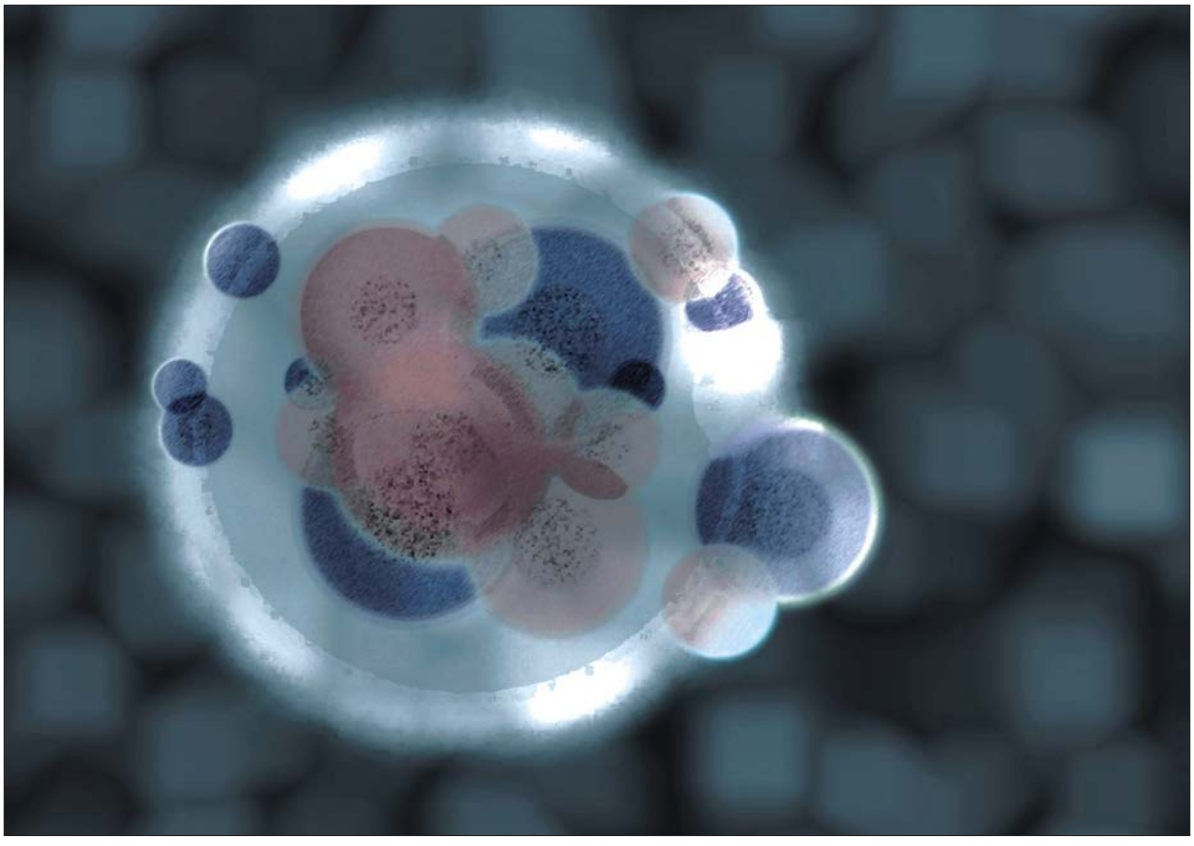

Journal of Biological Sciences

ISSN 1727-3048

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2026.1.17

Research Article
Influence of Mediating K2P and NALCN Channel Function on Cardiac Pacing with Alterations in Ionic Concentrations, Temperature and Expression

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Abstract

Background and Objective: The Sodium Leak Channel (NALCN) and the two-pore-domain K^+ (K2P) channel are known leak channels responsible for maintaining cell resting membrane potential through, respectively, the regulation of sodium and potassium ion flux across the cell membrane. Though some characterization of these channels exists, very little is known about them or the effects of abnormal expression beyond a connection to various human pathologies (e.g., cancer, neurological disorders). This study further examines how cardiac pacing behaviors are affected by altered ion channel expression and environmental conditions within *Drosophila melanogaster*. **Materials and Methods:** Several *D. melanogaster* strains were utilized to observe abnormal channel expression in the larval heart: One line to demonstrate K2P channel overexpression and two RNAi lines to exhibit reduced NALCN expression. Third-instar larvae were dissected to reveal the larval heart tube for observation and cardiac behaviors were observed in response to variations in temperature, ion concentrations in the surrounding saline and exposure to NALCN blocker CP-96345. **Results:** The use of RNAi techniques to decrease NALCN function resulted in a substantial increase in heart rate with higher external Ca^{2+} and increased temperature, as compared to control strains or increased cardiac K2P expression. The DMSO was used to solubilize CP-96345 and control experiments with the solvent alone yielded decreased heart rate, especially with K2P overexpression or NALCN knockdown. The CP-96345 exposure decreased heart rate in all strains tested. **Conclusion:** The data reported herein represent a preliminary examination into the effects of altered channel expression and, thus, provide further information into human pathologies and the treatment thereof.

Key words: Sodium Leak Channel (NALCN), K2P channels, resting membrane potential, cardiac sensitivity, ion channel regulation, RNA Interference (RNAi), calcium signaling, cardiac physiology

Citation: Taul, A.C., E.R. Elliott and R.L. Cooper, 2026. Influence of mediating K2P and NALCN channel function on cardiac pacing with alterations in ionic concentrations, temperature and expression. J. Biol. Sci., 26: 1-17.

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Funding: Research funding was provided by the University of Kentucky Cardiovascular Priority Area (A.C.T.), Beckman Scholarship (E.R.E), Chellgren Endowed Funding (R.L.C) and alumni of the research group.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

To maintain normal function, cells must balance a wide variety of variables, especially when united into complex systems like the syncytia of myogenic nodal pacing regions. Pacing cells like these require rapid and continuous ionic balancing (via coordination of passive and voltage-gated ion channels, pumps and exchangers), ready adaptability to speed alterations induced by external inputs (e.g., neural or hormonal control) and effective balance of ions and energy within the contractile machinery. In turn, the metabolic processes involved in the production of that energy affect cell function as well; specifically, ATP production through cellular metabolism within even a small group of nodal cells results in waste products that must be managed, potentially causing protonation of certain pertinent channels- given that carbonic anhydrase rapidly converts CO_2 into HCO_3^- and H^+-and altered temperature. The scope and variety of variables for which cells must adjust render it difficult to address the impact and interactions of all of them in unison; experimentally, altering any one factor to examine its impact on cellular function will also influence others.

However, examining how a biological system compensates for or responds to controlled manipulation nonetheless provides more information about the impact of the initial alteration. Addressing those variables likely most influential in driving pathological conditions may help develop a better understanding of potential therapies or treatments. Additionally, some genetic defects are silent unless enhanced (i.e., exacerbated) under stressful conditions like exercise or hormonal changes[1,2], while other pathological conditions are characterized by altered protein expression that is beneficial in some circumstances and harmful in others[3-6]. Both possibilities bear further investigation.

Since membrane electrical potential in pacing cells is continually changing, ionic flux regulation is very significant; any alteration in a pump, exchanger, voltage-gated channel, or ionic leak channel can perturb the pacing. Of note is the fact that key proteins in regulation of cell membrane potential-namely, Na^+ Leak Channels (NALCN)[7] and K^+ leak (K2P) channels[8]-are known to bear altered expression levels in pathologies associated with neuronal and cardiac function. Additionally, passive membrane potential is driven by ionic permeability, ionic concentration differences and temperature; however, temperature has a particularly diverse influence, as it affects not only the proteins involved in exchangers, pumps and voltage-gated channels, but even the permeability of leak channels and the pH of the medium.

Many investigations into mammalian channels occur at room temperature for ease of experimentation; however, these channels may function differently at body temperature *in vivo* and, thus, the effects of temperature alteration on regulation of membrane and pacing potentials may not be fully addressed by current literature. The kinetics of voltage-gated channels are generally increased as temperature increases[9] and can affect sensation perception[10]. In general, as temperature rises, so does invertebrate heart rate[11], yet, if the synchronization of ionic flux and contraction gets skewed, heart function may slow or flutter abnormally[12-14]. For example, optogenetically enhanced Cl^- flux in the *Drosophila* heart resulted in increased heart rate, or, in many cases, fluttering and then a cessation of beating altogether[14]. Even NALCN and K2P channels can undoubtedly be modulated by temperature, given their various associated accessory proteins, but the scarcity of studies into NALCN temperature dependence means that temperature sensitivity has only been demonstrated in K2P channels[15]. However, accessory proteins are known to be key modulators of NALCN function, which is critically dependent on the presence of proteins UNC79, UNC80 and FAM155A/B[16].

Little is known about how alterations in K2P/NALCN expression might impact membrane potential under varied environmental conditions (i.e., varied $[K]_o$, $[Ca^{2+}]_o$ and temperature). Given that Ca^{2+} is known to block NALCN[17-20], one would expect it to also affect membrane potential in pacing cardiac tissue, which is dependent on calcium ion influx; however, confounding variables in intact organisms must be considered. Both pharmacological manipulation and ion concentration alteration in the extracellular media constitute common approaches by which to address these effects. A number of genetic pathologies are also known to affect pacing in human cardiac tissue and have been modeled in rodents and *Drosophila*[21,22]. In some pathological cases, such as cancer and autism, NALCN and K2P channels are known to be altered in expression[23,24], such that the cells' electrical properties and response to environmental changes (e.g., temperature) are likely abnormal. It is unknown whether these expression alterations are the cause of the disease or a result of the disorder; regardless, it is of interest to know how tissues respond to alterations in channel function or expression, or to the presence of a psychological/environmental stressor. Additionally, since the effects of some pathologies are only uncovered with such environmental changes, investigations into the effects of altered temperature or ionic concentration (such as slight alterations to $[K^+]_o$ and $[Ca^{2+}]_o$) require that the effects of these parameters be addressed first individually, then in combination.

In past investigations with *Drosophila melanogaster*, a particular line (sensitive to volatile anesthetics) was noted to produce adults with more slender and elongated abdomens[25,26] and was thus christened "narrow abdomen." It was later shown that this phenotype is related to reduced NALCN expression in specific *Drosophila* cells, altering function and (depending on the tissue) potentially impacting the whole organism[27]. In this study, *na* expression was reduced in cardiac tissue with the use of RNAi strains. Another protein, observed in yeast and called Mid1 (from "mating-induced death" phenotype), is known to be key for calcium channel and (due to similarities in the relevant accessory proteins) NALCN function[28]. In *Drosophila*, the Mid1 protein is also referred to as Nif1 and has been shown to localize NALCN in the plasma membrane[29,30], while knockdown of Nif1 via RNAi techniques decreases NALCN expression[31].

The investigation reported herein aimed to investigate how alterations to temperature and/or to the ionic medium affected function of NALCN and K2P channels and, in turn, cardiac activity. *Drosophila* represent an ideal model by which to investigate these effects, as the organisms are readily amenable to genetic manipulation (allowing mimicry of human disorders and examination of subsequent effects) and can be dissected to reveal *in situ* exposed larval heart rates for investigation of compound/pharmacological agent exposure[32-34] without confounding from an uncontrolled surrounding medium. The larval hearts also serve as good representations of pacing myogenic hearts because they lack cardiac innervation, thus eliminating confounding caused by neural effects. Alterations in the experimental parameters ($[K^+]_o$, $[Ca^{2+}]_o$ and temperature) were assessed through evaluation of larval heart rate in wild-type flies (normal *Drosophila* strains), those overexpressing a *Drosophila*-specific K2P channel subtype (dORKA1) and those with either type of NALCN knockdown (in cardiac-specific tissue). To compare the pharmacological effects of decreasing NALCN function to the effects of both RNAi knockdown procedures, the NALCN blocker CP-96345 was utilized[27,35].

MATERIALS AND METHODS

Study design: To begin, the effects of temperature alteration on heart rate were examined for UAS parental strains (UAS-ORK1, UAS-mid1 RNAi, UAS-NA RNAi, UAS-TrpA1), ORK1 over-expressors (HAND>ORK1), NALCN knock-downs (HAND>mid1-RNAi and HAND>NA-RNAi) and TrpA1 over-expressors (HAND>TrpA1). Then, three phases of experimentation were conducted in HAND>ORK1, HAND>NA RNAi, UAS-ORK1 and UAS-NA RNAi organisms. The first phase investigated the independent cardiac effects of raised extracellular K^+ and varied extracellular Ca^{2+}. The second phase examined the cardiac effects of simultaneous variation in temperature and, respectively, $[K^+]_o$ or $[Ca^{2+}]_o$. The third phase was a combination study, investigating the effects of simultaneous variation in temperature, Ca^{2+} and K^+. Finally, in these same lines (HAND>ORK1, NA-RNAi, UAS-ORK1, UAS-NA RNAi), the effects of NALCN blocker CP-96345 (50 μM solution in DMSO) were examined, along with controls using DMSO solvent alone to reduce confounding.

Study area and duration: This research was conducted at the University of Kentucky in Lexington, Kentucky, USA, between January 2023 and May 2026.

***Drosophila* strains and maintenance:** Larval heart-specific overexpression of the ORK1 protein, a two-pore-domain potassium (K2P) channel, was achieved by crossing homozygous males of the heart-specific Hand4.2-Gal4 strain (chromosome II), provided by Dr. Anthony Cammarato, with virgin females from the respective UAS and RNAi stocks. The UAS-ORK1 strain (BDSC stock #6586) was crossed with Hand4.2-Gal4 males and the resulting progeny were designated HAND>ORK1.

Two RNAi lines were used to reduce the expression of NALCN-related channels. Virgin females of the mid1 RNAi strain (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8] = TRiP.HMS03014}attP2/TM3, Sb[1]; BDSC stock #36754) were crossed with Hand4.2-Gal4 males and the progeny were designated HAND>MID1 RNAi. Similarly, virgin females of the NA RNAi strain (y[1] v[1]; P{y[+t7.7] v[+t1.8] = TRiP.JF01826}attP2; BDSC stock #25808) were crossed with Hand4.2-Gal4 males and designated HAND>NA RNAi.

To activate the temperature-sensitive TRPA1 channel in the larval heart, the UAS-TrpA1 strain (w[*]; P{y[+t7.7] w[+mC]=UAS-TrpA1(B).K}attP16; BDSC stock #26263) was crossed with Hand4.2-Gal4 males. The resulting progeny were designated HAND>TrpA1.

All larvae were maintained at approximately 21°C in vials containing cornmeal-agar-dextrose-yeast medium under standard laboratory conditions.

Ethical consideration: Invertebrate animal care was approved by the Institutional Animal Care and Use Committee (University of Kentucky).

Dissection and heart preparation: To expose the larval heart tube *in situ*, procedures were followed as described in de Castro *et al.*[36] and in video format[37]. In brief, the larvae were dissected via a ventral incision and pinned to expose the heart tube. This type of dissection and recording dish has been previously described for pinning ganglia isolated from the leech ventral nerve cord[38]. The surrounding hemolymph was flushed away and replaced with saline controlled to resemble physiological conditions[36,39].

Heart rate assessment: Upon dissection, a microscope can be used to directly observe the *Drosophila* heart as it beats, whereupon comparisons of cardiac function across experimental conditions can be produced by determining the number of beats performed per minute in each condition. These determinations were conducted by counting (through visual observation) the number of beats performed in 15 sec, then quadrupling that number. Heart rate was calculated in this manner under each experimental condition.

Experimental solutions and reagents: The saline was a modified HL3 saline[36,39] containing (mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5-N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES). The saline was aerated and the pH corrected to 7.1 with NaOH (1 M) or HCl (1 M) at room temperature of 20-21°C. An Accumet model 10 pH meter (Fisher Scientific) and Ag/AgCl glass electrodes were used. All chemicals listed above were obtained from Sigma-Aldrich (St. Louis, MO). The NALCN blocker CP-96345 (Tocris Bioscience) was dissolved in DMSO and then placed into warmed saline at quantities, respectively, of 1.0 mg, 50 µL and 50 mL, yielding a solution of 50 µM. Alterations in the media were dependent on the experimental paradigm used, with each condition described individually in the Results.

Temperature regulation and experimental conditions: Regulation of experimental temperature was performed by placing the dissection dishes (thin glass slides) on the surface of a platform submerged in a temperature-regulated water bath. The platform comprised a test tube rack anchored to the bottom of the water bath, thus allowing the water to circulate freely around the bottom of the glass plate.

It should be noted that, for lack of a better option, solution temperatures were monitored with an infrared thermometer, which could have affected the temperature readings. It is known that infrared does not pass through water, so-given that most of the solutions used herein are saline, thus representing high water content-the temperatures reported refer to the temperatures observed at the surface of the water. Given that all saline solutions were observed in this same manner, the relative temperature differences remain unaffected and so the results reported here are equally preserved.

Statistical analysis: Significance was observed at $p<0.05$. The significance tests performed under each specific condition are listed in the Results section. When necessary, normality was assessed using the Normality Test by Shapiro-Wilk.

RESULTS AND DISCUSSION

Effects of temperature on heart rate in standard saline: The increased temperature produced variable responses in all the UAS strains (Fig. 1a-g). The only significant difference at room temperature (~21°C) was between UAS-ORK1 and UAS-TrpA1 (Fig. 1a-g; unpaired t-test, $p<0.05$, $n = 10$). Temperature change from ~21 to ~34°C in HAND>ORK1 and HAND>mid1 lines produced variable results even within strains, with heart rate increasing for some larvae and decreasing for others; it almost seemed as though there were two different groups within each strain, each responding in opposite manner to the other (Fig. 1b-d). Upon returning to ~21°C, the larval hearts that had previously stopped beating resumed cardiac function, while those that bore increased heart rates returned to a lower rate, indicating that their hearts were not damaged by the increased temperature and could regain normal function. In contrast, the HAND>NA and HAND>TrpA1 strains both saw significantly increased cardiac rates with increased temperature (Fig. 1f-h; paired t-test, $p<0.05$, $n = 10$) and a return to decreased rate following a return to the initial temperature (~21°C).

The overall heart rates exhibited by each strain at the various experimental temperatures are displayed as mean (±SEM) rates (Fig. 2a). The two strains significantly affected by temperature are shown with asterisks above the mean values. Comparison among the strains and normalization of the variability in the initial rates allowed for determination of each larva's percent change (between the initial and increased temperatures). The mean percent changes for each strain indicated that only the HAND>TrpA1 larvae were significantly different (Fig. 2b; ANOVA, $p<0.05$, $H = 12.269$, 6 degrees of freedom); however, the data were not normally distributed (Normality Test by Shapiro-Wilk).

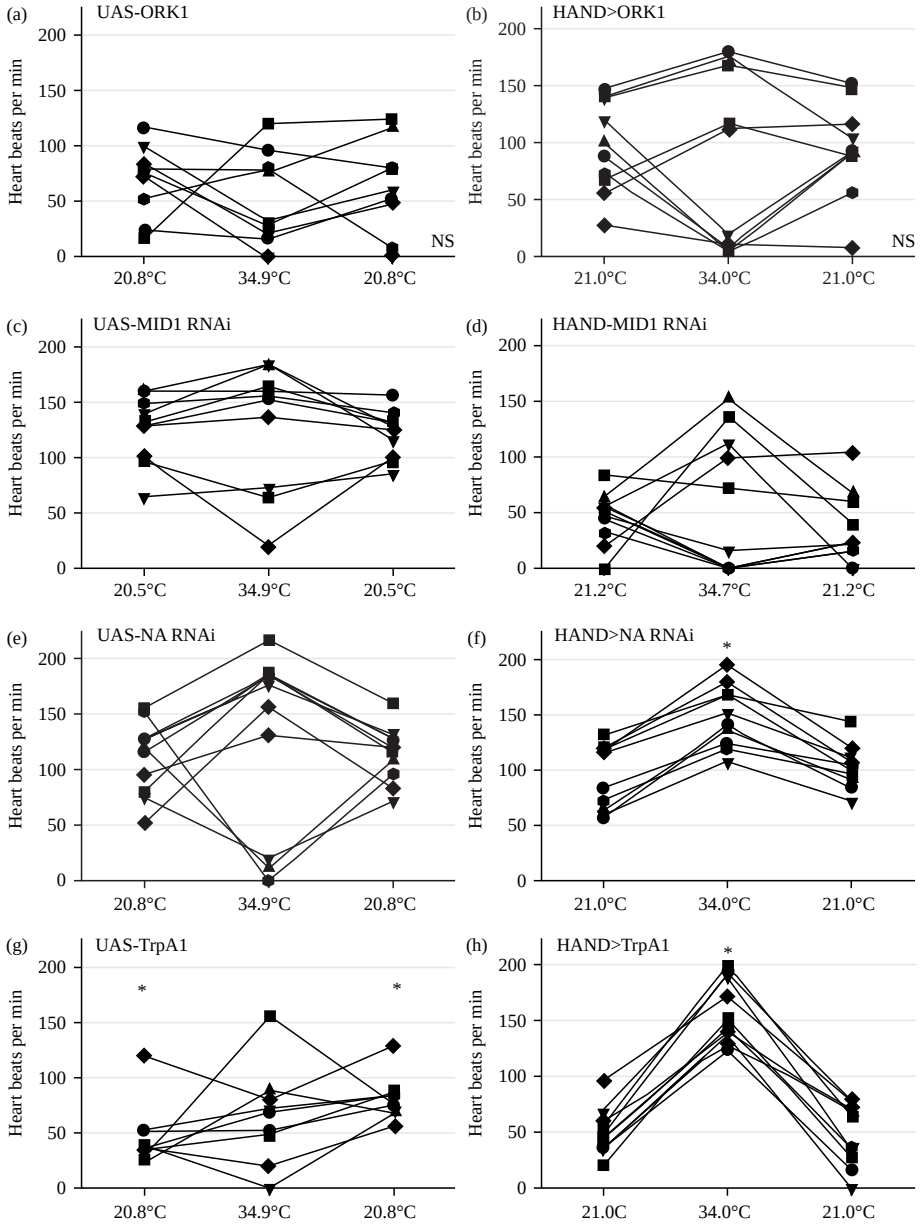

Fig. 1(a-h): The effects of temperature alteration on heart rate in lines with cardiac expression demonstrating (a, c, e, g) control levels, (b) K2P channel overexpression, (d) MID1 protein knock-down, (f) *na* knock-down and (h) TrpA1 overexpression

The asterisks represent significant changes ($p<0.05$, paired t-test, $n = 10$). Each line represents a single larva across the various conditions

Effects of K^+ on heart rate: The effects of raised extracellular potassium (i.e., $[K^+]_o$) on larval heart rate, as observed with ORK1 overexpression, RNAi-induced *na* knockdown and parental UAS strains, indicated that all featured decreased heart rates with increased $[K^+]_o$ (Fig. 3a-d). Changing $[K^+]_o$ from 1 mM to 5 mM over a short window (1 minute) induced varied responses among the strains, with most larvae illustrating decreased heart rate and a few showing an increase. Only the HAND>ORK1 larvae bore a significant change between 1 and 5 mM conditions (paired t-test, $p<0.05$, $n = 10$). All strains studied showed a significant change from 1 mM to all experimental conditions 10 mM and greater (paired t-test, $p<0.05$, $n = 10$); additionally, all strains featured significant increases in the rates upon return to 5 mM from 35 mM, indicating that the high $[K^+]_o$ exposure had not damaged the heart tissue beyond recovery. It should be noted that only the HAND>ORK1 larvae did not completely cease cardiac activity with $[K^+]_o$ increased to 35 mM (Fig. 3b).

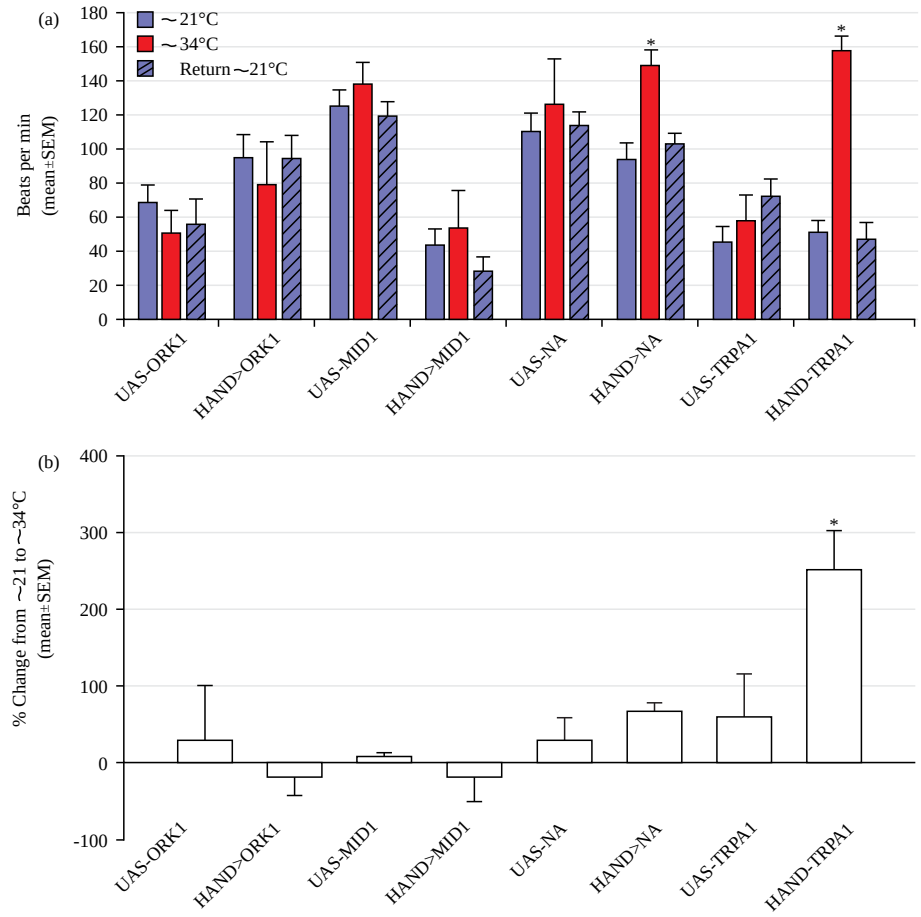

Fig. 2(a-b): Heart rates for each strain during exposure to temperature changes, (a) Mean (±SEM, n = 10) values in beats per minute (bpm) of larval heart rates for strains exposed to 21°C, then 34°C and back and (b) Percent change between initial heart rate and that measured at 34°C

Early 3rd instar *in situ* hearts were used. The raw data values used for these histograms are depicted in Fig. 1. The asterisks represent significant changes (a) $p<0.05$, paired t-test, n = 10 and (b) ANOVA, $p<0.05$

To compare the effects of raised $[K^+]_o$ on heart rate among the strains and to normalize the various initial rates, percent change was determined from the heart rates measured with 1 mM $[K^+]_o$ to those measured with each increasing $[K^+]_o$ solution (Fig. 4a-b). The percent differences used for the paired t-tests conducted here were for each increase of $[K^+]_o$ from 1 mM to the higher experimental $[K^+]_o$ concentrations. With the exception of the change from 1 mM to 10 mM observed in HAND>NA RNAi preparations (Fig. 4a), no significant differences were observed in either the UAS-NA RNAi or HAND>NA RNAi lines, regardless of the $[K^+]_o$ concentration being observed (Fig. 4a; unpaired t-test, $p>0.05$, not normally distributed because rates dropped to zero in some cases). However, a significant difference was observed between the UAS-ORK1 and HAND>ORK1 lines, as determined through percent changes from 1mM to the higher $[K^+]_o$ concentrations (Fig. 4b). Even though the HAND>ORK1 larvae did not exhibit a cessation of heart rate, the percent changes observed were larger because the initial rates (i.e., at $[K^+]_o = 1$ mM) were higher.

Effects of Ca^{2+} on heart rate: The cardiac effects of altering extracellular calcium (i.e., $[Ca^{2+}]_o$) to concentrations ranging from 0.5 mM to 3 mM were only significant for the decrease from 1 mM to 0.5 mM, as observed in both UAS-ORK1 and HAND>ORK1 larvae (Fig. 5a-b; paired t-test, $p<0.05$, n = 10). The UAS-NA RNAi and HAND>NA RNAi strains did not show consistent trends in heart rate alteration over the range from 0.5 to 3 mM $[Ca^{2+}]_o$ (Fig. 5c-d).

To compare the cardiac effects of altered $[Ca^{2+}]_o$ among strains and to normalize the various initial rates while exposed to 1 mM $[Ca^{2+}]_o$, percent changes were determined at each stage of the various $[Ca^{2+}]_o$ alterations (Fig. 6a-b). Significant percent differences were observed with the

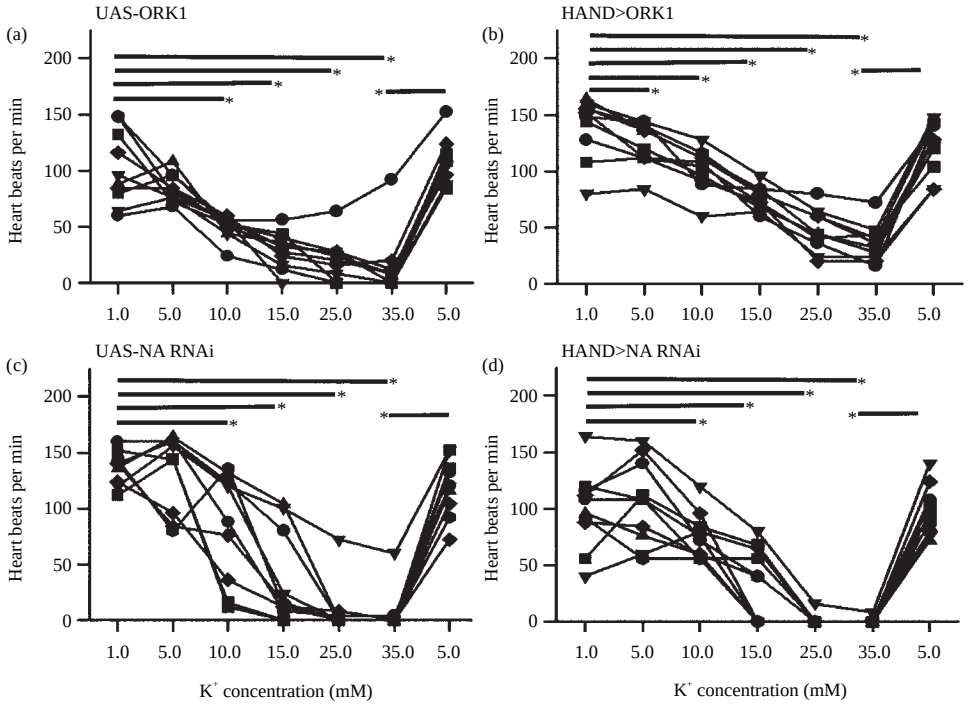

Fig. 3(a-d): Effects of raised extracellular K^+ on heart rate in lines with cardiac expression demonstrating (a, c) Control levels, (b) ORK1 overexpression and (d) RNAi-induced *na* knockdown

Each line represents a single larva in the various conditions. The asterisks represent significant changes ($p<0.05$)

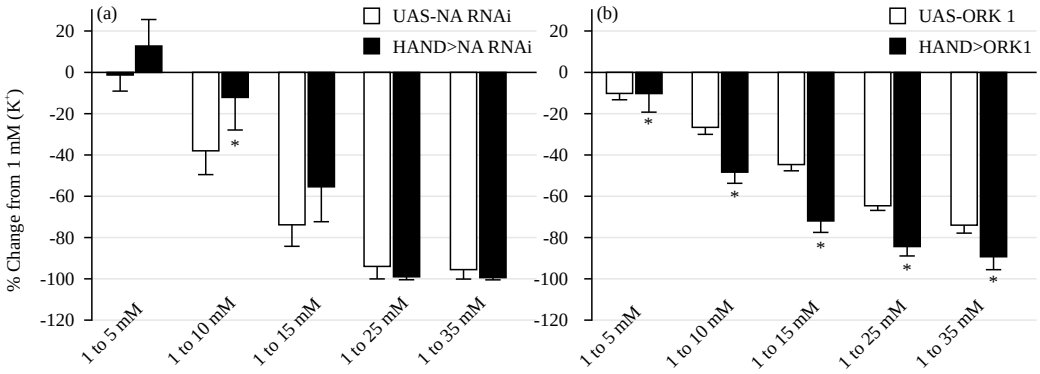

Fig. 4(a-b): Percent changes from 1 mM to varying raised extracellular K^+ in lines with cardiac expression demonstrating (a) *na* knock-down via RNAi techniques and the relevant control line or (b) ORK1 overexpression and the relevant control line

Values are displayed as means (±SEM). The asterisks represent significant changes ($p<0.05$). The raw data values used for these histograms are depicted in Fig. 3

change from 1 to 0.5 mM $[Ca^{2+}]_o$ for UAS-ORK1 and HAND>ORK1 (paired t-test, $p<0.05$, n = 10). For the parental strains, UAS-ORK larvae exhibited a significantly larger percent decrease from 1 mM to 0.5 mM $[Ca^{2+}]_o$ than the UAS-NA RNAi larvae (unpaired t-test, $p<0.05$, n = 10).

Effects of K^+ and temperature on heart rate: The effects of simultaneously raised $[K^+]_o$ (between 1 and 15 mM) and altered temperature (from ~21 to ~35°C) on larval heart rate in strains with ORK1 overexpression, the RNAi *na* strain, or control (parental UAS) expression did not include any significant trends (Fig. 7a-d). Even normalizing the initial rates for ~21 and ~35°C at each $[K^+]_o$ (1 to 15 mM) through calculation of percent changes did not reveal any significant changes (Fig. 8a-d). The lack of trends reported here is largely due to the wide variation in

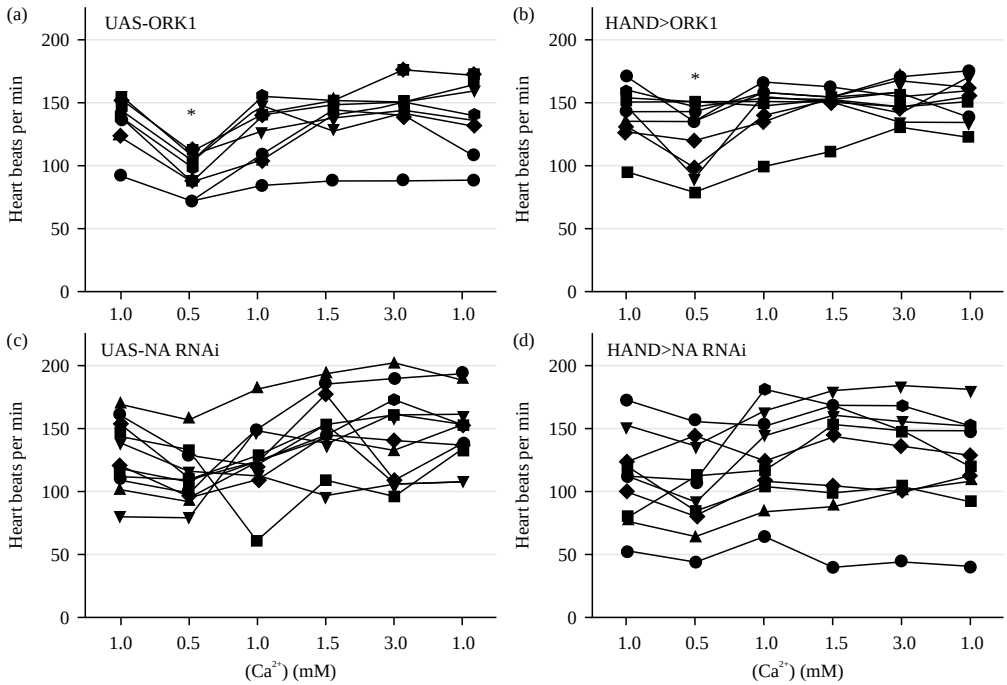

Fig. 5(a-d): Effects of varied extracellular Ca^{2+} on heart rate in lines with cardiac expression demonstrating (a, c) Control levels, (b) ORK1 overexpression and (d) RNAi-induced *na* knockdown

Each line represents a single larva in the various conditions. The asterisks represent significant changes ($p<0.05$)

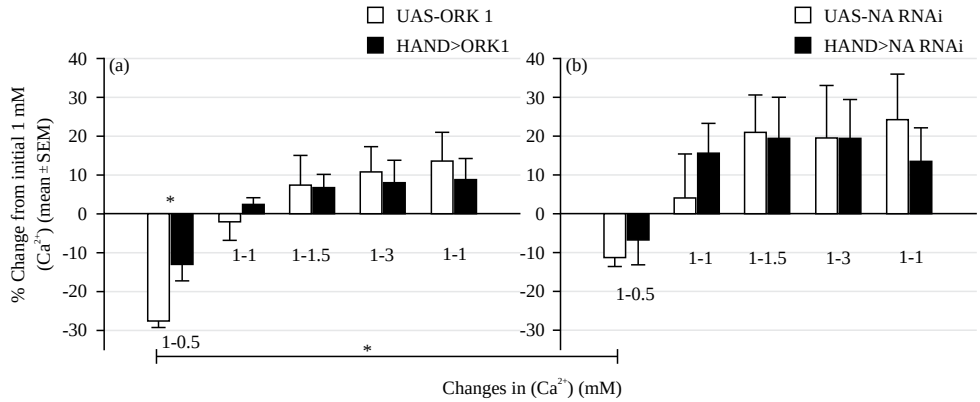

Fig. 6(a-b): Percent changes between 1 mM and the various extracellular Ca^{2+} concentrations. The asterisks represent significant changes ($p<0.05$), percent changes observed with (a) ORK1 overexpression and the relevant control levels and (b) RNAi-induced knockdown of *na* and the relevant control values

The values are depicted as means (±SEM). The raw data values used for these histograms are depicted in Fig. 5. Each set of percent changes is marked to illustrate which experimental conditions were involved; for example, the bars marked 1-0.5 represent percent changes from 1 to 0.5 mM

effects observed with the various variable changes; while larval heart rates were affected by temperature changes, they did not illustrate a consistent direction of change.

Effects of Ca^{2+}, K^+ and temperature on heart rate: The effects of simultaneous alteration of $[Ca^{2+}]_o$ (between 0.5 and 1 mM), $[K^+]_o$ (between 5 and 10 mM) and temperature (between ~21 and ~35°C) were examined in Fig. 9a-h. Significant effects were observed for: HAND>ORK1 larvae at 0.5 mM $[Ca^{2+}]_o$ and 5 mM $[K^+]_o$ (Fig. 9c, paired t-test, $p<0.05$, $n = 10$); HAND>NA RNAi larvae at 1 mM $[Ca^{2+}]_o$ and 5 mM $[K^+]_o$ (Fig. 9h, paired t-test $p<0.05$, $n = 10$); and both UAS-ORK1 and HAND>NA RNAi larvae exposed to 10 mM $[K^+]_o$ and 0.5 mM $[Ca^{2+}]_o$ (Fig. 10a-d, paired t-test, $p<0.05$, $n = 10$). All other examined crosses exhibited no such significant effects upon temperature change (Fig. 9a-g and Fig. 10b-c).

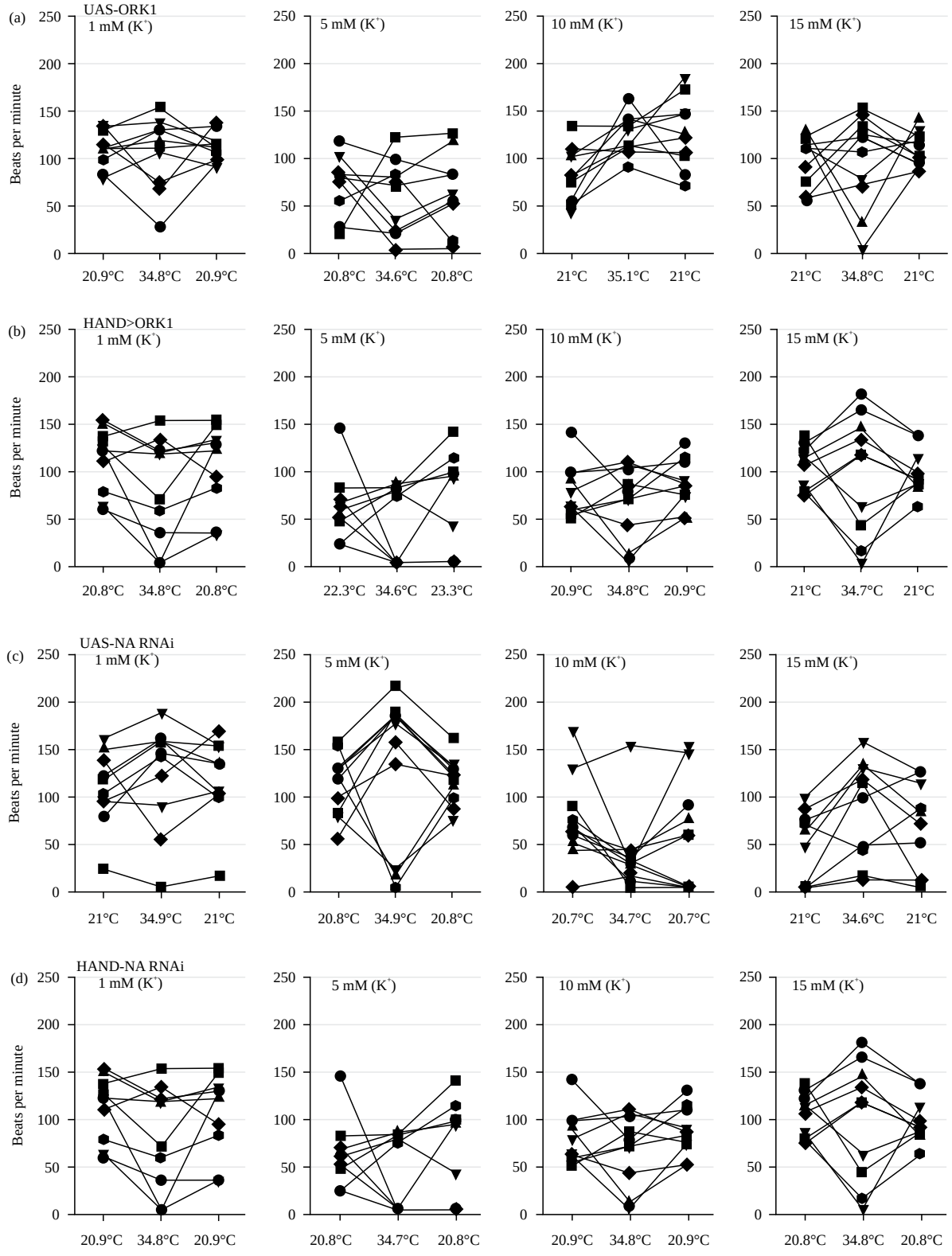

Fig. 7(a-d): Effects of simultaneous variation in extracellular [K^+] and temperature on larval heart rate in lines with cardiac expression demonstrating (a, c) control levels, (b) ORK1 overexpression and (d) RNAi-induced knockdown of *na*
Each line represents a single larva in the various conditions

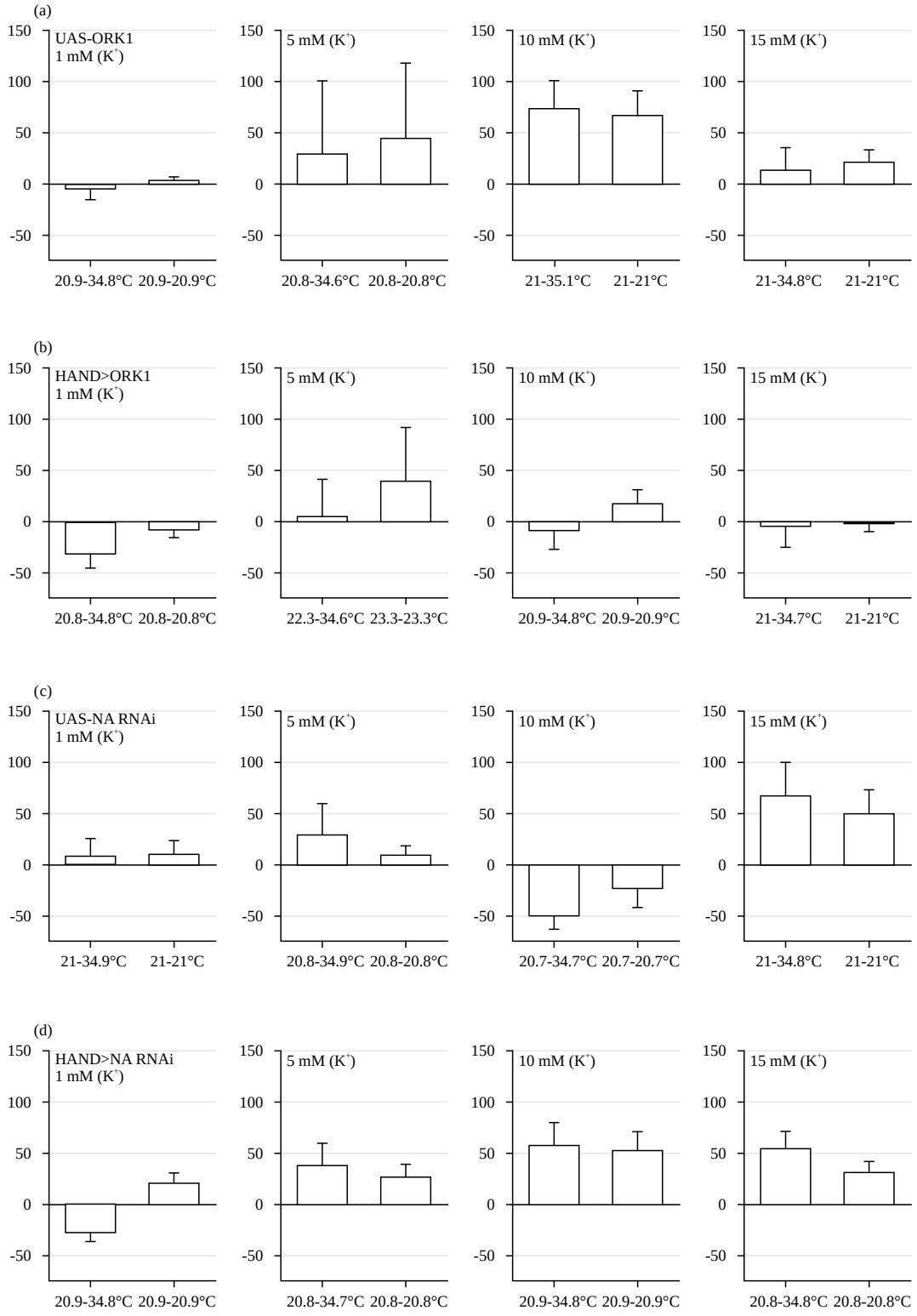

Fig. 8(a-d): The percent changes with varied extracellular K^+ concentration and temperature in lines with cardiac expression demonstrating (a, c) control levels, (b) ORK1 overexpression and (d) RNAi-induced knockdown of *na*

The values are displayed as means (±SEM). The raw data values used for these histograms are depicted in Fig. 7

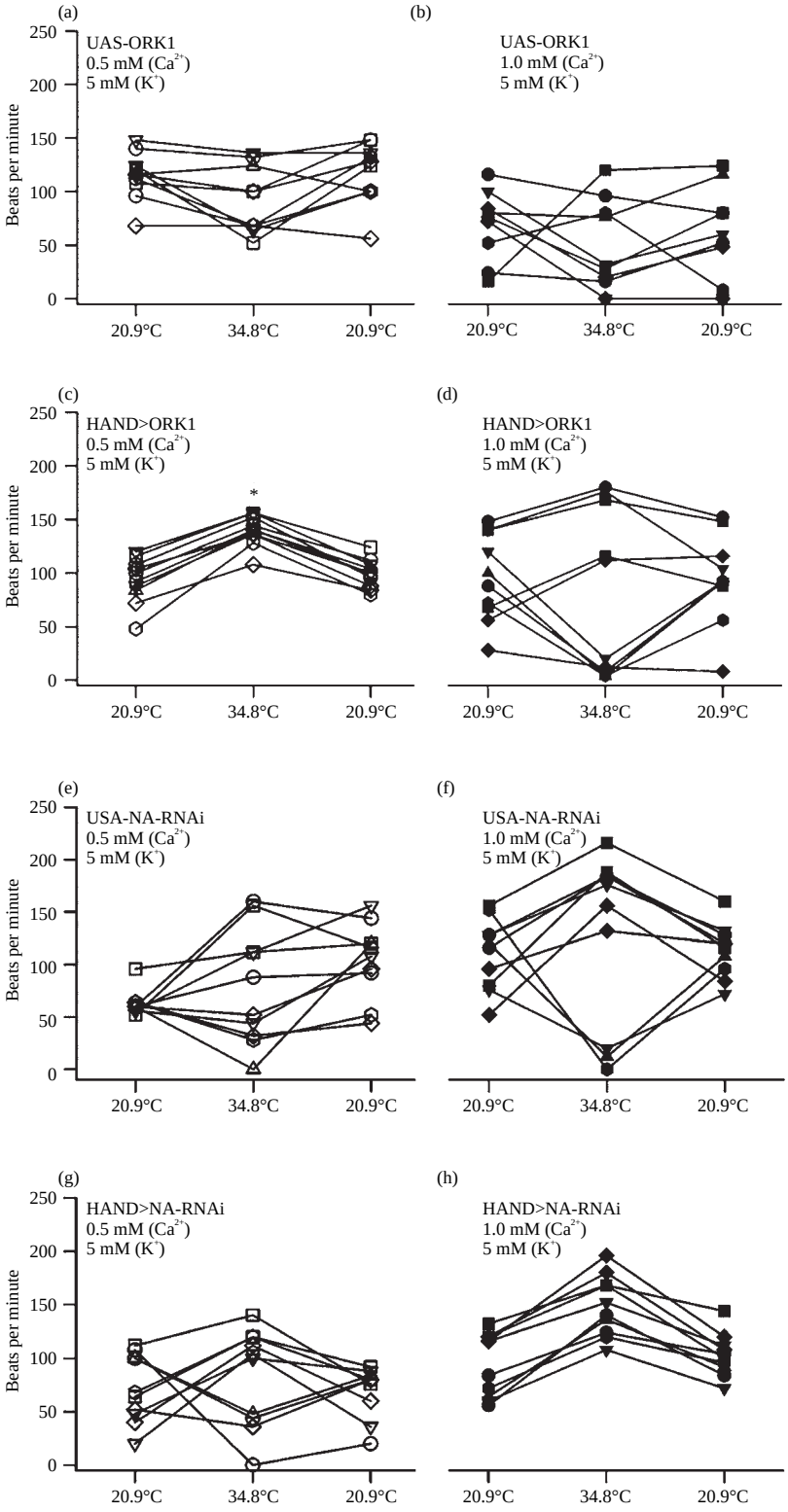

Fig. 9(a-h): Effects of simultaneous variation of extracellular Ca^{2+} and temperature (~21 to ~35°C) with 5 mM extracellular K^+ concentration in lines with cardiac expression, demonstrating (a, b, e, f) control levels, (c, d) ORK1 overexpression and (g, h) RNAi-induced *na* knock-down

Each line represents a single larva under the various conditions. The asterisks represent significant changes ($p<0.05$)

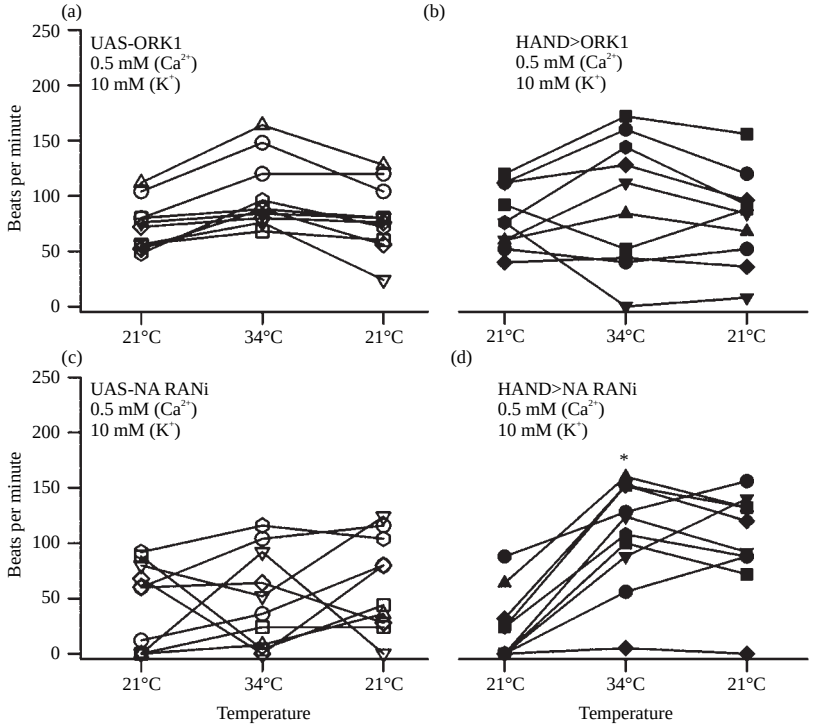

Fig. 10(a-d): Effects of temperature variation (~21 to ~35°C) with extracellular conditions of 0.5 mM Ca^{2+} and 10 mM K^{+}. The effects of this variation were observed in lines with cardiac overexpression demonstrating (a, c) control levels, (b) ORK1 overexpression and (d) RNAi-induced *na* knock-down

Each line represents a single larva under the various conditions. The asterisks represent significant changes ($p<0.05$)

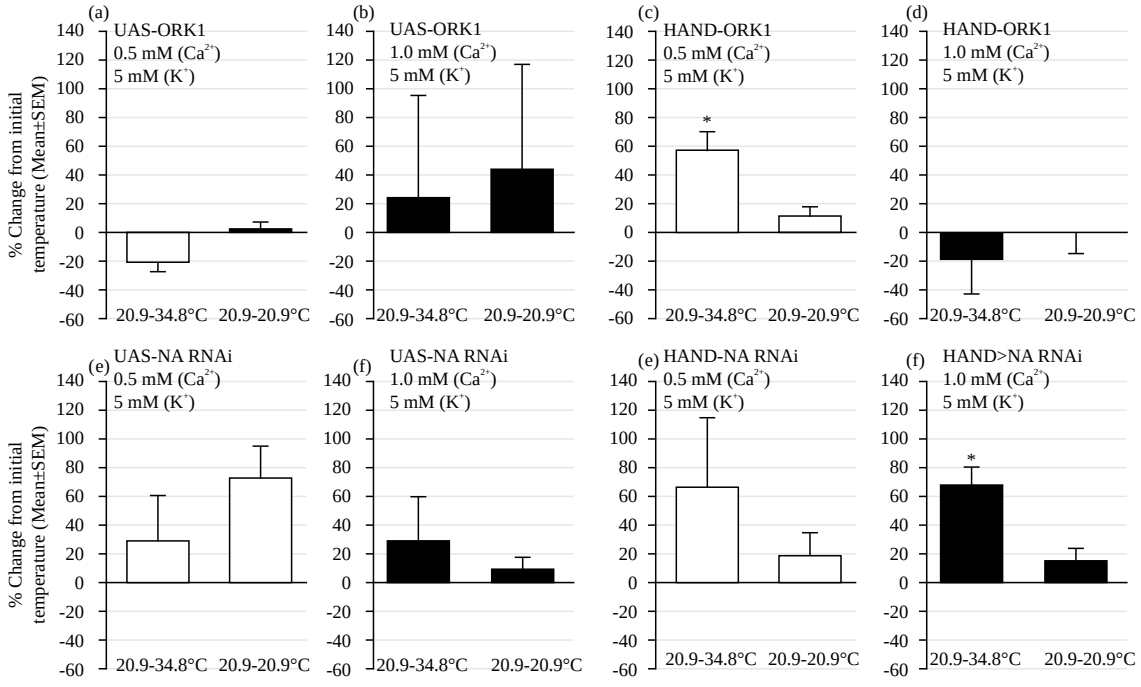

Fig. 11(a-h): Percent changes observed with simultaneous temperature alteration (~21 to ~34°C) and varied extracellular Ca^{2+} concentrations given 5 mM extracellular K^{+}, The percent changes were observed in lines with cardiac overexpression demonstrating (a, b, e, f) control levels, (c, d) ORK1 overexpression and (g, h) RNAi-induced *na* knockdown

The values are displayed as means (±SEM). The raw data values used for these histograms are depicted in Fig. 9. The asterisks represent significant changes ($p<0.05$)

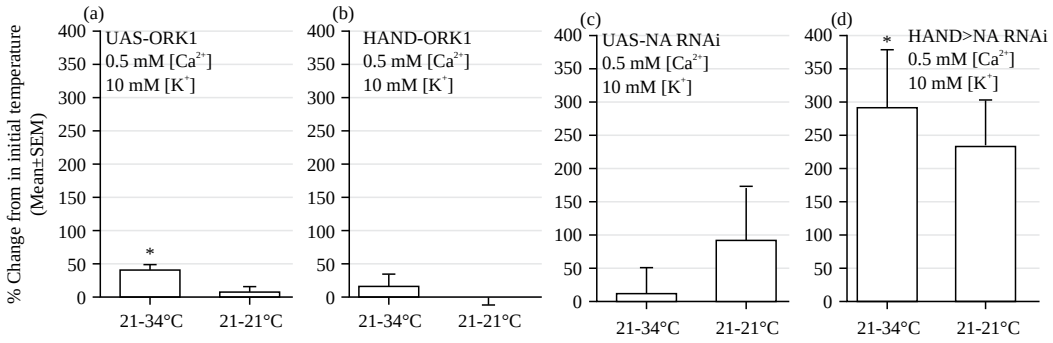

Fig. 12(a-d): Percent changes between ~21 and ~34°C given extracellular conditions of 0.5 mM Ca^{2+} and 10 mM K^{+} in lines with cardiac overexpression demonstrating (a, c) control levels, (b) ORK1 overexpression and (d) RNAi-induced *na* knockdown

The values are shown as means (±SEM). The raw data values used for these histograms are depicted in Fig. 10. The asterisks represent significant changes ($p<0.05$)

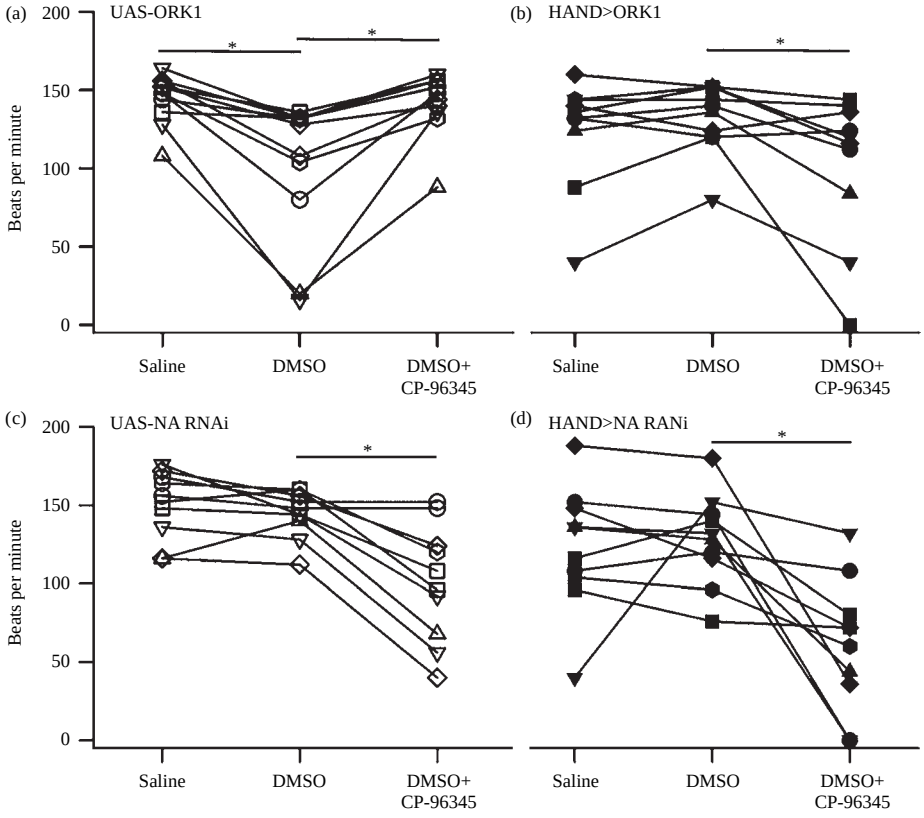

Fig. 13(a-d): Cardiac effects of NALCN blocker (CP-96345) exposure at 50 µM, as well as the solvent (DMSO) alone, with cardiac expression demonstrating (a, c) control levels, (b) ORK1 overexpression and (d) RNAi-induced *na* knock-down

The extracellular Ca^{2+} concentration was kept at 1mM and the extracellular K+ concentration at 5 mM. Each line represents a single larva under the various conditions. The asterisks represent significant changes ($p<0.05$)

Percent changes for each larva's heart rate under these varied conditions were also determined for normalization of initial rate variation. As is illustrated with the individual line graphs, the only notable significant effects observed were for HAND>ORK1 and HAND>NA RNAi (Fig. 11c-h, paired t -test, $p<0.05$, $n = 10$) and for UAS-ORK1 and HAND>NA RNAi (Fig. 12a-d, paired t-test, $p<0.05$, $n = 10$). The remaining percent changes did not exhibit significant alteration (Fig. 11a-g and Fig. 12b-c).

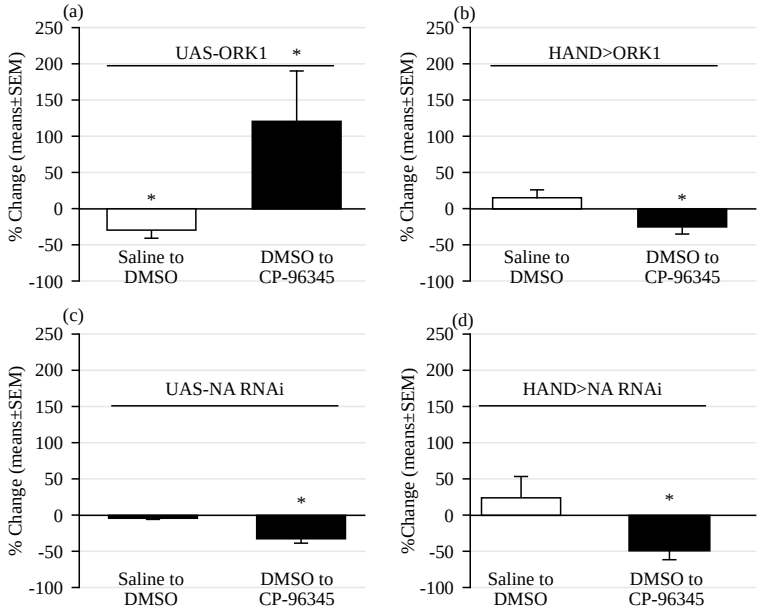

Fig. 14(a-d): Heart rate percent changes from saline to DMSO and from DMSO to NALCN blocker (CP-96345) with cardiac expression demonstrating (a, c) control values, (b) ORK1 overexpression and (d) RNAi-induced *na* knock-down

The values are displayed as mean (±SEM). The raw data values used for these histograms are depicted in Fig. 13. The asterisks represent significant changes ($p<0.05$)

Effects of the CP-96345 on heart rate: To examine the effects of NALCN blocker CP-96345 on ORK1 and NA RNAi lines, heart rates were observed first in DMSO alone (held at an equal concentration to the experimental solution to control for interference by the drug's solvent) and then with the CP-96345 (50 μM). Relative to control saline, exposure to DMSO alone produced a significant decrease in the heart rates of UAS-ORK1 larvae (Fig. 13a, paired t-test, $p<0.05$, $n = 10$) but not in HAND>ORK1 larvae (Fig. 13b). Exposure to the CP-96345 compound, on the other hand, resulted in an increased heart rate from that observed in DMSO alone for the UAS-ORK1 preparations (Fig. 13a, paired t-test, $p<0.05$, $n = 10$) and a decreased rate in the HAND>ORK1 larvae (Fig. 13b, paired t-test, $p<0.05$, $n = 10$). Of particular interest were the effects associated with the UAS-NA RNAi and HAND>NA RNAi strains; even though the latter would be expected to have compromised NALCN function not present in the former, CP-96345 resulted in decreased heart rates for both strains as compared to DMSO exposure alone (Fig. 13c-d; paired t-test, $p<0.05$, $n = 10$).

Percent changes were also determined to normalize the effects of the varied initial rates across the preparations, elucidating the effects of replacing the saline medium with DMSO alone and, from there, to CP-96345 exposure (Fig. 14a-d). As is also shown via the individual line graphs, exposure to DMSO alone resulted in a significant decrease in UAS-ORK1 heart rate relative to saline (Fig. 14a, paired t-test, $p<0.05$, $n = 10$), while CP-96345 produced a significant increase from that observed in DMSO (Fig. 14a, paired t-test, $p<0.05$, $n = 10$). The remaining lines-HAND>ORK1, UAS-NA RNAi and HAND>NA RNAi larvae-demonstrated significant decreases in cardiac activity between DMSO alone and CP-96345 exposure (Fig. 14b-d; paired t-test; $p<0.05$; $n = 10$ each).

The experiments reported herein have shown that using the RNAi MID1 and RNAi NA strains results in differential heart rate responses to temperature increase. The NALCN RNAi strain exhibited a consistent increase in heart rate, paralleling the effects observed with overexpression of TRPA1 channels under increased temperature. Temperature is known to influence cardiac activity in invertebrates and altered TrpA1 expression can modify the temperature-dependent heart rate response in *Drosophila* larvae[11-13]. Thus, NALCN may contribute to maintaining membrane electrical properties during temperature changes, consistent with its established role as a sodium leak channel involved in membrane excitability[19,23-27]. On the other hand, the RNAi MID1 strain demonstrated a biphasic response to temperature increase, which may indicate that 34°C represents a borderline temperature. At this temperature, most preparations showed complete cessation of the heartbeat. A greater increase in

temperature or longer exposure may therefore have resulted in cessation of cardiac activity in all preparations. The accessory protein MID1 may contribute to stabilizing NALCN function, or that of other ion channels, during environmental stress. The conserved association between MID1 proteins and cation leak channels further supports a potential functional relationship between MID1 and NALCN[28].

The HAND>MID1 RNAi strain was difficult to maintain because most larvae died as embryos or during the first larval stage. We had planned to supplement the reported NA strain studies with parallel investigations using the MID1 strain; however, too few survivors across repeated attempts rendered this approach impractical and we therefore continued with the NA strain only. The MID1 RNAi strain may also exhibit off-target effects, as embryos and larvae have previously been reported to survive to the pupal stage even in the absence of normal heart-tube development[21]. These observations highlight the importance of considering developmental and strain-specific effects when interpreting RNAi-based cardiac phenotypes in *Drosophila*.

It was not surprising that increases in $[K^+]_o$ produced similar effects across all strains, including controls, K2P channel-overexpressing strains and strains with NALCN knockdown through RNAi targeting MID1 or NA. Increased extracellular K^+ would be expected to depolarize the membrane potential and, consequently, alter cardiac contraction rate. These predictions were consistent with the experimental results, as increased $[K^+]_o$ relatively consistently decreased heart rate in every strain. However, the decrease was more pronounced in HAND>NA RNAi organisms than in HAND>ORK1. The K2P channels are important regulators of resting membrane potential and cellular excitability, while NALCN contributes to sodium leak currents and membrane depolarization[15,16,19,27]. Therefore, changes in extracellular K^+ may produce a more predictable depolarizing influence than temperature, whose effects can involve multiple ion channels, pumps and exchangers.

The effects of altered $[Ca^{2+}]_o$-namely, the lack of a clear effect on heart rate in either UAS-NA RNAi or HAND>NA RNAi and the similar responses observed in UAS-ORK1 and HAND>ORK1-were unexpected. However, the combination of altered $[K^+]_o$, $[Ca^{2+}]_o$ and temperature produced larger and more consistent increases in heart rate in HAND>NA RNAi flies than in HAND>ORK1 at $[K^+]_o$ of 5 and 10 mM, $[Ca^{2+}]_o$ of 0.5 and 1.0 mM and 34°C (Figs. 9, 11). These changes paralleled the effects of increased temperature alone (Fig. 1). The response to extracellular Ca^{2+} is particularly relevant to NALCN because extracellular Ca^{2+} can directly modulate the NALCN channel complex[20]. Given that the stressors examined in this study (altered $[K^+]_o$, $[Ca^{2+}]_o$ and temperature) represent environmental changes that may accompany pathological conditions, it would be useful to determine whether similar differential responses occur in other animal models exposed to comparable physiological stressors.

When examining the pharmacological action of CP-96345 following solubilization in DMSO, it was important to account for the effects of DMSO alone. These effects were particularly strong in the background UAS-ORK1 strain and tended to increase variability among the strains. Addition of the DMSO/CP-96345 solution generally tended to reverse the effect of DMSO alone, except in the HAND>NA RNAi strain, in which CP-96345 exposure decreased heart rate. One possible interpretation is that decreasing NALCN expression may produce an effect similar to increased K2P expression because both conditions could alter the balance of membrane currents toward hyperpolarization. Conversely, K2P overexpression may partially compensate for or override changes resulting from a reduction in functional NALCN channels. The functional interaction between NALCN and K2P channels provides a plausible basis for this interpretation[16].

In all cases, it is important to note that even within-strain data demonstrate considerable variation, which is a common challenge in physiological experiments using *Drosophila* preparations. Differences in physiological saline composition, developmental timing, preparation quality and other experimental variables can influence cardiac activity and baseline heart rate[36,37]. Even under controlled experimental conditions, it is difficult to account for every source of biological variation, and differences in RNAi penetrance and developmental timing may contribute to baseline fluctuations unrelated to the experimental treatment. This limitation should therefore be considered when interpreting the magnitude of the observed responses.

In future studies, it would be useful to monitor the membrane potential of cardiac myocytes in the HAND>ORK1, HAND>MID1 RNAi and HAND>NA RNAi strains during alterations in $[K^+]_o$, $[Ca^{2+}]_o$ and temperature. Direct electrophysiological measurements could help distinguish whether the observed changes in heart rate arise from alterations in membrane potential, ion-channel activity, or downstream excitation-contraction mechanisms. Such experiments would be particularly informative given the established roles of NALCN and K2P channels in regulating membrane excitability[15,19,20,27]. Cooling the bath and slowing the heart rate may also permit more stable intracellular recordings from these small contractile cells.

CONCLUSION

Temperature produced strain-dependent effects on larval cardiac function, with increases in temperature resulting in either increased or decreased heart rates depending on ion-channel expression. In contrast, elevated extracellular K^+ produced a more consistent reduction in heart rate, particularly in ORK1-overexpressing and *na* knockdown larvae. Combined temperature and K^+ alterations did not produce significant additional effects. Changes in extracellular Ca^{2+} generally had limited effects on cardiac rate, except for a significant reduction in heart rate in UAS-ORK1 and HAND>ORK1 larvae when Ca^{2+} decreased from 1.0 to 0.5 mM. Pharmacological inhibition with CP-96345 also reduced heart rate in most crosses, providing support for the proposed role of NALCN-dependent currents in cardiac pacing. Overall, the findings demonstrate that cardiac activity in *Drosophila* is strongly influenced by the interaction between environmental conditions and ion-channel expression. Future studies using direct electrophysiological measurements and mammalian models are warranted to determine how NALCN, K2P and related ion channels regulate cardiac excitability under physiological and pathological stress conditions.

SIGNIFICANCE STATEMENT

This study demonstrates that temperature and extracellular ion concentrations can differentially influence cardiac pacing depending on ion-channel expression in *Drosophila* larvae. The findings highlight potential functional interactions between NALCN and K2P channels in maintaining cardiac excitability under changing environmental conditions. These results provide a basis for further electrophysiological and mammalian studies investigating how ion-channel dysregulation may contribute to altered cardiac function and disease.

ACKNOWLEDGMENT

Drosophila stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. We thank Dr. Anthony Cammarato for supplying the heart-specific *Drosophila* line.

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