat.

The cellular origin of these defects could either be in the cardiomyocytes themselves or potentially in the

Table 1 Table of frequency (FR) and rhythmicity index (RI) values for various genotypes recorded without or with a heat pulse to 41°C

Genotype	20°	25°	30°	35°	37°	Mean across temp
No Temperature Shock						
CS	2.0 ± 0.1	2.4 ± 0.1	2.8 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	^A 2.8 ± 0.07
	0.45 ± 0.06	0.48 ± 0.04	0.43 ± 0.05	0.37 ± 0.05	0.33 ± 0.05	^B 0.41 ± 0.02
	19	20	19	20	20	98
All	1.8 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	^B 2.4 ± 0.05
	0.35 ± 0.05	0.51 ± 0.05	0.53 ± 0.05	0.51 ± 0.05	0.46 ± 0.04	^A 0.47 ± 0.02
	20	20	20	20	20	100
295	1.4 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	^C 1.9 ± 0.05
	0.37 ± 0.05	0.53 ± 0.05	0.58 ± 0.04	0.55 ± 0.05	0.54 ± 0.06	^A 0.52 ± 0.02
	16	19	20	20	20	95
170	1.5 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	^C 2.0 ± 0.05
	0.44 ± 0.04	0.51 ± 0.04	0.51 ± 0.03	0.40 ± 0.03	0.39 ± 0.03	^A 0.45 ± 0.02
	19	20	20	20	20	99
Temperature shocked						
CS	2.0 ± 0.1	2.4 ± 0.1	2.8 ± 0.2	3.3 ± 0.1	3.3 ± 0.1	^A 2.8 ± 0.07
	0.40 ± 0.05	0.38 ± 0.05	0.40 ± 0.05	0.33 ± 0.05	0.37 ± 0.05	^A 0.38 ± 0.02
	19	20	20	19	20	98
All	1.6 ± 0.2	1.8 ± 0.1	2.2 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	^B 2.3 ± 0.07
	0.24 ± 0.08	0.37 ± 0.07	0.39 ± 0.08	0.36 ± 0.06	0.40 ± 0.04	^A 0.37 ± 0.03
	8	12	15	19	19	73
295	1.7 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	^B 2.1 ± 0.06
	0.29 ± 0.04	0.40 ± 0.05	0.43 ± 0.05	0.41 ± 0.05	0.40 ± 0.05	^A 0.39 ± 0.02
	14	18	20	20	20	92
170	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.02	1.2 ± 0.2	1.7 ± 0.3	^C 1.0 ± 0.09
	0.17 ± 0.07	0.25 ± 0.07	0.18 ± 0.08	0.20 ± 0.06	0.13 ± 0.03	^B 0.19 ± 0.03
	11	11	11	11	14	58

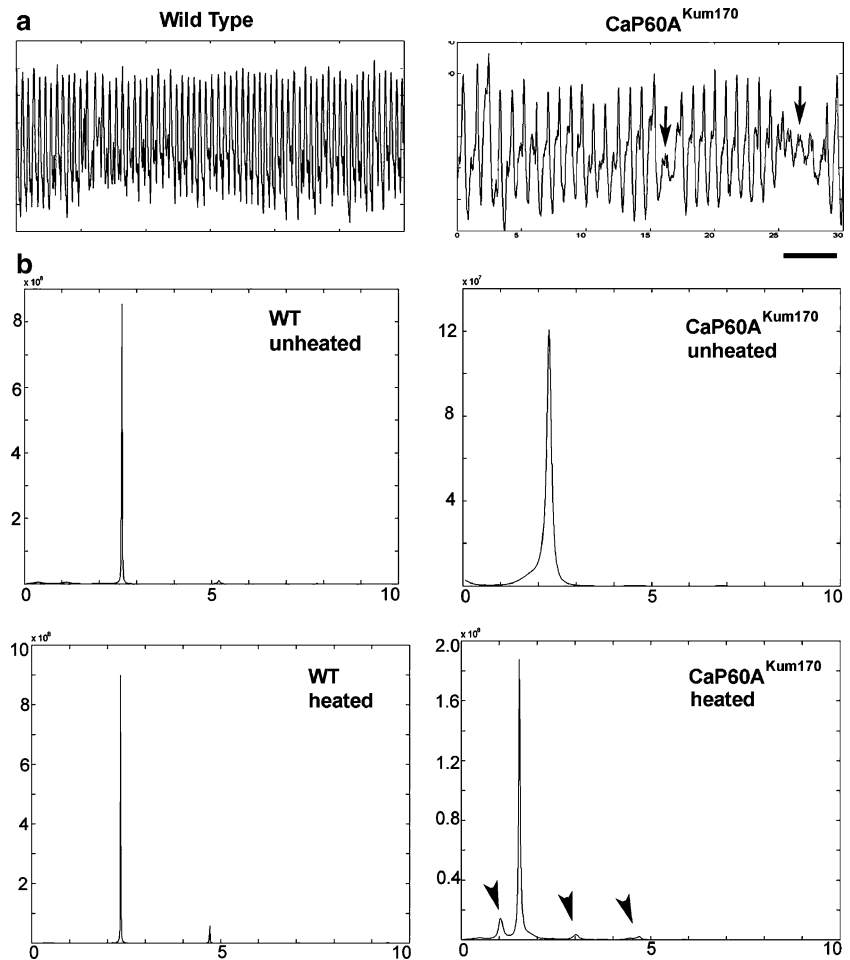
The first line in each cell contains FR ± SEM, the second contains RI ± SEM, and the third notes the number of recordings at that temperature that showed functioning hearts. The rightmost column contains data summed across all temperatures. Means that share a superscript are not significantly different. The heat pulse regimen, when applied, is shown schematically above the table. All mutant alleles had lower FR than WT with that of 170 being the worst. Two mutant SERCA alleles (295 and 170) in the heat-shocked group had reduced FR compared to the unpulsed group, and the third (All) had a lower FR than WT after the pulse but was not significantly changed. Wild type flies were unchanged by the heat treatment. The heat pulse did not alter regularity of beat (RI) in wild-type, but it was reduced in all three strains in the heat-shocked group. The number of temperature X animal cells that contained recognizable heartbeating was reduced to an extent in all the mutant strains in the heat shocked group, as indicated by N

neurons that innervate these muscles, although it is important to note that the pupae have hearts without innervation (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 mutant 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). Preliminary experiments using a collection of GAL4 drivers that express in neurons, muscle or all tissues revealed that strong muscle expression of the mutant transgene gives rise to discernible cardiac defects (Sanyal et al. 2005 and data not shown). For instance, when the transgene was expressed using a pan-neuronal driver (*elav^{C155}-GAL4*; Robinow and White 1988), no changes in heartbeat were observed. Thus, 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 170 flies. Flies showed a significantly lower heart rate after being heat pulsed ($F=10.8$, $P=0.01$). As with the *CaP60A^{Kum170}* mutant strain, the number of rhythmic animals was reduced by 50% post pulse. 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.

Further to investigate the effects of heating on the hearts of WT, mutant and transgenic flies, we employed an extended temperature protocol (Materials and Methods). WT, 170, and doubly transgenic flies carrying *UAS-CaP60A^{Kum170}* along with the *mef2-GAL4* driver (TR) were tested in this manner. This appeared to be a gentler procedure, as all flies had restored heart function after the test as affirmed by simple observation. When tested across all temperatures, genotype had an effect on FR ($F=13.2$, $P=0.0001$) (WT: 2.2 ± 0.08 = TR: 2.0 ± 0.08 > 170: 1.6 ± 0.07 ; $N=100$ cells for each strain). RI was similarly affected ($F=4.1$, $P=0.017$) (WT: 0.38 ± 0.02 = TR: 0.37 ± 0.02 > 170: 0.29 ± 0.02). Both FR and RI results thus corroborate the findings of the earlier protocol. 170 was significantly lower than either WT or TR, with TR intermediate, however neither FR

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 s.* **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



or RI of TR were significantly lower than WT in this test.

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. For this, we first demonstrated that larval hearts, similar to pupal hearts, also show dramatic effects of SERCA perturbation (Fig 3a). While heated dSERCA mutants displayed profound alterations in beat frequency, both wild type animals and another temperature sensitive paralytic mutation, *comatose*, showed no changes following an identical heating regimen (5 min at 40°C). We selected *comatose* because upon exposure to restrictive temperatures, these larvae display prolonged paralysis and are thus phenotypically somewhat

similar to the SERCA mutants. Typically, the heart of a dissected larva continues to beat for up to 30 min 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 (Fig. 3c shows representative recordings from two animals in each category). Although dissected larvae display greater variability than pupal hearts, it was obvious that beats in the mutants typically became infrequent and erratic with no discernible periodicity. While the amplitude of beats remained comparable (it is impossible to accurately measure beat amplitude with extracellular recordings), individual beats appear slowed down. These changes correlate well with observed changes in the physical beating of the heart. Taken together, these experiments provide compelling evidence that dSERCA dependent calcium sequestration directly affects excitability and permits repetitive contractions of the fly heart.

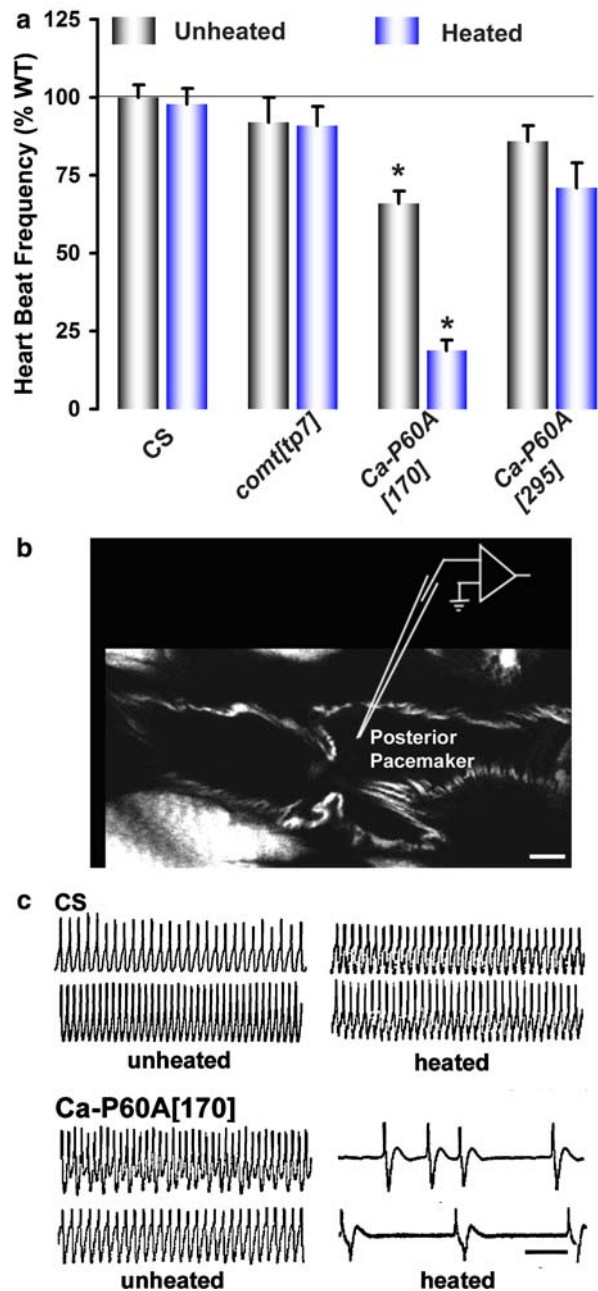
Fig. 3 SERCA regulates electrical activity in the heart pacemaker. **a** Effect of heating on larval hearts from canton-S, *comt^{tp7}*, *Ca-P60A^{Kum170}* and *Ca-P60A^{Kum295}*. SERCA mutant hearts, particularly *Ca-P60A^{Kum170}*, show reduced beat frequency that is further lowered upon heating. Heart beat is manually measured in intact animals with or without treatment (heating at 40°C for 5 min). A temperature sensitive paralytic mutation, *comatose*, displays unaltered heart beat even when the animal is paralyzed. *comatose* animals were selected since they show prolonged paralysis like dSERCA mutants. **b** Schematic of recording configuration. Extracellular recordings are made from the posterior contractile elements (pacemaker) of the larval heart. **c** 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. Representative recordings from two different animals for each category are shown. There is considerable variability in these recordings, however, the effect of heat on the SERCA mutant hearts is unequivocal. In all cases, frequency is severely altered with several hearts stopping for extended periods of time. Though beat amplitudes cannot be measured accurately from extracellular recordings, visual inspection indicated essentially unaltered beat amplitudes. Beat durations in heated mutants were also typically prolonged. Scale 5 s

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, 1997, 2000, 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 (Fig. 1). Specifically, SERCA shows strong co-localization with alpha-actinin, an integral component of muscle Z-line (Fig. 1d). Similar specialized zones of sarcoplasmic reticulum have been previ-



ously described in vertebrates (Drago et al. 1998). 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 noteworthy. 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*^{K^{um}170} mutants show clearly reduced FR that is further aggravated upon incubation at restrictive temperatures coupled with a reduced number of rhythmic animals. This is seen in the two other independently isolated alleles, but to a less pronounced degree, and FR is unaffected in A11. Both the interval between two subsequent beats and the duration of each beat are increased. Accompanying reduced heart rate, the hearts of all three mutant alleles beat less regularly after being heat shocked with the 170 allele again being most profoundly affected. The strong temperature-dependence of both the FR and RI phenotypes 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.

The possible role of SERCA is best discussed against the background of a model for *Drosophila* pacemaking formulated by Johnson et al. (1998) (expanded and discussed in Bodmer et al. 2004; Ray and Dowse 2005). The pacemaker is envisioned to involve a generic leak current which slightly depolarizes the plasma membrane. This triggers a strongly depolarizing Ca²⁺ spike shown to be necessary for pacemaking in the fly (Dowse et al. 1995; Gu and Singh 1995; Johnson et al. 1998). This spike may rely on cooperative activity of two channels, one a T-type (Tiny and

transient) contributed by an as yet to be identified channel, followed by a more substantial inward Ca²⁺ current admitted by the channel encoded by the *cacophony* gene (Ray and Dowse 2005). This interaction is similar to that seen in vertebrates (Hagiwara 1988). The Ca²⁺ channels are likely to inactivate as a result of increased cytosolic Ca²⁺ concentration (cf: Peterson et al. 2000). Recent work indicates that release of Ca²⁺ into the cytosol from sarcoplasmic reticulum stores via ryanodine receptors is critical to pacemaking in vertebrates (Rigg et al. 2000; Terrar and Rigg 2000; Bers 2002), with evidence from *Drosophila* as well (Sullivan et al. 2000 E. Johnson and H. Dowse, unpublished results). Indeed, tight regulation of cytosolic Ca²⁺ level may be one of the most critical mechanisms in vertebrate pacemaker function and regulation, involving additionally an interaction with a cell surface Na⁺-Ca²⁺ exchanger (e.g. Lakatta et al. 2002). Repolarization is by a K⁺ current triggered by increased Ca⁺ concentration through a *slowpoke*-encoded Ca⁺-gated K⁺ channel. A delay in the restoration of low resting calcium levels after a sharp rise during pacemaker cycling, owing to defective internal sequestration by SERCA would severely impact pacemaker function. 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). Also of direct interest is work related, as discussed above, to the control of cytosolic Ca²⁺ concentration. It has been shown that unloading of Ca²⁺ stores in the rabbit sarcoplasmic reticulum (in this case by upregulating the Na⁺-Ca²⁺ exchanger) results in ventricular arrhythmias and contractile dysfunction (Pogwizd et al. 2001; Bers 2002). 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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