Roles of the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase, Plasma Membrane Ca^{2+}-ATPase and Na^{+}/Ca^{2+} Exchanger in Regulation of Heart Rate in Larval *Drosophila*

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Abstract: We investigated the roles of three regulatory proteins that impact $[Ca^{2+}]_i$ within cardiac myocytes of *Drosophila melanogaster*. The NCX (Na^{+}/Ca^{2+} exchanger), PMCA (plasma membrane Ca^{2+}-ATPase) and SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) were compromised by ionic, pharmacological, mutational-manipulation, and with a combination of approaches, while heart rate (HR) was monitored. A decrease in SERCA function reduced HR more for intact larva in comparison to a dissected larva. Dissected preparations were used to expose the heart directly to agents. A compromised PMCA also reduced HR; however, attenuated NCX function by low $[Na^{+}]_o$ increased HR. KBR7943, a blocker of Ca^{2+} entry via NCX, exposure increased HR. A combined loss of function in all three channels did not show a significant change in HR. The results indicate that NCX and PMCA are important in regulating HR, whereas SERCA does not have as pronounced role for dissected preparations. However, with intact preparations the loss of SERCA function by a mutation does have a significant impact on HR. Pharmacological approaches to alter PMCA and SERCA paralleled the results obtained by ionic and mutational approaches. To further understand the pacemaker activity, intracellular recordings were obtained. Mapping of action-potentials in myocytes revealed that the caudal region of the heart has large amplitude potentials and is likely to contain the pacemaker cells. The *Drosophila* heart can serve as a genetic model in understanding regulation of ionic currents for pacing cells of various types.

Keywords: NCX, PMCA, SERCA, heart, calcium, heart rate.

INTRODUCTION

Insects like vertebrates use a heart to promote circulation of the hemolymph or blood. The *Drosophila melanogaster* cardiac muscle is similar in certain functions that are relevant to model cardiac function and disease states in other animals including humans [1]. Besides being a strong model for genetic studies of deleterious genes or malformations for development in morphology, *Drosophila* also serve as a good model for physiological function at a cellular level. A number of ion channel mutations discovered in mammals also have homology with ones found in skeletal muscle and neurons of *Drosophila*. Disease states can be mimicked in the tractable genetic organism of *Drosophila melanogaster* [1-6]. There is little known about the Ca^{2+} regulation in the *Drosophila* heart, as only a few studies have used the larval heart in this regard.

Here, we have made an attempt to elucidate the role of the different Ca^{2+} ion regulators namely the plasmalemmal Na^{+}/Ca^{2+} exchanger (NCX), the Ca^{2+}-ATPase (PMCA) as well as the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) on the endoplasmic reticulum on larval heart rate (HR).

A majority of studies on *Drosophila* heart have been targeted toward development in the embryo [7-10] and physiological function in the pre-pupal, pupal or adult stages [11-16]. The pupal stage is very dynamic during metamorphosis since hormones and biogenic amines are fluctuating and the heart is undergoing structural transformation. Thus, this stage is hard to control for variables while investigating physiology of the myocytes [17-20]. Likewise, since the adult heart is modulated by neuronal inputs, this complicates addressing the function of the intrinsic cardiac pacemaker and ionic regulation in the intact heart [17,21]. The heart is known to be myogenic in the larval stage and can readily be removed or left *in situ* while being bathed by a defined physiological saline to limit compounding variables such as hormones or neural modulation [2, 22-25]. The myogenic nature of the larval heart is comparable to the mammalian heart in that there are pacemakers that drive the rest of the heart to pump fluid in a direction to be effective in bathing organs, despite the fact that *Drosophila* has an "open" circulatory system. The chronotropic and inotropic nature of the *Drosophila* larval heart could serve as a rapid means to test fundamental principles and pathological effects of mammalian heart function as well as help in developing a comparative model for cellular physiology such as ionic regulation of pacemaker cells.

The *Drosophila* heart, known as the dorsal vessel, is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. The heart is divided into anterior aorta and posterior heart [22,26]. There are a variety of cell types in the heart and they are differentially regulated [10,13,27]. The HR varies throughout larval stages depending on whole animal activity (feeding and crawling) and the HR tends to slow down during pupation [15,28]. The larval heart is very susceptible

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to biogenic amines and peptides which could vary in the hemolymph depending on food source or intrinsic state of the animal [22,24,25,29,30].

The mechanism of action for neurotransmitters and cardiac modulators on the larval heart has not been described at the cellular level to date. This is due to the fact that there has not been a sufficient understanding of the ionic currents and channel types present in larval heart that contribute and regulate pacemaker activity. This highly tractable genetic system and potential model for human diseases can be utilized to study the significance of calcium regulation in myocytes with regard to combined importance of the NCX, PMCA and the SERCA. A number of ailments due to dysfunction of mammalian atrial pacemaker cells and myocytes, in general, are known to be related to an altered function in one or more of these calcium regulatory processes [31-33].

The general notion is that the NCX has a low affinity but high capacity for Ca^{2+} turnover, whereas the PMCA has a high affinity but a low capacity. The NCX comes into play with large $[Ca^{2+}]_i$ and the PMCA fines tunes $[Ca^{2+}]_i$ [34]. As noted in a recent review [35], inhibiting NCX can improve function in mammalian heart failure, while other studies demonstrated overexpression of NCX decreases the progression of systolic and diastolic contractile dysfunction. This implies that the approach to manipulate the NCX function at expression level by gene therapy for human heart failure requires further investigation. Even the role of the PMCA and density of expression are not understood in regulation of pacemaker activity for mammalian cardiomyocytes. The current view is that the PMCA does not have a significant role in the excitation-contraction coupling of cardiomyocytes [36], but is presumed to have an important role in the development of the heart [37,38]. On the other hand, the SERCA plays a role as providing an ionic current that can shape the membrane action potential and contractility [39-41]. The density of the SERCA is known to regulate the rate of muscle relaxation-contraction by determining the extent of removing $[Ca^{2+}]_i$ in heart muscle and also in slowly and rapidly contracting skeletal muscle cells [42].

The scheme of ionic currents within a cardiac cycle for a mammalian pacemaker cell (i.e., SA node) is generally described with the background $[Ca^{2+}]_i$ continually increasing and decreasing. Starting in diastole, depolarization and a slow release of Ca^{2+} from the sarcoplasmic reticulum (SR) by ryanodine receptors (RyR) leads to a rise in $[Ca^{2+}]_i$. The SERCA pumps Ca^{2+} back into the SR and the NCX removes $[Ca^{2+}]_i$ in exchange for Na^+ ions across the plasma membrane of the cell. The influx of Na^+ ions leads to a depolarization of the plasma membrane, thus opening low voltage-gated T-type Ca^{2+} channels (V_{Ca}) [43] and potentially voltage-gated Na^+ channels. The influx of Ca^{2+} acts on the RyR (ryanodine receptors) to cause the ER (endoplasmic reticulum) to dump Ca^{2+} which results in a calcium induced inhibition of the RyR. Until the $[Ca^{2+}]_i$ is reduced by the SERCA and NCX, the RyR stays inhibited but will start leaking Ca^{2+} as $[Ca^{2+}]_i$ returns to a low level to then repeat the cycle [44]. In the mammalian heart, the sinus node cells do not contain a K^+ current (I_{K1}) which is thought to be one reason the pacing cells do not show a resting membrane potential [45]. However, we speculate based on gross anatomy of the *Drosophila* heart [46] that the pacing cells act as contracting myocytes and that they can also generate action potentials. This would suggest these heart cells likely have pronounced voltage-gated Na^+ currents. This remains to be examined as larval skeletal muscle appears to lack voltage-dependent sodium channels. The influx of Na^+ as well as Ca^{2+} must be tightly regulated to prime the cell for the next cycle. In short, as in mammalian hearts [47,48], the NCX and SERCA in *Drosophila* likely have major roles in the cardiac electrical activity of the pacemaker and are key in coordination with each other [49-51]. It has been recently proposed that two clocks actually regulate pacemaker function. One clock is comprised of the membrane voltage-gated ion channels (M clock) and the other is a Ca^{2+} clock regulated by the sarcoplasmic reticulum [52].

The approach used in this study was to compromise the NCX, PMCA, and SERCA individually and then in combination, to observe the interplay of the three main contributors of intracellular calcium regulation on pacemaker activity and contractility. We also used a heat shock sensitive fly line (Kum^{170}) developed by Sanyal et al., [53, 54] to determine the role of the SERCA when it is made nonfunctional. Learning more about the underlying ionic currents that shape the action potentials in myocytes in various species, one can hope to get a handle on the known ionic dysfunctions associated to specific genes responsible for various diseases [16,55,56].

METHODS

Staging of the Flies

Canton S (CS) flies, a wild type of *Drosophila*, were used for comparison to Kum^{170TS} (referred to as Kum from now on) [54]. Both strains were maintained on a standard cornmeal-dextrose-agar-yeast medium. The Kum^{170TS} strain was provided by Dr. Sanyal (Emory University, School of Medicine). To obtain staged larvae, flies were pulsed to lay eggs for 2 hours to clear eggs in the oviduct. Then newly laid eggs were incubated at either 18°C, 21°C or 28°C.

Monitoring Heart Rate in the Intact larva

A detailed methodological approach is available in video format online [57]. In brief, tracheal movement is commonly used to monitor *Drosophila* larval HR because of the clear contrast of the structures [22,58,59]. Early 3rd instar larvae were glued ventrally on a glass cover slip using super-glue in such a way that mouth hooks are free to move. Care was taken not to glue the spiracles so that only the ventral aspect within the mid-length was adhered to the glass. Food was placed over their head as to keep the larva busy eating [60]. The larvae were glued down and HR was measured for 1 min. For heat shock experiments, the larva and glass cover slip were placed in a glass Petri dish that floated on top of water in a temperature controlled water bath.

Monitoring Heart Rate in the Dissected Larva

The general dissection technique and HL3 saline content has been previously reported [22,58]. The HL3 saline was derived from direct measures with ion sensitive electrodes of

larval hemolymph. This saline maintains normal function of larval neuromuscular junctions and the CNS [61]. In brief, the HL3 saline was prepared from component reagents (Sigma) and contained: 1 mM $CaCl_2.2H_2O$, 20 mM $MgCl_2$, 70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM BES (N,N-bis [2-Hydoxy-ethyl] -2- aminoethane-sulfonic acid). The HL3 was freshly controlled for pH and temperature prior to experimentation, as the pH will drift during storage. In experiments where the $[Na^+]_o$ was reduced, the HL3 saline was made with TRIZMA base and the pH was adjusted with HCl (1 M) in order not to alter the $[Na^+]_o$ by adjusting pH with NaOH.

The modifications to the HL3 saline for specific experiments are described in the 'Results'. For heat shock experiments, the dissected larvae were pinned out on the dissection dish covered with the HL3 saline and placed on a glass Petri dish that floated on top of water in a temperature controlled water bath. Within the time frame used in these experiments no changes in pH of the HL3 saline were noted during the heat shocks.

Varying External Calcium Concentration

Varying the $[Ca^{2+}]_o$ to higher levels in order to load the myocytes with Ca^{2+} during electrical depolarization and contraction allowed us to examine how the NCX, PMCA and SERCA would process the increased $[Ca^{2+}]_o$ load placed on them. The basal HR was monitored in both CS and Kum lines while the bathing media was exchanged to increasing $[Ca^{2+}]_o$ (1, 2, 4, and 8 mM). Each concentration was allowed to bathe the preparation for 1 min prior to recording the HR, and exchanging the media for the next higher concentration.

Chemicals

All chemicals were obtained from Sigma chemical company (St. Louis, MO) with the exception of thapsigargin and KB R7943 (Tocris; Ellisville, MO, USA) and 5,6-Carboxyeosin (Marker Gene Technologies Inc.; Eugene, OR, USA).

Intracellular Recordings

Intracellular recordings were performed in cardiomyocytes with a 30-60 MΩ resistance, 3 M KCl-filled microelectrode. The amplitudes of the depolarizing potentials and the resting membrane potential were monitored with a 1 X LU head stage and an Axoclamp 2A amplifier. Various regions of the heart and the aorta were surveyed for electrical events. Electrical signals were recorded on-line to a PowerLab/4s interface (ADInstruments, Australia) at an acquisition rate of 20 kHz. Some preparations were dye filled by pressure injection of Texas Red-dextran 3,000 KD (Molecular Probes; Eugene, OR). Blebbistatin (10 μM; Sigma) was used in some preparations to stop muscle contraction while recording electrical events. Since it is suggested that blebbistatin is a light sensitive compound [62] we tried to minimize light exposure as much as possible. There is little likelihood of neural innervation on the true heart region, but there could be some neural influence in the aortic region. Our lab documented neural endings in the very distal aorta which might be the beginning of neural innervation in the 3rd instar [63].

Statistical Analysis

Depending on the experimental design either a Student's *t*-test (paired or unpaired) or a two-way ANOVA followed by various *posthoc* analysis were used. Some data is presented as a percent change to compare an experimental manipulation or differences over time as explained within the Results section for each given experiment.

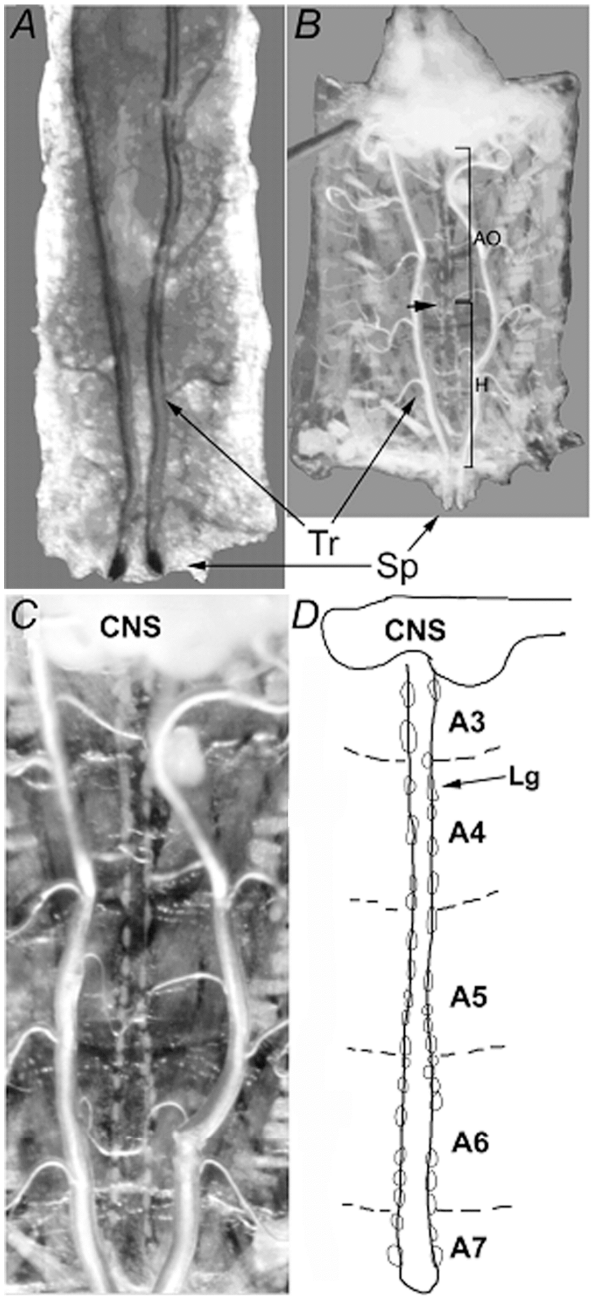


Fig. (1). (*A*) Dorsal view of an intact 3rd instar *Drosophila* larva. The movement of trachea due to pulling of the attachments from heart is used to observe the heart rate. (*B*) Ventral dissection of a 3rd instar to view the heart directly. Pinning of the animal on its back after dissection is used to directly apply the compounds on the heart with or without the CNS intact. The arrow indicates the division of the heart and aorta. (*C*) Enlarged view of the *in situ* heart tube and (*D*) a schematic view of the heart and aorta as shown in (*C*) for the larval segments. The (Tr, Trachea; Sp, Spiracles; H, Heart; AO, Aorta; Lg, lymph gland).

RESULTS

Heart Rate Recording

The anatomical arrangement of the heart tube in larvae is very conducive for monitoring heart rate within the intact animal since it can readily be observed on the dorsal midline (Fig. **1A**). The exposed preparation is also anatomically advantageous since one can readily expose the heart tube by ventral dissection of the animal. Exposing the heart is essential in order to examine the effects of pharmacological agents and to directly measure the electrophysiological properties of myocytes. The internal organs are easily removed in a filleted larva that is pinned with the dorsal side down (Fig. **1B**). The division between the heart and the aorta can be easily seen by the narrowing of the heart (Fig. **1C**). The lymph glands provide a good demarcation of the heart tube for aiding in monitoring heart rate. The heart and aorta are outlined in Fig. (**1D**) for clarification.

The first set of experiments were to examine if the HR within the intact animal is substantially different in the Kum strain as compared to controls (CS strain) at room temperature and following heat shock (41°C) for 5 min. We also tested if there is a difference in recovery after heat shock by measuring HR again 30 min after the animals were returned to room temperature. In these experiments, changes within individual larvae were monitored for statistical analysis as a percent change. There is a wider degree of variation of the Kum group (Fig. **2B1**) as compared to the controls (Fig. **2A1**, Canton-S; CS) after heat shock (HS) (coefficient of variation: for CS= 0.19; CS after HS= 0.26; CS 30 min after HS = 0.14; Kum= 0.13; Kum after HS= 0.53; Kum 30 min after HS= 0.4). Comparing the percent difference of the initial value to heat shock for both groups, there is a greater decrease in the Kum larvae than for controls (Fig. **2C1**; $P<0.02$; Student's un-paired *t*-test). There is also a significant decrease in HR in both fly lines after heat shock. (Fig. **2C1**, P<0.001 for CS and Kum, Student's paired *t*-test).

The whole larvae assay of Kum and controls allowed us to examine the effect of an altered SERCA function on HR. However, to address the role of the PMCA and the NCX, the heart has to be exposed so that the bathing media can be exchanged. Since dissection and exposure to the specially designed *Drosophila* physiological saline (e.g. HL3 saline) could have consequences on the initial HR, the rate was measured before and after heat shock as performed for intact larva with the exception that the larvae were dissected and bathed in HL3 saline.

Exposing the *in situ* heart to saline resulted in increase in the variation of initial HR in both control and Kum larvae. In addition, the initial rate is lower than for the intact larvae ($P<0.05$, Student's unpaired *t*-test, N=20; Fig. **2** top and bottom rows). During heat shock there was little change in the mean HR for the Kum strain which was different from intact animals (Fig. **2B1** and **2B2**). There was also a smaller

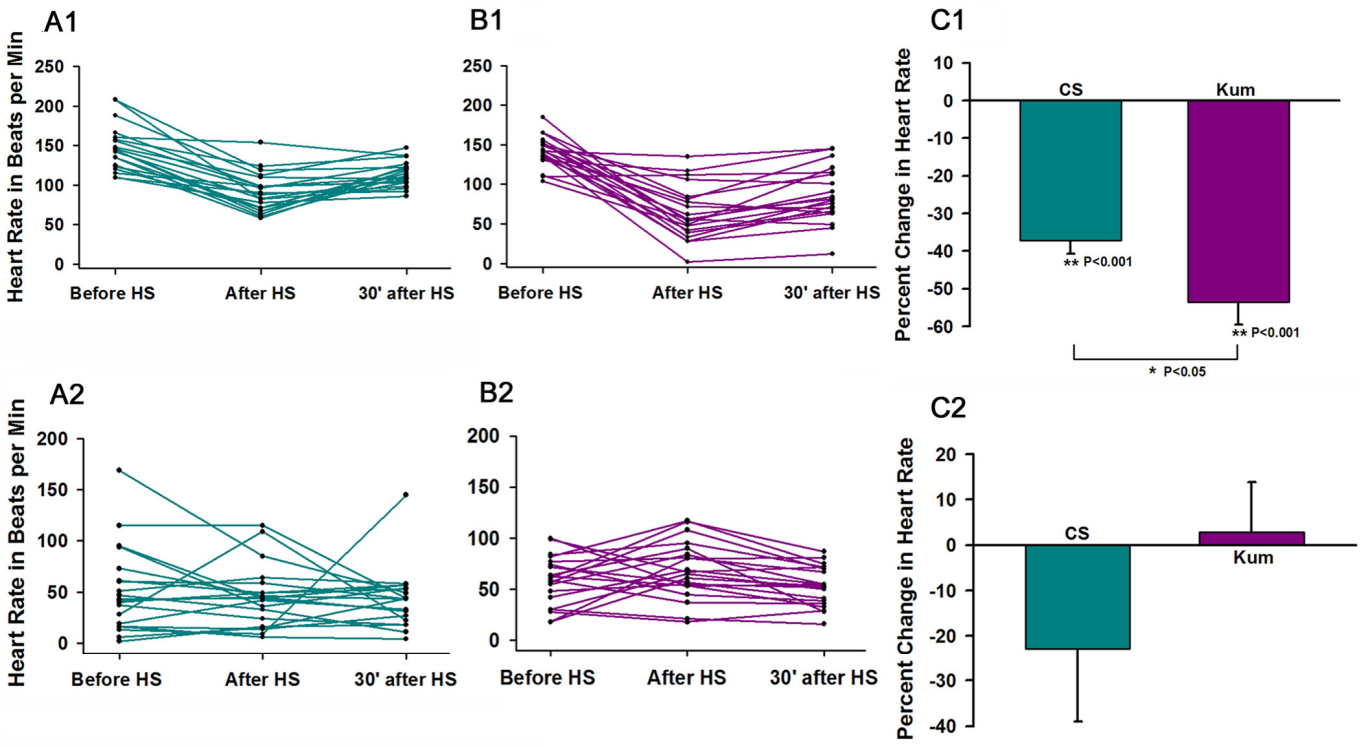


Fig. (2). (Top row) Effect of heat shock on HR for CS (A1) and Kum (B1) strains. HR in intact preparations of flies grown at 21°C (n=20 each) before heat shock, immediately after heat shock and 30 min following heat shock (recovery).Instead of highlighted text: In the figures blue lines/bars indicate CS flies and purple lines/bars indicate Kum flies. There is a significant decrease in HR of both CS and Kum flies after heat shock ($P<0.001$ for both CS and Kum, Student's paired *t*-test, n=20 for each set). Moreover the Kum has a significantly greater drop in HR than the CS after heat shock (C1; $P<0.05$, Student's unpaired *t*-test). (Bottom row, A2 and B2) HR in dissected preparations of flies grown at 21°C and bathed in HL3 saline. HR in intact preparations of flies grown at 21°C (n=20 each) before heat shock, immediately after heat shock and 30 min following heat shock (recovery). (C2) The percent change in HR rate after heat shock (not significant; paired and unpaired Student's *t*-tests; n=20 for each CS and Kum sets).

reduction of HR upon heat shock for the control in these exposed heart preparations as compared to intact control larvae (Fig. **2C1** and **2C2**). As a result of these changes the dissected preparations are not significantly different between the controls and Kum strain in the percent change during heat shock exposure.

Since the Kum strain is a heat shock line that reduces the SERCA function, room temperature (21°C) might be high enough to alter the function of SERCA protein while the larvae were developing. In order to address this potential effect, flies were raised at 18°C for 2 generations and these larvae were used to examine the effects of heat shock in intact as well as dissected (saline exposed) larval groups. The percent change to heat shock (41°C) for intact larvae is significantly different between control and the Kum strain when they were raised at 18°C (Fig. **3A**; $P<0.001$, Student's unpaired *t*-test, N=20). But no significant difference is noted after HS between larvae grown at 21°C and those grown at 18°C (P=0.55, Student's un-paired *t*-test). However, the dissected preparations, grown at 18°C, also show no significant difference between the controls and the Kum strain. Moreover the dissected controls and Kum larvae do not show any difference before and after HS (Fig. **3B**).

The effect of chronic exposure at the permissive temperature to activate the heat shock effect in the SERCA mutation was also tested by raising adult flies at 28°C and examining the larvae of their offspring. As in previous experimental paradigms, both intact and dissected preparations were examined for the effect of heat shock at 40°C. As for flies raised at the other temperatures, heat shock decreased HR in intact flies raised at 28°C ($P<0.001$ for both CS and Kum, Student's paired *t*-test, n=20 for both).

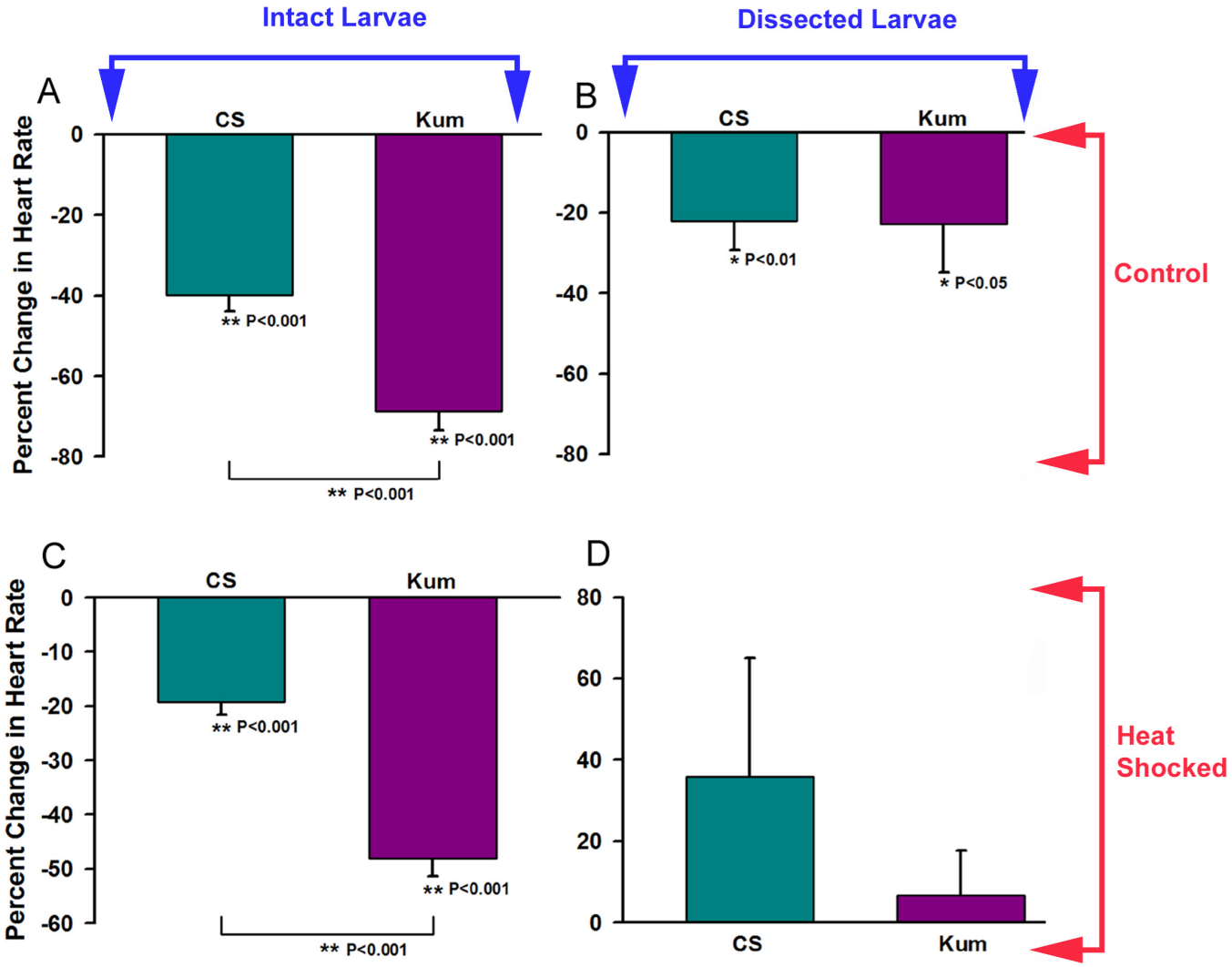
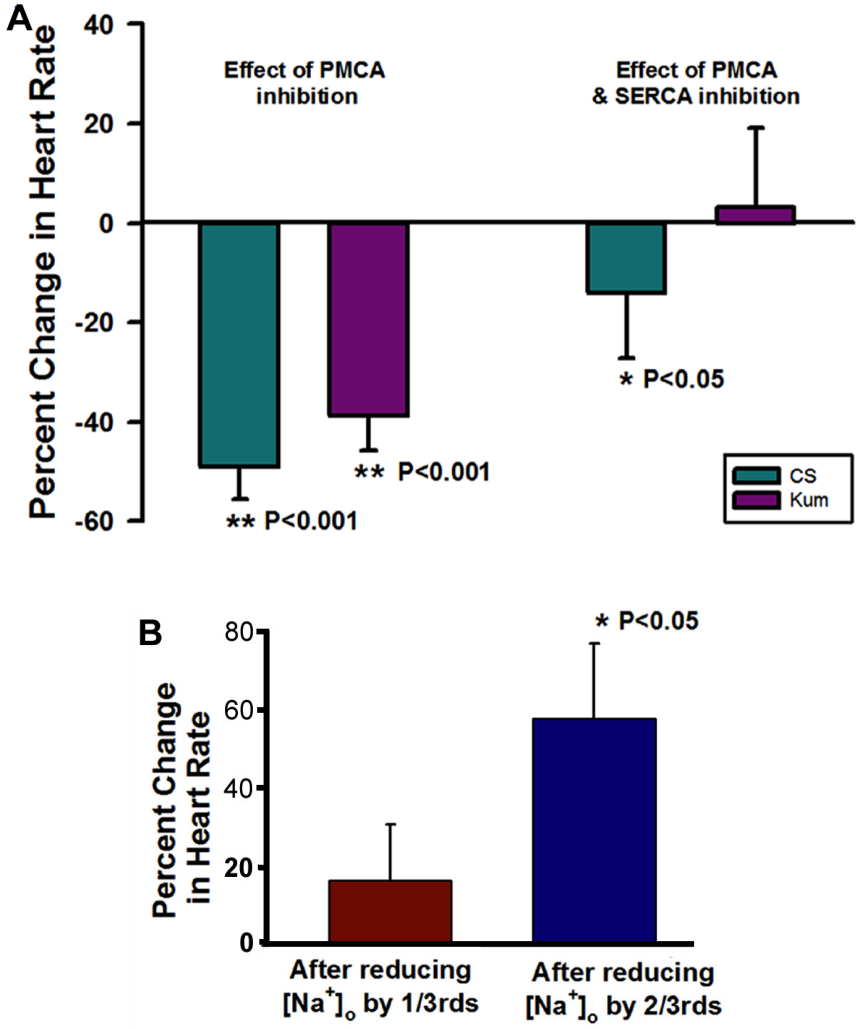


Fig. (3). Larvae cultured at 18°C or 28°C to address effects on HR for the temperature sensitive Kum. (A,B) The percent change in HR after heat shock for flies grown at 18°C: (A) intact preparations, (B) dissected preparations. A significant decrease in HR is present for CS and Kum flies after heat shock in both intact and dissected preparations (Student's paired t-test, n=20). A significant decrease is present in the Kum as compared to CS after heat shock in the intact preparations (A) ($P<0.001$, Student's unpaired t-test), where as there is no significant change in Kum compared to CS in the dissected preparations. (C, D) Effect of heat shock on HR for larvae cultured at 28°C (C) intact preparations and (D) dissected preparations. Here a significant decrease in HR is observed for both CS and Kum flies after HS in intact preparations (C) (Student's paired t-test, n=20) but not in the dissected preparations (D). Moreover there is a significant decrease in the HR in Kum flies as compared to the CS in the dissected preparations (C) ($P<0.001$, Student's unpaired t-test).

Moreover, this decrease in HR was significantly larger in intact Kum flies than in intact CS flies (Fig. **3C**) (P<0.001, Student's unpaired t-test). Heat shock failed to decrease HR in dissected preparations of Kum or CS flies reared at 28°C (Fig. **3D**).

To address the role of PMCA and SERCA on HR, a series of experiments were conducted to compromise one or both of these proteins. Only dissected preparations are feasible to block the PMCA as the bathing media either requires pharmacological agents introduced or adjusting the pH to 8.8. For this set of experiments, the larvae were raised at 21°C and dissected open. There was a significant reduction in HR for both controls and Kum (P<0.001; Student's paired t-test) when the bathing saline is changed from pH 7.2 to pH 8.8 (Fig. **4A**; first two bars). Moreover, there is no significant difference between controls and Kum at pH 8.8. The same preparations were heat shocked at 40°C for 5 min to combine the effects of blocking both the PMCA and the SERCA. There is a significant increase in HR for CS after heat shock as compared with exposure at pH 8.8 (P<0.05; Student's paired t-test Fig. **4A**, 1st bar and 3rd bar). However, the rate is lower than at pH 7.2. Thus, for the CS flies there is a greater decrease in HR with inhibiting the PMCA but the HS actually helped dampen the extent of the decrease. In the Kum flies, the HR returned to near normal rate (No significant change; Student's paired t-test) after the combined treatments. Furthermore, there is no significant difference between controls and Kum after the combined treatments intended to reduce the SERCA and PMCA function.

Since we addressed the effects of PMCA and the SERCA on HR, we next examined the role of the NCX on HR. To start off, we examined the effect of a compromised NCX by itself before combining a compromised PMCA and SERCA. The NCX is compromised by reducing the driving gradient of Na^+ into the cell by lowering the extracellular Na^+. A reduced $[Na^+]_o$ of 1/3rd and 2/3rd on HR was investigated. The NCX is compromised in its normal function (exchanging Na^+ into the cell for Ca^{2+}) when the driving gradients are altered. However, when $[Na^+]_o$ is reduced, the resultant gradient is also reduced resulting in increased $[Ca^{2+}]_i$ over time. The effect on HR can be addressed under short intervals or over longer periods. One issue with reduced $[Na^+]_o$ is that if the level is reduced drastically then the excitability of the myocytes might not be sufficient enough to allow the pacemaker potential to sustain function. Ca^{2+} enters the myocytes though voltage-gated calcium channels (Ca^{2+}_V). Thus, the cells need to be sufficiently depolarized in order to activate these channels. Even with 2/3rd decreased $[Na^+]_o$, the heart beat was vigorous. There was no significant increase in HR in 1/3rd reduced $[Na^+]_o$; however a 2/3rds reduction in $[Na^+]_o$ induced a significant



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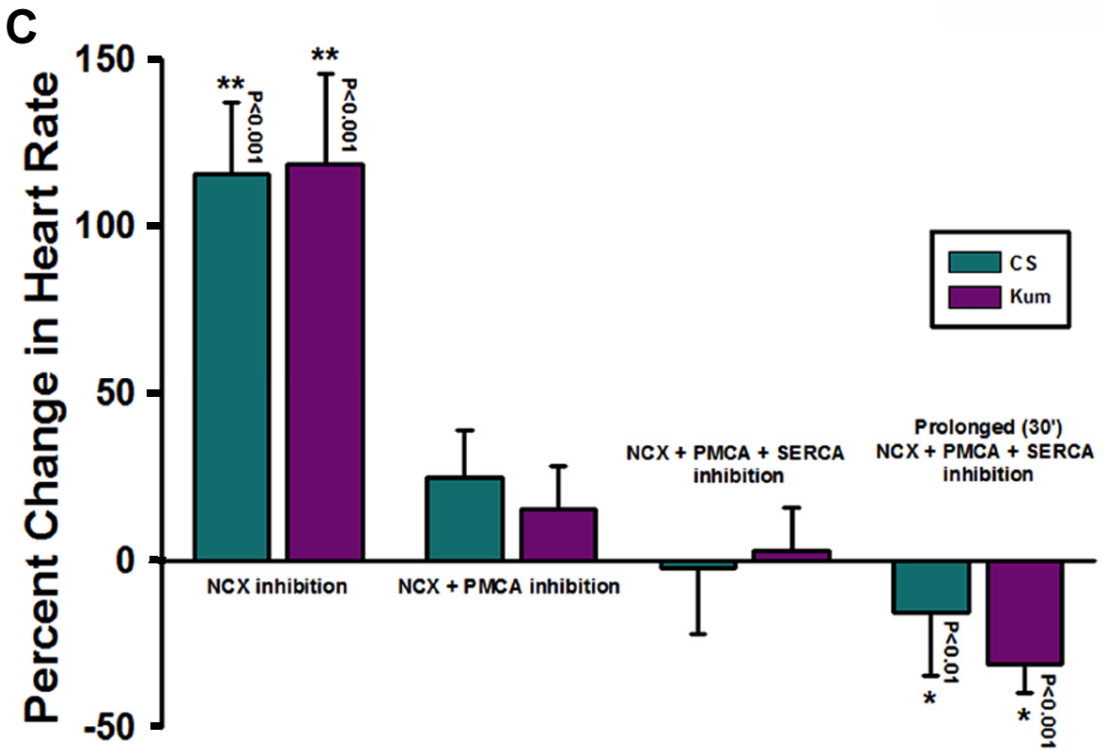

Fig. (4). The effect of inhibiting the PMCA, NCX and the combined effect of inhibiting the PMCA, SERCA and NCX on HR. (A) The first pair of bars, on the left, depicts the change in HR for CS (blue) and Kum (purple) after blocking the PMCA at pH 8.8. A significant decrease in HR occurs for both CS and Kum larvae after inhibiting the PMCA ($P< 0.001$ for both CS and Kum, Student's paired *t*-test, n=20). Only in the CS is the effect of reducing both the SERCA and PMCA significant (right set of bars, Student's paired *t*-test,). (B) Change in heart rate after reducing the $[Na^+]_o$ by 1/3rd and 2/3rds. There is a significant increase in heart rate when $[Na^+]_o$ is reduced by 2/3rd ($P<0.05$, Student's paired *t*-test, n=10). (C) The combined effect of inhibiting all three channels. The first pair of bars indicates the percent change in HR of CS and Kum on reducing the NCX function and as before there is a significant increase in HR for both. There is no difference between CS and Kum. The second set of bars indicates the change in HR when both the NCX and the PMCA are inhibited. Surprisingly CS and Kum are not significantly different. The third set of bars indicates the percent change in HR when the NCX, the PMCA and the SERCA are inhibited. There is no significant change in HR between CS and Kum and no significant effect as compared to baseline. The last set of bars shows the percent change in HR 30 min after heat shock in saline with low $[Na^+]_o$ and pH 8.8. There is no significant change in HR between CS and Kum but there is significant difference as compared to baseline (Student's paired *t*-test for comparing HR before and after treatment and Student's unpaired *t*-test for comparing between CS and Kum, *P* values as indicated in graph, n=20 for each set).

increase in HR (Fig. **4B**, $P<0.05$, Student's paired *t*-test, n=10).

To address the combined effect of inhibiting NCX, PMCA, and SERCA -all the three above mentioned methods were implemented. In dissected preparations, the NCX was compromised with 2/3rd reduced $[Na^+]_o$ saline. Then saline with 2/3rd reduced Na^+ and pH 8.8 was added to inhibit both the NCX and the PMCA. Lastly, the preparation with inhibited NCX and PMCA was heat shocked for 5 min at 41°C to also inhibit the SERCA (Fig. **4C**).

Varying External Calcium Concentration

Prior to examining the effect of altered $[Ca^{2+}]_o$ on HR, the variation in HR over time was investigated when exposed to 1 mM $[Ca^{2+}]_o$ for up to 40 min. Twelve larvae were monitored every 10 min and the distribution of absolute rates were compared. No significant differences were detected among the various time points over the 40 min period (data not shown).

$[Ca^{2+}]_o$ was increased to higher levels in order to load the myocytes with Ca^{2+} during electrical depolarization-contraction coupling to examine the response of NCX, PMCA and SERCA in processing the increased $[Ca^{2+}]_o$ load placed on them. The basal HR was monitored in both CS (Fig. **5A**) and Kum (Fig. **5B**) lines while the bathing media was exchanged with decreasing as well as increasing $[Ca^{2+}]_o$ (1, 0.5, 2, 4, and 8 mM; with 1 mM being the normal HL3 concentration). Each concentration was allowed to bathe the preparation for 2 min prior to exchanging the media to the next concentration. There is a substantial dose-dependent effect of increasing HR with rising $[Ca^{2+}]_o$ (ANOVA $P<0.05$; n=20) except when exposed to 8 mM (Fig. **5**; 12.23 ±13.15 % change for Ca^{2+}). Upon initial exposure to 8 mM the HR increased but subsequently decreased. In some cases, when exposed to 4 or 8 mM the heart would rapidly beat and flutter before slowing down. The HR was monitored for 2 min and an average beat per minute is reported. The same effect on HR was observed for the various $[Ca^{2+}]_o$ in both CS (Fig. **5A2**) and Kum (Fig. **5B2**).

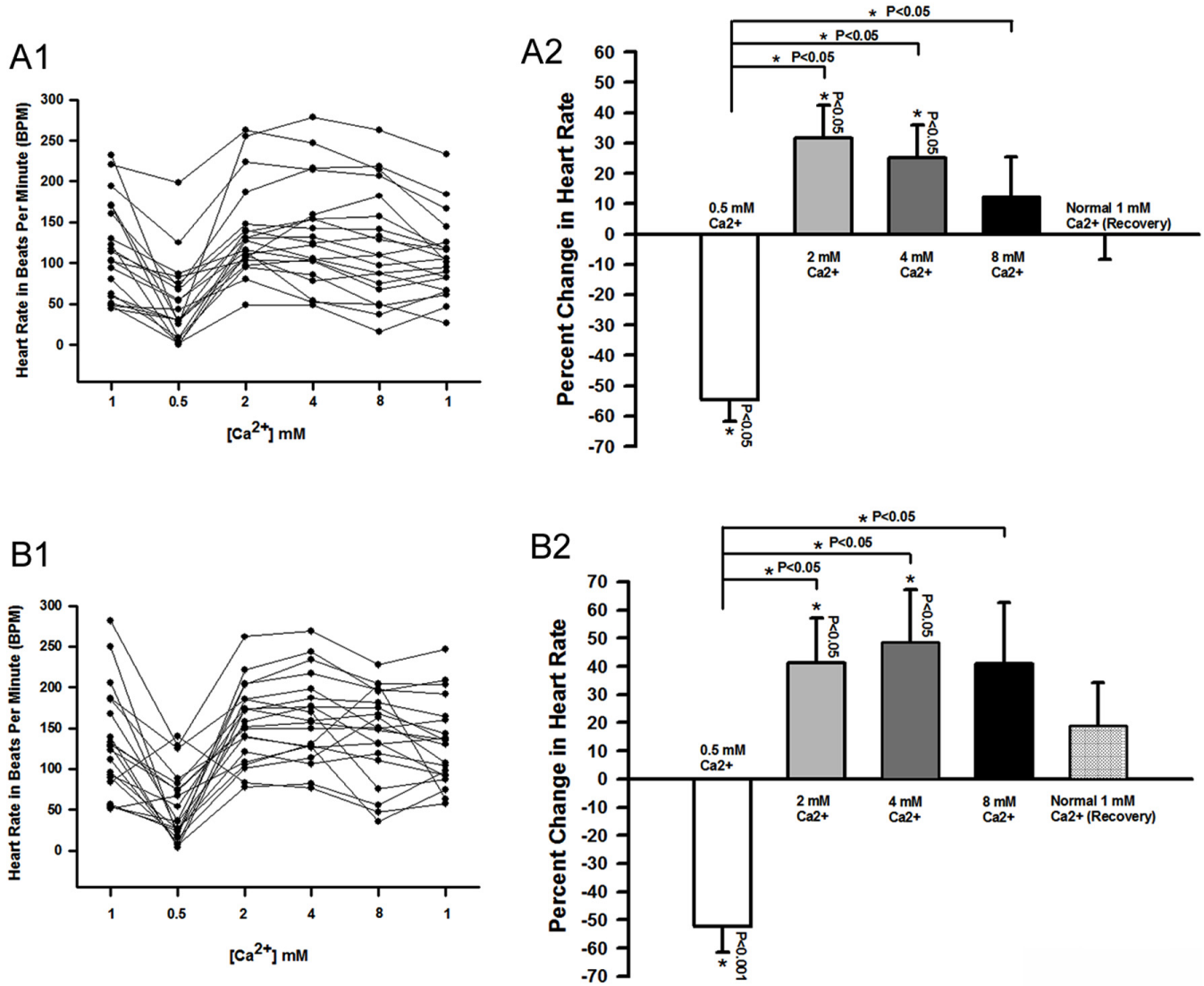

Fig. (5). The effects of varying $[Ca^{2+}]_o$ on HR in CS (A) and Kum (B). The individual larvae were monitored for the effects of $[Ca^{2+}]_o$ on HR. The left panels show the HR in individuals for each change in $[Ca^{2+}]_o$. The right panels depict the percent change in HR in various $[Ca^{2+}]_o$ as compared to HR when exposed to the initial 1 mM $[Ca^{2+}]_o$. The asterisks/*P* values on top of the individual bars indicate a significant difference between that particular $[Ca^{2+}]_o$ and normal 1 mM $[Ca^{2+}]_o$. Both the CS (A2) and Kum (B2) show a significant difference when the $[Ca^{2+}]_o$ is reduced to 0.5 mM as well as when increased to 2 mM and 4 mM (Student's paired *t*-test, *P* values as indicated in graph, n= 20, for each CS and Kum). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes. As indicated there is a significant difference between the percent change in HR at 0.5 mM $[Ca^{2+}]_o$ and that at 2 mM, 4 mM and 8 mM $[Ca^{2+}]_o$ (One way RM ANOVA with *Tukey's post-hoc* analysis, *P* values as indicated in graph). The far right hand bar shows the recovery back to 1 mM $[Ca^{2+}]_o$.

Pharmacological Approach to Examine NCX, PMC and SERCA

In order to examine if the Kum mutation, with a defective SERCA, was mimicked by a pharmacological approach of blocking the SERCA, we used thapsigargin (TG) at 1 μM and 10 μM with several minutes of incubation. TG is known to block SERCA function at 100 nM to 1 μM range [64]. Within the first 2 min there was no significant difference in HR; however, every time measured afterwards for up to 17 min there was a significant reduction in HR for both concentrations of TG (Fig. **6A**, **B**; $P<0.05$). Further studies were conducted with 10 μM TG for up to 42 min (Fig. **6C**). The effect of TG continued to produce a reduction in HR; however, the decrease was not as pronounced over the longer incubation times.

We also compared the approach of 2/3rd reduced $[Na^+]_o$ to application of the NCX blocker (KB R7943) at two different concentrations (Fig. **7**; 20 μM-top panel and 40 μM-middle panel). KB R7943 is known to block the influx but not the efflux of Ca^{2+} through the NCX in rat myocytes (5 μmol/L within 15 sec; [65]). In frog oocytes, the expressed *Drosophila* NCX form is inhibited by KB R7943 for both inward and outward exchange currents [66]. The difference in the effects between 20 and 40 μm is not significant in our experiments. However, the low concentration initially caused a slight decrease in HR after 5

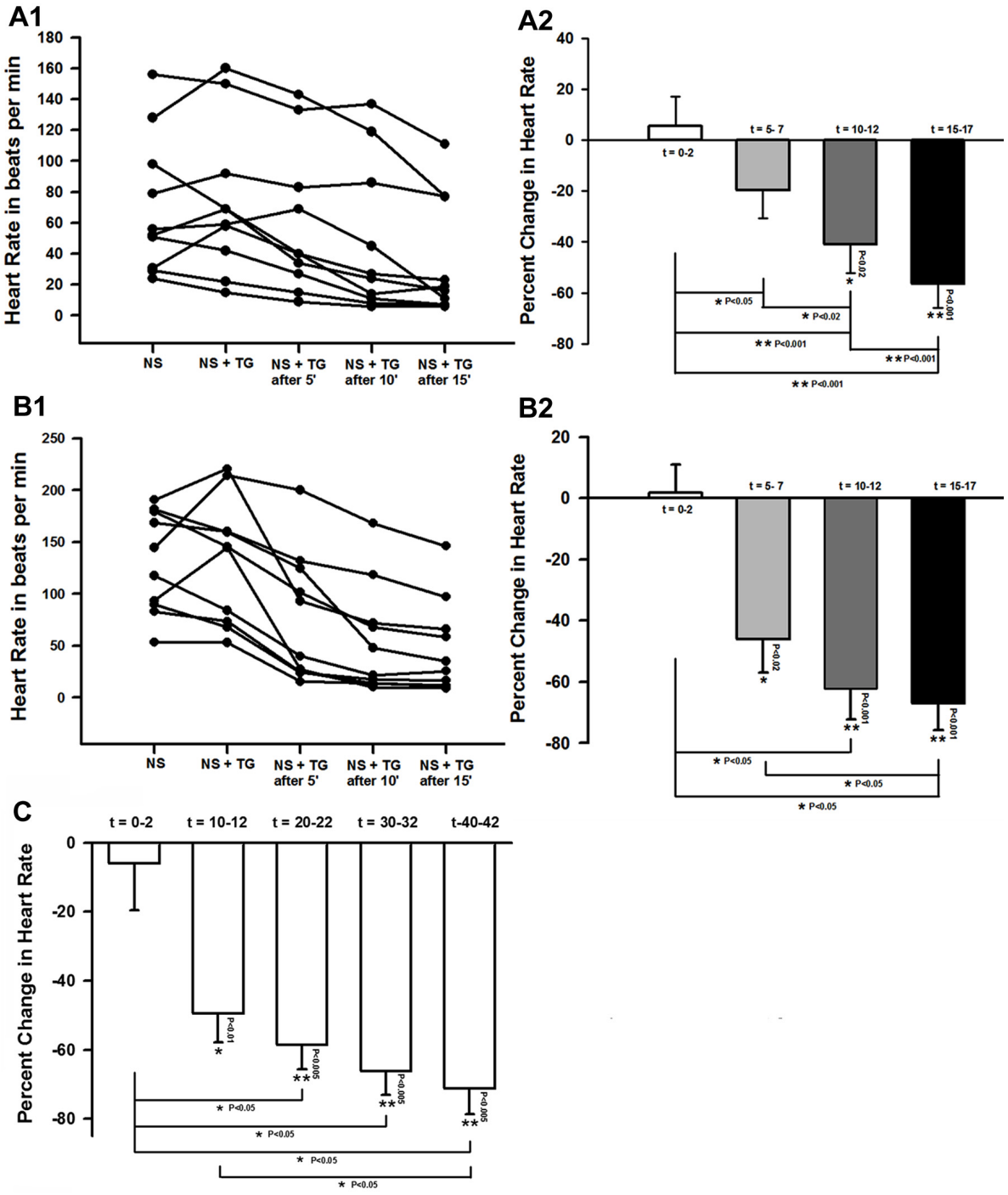

Fig. (6). The effects of the SERCA blocker thapsigargin (TG) 1 μM (A) and 10 μM (B) on HR for individual CS larvae over time for up to 17 min. Right panels indicate the percent change in HR before and during exposure to TG over a time periods. The change is compared to the HR prior to exposure of TG. The various time periods in which measures were taken were calculated as an average BPM. The asterisks/*P* values on top of the individual bars indicate a significant difference between HR at that particular time point in presence of TG to normal saline. There is a significant decrease in HR as compared to normal saline after 10 min of adding 1 μM TG and the decrease becomes more prominent with time. With 10 μM TG a significant decrease occurs after 5 min and the HR decreases even further with time. (Student's paired *t*-test, n=10). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes. (*P* values as indicated in graphs are determined by one way repeated measures ANOVA with *Holm-Sidak posthoc* analysis in *B* and *Tukey's posthoc* analysis for *D*; n =10 for both 1 μM and 10 μM TG). (C) Prolonged exposure of 10 μM TG for up to 42 min. The asterisks/*P* values below the individual bars indicate a significant difference between HR at that particular time point in presence of TG to normal saline. There is a significant decrease in HR after 10 min of adding 10 μM TG and the decrease becomes more prominent with time. (Student's paired *t* test, *P* values as indicated in graph, n=10). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes. (*P* values as indicated in graphs are determined by one way repeated measures ANOVA with *Tukey's posthoc* analysis; n =10).

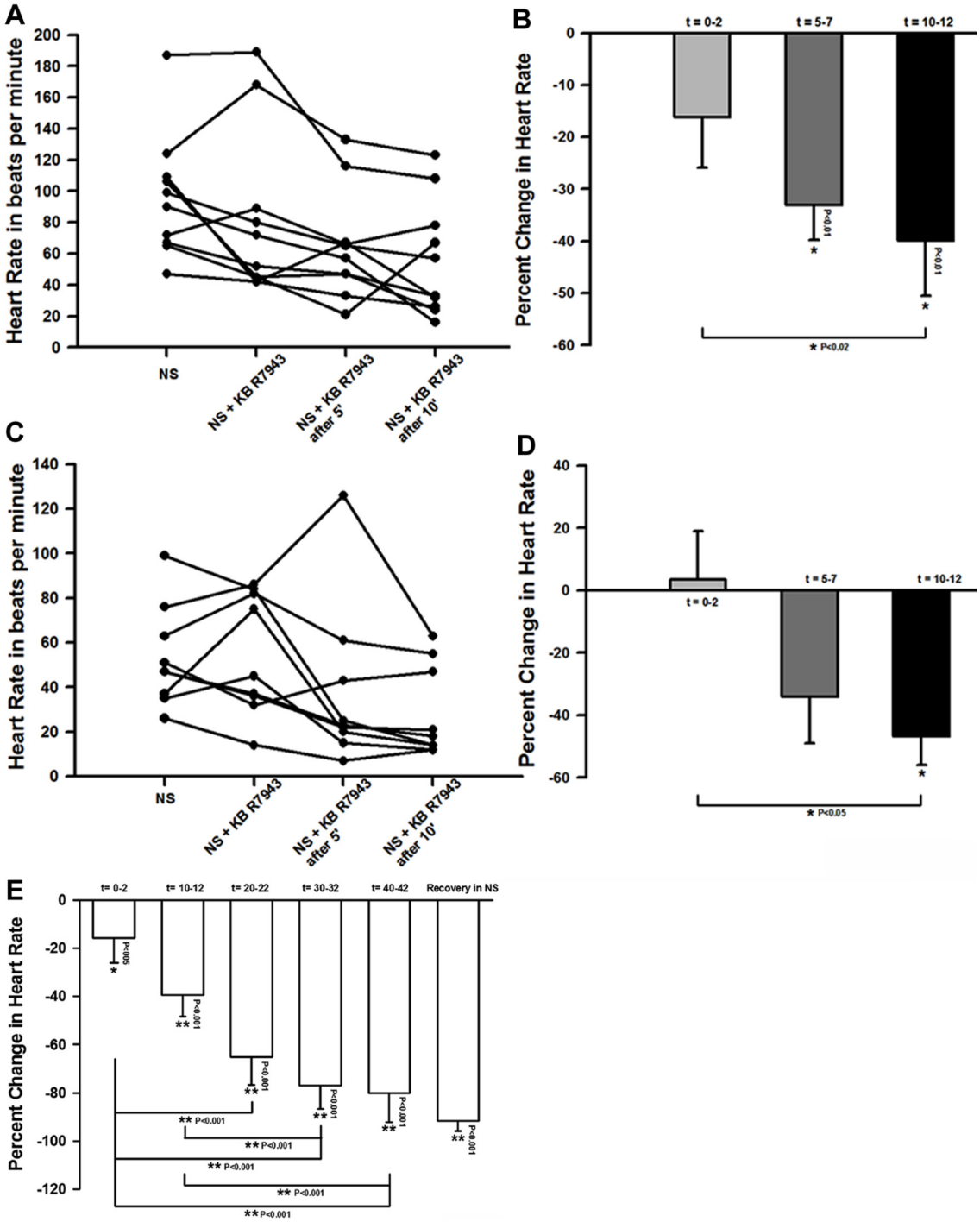

Fig. (7). The effects of the reverse NCX blocker KB R7943 (20 μM and 40 μM) on HR for individual CS larvae over time. Top panel is for 20 μM and middle panel for 40 μM. Right side indicates the percent change in HR before and during exposure to KB R7943 over a time period of 12 min. The change is compared to the HR prior to exposure to KB R7943. The various time periods in which measures were taken were calculated as an average BPM. The asterisks/*P* values on top of the individual bars indicate a significant difference between HR at that particular time point in presence of KB R7943. Student's paired *t*-test, n=10 for 20 μM and n=9 for 40 μM. The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes. *P* values as indicated in graphs are determined by one way repeated measures ANOVA with *Holm-Sidak posthoc* analysis for both 20 μM and 40 μM, n=10 for 20 μM and n=9 for 40 μM.) (E) Prolonged exposure of 40 μM KB R7943 for up to 42 min. The asterisks/*P* values below the individual bars indicate a significant difference between HR at that particular time point in presence of KB R7943 and that in normal saline. There is a significant decrease in HR as compared to normal right after adding 40 μM and the decrease becomes more prominent with time. (Student's paired *t* test, *P* values are indicated in graph, n=10). The asterisks/ *P* values on lines between bars indicate a significant difference between the different percent changes as determined by one way repeated measures ANOVA with *Holm Sidak's posthoc* analysis; n =10.

min, where as the higher concentration did not show a significant effect until 10 min later (Fig. **7B** and **D**). Since the decrease in the HR continued to occur after 10 min, further studies were conducted with 40 min of incubation (40 μM of KB R7943; Fig. **7E**). There is a significant decrease in HR after 10 min. Potentially $[Na^+]_i$ might be increasing over time with the exposure to KB R7943 as the drug's main action is presumed to block efflux of Na^+ and the influx of Ca^{2+} but this should only occur when the gradients are sufficient to promote this reversed NCX action.

To examine the difference between inhibiting the PMCA by pH 8.8 and a pharmacological approach, carboxyeosin (CE) was used. It was shown that 5 μM is sufficient to inhibit PMCA in rats and in guinea pigs [67]. Therefore, this concentration was used and was incubated for 40 min. There is significant decrease in HR after 10 min (Fig. **8**). The decrease was directly proportional to the duration of incubation. Exchanging the bathing medium back to saline without CE did not produce a reversal of the effect within a 10 minute window.

Comparative effects of pharmacological agents, ionic treatments and a mutational approach to the altered function of calcium regulation on HR are shown in Fig. (**9**). Inhibiting PMCA by pH 8.8 treatments and exposure to CE produced the same effect in reducing HR for CS larva. Both treatments significantly reduce HR. The changes in HR were noted between normal $[Na^+]_o$ and 2/3 reduced $[Na^+]_o$ compared to the change induced by normal $[Na^+]_o$ and treatment with KB R7943 (40 μM). The treatments with KB R7943 produced an opposite result from the 2/3 reduced $[Na^+]_o$ paradigm. Lastly, comparing the change in HR for Kum with heat shock and CS treated with TG only, in bathing media containing 1 mM $[Ca^{2+}]_o$, there was no difference in the treatment groups and the treatment itself.

Mapping of the Heart for Electrical Responses

Our objective was to examine the influence of the PMCA, NCX and SERCA on the shape of the action potential in pacemaker myocytes. The myocytes were first mapped for regions that were presumed to produce pacemaker potentials in the heart tube. Surveying the heart has not been tackled in prior experiments of a contracting *Drosophila* larval heart. Lalevée *et al.* [68] did examine the action potentials produced in the caudal heart using Schneider's insect culturing medium. This medium is known to damage skeletal muscle and produce abnormal shaped excitatory postsynaptic potentials. A defined physiological saline HL3 was used in our study, which is known to match the larval hemolymph in ionic composition [61]. In order to understand the variation of electrical potentials generated and conducted along the heart, several larval hearts were mapped for electrical responses during rhythmic contraction. As shown in Fig. (**10**), for 3 larval hearts there is substantial variation in the amplitude and shapes of the action potentials. Following the survey of potentials, it is apparent that the large spiking action potential occurs in the most caudal

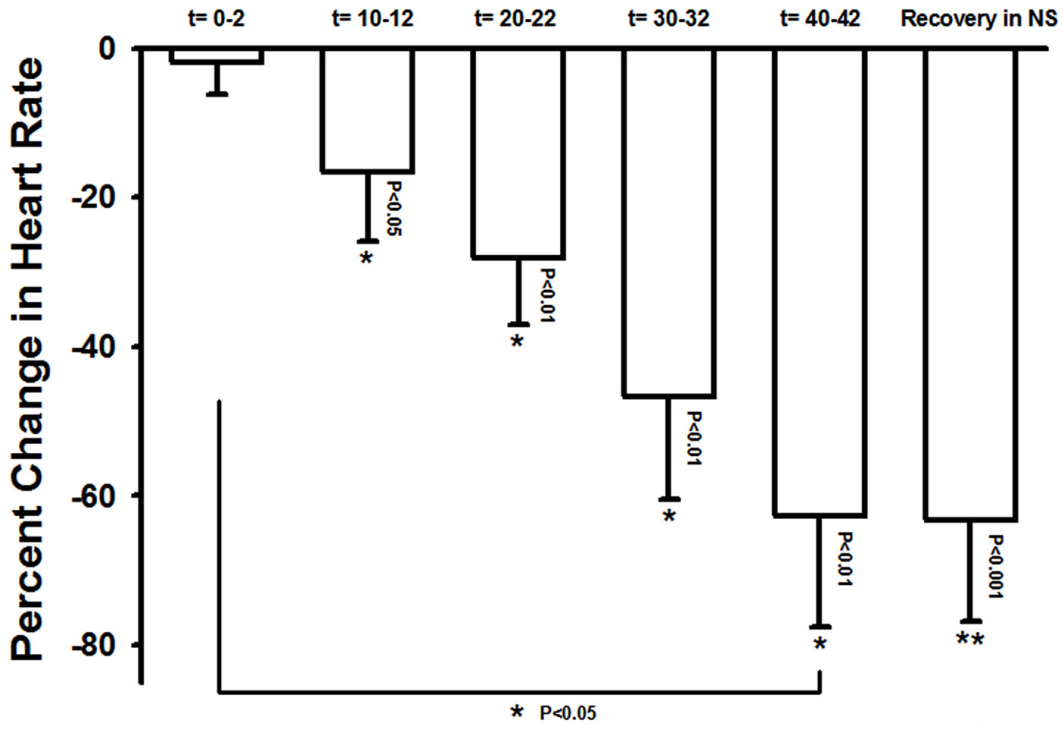

Fig. (8). Prolonged exposure of 5 μM of 5,6-Carboxyeosin (CE) for up to 42 min. The asterisks/*P* values below the individual bars indicate a significant difference in HR before and after a particular time point in the presence of CE. There is a significant decrease in HR as compared to normal after 10 min of adding 5 μM CE and the decrease becomes more prominent with time (Student's paired *t*-test, *P* values as indicated in graph, n=10). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes as determined by one way repeated measures ANOVA with *Tukey's posthoc* analysis; n =10.

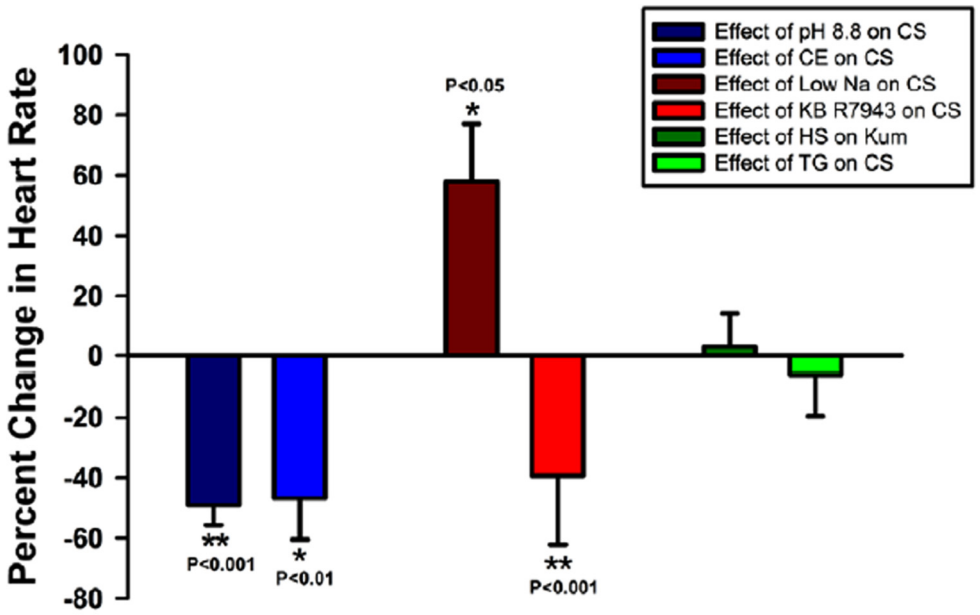

Fig. (9). Comparing pharmacological inhibition of channels with other methods of inhibition. The blue bars show the two different methods used for inhibiting the PMCA. The dark blue bar shows the percent change in HR from normal after inhibiting the PMCA at pH 8.8 and the lighter blue bar shows the percent change in HR from normal after inhibition of PMCA using 5 μM CE. There is no significant difference between the changes in HR by these two methods. (Student's unpaired *t*-test, n=20 for inhibition with pH 8.8 and n=10 for inhibition with CE, *P* values>0.05 therefore not significant). The red bars show the two different methods used for inhibiting the NCX. The dark red bar shows the percent change in HR from normal after compromising the NCX at low $[Na^+]_o$ and the lighter red bar shows the percent change in HR from normal after exposure to 40 μM KB R7943. Low $[Na^+]_o$ results in a significant increase in HR while KB R7943 causes a significant drop in HR. The green bars show the two different methods used for inhibiting the SERCA. The dark green bar shows the percent change in HR from normal after inhibiting the PMCA in Kum flies by HS and the light green bar shows the percent change in HR from normal after inhibiting the SERCA using 10 μM TG. There is no difference between the change in HR by these two methods (Student's unpaired *t*-test, n=20 for SERCA inhibition in Kum using HS and n=10 for inhibition with TG, *P* values>0.05 not significant). The asterisks/*P* values by the individual bars indicate a significant difference between HR with that particular effect to HR in normal saline.

region of the heart tube and the smaller potentials arise from both the caudal and distal regions of the heart, while only smaller potentials were observed in the aorta (data not shown).

The resting membrane potentials were around -20 mV for most recordings. The resting membrane potential from various regions of the heart was taken while the cells were not contracting. The problem while recording during the contractions is that the cells become damaged and they lose their resting membrane potential rather quickly. The composite map (Fig **10**) shows the range of resting membrane potentials that were deemed to be reliable in non-contracting myocytes or from initial recordings prior to the cell starting to contract.

In about 50% of the preparations, immediately after impaling the myocyte with the microelectrode the cell stopped pacing. In order to help increase the ability to maintain pacing, we examined the effect of switching the bathing media from 1 to 8 mM $[Ca^{2+}]_o$ (Fig. **11A**). The action potentials did not show a consistent trend in alteration of the amplitude with the switch to the 8 mM. The shape of the action potentials was compared by normalizing the peak amplitudes. No consistent trend in altering the width or rise time is observed by switching to a higher $[Ca^{2+}]_o$ bathing medium (Fig. **11B**). The slow gradual depolarization (#1 on the traces), the rapid rise time (2 to 3), the amplitude (3 before amplitude normalization), the decay time (3 to 4), and after polarization time (5) showed variations among the preparations. In some preparations, the general normalized shape did not vary from 1 to 8 mM $[Ca^{2+}]_o$ despite differences in their amplitude (see preparations 3 and 4 in Figs. **11A** and **B**).

Changes in the inter-pulse interval in mammalian pacemaker potentials are indicative of alterations in the ionic currents that impact chronotropy. One postulation is that activation of the voltage-dependent T-type Ca^{2+} current in neonatal ventricular myocytes stimulates the sacroplasma to release Ca^{2+} during the diastolic phase. However, the voltage-dependent T-type Ca^{2+} current appears to be reduced in adult myocytes, so the higher threshold L-type Ca^{2+} channels have a larger role for pacing [69,70]. The types of voltage-gated calcium channels present in the *Drosophila* larval heart are not differentiated in this experiment. So, if $[Ca^{2+}]_o$ is raised then Ca^{2+} current (I_{Ca}) would be enhanced and the diastolic phase should be shortened, but a consistent trend was not observed due to change in $[Ca^{2+}]_o$ from 1 to 8 mM (Fig. **12**). Preparation 2, shown in Fig. **13**, produced a nice slow depolarization with a clear threshold for initiating an action potential even in 8 mM $[Ca^{2+}]_o$. Preparation 1 showed an increase in heart rate and preparation 2 a decrease in rate with higher $[Ca^{2+}]_o$. Preparations 3 and 4 showed no significant change with altered $[Ca^{2+}]_o$. It should be noted that the membrane potential shapes may also contain artifactual shapes in these recordings as the heart is pulsating causing movements of the intracellular electrode tip.

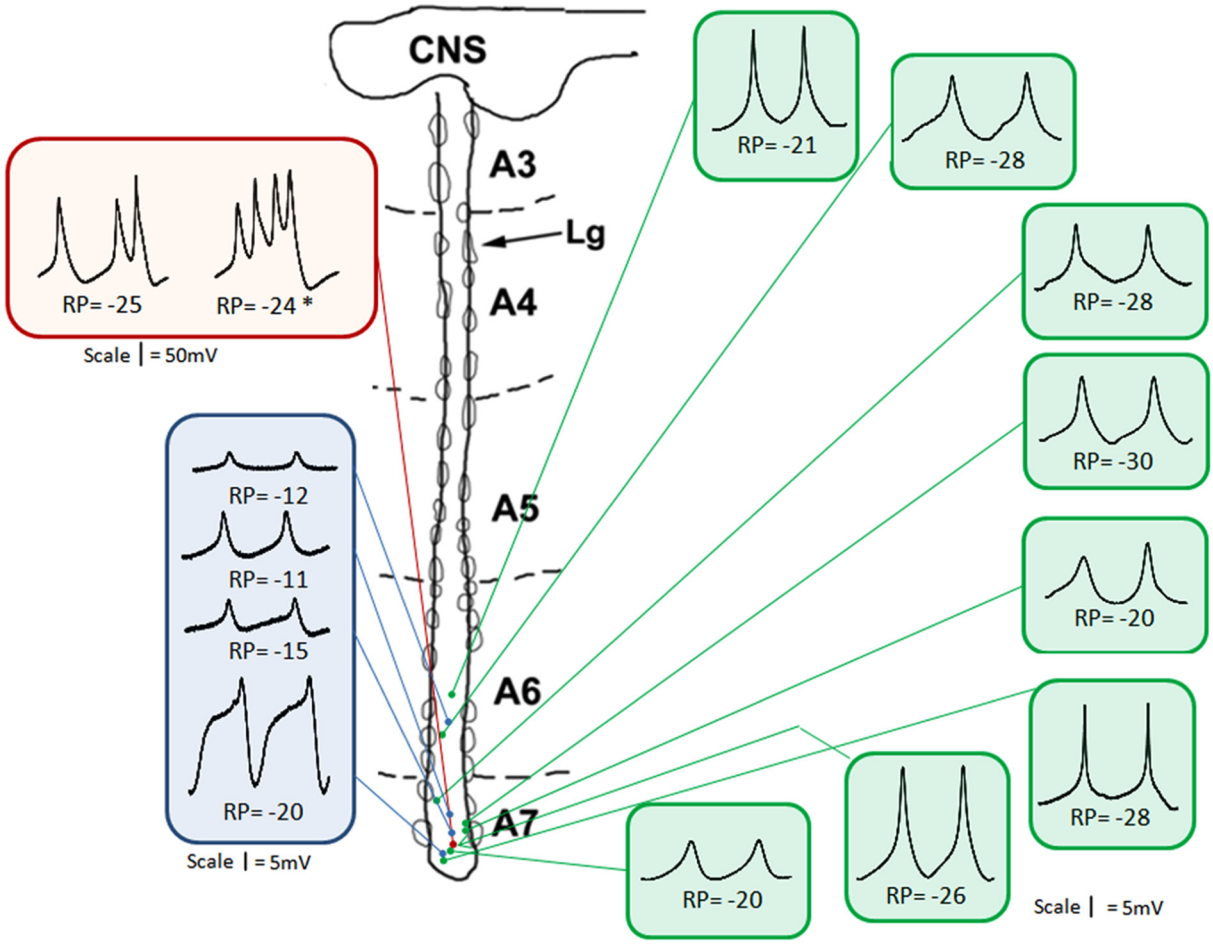

Fig. (10). Traces show intracellular recordings (action potentials) from *Drosophila* larval heart with the membrane potentials noted below them. The lines point to the region of the heart from where the recordings were obtained. Each individual box indicates recordings from a different larva. All the recordings are in normal saline except the one marked with asterisk which is in presence of blebbistatin containing normal saline to uncouple excitation and contraction. It is evident that different regions in the caudal heart show action potentials of different shapes as well as amplitudes and have different membrane potentials. (Amplitude scales are indicated for each preparation).

To determine if the NXC, PMCA and the SERCA have a role in shaping the action potential, the muscle contractions need to be blocked while recording from pacing myocytes. Since it appears that waves of contractions start in the caudal end of the heart for intact larvae (non-dissected) these caudal cells were targeted for recording in dissected preparations. The recordings was obtained after observing a response in blebbistatin incubated preparations. Blebbistatin uncouples the myosin and actin in the contractile unit. The resting membrane potential before and after application of blebbistatin did not change in our study.

In the next series of experiments, the dissected preparations were first exposed to normal HL3 saline containing blebbistatin (note: the buffer was TRIS-base to avoid adding NaOH to obtain a pH of 8.8 when needed). Only CS preparations were used in these experiments. As shown in Fig. (**10**) (top left panel) the potentials are similar in threshold and amplitude before and after blebbistatin. In order to see if an intracellular recording would alter the cell contraction rate and responses to altered ionic environments, the electrical pacing was measured for the same ionic manipulations (Fig. **13**). After the heart stopped beating electrically pacing cells were obtained in the most caudal region of the hearts. When the bathing solution was exchanged to solution with 2/3rd reduced $[Na^+]_o$ some preparations showed an increase while others showed a decrease in amplitude of the action potential. In addition, all preparations shortened the inter-pulse interval (diastolic time) (P<0.05, Wilcoxon rank-sum). After switching the media to pH 8.8 and 2/3rd reduced $[Na^+]_o$ (to block PMCA and reduce the NCX function), the inter-pulse interval (diastolic time) increased as compared to the reduced $[Na^+]_o$ (P<0.05, Wilcoxon rank-sum), but there is no consistent trend in an alteration in the amplitude or shape of the action potential (Fig. **13**).

DISCUSSION

In this study, we have shown compromising the SERCA in intact *Drosophila* larvae, of a heat sensitive mutant strain (Kum^{170}), HR is reduced as compared to control larvae at

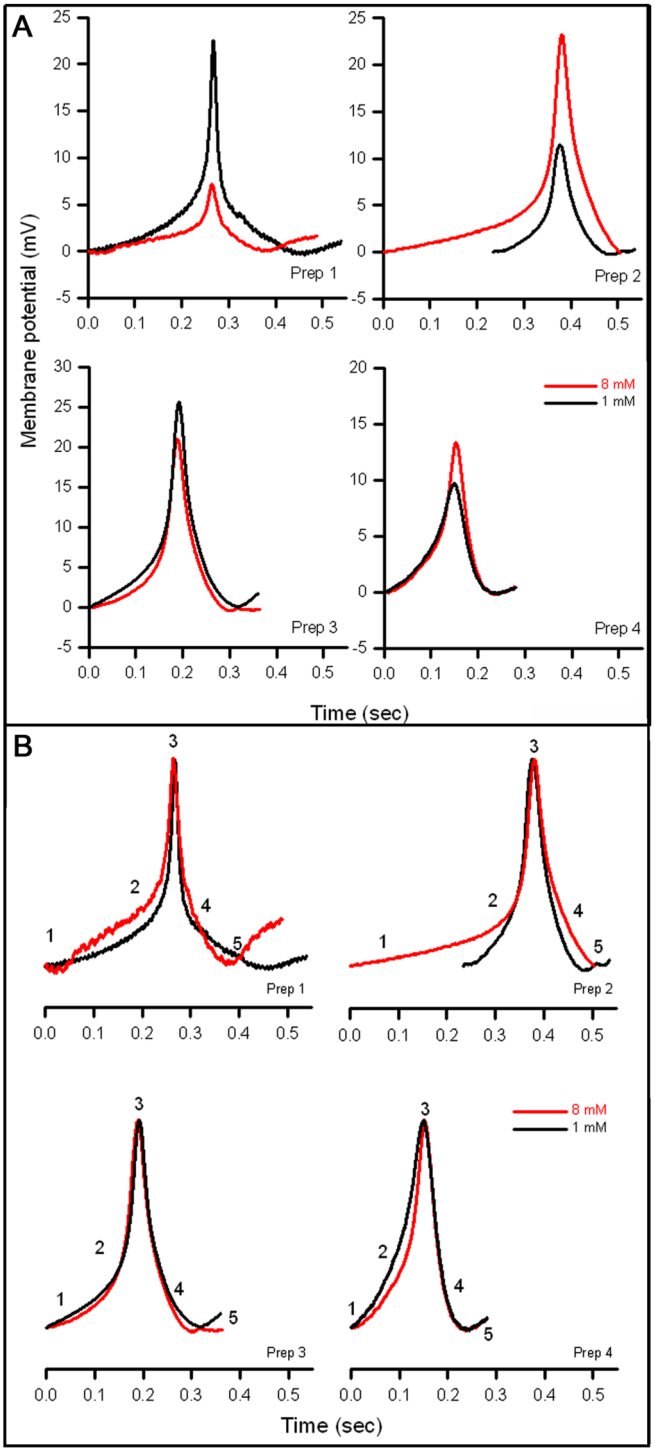

Fig. (11). (A, Top Box) Four representative recordings of action potentials recorded in four different larval hearts in the caudal region of the heart. The initial recordings were made in 1 mM $[Ca^{2+}]_o$ (black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the action potential amplitude with an increase in $[Ca^{2+}]_o$. Also the initial action potential amplitude varies within and among preparations. The resting membrane potential also varied among preparations. Each is adjusted to start at 0 mV for comparisons in the magnitude of the action potential. For these recordings the resting membrane potentials were: preparation 1 -27 mV; preparation 2 -30 mV; preparation 3 -26 mV; preparation 4 -28 mV. (B, Bottom Box) The same four representative recordings of action potentials shown in A; however, the amplitudes are normalized for comparisons in the characteristic shapes of the action potential with varying $[Ca^{2+}]_o$. The initial recording is made in 1 mM $[Ca^{2+}]_o$ (black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the change in shape of the action potential amplitude with an increase in $[Ca^{2+}]_o$. In preparation 2, the increase in $[Ca^{2+}]_o$ resulted in a prolonged rise time from rest (1) to the threshold (2) but in preparation 1 the rise time is slower. From threshold (2) to the peak amplitude (3) the rise times are very similar as well as the decay time from the peak (3) to the repolarization (4). There is no consistent trend for the effect of a exposure to a higher $[Ca^{2+}]_o$. The duration of the action potential in all recordings is similar (100 msec). The time from repolarization (4) to initiation of the next action potential (5) depended on the heart rate.

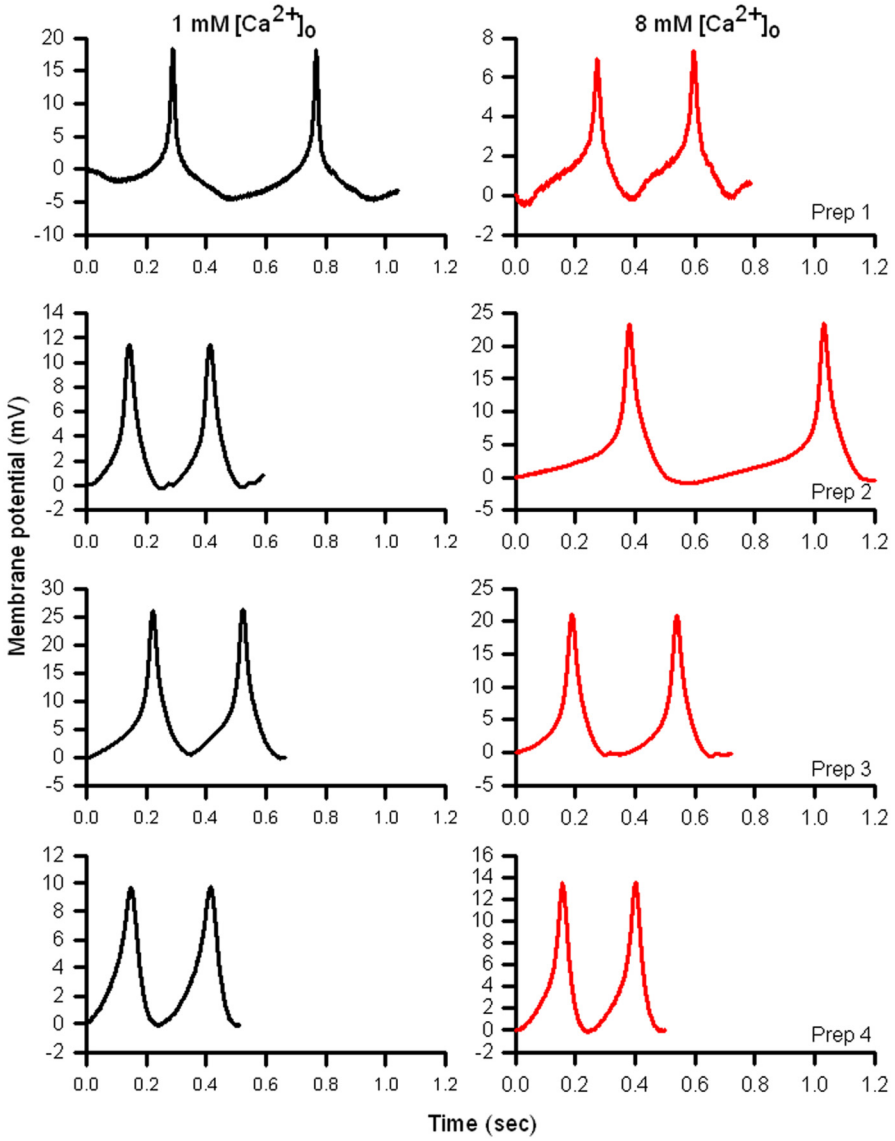

Fig. (12). The same four representative recordings of action potentials shown in Figs. **11A** and **B**; however, plotted at the same time scale with different scales for membrane potential as to enhance comparisons in intrapulse potentials depending on heart rate. The heart rate with varying $[Ca^{2+}]_o$ is noted not to produce consistent results while maintaining a intracellular recording. The initial recording is made in 1 mM $[Ca^{2+}]_o$ (black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the shape of the interpulse potential depending on heart rate nor in altered $[Ca^{2+}]_o$.

21°C. In order to expose the heart directly to pharmacological agents, without the complications of endogenously released compounds within the intact larva, dissected preparations were used. HL3 bathing media is used primarily for maintenance of larval skeletal NMJ but we found HL3 might not be optimal for larval hearts, since substantial variation was observed among dissected preparations as compared to intact larvae. The approach of heat shocking dissected preparations also did not mimic the results for intact larva. In examining, the potential long term effects of a dysfunctional SERCA on HR, larvae raised at 18, 21 and 28°C were examined as intact and dissected preparations. The intact preparations of larvae raised at the three temperatures all showed a greater reduction in HR for the Kum line as compared to controls. Due to the large variations observed in dissected preparations no conclusive findings were apparent for the Kum as compared to controls upon heat shocking. In compromising the PMCA, by exposure to pH 8.8, there is a significant reduction in HR for both the control and Kum lines without any difference among controls and Kum strains before or after heat shock. Thus, inhibiting the PMCA results in a decrease in HR, where as the SERCA, in dissected preparations, does not appear to have a substantial effect. In reducing the function of the NCX (physiological function is an efflux of $[Ca^{2+}]_i$ for an influx of $[Na^+]_o$) both controls and Kum strains had an increased HR with reduced $[Na^+]_o$ by 2/3; however, upon heat shocking controls and Kum strains no additional effects were apparent. From these results, it appears both the NCX and PMCA are important in regulating HR, where as the SERCA does not have as pronounced role. However, in the intact preparations the loss of the SERCA function does have

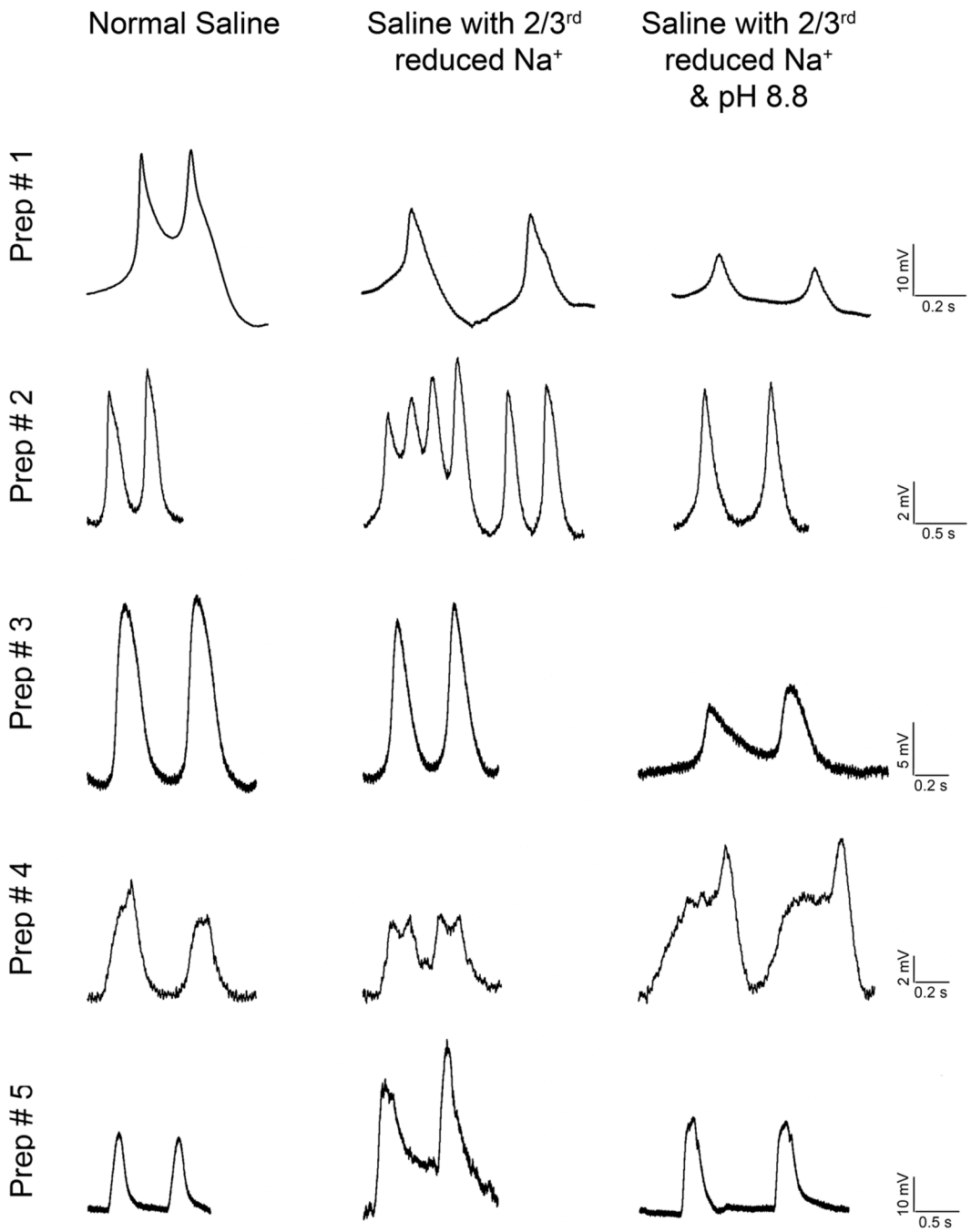

Fig. (13). Intracellular recordings in 5 different CS *Drosophila* larval hearts. The recordings were initially carried out in normal HL3 saline (left panels) then switched to a saline containing 2/3rd reduced Na^+ (to compromise the NCX; middle panels). Thereafter the saline was exchanged with one at pH 8.8 and 2/3rd reduced Na^+ (to compromise the NCX as well as inhibit the PMCA; right panels). HL3 saline contained blebbistatin. The time scale and amplitudes are consistent for each row (each preparation; scale bars shown on right for each row).

an impact on HR, which might be accounted for by other modulatory roles in contents within the hemolymph for intact animals. In examining a range of $[Ca^{2+}]_o$ on HR we noted an increase in HR as extracellular Ca^{2+} increased to 8 mM. Pharmacological approaches to alter the PMCA and SERCA paralleled the results obtained by ionic and mutational approaches. This suggests that the effects or lack of effects, by one or another approach is real in the dissected preparations. For reasons we can not account for, the exposure to KB R7943 decreased HR while retarding NCX by lower $[Na^+]_o$ increased HR. Mapping electrical events of the larval heart revealed that myocytes in the caudal region have both large and small amplitude action potentials. The myocytes proved to be difficult to hold intracellular recordings for prolong times due to the movements; however, stopping contractions by the use of blebbistatin revealed that the myocytes continued to pace in the absence of contraction and various ionic manipulations are possible.

The novel findings in this study are that reduction in the PMCA (by pH 8.8) and the SERCA (by heat shock in the Kum mutation) in dissected preparations did not result in an increase in HR and stronger contractions but a reduction in HR. The calcium exchanger and pumps are meant to lower

$[Ca^{2+}]_i$, so when they are not fully operational, one would assume $[Ca^{2+}]_i$ to increase even in the acute time periods as used in this study. Possibly the dynamics of $[Ca^{2+}]_i$ has some indirect effects on rate via an influence on the NCX or coordination of the various underlying ionic currents to cause the rate to slow down. As expected, a compromised NCX (by low $[Na^+]_o$) caused an increase in HR. However, if the PMCA and/or the SERCA is subsequently inhibited this increase in HR is drastically reduced. The reduction is not as great when PMCA or SERCA are inhibited individually. Thus, the rise in HR when NCX is reduced attenuates the decrease caused by inhibiting the SERCA and PMCA. This raises the question on how selective raising pH to 8.8 targets the PMCA and not other cellular functions. Heat shock itself reduced HR even in dissected controls, which implies a possibility of other effects in the Kum line than just actions on the temperature sensitive nature of the SERCA function. Even in intact larva, heat shocking resulted in a decrease in HR for CS and Kum lines. Thus, why heat shock reduces HR in CS and does not increase HR in the Kum line is yet to be determined. However, Sanyal *et al.* [54] also reported a decrease in HR in intact Kum larva with heat shock. This SERCA mutant had unexplained actions in adult *Drosophila* muscle with spike broadening. Sanyal *et al.* [54] suggested that the SERCA may alter the function of the voltage-gated calcium channels. The effect of heat shock was unexpected as HR generally increases with temperature for invertebrates [11,24]. It becomes increasingly debatable on using heat shock mutants in general for manipulating other physiological functions commonly used in the *Drosophila* research community if HR is reduced since locomotive assays could be compromised or possibly perfusion to the head and limbs in adults might be reduced.

The pharmacological approach used was meant to target the same Ca^{2+} transport proteins as compared to the effects by lowering $[Na^+]_o$, raising pH to 8.8 as well as using the Kum mutant line. One reason to test TG to block SERCA is that heat shocking the Kum larvae might not be fully knocking out the function of SERCA. In addition, pH 8.8 might only partially compromise PMCA as compared to action of CE. The amount of reducing $[Na^+]_o$ has a dose-dependent effect on NCX. We used KB R7943 as an independent measure of altering NCX function; however, its action is not meant to be the same as a reduced $[Na^+]_o$ since this compound blocks the reversed function the NCX. The reversed action of NCX would likely not be present since the gradients do not promote this role in the physiologically range of the myocyte. The reduction in HR by KB R7943 suggests possible other actions of the drug. It is known that this drug can block a nonselective sodium-gated cation channel on lobsters [71]. We have no explanation yet to account for non-selective effects by KB R7943. This needs to be further examined. Using CE and KB R7943 in the *Drosophila* larva also provides information on the potency in *Drosophila* as a species. So these applications should be considered when examining other *Drosophila* tissues (i.e., NMJ, CNS).

The varied methodological approaches used in this study for addressing the role of the SERCA and PMCA were beneficial as one can be more confident of the results being specific to the targeted site in question since the results are similar among the methods used. We did find that all the procedures, with the exception of varying $[Ca^{2+}]_o$, among dissected larvae, produced a large variation in the absolute rate but a trend to increase or decrease HR was consistent. The absolute differences in an initial rate is one of the reasons to report percent changes in compromising the Ca^{2+} channel.

The variation in HR is substantial but each preparation was followed individually to determine the effects of NCX, PMCA as well as for the SERCA. The consequence of variable HR in altering channel function is still not known. If the rate was fast initially and by inhibiting the NCX then $[Na^+]_i$ should build up rapidly as compared for a preparation with an initially low HR. This could cause more Ca^{2+} influx, via the NCX. The Ca^{2+} leak from ER would also promote NCX to work by driving Ca^{2+} out and Na^+ into the cell. So, in theory inhibiting the NCX, PMCA or SERCA with a high or a low HR should have differential effects. Evaluating HR in individual larva with each manipulation was done in order to allow this to be assessed. However, such substantial differences were not observable among these manipulations based on the initial HR.

It is apparent that the physiological saline (HL3) [61] is not optimal for the heart since the HR drops as compared to intact larvae. The heart is myogenic and does not require neural innervation in the larva, so one can rule out direct neural influence. The effect of the dissection and possibly the saline is surprising as the HL3 saline performs extremely well for skeletal NMJs. The numerous peptides and biogenic amines in the hemolymph, may influence the inotropic and chronotropic nature of the heart as observed in intact preparations. These factors that introduce uncontrolled variability in accessing the Ca^{2+} channel regulation on HR of intact larva. Thus, a greater effort is needed to develop a physiological bathing media that is specific to larval heart in order to facilitate various assays of pharmacological treatments. A variety of physiological saline solutions were screened for HR based on different ionic compositions and the one similar to HL3 did produce good heart rates [46]. Despite the HL3 saline producing a reduced HR, as compared to intact larva, the effects of perturbing calcium regulation on HR could be investigated with before and during exposure to pharmacological compounds as well as reducing $[Na^+]_o$ and/or altering pH. We were able to investigate the role of the SERCA along with the actions of a compromised NCX and PMCA which was not previously attempted. Sanyal *et al.* [54] did examine Kum^{170} in larval hearts and found similar results that heat shock reduced HR in Kum^{170}. The CS control larva had about 60 beats per minute (BPM) in HL3 saline; however, in this previous report there is no mention about the substantial variation among preparations as observed in our study where fresh HL3 saline was used. As was noted in an earlier study [22] when the heart tube is transected into the true heart and aorta the rate of beating for each tissue becomes very sensitive to changes in the bathing media. Reasons that heart and aorta might have different pacing action potential shapes and rhythm could be due to some cells not acting as a dominant pacemaker but as latent pacemaker cells, as postulated to occur in mammalian hearts [70]. These latent pacemaker cells may even be a reason for atrial arrhythmias in mammals [70]. Since the saline lacks hormones, biogenic amines as well as other compounds, this could be a reason

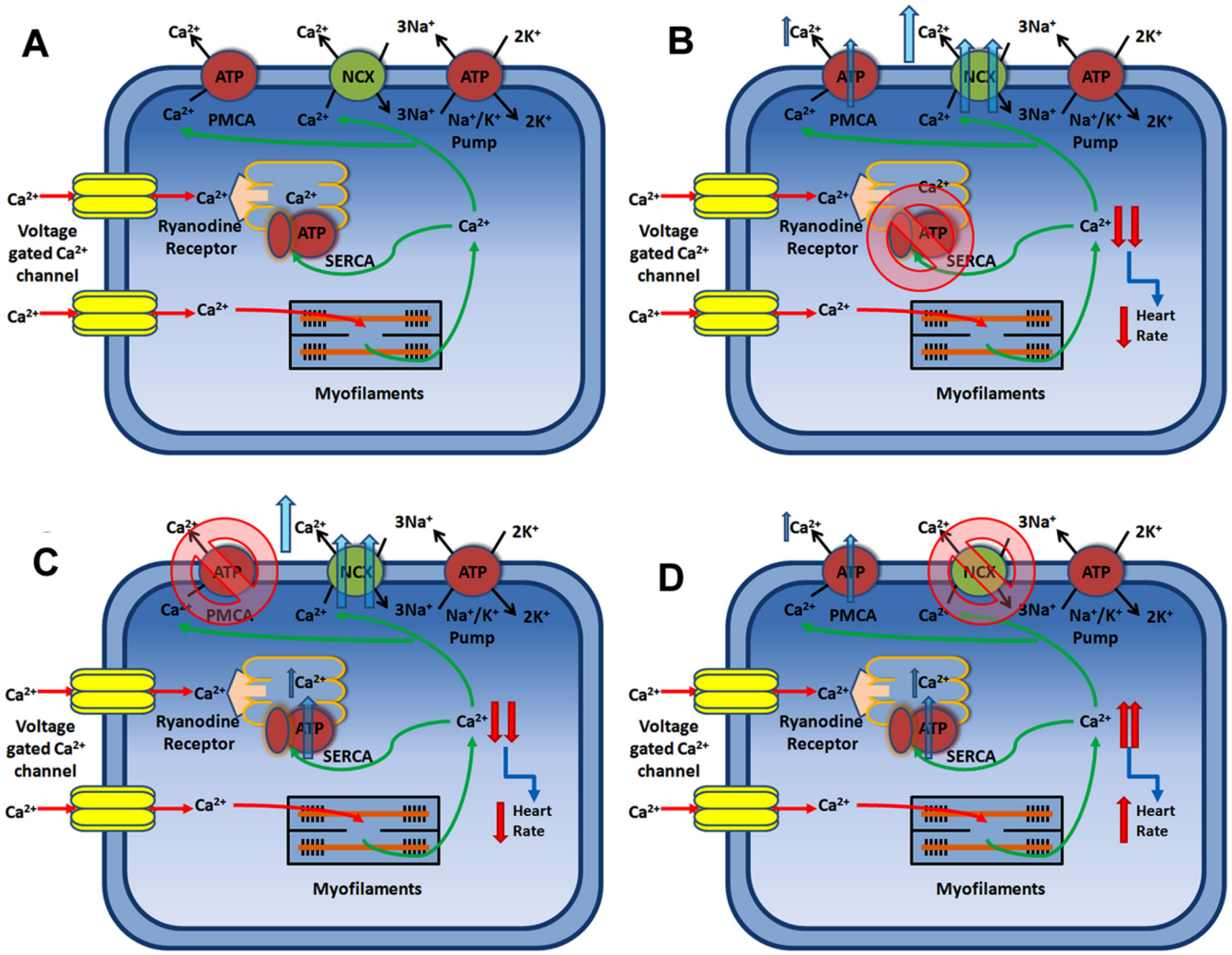

Fig. (14). Schematic model for the effects of blocking the NCX, PMCA and SERCA on HR (A). When either of the three Ca^{2+}-ion channels is blocked the other two channels have an increased load to reduce the $[Ca^{2+}]_i$ in order to bring the HR back to normal or below a normal rate. (B) When the SERCA is blocked in Kum mutants, by means of heat shock, the increase in $[Ca^{2+}]_i$ causes the NCX and the PMCA to work even more efficiently and reduces the $[Ca^{2+}]_i$ below normal levels causing a decrease in HR. (C) In case of the compromising the PMCA, the reduction of HR is large, possibly indicating that the NCX and SERCA compensate more than necessary as the PMCA plays an important role in regulation of HR. This might suggest that there could be a rapid signaling mechanism among SERCA and the PMCA when one is inhibited so that the other can work more efficiently for $[Ca^{2+}]_i$ removal. (D) When the NCX is partly blocked, the other channels are not signaled to remove the $[Ca^{2+}]_i$ as quickly. But when the drug KB R7943 is used, the HR decreases suggesting that now either there is some activity of NCX working in reverse or a nonspecfic action of the drug. Poteniatlly, the PMCA and SERCA increase their efficiency when the KB R7943 is present to reduce $[Ca^{2+}]_i$ and thus HR.

for the drop in HR in dissected preparations. In saline there maybe a lack of driving (i.e., modulating) the dominant pacemaker cells while latent ones are unmasked. Another factor that could be responsible for a reduced HR in the dissected preparations as compared to the intact preparations is that back pressure on the heart is reduced in the dissected larvae but the presence of stretch activated channels in the *Drosophila* heart has not been documented.

Several other factors that could have a significant role on regulation of HR could be addressed. One should consider if other exchangers are present in the cardiomyocytes, such as the NCKX [72] known to be in the fly genome. Likewise, different isoforms of the PMCA could be present which might result in some being inhibited at pH 8.8 while others are not [73]. In fact, activity could have a role in phosphorylation of key proteins, such as the NCX, which then would impact HR [74]. In rodents, the unphosphorylated Phospholamban (PLM) form will inhibit the Na^+-K^+-ATPase [75]. The earlier report states that PLM impacts cardiac contractility by modulating the function of the NCX and Na^+-K^+-ATPase. The pH shift we used to 8.8, in order to inhibit the PMCA, could have indirect effects on other ion channels. It is known that switching pH from 7.4 to pH 9.5 produces a negative voltage shift in the I-V relationship for I_{Na+} currents without altering the amplitude in isolated rat ventricular cells [76]. Identifying the subtypes of ion channels present in the myocytes would be helpful. Since TTX does not even block the Na^+ channels in the *Drosophila* heart this could indicate that the voltage gated Na^+ channels might not have a significant role in cardiac

pacing [46] or that they are not even present. Alternatively, there might be of a TTX insensitive channel subtype. It is known that the L-type Ca^{2+} channel blocker in mammals also reduced HR in *Drosophila* and K^+ channel blockers that function on larval muscles also reduced HR in larva [46; but also see 4]. Some of the channels can be identified by classical pharmacological approaches but it is more interesting to understand how they are modulated. In fact, one does not even know if Ca^{2+} activated K^+ channels are present in these myocytes; however, they are not even known to be present in cardiac myocytes of mammals [69]. Since the L-type Ca^{2+} channel blocker has an effect of *Drosophila* heart, this would support the notion that the larval heart functions as the mammalian adult heart in that L-type Ca^{2+} channels are key in pacing [69] as compared to the T-type Ca^{2+} channel. It is still unknown to what starts the beating after a quiescent period in the *Drosophila* heart. Modulating the degree of the ER's background leak of Ca^{2+} through RyR or even IP3 mediated receptors on ER could be the key. Since the larval heart is sensitive to 5-HT modulation [22], IP3 receptors may have a role in the background $[Ca^{2+}]_i$. Quantitative genetic analysis effects on HR were examined for multiple deficiencies. Some mutations were shown to increase HR while others decreased HR [11].

The large action potentials recorded in the caudal myocytes are likely responsible for the pacemaker activity of the larval heart as this is the location where waves of contractions originate. Based on cross sections and SEM topography [77] there are a few cells that make up the heart tube in this region. Such large action potentials would suggest a large Ca^{2+} entry if voltage-gated Ca^{2+} channels are present on the plasma membrane as in mammalian cardiomyocytes that can pace (i.e., purkinje cells) [78]. This would also suggest that these cells have a sufficient means to return $[Ca^{2+}]_i$ to resting stores in order to maintain a rhythm. The initialization of the Na^+ spike could be explained as for ventricular cells of mammals. As mentioned in the Introduction, the slow leak of Ca^{2+} by ryanodine receptors (RyR), can be a trigger for NCX to function and then produce a feedback inhibition of the SR release. Since inhibiting the PMCA at *Drosophila* NMJ in larvae by pH 8.8 produces a longer lasting $[Ca^{2+}]_i$ from single action potentials, [79] it would be of interest to directly measure $[Ca^{2+}]_i$ with indicators in myocytes. Given that the resting membrane potential is around -20 mV would indicate that the myocytes are permeable to Na^+ and that the Na^+-K^+ ATP pump is not working at a level to keep the cell membrane at a more negative potential. It would be of interest to determine the value for E_K in these myocytes in order to learn why these cells have a low resting membrane potential. In fact, the membrane potential can influence Na^+-Ca^{2+} exchange and if the $[K^+]_o$ is not optimal in the HL3 this could explain a depolarized state for the myocytes [80]. It is known that cardiac fibroblasts of human heart are not electrically excitable but mechanosensitive. The resting membrane potential of these cells is around -16 mV [81]. It is unlikely that the potentials we recorded are from supportive fibroblasts as when the heart is not contracting, during blebbistatin treatment, the pacing potentials are still detected.

Based on the results obtained in this study, we propose a model for the role of NCX, PMCA and SERCA within the heart cycle (Fig. **14**). The basic mechanism is hypothetically explained by an overcompensation of PMCA or SERCA when one or the other is compromised. The potential Na^+ and K^+ ionic currents were not included in the model to account for the cardiac cycle, nor the feedback inhibition on the RyR or the voltage-gated Ca^{2+} channel by raised $[Ca^{2+}]_i$. Many possible scenarios exists from the observed measures but until direct measures of $[Ca^{2+}]_i$ can be made with Ca^{2+}-sensitive indicators or isolated ionic currents they would be very speculative. Possible computational models [82,83] might be of help here in postulating feasible methods based on selective blocking of one or a combination of these Ca^{2+} channels as approached in this study.

Several follow up studies to these results would help in delineating the mechanisms of interaction of the various Ca^{2+} buffering systems. One idea is to use cultured myocytes for whole cell patch recordings and better isolate ionic currents involved primarily in pacing. Also determining specifically the dominant and latent pacemaker cells as postulated to occur in mammalian hearts is also important [70]. Moreover responses to chronically alter one or all of the key proteins (i.e., NCX, PMCA, SERCA) and various treatments ranging from gene therapy to pharmacological agents would be insightful. It would be interesting to determine if the larval heart is still sensitive to 5-HT when CaM Kinase-II is inactivated to learn how the HR is regulated by this modulator. Increased levels of cGMP increases HR so it is likely that cGMP would be a potentially activated cascade for a modulator role [18]. It is interesting that not all the pacemaker cells in mammalian heart are sensitive to vagal stimulation (i.e., Ach) since this could induce cardiac arrest. Thus, having some cells not responsive allows them to be driven but not turned off when pacing cells are in a "standstill" by inhibitory modulation [45]. It would be of interest to examine if there is uniformity in responsiveness to modulators for individual myocytes along the entire dorsal tube in larval *Drosophila*. It would also be of interest to investigate developmental regulation to chronotropic and ionotropic modulations of HR. Such differences my help to explain why HR slows from 1st instar to adult [15]. As specific ionic current recordings are forth coming in myocytes, it is hopeful that the cellular responses of inhibiting NCX, SERCA and PMCA can be determined. A similar investigation in the function of these three proteins has just been reported on in relation to synaptic transmission at skeletal neuromuscular junctions in *Drosophila* and crayfish [84]. Diseases that inflict humans are becoming more common to be genetically modeled in *Drosophila melanogaster* [1-6], and thus the physiological alterations continually need to be assessed.

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