applied biosciences

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Effect of 2-Aminoethoxydiphenyl Borate (2-APB) on Heart Rate and Relation with Suppressed Calcium-Activated Potassium Channels: Larval *Drosophila* Model

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Highlights:

- 2-APB slowed heart rate at a high concentration (>10 μM) in the *slo* mutant line.
- The *slo* strain and CS strain both increased heart rate when exposed to 5-HT.
- The *slo* strain and CS strain both increased heart rate when exposed to 5-HT following 2-APB incubation.
- No differences were measured between the use of thapsigargin or 2-APB on heart rate with exposure to 5-HT between the *slo* and CS strain.

Abstract: Cardiac contractile cells depend on calcium in order to function. Understanding the regulation of calcium influx, efflux, and release from the sarcoplasmic reticulum is essential. The focus of this investigation is to address how a reduction of functional Ca^{2+}-activated K^+ (K_{Ca}) channels, via a mutational line, might impact the heart rate in larva when the SER is also modulated through Ca^{2+} loading and stimulation. The larval heart tube is exposed in situ and flushed with saline. With a known saline composition, a potential therapeutic pharmacological agent, 2-Aminoethyl diphenylborinate (2-APB), was examined for its effect on heart rate, as well as to determine the contribution from K_{Ca} channels. In this study, it was determined that mutation in the K(Ca) channel (i.e., *Slo*) showed a different trend than the wild-type CS strain. Exposure to high concentrations of 50 μM 2-APB decreased heart rate in the *Slo* strain and increased it in the wild-type CS strain. Serotonin increased heart rate in both thapsigargin- and 2-APB-treated larvae, with no significant difference between the strains.

Keywords: calcium; *Drosophila*; heart rate; pharmacology; potassium channel

Citation: Hensley, N.; Elliott, E.R.; Abul-Khoudoud, M.O.; Cooper, R.L. Effect of 2-Aminoethoxydiphenyl Borate (2-APB) on Heart Rate and Relation with Suppressed Calcium-Activated Potassium Channels: Larval *Drosophila* Model. *Appl. Biosci.* **2023**, *2*, 236–248. https://doi.org/10.3390/applbiosci2020017

Academic Editor: Demetrios A. Arvanitis

Received: 12 February 2023
Revised: 30 April 2023
Accepted: 17 May 2023
Published: 23 May 2023

1. Introduction

Cardiac pathologies are diverse [1], and those with abnormalities in rhythm or output are commonly associated with therapies to manage intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [2]. As cardiac contractile cells depend on $[Ca^{2+}]_i$ in order to function, understanding the regulation of calcium influx, efflux, and release as well as uptake from the sarcoplasmic reticulum is essential. The action of digitalis is a prime example of how the control of ionic homeostasis with ions other than Ca^{2+} (i.e., Na^+ and K^+) has an indirect impact on $[Ca^{2+}]_i$ [3–5]. Thus, pharmacological approaches to the direct or indirect management of $[Ca^{2+}]_i$ in heart tissue should be investigated [6,7].

Various diseases originating from genetics or from random alterations in protein expression have been shown to impact cardiac Ca^{2+} dynamics [8,9]. One particular channel, which is a large-conductance Ca^{2+}-activated K^+ channel (K_{Ca}), is known as $K_{Ca}1.1$. This channel is associated with the gene *KCNMA1*. The screening of human families with histories of atrial fibrillation has found a strong association between the disorder and alterations in this gene [10].

An altered K_{Ca} channel is also associated with neurological disorders [11] (Bailey et al., 2019). In order to better understand the role of the K_{Ca} channel in cardiac functioning, experimental models using rodents, zebra fish, and *Drosophila melanogaster* have been used [10,12–15]. The *Drosophila* heart continues to serve as a proof of concept for examining the mutations and pharmacological agents that affect mammalian cardiac functioning [16,17]. Modulating $[Ca^{2+}]_i$ in the cardiac tissue of mutational models could allow a better understanding of how to manage such pathological conditions.

Mutational studies into different forms of K_{Ca} channels have provided a plethora of information on the role in cardiac functioning and other tissues. Examining the dysfunction of K_{Ca} in cardiac physiology through the modulation of other cellular responses and, thus, the alteration of free Ca^{2+} allows for investigation into potential therapeutic interventions, as well as a better general understanding of the interconnection within myocyte systems. 2-Aminoethyl diphenylborinate (2-APB) is one compound that alters $[Ca^{2+}]_i$. 2-APB can rapidly pass into cells and has been recognized as an inhibitor of Ca^{2+} release from ryanodine receptor-sensitive internal stores (RyR). However, it is not yet known whether the inhibition of RyR actually occurs, or if the compound's primary effect is to prevent loading of the endoplasmic reticulum (ER) through the Ca^{2+} ATP-dependent pump such that activation of the RyR nonetheless results in a reduced quantity of Ca^{2+} being released. The actions of 2-APB have also been reported via the rapid alteration of intracellular Ca^{2+} dynamics for rat RBL-2H3 (basophilic leukemia cell line) and MDA-MB-231 breast cancer cells [18,19]. In these studies, the actions of 2-APB were shown to be primarily based around the blockage of intracellular Ca^{2+}-build-up within the ER. 2-APB was shown to inhibit Ca^{2+} release from rat primary cortical neurons [20]; however, it is possible that the neurons simply did not take up the Ca^{2+} in order to be released. The effects on Ca^{2+} uptake in the intracellular stores of DT40 chicken B lymphocytes were found to be dose-dependent [21]; low concentrations (1–10 μm) of 2-APB seem to stimulate an uptake of Ca^{2+}, while larger concentrations of the compound (25–75 μm) inhibited that uptake [21]. Studies also suggest that 2-APB might both inhibit InsP3-induced Ca^{2+} release from RyR and block the uptake to Ca^{2+} stores [22–24]. 2-APB was also found to activate the transient receptor potential (TRP) family of cation channels, such as TRPV1, TRPV2, and TRPV3 [25]; however, the TRP channels TRPC3, TRPC5, TRPC6, TRPM2, TRPM3, and TRPM7 are all blocked. Overall, 2-APB is considered a blocker of Ca^{2+} uptake into cellular stores, as well as through some ion channels [26].

Given the rapidity with which 2-APB alters Ca^{2+} levels in active cells, one would expect the contraction of muscle cells, which depend on intracellular Ca^{2+} changes and, thus, should show significant effects upon exposure to 2-APB. Interestingly, the smooth muscle of the rodent oviduct has been shown to depolarize and decrease spontaneous contraction with high quantities of 2-APB [27]. This response is contrary to what might be expected, indicating that further studies are needed to fully understand 2-APB action; in particular, an investigation of different cell types would possibly garner a larger understanding of the mechanisms of action. However, if ER stores are depleted and Ca^{2+} is exchanged or pumped out of the cell, the Ca^{2+} cycling levels may be too low to maintain contractions.

Cardiac myocytes can regulate Ca^{2+} ions in various ways. Primarily, key components include: the plasmalemmal Na^+/Ca^{2+} exchanger (NCX), the plasma membrane Ca^{2+}-ATPase pump (PMCA), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA), which are key components on the endoplasmic reticulum. Changing the $[Ca^{2+}]_i$ by blocking the release of Ca^{2+} from the RyR or by blocking Ca^{2+} uptake through SERCA can have an impact on the membrane potential through action on the K_{Ca}. Alterations in the functions of these key proteins (normally expressed within cardiac tissue) are known to produce pathological conditions [3–5,10]. Not only does a lower cytoplasmic Ca^{2+} harm normal functioning, but an inability to extrude or buffer intracellular Ca^{2+} may lead to abnormally high Ca^{2+} and, thus, damage to the myocyte [28–32].

The myogenic larval *Drosophila* heart serves as a model by which to screen pharmacological agents that alter heart rate. Investigating the actions of pharmacological agents, both in

normal circumstances and under the effects of compromised physiological processes, provides an insight into cellular responses [33–37]. 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, the combination of 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 (Ca_V) [38] and, potentially, voltage-gated Na^+ channels. The influx of Ca^{2+} acts on the RyR 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 for the cycle to be repeated [39]. 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 [40]. However, we speculate that, judging by the anatomy of the *Drosophila* heart [41], the pacing cells act as contracting myocytes, and that they can generate action potentials. This would suggest that these heart cells are likely to have pronounced voltage-gated Na^+ currents. This remains to be examined, as the larval skeletal muscle appears to lack voltage-dependent sodium channels. The influx of Na^+ in addition to Ca^{2+} must be tightly regulated to prime the cell for the next cycle. In short, as in mammalian hearts [42,43], 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 [44–46]. It has been recently proposed that two clocks can actually regulate pacemaker functioning. 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 [47].

The fine regulation In the pacemaker potential driving the heartbeat provides an index of the heart rate against which to assess functional changes. In *Drosophila*, as in mammals, NCX and SERCA play major roles in coordinating the cardiac pacemaker's electrical activity. The *slowpoke* (*slo*) gene in *Drosophila* is a mammalian homolog of *KCNMA1* and, due to the "Big" conductance of K^+, the protein is referred to as a BK channel [48]. The channel was initially identified in the skeletal muscle of adult *Drosophila* as the ortholog *slowpoke* [49–51]. The related *slowpoke* gene expression was shown to occur in the adult *Drosophila* heart by Reverse Transcriptase-PCR and qPCR [10]. In adult *Drosophila*, various mutations in the slowpoke gene (slo^1 and slo^4) produced longer heart periods, systolic intervals, diastolic intervals, and arrythmia indices, as well as increased electrical dysfunctioning [52]. In mammalian neurons, the BK channels function in relation to Ca^{2+} dynamics within the cell, which is itself impacted by the Ca^{2+} flux from the ER through RyR [53]. This is likely even more tightly regulated in cardiac tissue, with the SER having a large role in affecting the pacemaker potential and contraction/relaxation of the contractile muscle.

Using a pharmacological approach to assess how unloading Ca^{2+} stores from the SER and preventing Ca^{2+} re-uptake might impact BK channels of non-mutant and mutant lines would provide more information about the interconnected relationship of the Ca^{2+} cycle and cardiac functioning. The current literature suggests that 2-APB has an impact on loading the SER and, thus, depletion of Ca^{2+} within it, so one would expect that stimulation of the RyR would not produce any additional effects after exposure to the compound. Testing this would provide insight into the workings of 2-APB and whether the afore-described effects might plausibly occur upon exposure to the compound.

Serotonin (5-HT) increases the heart rate in larval *Drosophila* and appears to work by triggering a release of Ca^{2+} from the SER via inositol 1,4,5-triphosphate (IP3), a second messenger activated by 5-HT receptors on the plasma membrane of the heart [54]. Additionally, addressing how a transient increase in $[Ca^{2+}]_I$, either by 5-HT releasing Ca^{2+} from the SER or by the 2-APB blocking Ca^{2+} uptake back into the SER, affects the heart rate in a mutational line with a non-functional K_{Ca} can speak to the role of the K_{Ca} channel's

contribution in Ca^{2+} regulation and heart rate control. Thapsigargin is also known to block Ca^{2+} uptake back into the SER and was used to parallel the actions of 2-APB.

The use of larval-stage *Drosophila* is beneficial to address the direct effects on the heart, as the heart tube is not yet innervated, is easily viewed in situ, and is easy to maintain using only a simple saline solution to remove any hormones and peptides that affect heart rate [55,56]. The larval heart is a dorsal vessel and is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. In the larvae, the origin of the heartbeat is known to be myogenic [57,58], with the pacemakers located in the caudal region [54,59]; however, the larval heart starts to be innervated from the anterior region in the late third instar [60] and is well innervated in adults [61]. The heartbeat is very sensitive to modulators such as serotonin (5-HT), dopamine, acetylcholine, octopamine, and peptides, as well as pH changes [54,58,62–67]. These variables are hard to control in an intact and restrained pupa or adult, but an early third instar larva flushed in a saline bath removes many of these uncontrolled variables and allows for a controlled environment (though this is also not as natural as a fully intact preparation).

The focus of this investigation is to address how a reduction of functional KCa channels by the use of a mutational line might impact larval heart rate when the SER is also modulated in Ca^{2+} loading and stimulation. Additionally, it focuses on examining 2-APB as an experimental compound to determine its effects in pacing of the larval heart. It is important to understand the actions of 2-APB, as chemical modifications might alter its therapeutic efficacy in cardiac functioning.

2. Methods

2.1. Animals

Drosophila melanogaster (Dmel), Canton S (CS) flies were used in all physiological assays. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington *Drosophila* Stock Center (BDSC). One line with a mutation in the gene coding for K_{Ca} was also used (st [1] slo [1] (BDSC 4587)). Slowpoke (slo) encodes the structural alpha subunit of a BK ('maxi K') calcium-activated potassium channel [50]. This mutation line is constituently active in all cells. The gene scarlet is referred to in FlyBase by the symbol Dmel\st. It is a protein coding gene from Dmel. All animals were maintained in vials partially filled with a cornmeal–agar–dextrose–yeast medium.

2.2. Dissection and Procedures

The same procedures for dissecting the *Drosophila* larvae and exposing the larval heart were used as described in Anyagaligbo et al. [68] and shown in video format [69]. Only early third instars were used, which had been taken from the cornmeal food without them yet occupying the sides of the vials.

The standard saline is HL3 saline (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5N, and N-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES) at a pH of 7.1 [70,71]. Exchanges in the saline bathing media are shown within the figures. The pharmacological agents used were 2-Aminoethyl diphenylborinate (2-APB) and serotonin (5-HT, 100 µM). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), save the thapsigargin which was obtained from Tocris Bioscience (Minneapolis, MN, USA).

2.3. Statistical Methods

In general, when normality was to be assumed, Shapiro–Wilks tests were used to validate assumptions and determine whether it would be suitable to use a *T*-test or a Wilcoxon Signed Rank Test. A percent change in the individuals from saline to a compound were compared in some conditions which normalized the differences in the initial values of the rates. A significant difference is considered to be $p < 0.05$.

3. Results

3.1. Effect of 2-APB on Heart Rate in CS and Slo Strains

Since it was previously shown that mechanical disturbance of the in situ heart tube could alter heart rate [71], both the CS and the *Slo* strains in this study were examined to serve as a control for exposure to the various compounds (including exchange of the saline bath). Heart rate was determined initially after dissection and after switching the bath for saline or a compound of interest. The heart rate was taken again after exchanging the bath back to fresh saline (Figure 1). The initial rates in saline were more variable in the *Slo* strain than for the CS strain after dissection (Figure 1A1,A2). There was a significant effect of exposure to 2-APB at 10, 30, and 50 μM for the *Slo* strain (Figure 1B2,C2,D2; N = 10 and N = 12 for 50 μM; $p > 0.05$, Paired *T*-test). There was only a significant effect of exposure to 2-APB at 10 and 50 μM for the CS strain (Figure 1,B1,D1; N = 10 and N = 12 for 50 μM; $p > 0.05$, Paired *T*-test). There was no significant difference between the CS and *Slo* strains for the % differences in the effects of exposure to 2-APB even though the 50 μM exposure showed opposite significant effects in the CS and *Slo* strains. As shown for the *Slo* strain, the baseline variability in the heart rate while exposed to saline is more pronounced as compared to the wild-type, indicating that there may be an effect of K_{Ca} channels on the basal heart rate.

3.2. Effect of 2-APB and 5-HT on Heart Rate in CS and Slo

In a previous study, it was shown that the exposure of in situ hearts of third instar larvae to 5-HT increased heart rate [54,55]. It was established that the effect of 5-HT was mediated through a 5-HT 2A receptor, which stimulated the release of Ca^{2+} from the SER [54,66]. Thus, it was of interest to expose the heart tube to 5-HT after incubation with 2-APB to determine if the compound blocked the uptake of Ca^{2+} in the SER and reduced the 5-HT-initiated heart rate increase as compared to the preparations not exposed to 2-APB. Intracellular Ca^{2+} is pumped across the plasma membrane of the myocyte by PMC and exchanged out of the myocytes by the NCX. Thus, if the sarcoplasmic reticulum was depleted of Ca^{2+} in time, it would be expected that 5-HT would not increase heart rate. However, the effect of 5-HT on the *Slo* strain treated by 5-HT could be different from that on the CS strain, depending on the residual $[Ca^{2+}]_i$, since concentration differences may promote K^+ efflux and alter myocyte membrane potential. Heart rate increased in both the CS and *Slo* strains to similar degrees (N = 10; $p > 0.05$, Paired *T*-test; Figure 2); however, there was no difference in the percent change to 5-HT exposure between the strains (N = 10; $p < 0.05$, *T*-test; Figure 2).

The CS and *Slo* strains incubated with 2-APB both showed a significant decrease in the rate of the heartbeat after 7 min and an increase when exposed to 5-HT (N = 24 each strain; $p > 0.05$, repeated measures ANOVA and a post-hoc Bonferroni *T*-test; Figure 3), but did not show differences between the two strains (N = 24 each strain; $p < 0.05$, Rank Sum ANOVA and a post-hoc Bonferroni *T*-test; Figure 3). Since the effect of 2-APB and 5-HT were the two conditions of interest, these were examined for significant differences. Two different sets of data were obtained for both the CS and *Slo* strains on two different days with each day obtaining 12 preparations with freshly made 2-APB and 5-HT each day and rotating between the strains when taking measures. Note that the rate increases at 1 min for the CS strain (Figure 1D1) but over time the rate decreases (Figure 3A2).

3.3. Effect of Thapsigargin and 5-HT on Heart Rate in CS and Slo

Thapsigargin blocks the SERCA pump on the sarcoplasmic reticulum, which is the same proposed mechanism of action as 2-APB. Thus, the two different pharmacological agents should show similar effects on the heart rate for the CS and *Slo* strains. Upon blockage of the SERCA, $[Ca^{2+}]_i$ in the larval heart rate is expected to rise and potentially decrease in the same manner as for the preparations exposed to 2-APB. Exposure to 5-HT was not expected to increase the heart rate after incubation with thapsigargin.

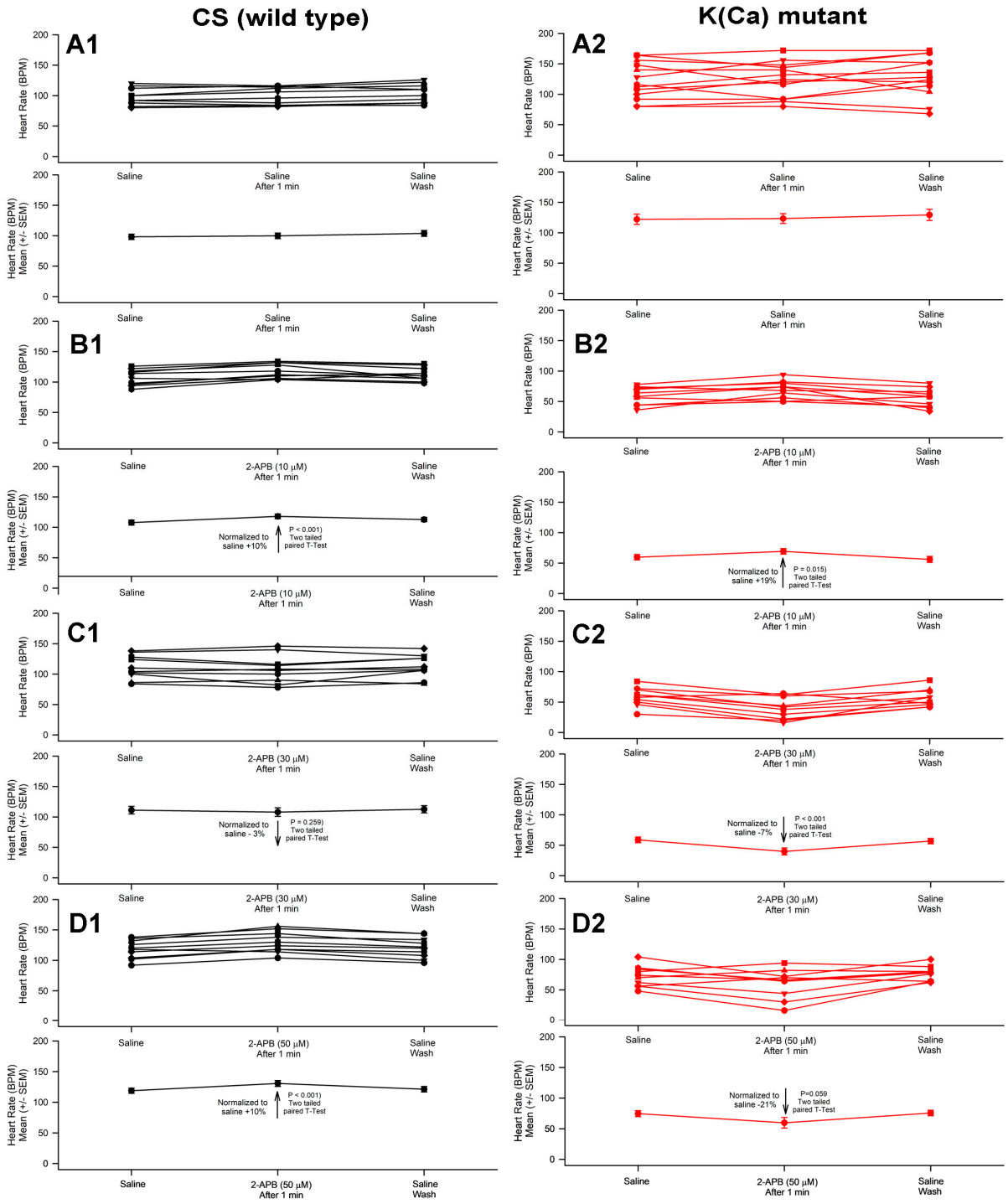

Figure 1. Effect on heart rate with exposure to 2-APB for CS and *Slo* (K(Ca) mutant) strains. (**A1**,**A2**) Controls for exchanging the saline bath. Saline was exchanged for saline and again for fresh saline. (**B1**,**B2**) The effect of 2-APB (10 μM) on heart rate after 1 min of incubation followed by saline wash. (**C1**,**C2**) The effect of 2-APB (30 μM) on heart rate after 1 min of incubation followed by saline wash. (**D1**,**D2**) The effect of 2-APB (50 μM) on heart rate after 1 min of incubation followed by saline wash. The left column represents responses for the CS strain and the right column for the *Slo* (K(Ca) mutant) strain. There is a significant decrease in heart rate to exposure of 2-APB at 10, 30, and 50 μM for the *Slo* strain (**B2**,**C2**), (N = 10, N = 12 for 50 μM, $p > 0.05$, Paired *T*-test; the CS strain presented with a significant effect for 10 μM and 50 μM, *T*-Test was used $p > 0.05$). A percent change in the individuals from saline to 2-APB which normalized the differences in the initial values of the rates.

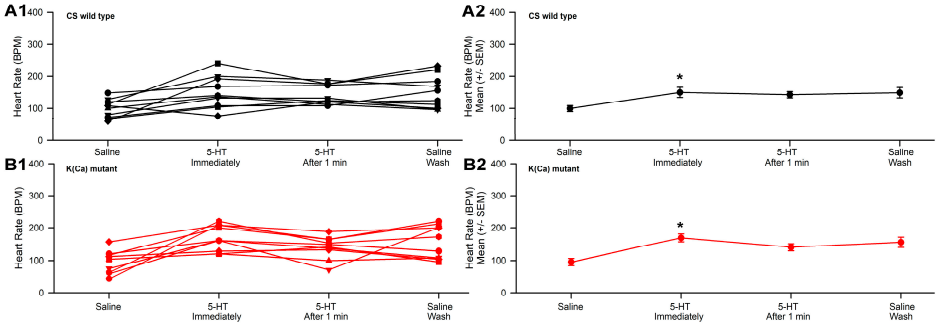

Figure 2. The effect of exposure to 5-HT on heart rate. (**A1**,**A2**). The CS larvae and the (**B1**,**B2**) *Slo* both increased in heart rate immediately upon exposure to 5-HT and the rates remained elevated for over a minute (N = 10; $p > 0.05$, Paired *T*-test). Upon exchanging the media back to fresh saline, the rates remained elevated. The * (asterisk) indicates a significant difference between saline and 5-HT.

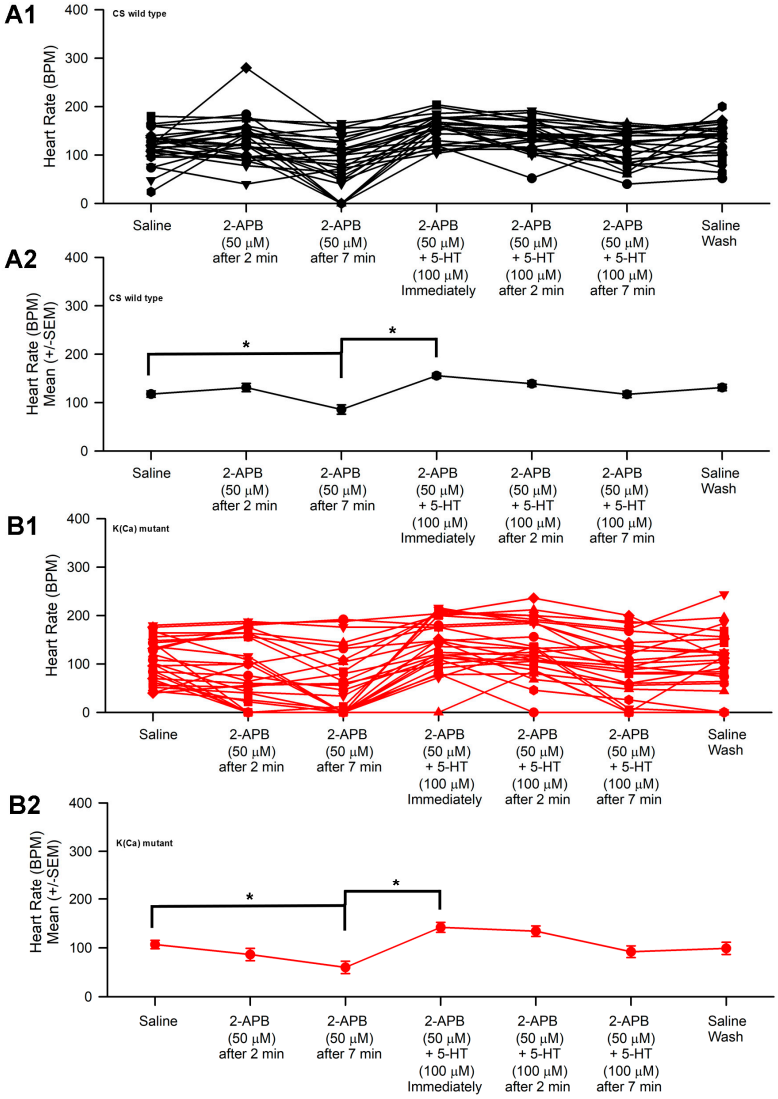

Figure 3. The effect of exposure to 5-HT on heartbeat rate after incubation with 2-APB. (**A1**) The CS larvae and the (**B1**) *Slo* both decreased the rate of the heartbeat with 2-APB after 7 min and increased in heartbeat rate immediately upon exposure to 5-HT and the rates remained elevated for over 2 min (N = 24 for each line; $p < 0.05$, ANOVA and a post-hoc Bonferroni *T*-test). (**A2**) Represents the mean (+/− SEM) of values shown in A1. (**B2**) Represents the and mean (+/− SEM) of values shown in B1. The * (asterisk) indicates a significant difference between groups.

The manufacturer's (Tocris Bioscience) recommendation for thapsigargin is that it can be stored for up to 6 months from the date of receipt at −20 °C. Our 1st batch had been stored for a longer period at −20 °C (Figure 4A1). Thus, we ordered fresh thapsigargin, using it within a week of obtaining it and making solutions fresh on the day of use (Figure 4A2). Upon comparing the twelve preparations within each batch to each other, there were no significant differences (ANOVA). However, in the new batch, one preparation with ten minutes of exposure to thapsigargin had its heart stop beating. That preparation resulted in the data set being non-normally distributed. When that one preparation was removed, the differences were noted between the two batches of thapsigargin. In combining the two data sets, there was an increased heart rate upon exposure to 5-HT and after the 20 min of incubation to thapsigargin (N = 12, $p > 0.05$, Paired *T*-test) as well as for the *slo* strain (N = 12, $p > 0.05$, Paired *T*-test; Figure 4B) with no significant difference occurring between the two strains concerning the effect of 5-HT (N = 24 and 12, $p < 0.05$, *T*-test; Figure 4A1,A2,B). Exchanging the saline bath for fresh saline did not alter the heart rate over the 20 min of incubation for the *slo* strain (i.e., K(Ca) mutational line) (Figure 4C).

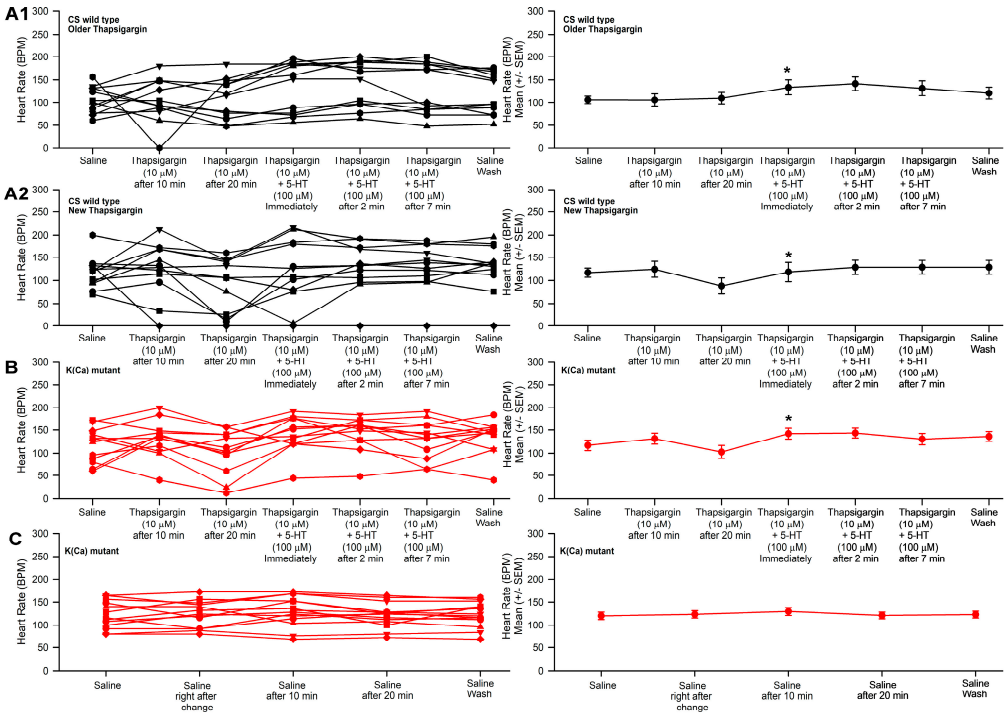

Figure 4. The effect of exposure to 5-HT on heart rate after incubation with thapsigargin for 20 min. (**A1**,**A2**) Two batches of thapsigargin were examined. One batch (**A1**) was stored for over 6 months as a lyophilized solid at −20 °C; the other was a newer batch used within a week (**A2**). There were no significant differences in the responses between the two groups (N = 12 for each group, ANOVA). The rate increased in heart rate immediately upon exposure to thapsigargin and 5-HT as compared to 20 min of thapsigargin exposure (Paired *T*-test $p < 0.05$, N = 24), and remined higher for the next 7 min. (**B**) The K(Ca) mutational line (*Slo*) increased in heart rate immediately upon exposure to thapsigargin and 5-HT as compared to 20 min of thapsigargin exposure (Paired *T*-test $p < 0.05$, N = 12), and remined higher for the next 7 min. (**C**) A control for exchanging the bathing media altering the heart rate was performed for the K(Ca) mutational line (*Slo*) without any significant changes for 20 min of incubation. The * (asterisk) indicates a significant difference between thapsigargin after 20 min to the immediate application of 5-HT. Traces on the right are means (+/− SEM) of the traces on the left.

A summary of the main findings in this study is presented in Table 1. As indicated, the most noticeable changes in heart rate between the strains occurs for the high concentration of 2-ABP at 50 µM.

Table 1. Summary of the effects on heart rate by pharmacological agents on wild-type CS and the *Slo* strain.

	2-ABP				2-APB 50 µM	Thapsigargin 10 µM
	10 µM	30 µM	50 µM	5-HT 100 µM	5-HT 100 µM	5-HT 100 µM
CS	↑	↓	↑	↑	↑	↑
Slo	↑	↓	↓	↑	↑	↑

4. Discussion

In this study, it was determined that mutations in the K(Ca) channel (i.e., *Slo*) did not illustrate a large contribution in regulating the in situ heart rate under normal laboratory conditions for larval *Drosophila*, with exposure to pharmacological agents resulting in heart rate differences compared to the wild-type CS strain. Wild-type and *Slo* both responded with an increase in heart rate upon 5-HT exposure, even if the preparations were incubated with 2-APB or thapsigargin. However, exposure to high concentrations of 50 µM 2-APB decreased the heart rate in the *Slo* strain and increased the heart rate in the wild-type CS strain. Comparing the percent change between the *Slo* and CS strains from saline to 2-APB, there were no significant differences.

The regulation of the cytoplasmic Ca^{2+} concentration in the cardiac myocytes of larval *Drosophila* is key to influencing heart rate. The balance of Ca^{2+} though PMCA, NCX, endoplasmic reticulum, and the plasma membrane voltage-gated Ca^{2+} channels are the major contributors to cardiac performance. It is possible that the larval K_{Ca} channel has a role in fine-tuning the rate under various environmental conditions, such as heat or cold stress, or particular ionic conditions in the hemolymph or cytoplasm. Even with a compromised K_{Ca} channel and 5-HT modulation of the endoplasmic reticulum to release Ca^{2+}, the heartbeat was able to be maintained.

There is a therapeutic interest in being able to modulate intracellular Ca^{2+} concentrations in cells, particularly within myocytes. One approach is to modulate the release and storage of Ca^{2+} within the SER as well as Ca^{2+} exchangers and pumps on the plasma membrane [72–74]. The compound 2-APB, as well as derivatives of its chemical structure, has been investigated over the years for altering the ER store of Ca^{2+} [19]. 2-APB shows some promise in cardio-protection from ischemia [75]; however, some of the derivatives may show more promising effects [76]. As mentioned earlier, the exact nature and various actions of 2-APB remain to be addressed [77]. Given the rapid-action effects of 2-APB reported for MDA-MB-231 breast cancer cells and mast cells [19,78], it may work more quickly than thapsigargin, which has been established as needing several minutes to an hour of incubation in order to be effective in blocking the SERCA [79,80].

It seems likely that 2-APB is able to pass through the plasma membrane more rapidly than thapsigargin, eliciting as-of-yet unidentified actions that would explain the differences in action between the two compounds. Even though 2-APB has been shown to have varied mechanistic effects, it is worthwhile to investigate its physiological actions in relation to other agents assumed to have the same mechanisms of action and, thus, determine the similarities and differences. Should this be done, the physiological effects of modifications in these agents' chemical structures can also be examined, with potential applications in the development of therapeutic compounds or experimental agents for addressing cellular processes [81].

The stability of 2-APB in the saline used for *Drosophila* may be compromised over time. In initial studies, the 2-APB was stored at 4 °C to be used on multiple days, as completing all the studies in one day was not possible. However, we noticed differences from one day to the next when using stored 2-APB. The stored compound reduced the rates more drastically than when it was made fresh on the day of experimentation, and the responses were more variable. The pH was noted to be the same across the days, when checked at room temperature. In addition, small weights of the 2-APB powder were initially measured to directly produce 10, 30, and 50 µM. To rectify this issue, we made fresh 2-APB at 500 µM on the day of experimentation and diluted to the concentrations used. In addition, the stock solution was vigorously vortexed for 5 min to ensure mixing. The 25 mM buffer of

BES or the salts in the physiological saline may interact with 2-APB in ways not established, but freshly made solutions on the day of experimentation appear to be required.

The use of *Drosophila* as a model can aid in screening novel pharmacological agents, allowing us to address not only the similarities and differences amongst organisms, but also basic cellular commonalties. It would be of interest to use this model preparation to screen other known agonists and antagonists of the SERCA and K_{Ca} channels used in mammals. A working model to highlight some of the mechanisms investigated in this study in relation to regulating Ca^{2+} within the larval *Drosophila* cardiac myocyte are illustrated in Figure 5.

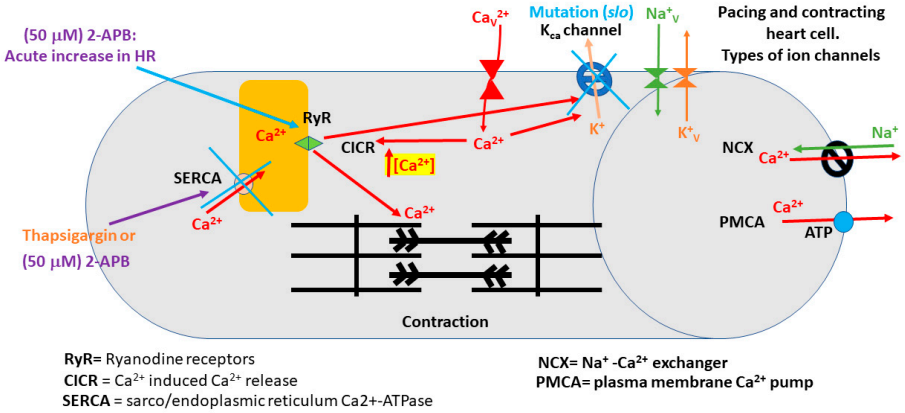

Figure 5. Schematic model in the effects on heart rate in larvae when altering intracellular Ca^{2+} by 2-APB in wild-type and *slo* strains of larval *Drosophila*.

A theoretical model of maintaining the pacing heart may start with the leak of Ca^{2+} from the SER via the ryanodine receptors (RyR) leading to the NCX exchanging Ca^{2+} out for Na^+ into the myocyte as well as Ca^{2+} being pumped out via the PMC (Figure 5). The Na^+ entry may lead to further member depolarization and opening of the voltage-gate Ca^{2+} channels on the membrane and potentially Na_V and K_V channels. The rise in $[Ca^{2+}]_I$ may even induce a calcium-induced calcium release of RyR. The increase rise in intracellular Ca^{2+} may then be taken up by the SER via a SERCA pump and even exchanged out of the myocyte via the PMCA. Depending on the threshold of the K_{Ca} channel for Ca^{2+}, a K^+ current would lead to hyperpolarization and the cycle repeating, with many of these actions happening simultaneously. Any disruption in the rapid balance of Ca^{2+} dynamics could then also affect the heart rate, as the myocytes are also pacemaker cells. This depression would reduce the presence of K_{Ca} channels. Blocking the SERCA or targeting the RyR would be expected to alter heart rate. Being able to image the rapid Ca^{2+} signals while perturbing various channels, pumps, and exchangers would be ideal, especially during the application of pharmacological agents. Capturing the Ca^{2+} sparks within the cycle requires rapid imaging when cells are not contracting [82].

Author Contributions: Conceptualization, N.H., E.R.E., M.O.A.-K. and R.L.C.; methodology, N.H., E.R.E., M.O.A.-K. and R.L.C.; software, N.H., E.R.E., M.O.A.-K. and R.L.C.; validation, N.H., E.R.E., M.O.A.-K. and R.L.C.; formal analysis, N.H., E.R.E., M.O.A.-K. and R.L.C.; investigation, N.H., E.R.E., M.O.A.-K. and R.L.C.; resources, R.L.C.; data curation, R.L.C.; writing—original draft preparation, N.H., E.R.E., M.O.A.-K. and R.L.C.; writing—review and editing, N.H., E.R.E., M.O.A.-K. and R.L.C.; visualization, N.H., E.R.E., M.O.A.-K. and R.L.C.; supervision, N.H., E.R.E., M.O.A.-K. and R.L.C.; project administration, R.L.C.; funding acquisition, R.L.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the alumni of the research group, personal funds (R.L.C.), and Chellgren Endowed Funding (R.L.C.).

Institutional Review Board Statement: Ethical review and approval were waived for this study due to use of insects.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are presented in the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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