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Extracellular Sodium and Potassium Levels Modulate Cardiac Conduction in Mice Heterozygous Null for the Connexin43 Gene

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Abstract

Several studies have disagreed on measurements of cardiac conduction velocity (CV) in mice with a heterozygous knockout of the connexin gene *Gja1* – a mutation that reduces the gap junction (GJ) protein, Connexin43 (Cx43) by 50%. We noted that perfusate ionic composition varied between studies and hypothesized that extracellular ionic concentration modulates CV dependence on GJs. CV was measured by optically mapping wild type (WT) and heterozygous null (HZ) hearts serially perfused with solutions previously associated with no change (Solution 1) or CV slowing (Solution 2). In WT hearts, CV was similar for Solutions 1 and 2. However, consistent with the hypothesis, Solution 2 in HZ hearts slowed transverse CV (CV_T) relative to Solution 1. Previously, we showed CV slowing in a manner consistent with ephaptic conduction correlated with increased perinexal inter-membrane width (W_P) at GJ edges. Thus, W_P was measured following perfusion with systematically adjusted $[Na^+]_o$ and $[K^+]_o$ in Solutions 1 and 2. A wider W_P was associated with reduced CV_T in WT and HZ hearts, with the greatest effect in HZ hearts. Increasing $[Na^+]_o$ increased CV_T only in HZ hearts. Increasing $[K^+]_o$ slowed CV_T in both WT and HZ hearts with large W_P but only in HZ hearts with narrow W_P . Conclusion: When perinexi are wide, decreasing excitability by modulating $[Na^+]_o$ and $[K^+]_o$ increases CV sensitivity to reduced Cx43. By contrast, CV is less sensitive to Cx43 and ion composition when perinexi are narrow. These results are consistent with cardiac conduction dependence on both GJ and non-GJ (ephaptic) mechanisms.

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CONFLICT OF INTEREST: RGG holds stock in FirstString Research.

Keywords

Conduction; Connexin43; Sodium; Potassium; Edema

INTRODUCTION

Gap junctional coupling is an important determinant of electrical impulse propagation through the heart and many other tissues. Gap junctions are low resistance pathways between myocytes that aid in the transmission of electrical impulses from one myocyte to the next [16]. Remodeling of the principal ventricular gap junction (GJ) protein Connexin43 (Cx43) is a hallmark of several cardiac diseases [21,5,6]. Due to its association with abnormal conduction and arrhythmogenesis, the conduction velocity–gap junction (CV–GJ) relationship has been the focus of intense study [19,10,14,27,30,31,8,9]. However, the results of these studies have not been in agreement, leading to interesting and as yet unresolved controversies.

In general, the conclusions of previous studies can be summarized in two categories: 1) substantial reductions in GJ expression (>50%) is required to change CV [19,28,30,31,4] or 2) CV slows secondary to an approximate 50% loss of Cx43 protein[10,14,9]. Comparing two specific studies [19,10] revealed that perfusate composition and conclusions drawn were different even though both groups used genotypically identical mice with heterozygous loss of GJa1 and a resultant 50% reduction in Cx43. It has been previously demonstrated that altering perfusate composition can change the interstitial volume (V_{IS}) in the heart and thereby modulate the CV-GJ relationship [33]. More recently, we found that variance in inter-membrane spacing within intercalated disk microdomains adjacent to GJ plaques termed the perinexus may modulate an alternative mode of electrical coupling that can explain how V_{IS} modulates the CV-GJ relationship [32]. We therefore hypothesized that perfusate ionic composition can modulate the CV-GJ relationships in a related manner. The specific aims of this study were to determine the differential effects of perfusate composition on perinexal spacing and how varying extracellular sodium and potassium modulates the CV-GJ relationship in the GJa1 heterozygous null mouse.

MATERIALS AND METHODS

The study protocols were approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University and conform to the NIH Guide for the Care and Usage of Laboratory Animals.

Langendorff Perfusion System

Mice from the same lineage as in the aforementioned two studies [19,10,14] were genotyped by Transnetyx (Cordova, TN). These mice, generously provided by Dr Jeffery Saffitz of Harvard University, were on a C57BL/6 background (original breeders from Jackson Laboratory) and were heterozygous for Cx43 null mutations (~50% Cx43 reduction compared to control [14]). Wild Type (WT) and heterozygous (HZ, ~50% Cx43) mice (10–30 weeks of age) were anesthetized by a lethal intraperitoneal injection of sodium

pentobarbital (~325 mg/kg) and the hearts were quickly excised. The aorta was cannulated and retrogradely perfused as previously described [10,19]. Hearts were perfused with solutions (pH7.4) described in Table 1 in random order at a flow rate of 1 to 1.5 ml/min maintaining the perfusion pressure at approximately 65 mmHg. The temperature of the perfusate and the bath were maintained at 37°C. The osmolarity of the solutions were measured using Precision Systems Micro Osmometer and are reported in Table 1.

All solutions were perfused in random order to minimize differences due to perfusion order and time. Each heart was perfused with a maximum of 4 solutions. Electrophysiology was quantified in hearts perfused with Solutions 1 and 2 (13 WT and 13 HZ animals). Electrophysiology was quantified in 5 hearts for every other solution except Solution 1A (n=9).

Histology

Hearts (n=8 per intervention, and N=32 total) were formalin fixed after one hour of perfusion and V_{IS} was quantified as previously described [33]. Fixed hearts were paraffin embedded, sectioned and Hematoxylin and Eosin (H&E) stained. Sections from the right ventricle were then digitally scanned using an Aperio ScanScope XT system (Vista, CA). A positive pixel algorithm was applied to whole slide images to segment cardiac myocytes and the interstitial (extracellular) volume (excluding blood and lymph vessels). The percent interstitial volume (% V_{IS}) was determined as follows.

$$\% V_{IS} = \frac{(\text{Total analysis area} - \text{Stained Area})}{\text{Total analysis Area}} \times 100$$

Blood and lymph vessels that were greater than 100 μm^2 in area were excluded from the selected region.

Transmission Electron Microscopy

In another separate set of experiments (n=3 per intervention, N=24 samples, with 15 images per sample), 1mm³ cubes of right ventricular tissue perfused for 1 hour were fixed in 2.5% Gluteraldehyde overnight at 4°C and then transferred to PBS at 4°C. The tissue was processed as previously described [32] initially in 1% Osmium Tetroxide (OsO₄) and 1.5% Potassium Ferricyanide (KFeCN) followed by rinse with H₂O. Samples were then transferred to ethanol at increasing concentrations (70, 95 and 100%) for 15 minutes at each concentration and then transferred to a 1:1 solution of 100% ethanol and acetonitrile for 10 minutes. Samples were then transferred to only acetonitrile for two 10 minute periods and then embedded in PolyBed 812 at increasing concentrations with Acetonitrile on a rotator. The samples were left in vacuum for ~ 3 hours and then left in PolyBed 812 overnight and transferred to flat molds and incubated at 60°C for 2 days. The blocks were then sectioned using a microtome onto copper grids and stained with Uranyl acetate (Aq) for 40 minutes followed by Hanaichi Pb stain. Images were then collected using a transmission electron microscope (JEOL JEM1400). The images of the gap junctions and the perinexus were obtained at 150,000X magnification. The perinexal width (W_p) in these images was measured using ImageJ. Mean $\pm$ standard error is reported.

Optical Mapping

Hearts were optically mapped with the voltage sensitive dye, Di-4-ANEPPS at a concentration of 4 μM for approximately 5 minutes. Motion was reduced with the electromechanical uncoupler, 2,3-butanedione monoxime (BDM). Specific BDM concentrations matching the previous studies are detailed in Table 1. Hearts were stabilized against the front glass of the bath by applying slight pressure to the back of the heart. The center of the anterior ventricular surface was paced with a unipolar silver wire with a reference electrode at the back of the bath. Hearts were stimulated at $\sim 1\text{V}$ for 1ms at a BCL of 150 ms.

The excitation light from a halogen light source (MHAB-150W, Moritex Corporation) was filtered by a 510 nm filter (Brightline Fluorescence Filter) before it reached the heart. The emitted light was filtered by a 610 nm filter (610FG01-50(T257), Andover Corporation) before it was recorded using a MiCam Ultima CMOS L-camera at a sampling rate of 1000 frames/sec. The camera captured optical signals from an area of 1 cm^2 in a 100×100 pixel array with an interpixel resolution of 0.1 mm.

Activation times were assigned to the maximum rate of rise of an action potential as previously reported[33] using the Bayly et al. algorithm [3]. In short, activation time was determined from the maximum rate of optical action potential rise at each pixel, and a parabolic surface was fit to activation times in order to determine a CV vector at each pixel. Longitudinal and transverse CV (CV_L and CV_T), and anisotropic ratios (ratio of CV_L/CV_T , AR) were quantified.

Statistical Analysis

Equal variance and sample size, one/two-tailed student's t-tests were performed on paired and unpaired data to detect significance using Matlab. Specific statistical analyses are indicated in Figure legends. Single factor ANOVA was performed to detect differences in perinexal width between solutions and WT/HZ mice, and t-tests were used as a posthoc test to determine significance in perinexal width at specific distances. Bonferroni correction was applied for multiple comparisons.

All statistical tests were performed on absolute data values except for data in Figure 5 and 6. Values reported in these figures are relative to Control solutions ($[\text{Na}^+]_o = 155.2 \text{ mM}$ and $[\text{K}^+]_o = 4.0 \text{ mM}$).

All data are reported as mean $\pm$ standard deviation unless stated otherwise. $p < 0.05$ was reported as significant.

RESULTS

Interstitial Volume

V_{IS} is the interstitial volume between myocytes in myocardial tissues excluding blood and lymph vessels. Previously, we provided evidence that altering V_{IS} , by varying the osmolarity of the perfusate, [33] modulated the CV-GJ relationship. In this study, differences in osmolarity correlated with $[\text{Na}^+]_o$ where increasing $[\text{Na}^+]_o$, on average, increased osmolarity

by 5% (Table 1). We previously reported that increasing osmolarity by approximately 40% increased histologically assessed V_{IS} by 61%. A similar analysis was performed in the current study to determine whether the 5% increase in osmolarity resulting from increased $[Na^+]_o$ altered V_{IS} .

H&E stained ventricular sections of WT and HZ hearts perfused with solutions published by Morley et al. (Solution 1) [19] and Eloff et al. (Solution 2), [10] are shown in Figure 1a and b. V_{IS} was not significantly different as a result of perfusate in WT ventricles. In contrast, V_{IS} in HZ ventricles was greater when perfused with Solution 1 than 2 (Figure 1b). The % V_{IS} was significantly reduced in HZ hearts perfused with Solution 2 (Figure 1c) compared to hearts perfused with Solution 1. Thus, a 5% increase in osmolarity was associated with a significant increase in V_{IS} (70%) only in HZ hearts.

Perinexus

The intercalated disc and specifically the perinexus- a specialized domain of cell membrane adjacent to gap junction (GJ) plaques, has been identified as a site of dense voltage-gated sodium channel ($Na_v1.5$) localization [23,24,32,1]. Representative electron micrographs of GJs and neighboring perinexi in Figure 2a and b demonstrate the effects of perfusate on perinexal width (W_P). W_P in both WT and HZ hearts was larger with Solution 1 than Solution 2 (Figure 2a Upper Panels versus Lower Panels). Solution 1 was then modified to contain the same $[Na^+]_o$ and $[K^+]_o$ as Solution 2 (Solution 1C) or vice versa (Solution 2C). Solution 1C also increased W_P relative to Solution 2C, as seen in representative Figure 2b. W_P , up to 105 nm from the edge of a gap junction for all experiments, is summarized in Figure 2c and d. For any given solution, W_P was not significantly different between WT and HZ hearts. Therefore, WT and HZ measurements were combined. Solution 1 and 1C increased W_P at all measured points relative to Solution 2 and 2C respectively. Additionally, Solution 1 was associated with a *larger* W_P and *higher* osmolarity relative to Solution 2. Interestingly, Solution 1C was also associated with a *larger* W_P but *lower* osmolarity relative to Solution 2C. This finding suggests that changes in perinexal width may not always correlate with osmolarity and that additional factors may be involved.

Interestingly, V_{IS} does not always correlate with W_P . Specifically, V_{IS} as quantified from gross histology in WT hearts was not different with Solution 1 or 2, but W_P was significantly larger in WT hearts perfused with Solution 1 than 2. On the other hand, V_{IS} correlated with W_P in HZ hearts.

Conduction Velocity

In order to directly test the hypothesis that perfusate composition underlies the CV-GJ relationship in *Gja1* heterozygous null hearts, Solutions 1 and 2 were serially perfused. Representative isochrones of epicardial conduction from optical maps are provided in Figure 3a, and CV is reported in Figures 3b and c as well as Supplemental Figure 1. For all experiments in WT animals with the native complement of Cx43, CV_L , CV_T , and anisotropic ratio (AR) were not different during Solution 2 perfusion relative to Solution 1 (Figure 3b–d, Left Panels). In HZ hearts, Solution 2 preferentially slowed CV_T relative to Solution 1 without significantly altering CV_L or AR (Figure 3b–d, Right Panels). However,

though we reproduce the Morley et al. results (no change in CV between WT and HZ hearts), CV slowing in HZ hearts relative to WT, as in the Eloff et al. study, was not statistically significant by comparison with a 2-tailed, unpaired t-test and Bonferroni correction.

Our previous studies suggested that *decreased* V_{IS} , and more specifically decreased W_P , is associated with *faster* conduction [33,32]. At first glance, the finding that Solution 1 is associated with *increased* W_P and *faster* conduction in HZ hearts appears inconsistent with these earlier findings. However, the concentrations of sodium and potassium in Solution 2 was also different from Solution 1, leading us to hypothesize that $[Na^+]_o$ and $[K^+]_o$ may further modulate the CV-GJ relationship.

Conduction Velocity and Perinexal width

We next compared conduction in the same hearts, with solutions containing similar $[Na^+]_o$ and $[K^+]_o$ but producing different perinexal spacing (Solutions 2 – Small W_P and 1C – Large W_P). While CV_L was not significantly different in WT or HZ hearts perfused with Solutions 2 and 1C (Figure 4a), CV_T was significantly slower in both WT and HZ hearts with wider perinexi (Solution 1C) than in hearts with narrower perinexi (Solution 2). The finding that increased W_P is associated with slower CV_T , but no change in AR, is summarized in Figures 4b and c. Importantly, Figure 4d demonstrates that increased W_P is associated with greater CV slowing in HZ animals relative to WT. This further supports our previous results that CV_T is more sensitive to GJ uncoupling when the perinexus is wide [32]. In summary, when we control for ionic composition, CV is inversely proportional to W_P , consistent with our previous study [32].

Taken together, these data suggest that the CV-GJ relationship may be modulated by other factors such as $[Na^+]_o$ and $[K^+]_o$ in addition to perinexal spacing. Interestingly, the ionic compositions of Solutions 1 and 2 are significantly different, and the following experiments focus on the first two cationic differences (sodium and potassium, due to their plausible effect on excitability), W_P , and the combined effects on the CV-GJ relationship.

- Solution 1 – Increased Perinexal Width: Effects of $[Na^+]_o$ and $[K^+]_o$ —In the following experiments, multiple solutions were perfused through the hearts. In order to reach a steady-state during each solution perfusion while limiting the entire experiment to 60 minutes, each heart was perfused with a maximum of 4 solutions in random order. Therefore, all data are normalized to a single solution that was constant in all experiments. Percent changes in CV by varying $[Na^+]_o$ and $[K^+]_o$ in Solution 1 to match those in Solution 2 are presented in Figure 5. Absolute CV values are provided in the Supplemental Figure.

WT: In WT hearts, decreasing $[Na^+]_o$ (Solution 1A) did not vary CV_L , CV_T , or AR relative to control (Solution 1). However, increasing $[K^+]_o$ (Solution 1B) uniformly slowed CV_L and CV_T without significantly altering AR. Reducing $[Na^+]_o$ and increasing $[K^+]_o$ (Solution 1C) had an effect similar to increasing $[K^+]_o$ alone, where CV_L and CV_T were uniformly slowed compared to control, and no change in AR was observed.

HZ: Cardiac conduction in HZ hearts is summarized in Figure 5 (Right Panels). Notably, reducing $[\text{Na}^+]_o$ (Solution 1A) did not alter CV_L but significantly slowed CV_T relative to control Solution 1. Again, increasing $[\text{K}^+]_o$ (Solution 1B) did not significantly alter CV_L , but slowed CV_T and significantly increased AR. The combined effect of reducing $[\text{Na}^+]_o$ and increasing $[\text{K}^+]_o$ in Solution 1C reduced CV_T relative to Solution 1 without changing CV_L or AR.

Though some of the perfusate combinations slowed CV_T in both WT and HZ hearts, Solution 1A was the only solution to significantly slow CV_T in HZ hearts relative to WT as determined by unpaired comparison (#, Figure 5). These data demonstrate that perfusate composition can confound whether a 50% reduction of Cx43 is associated with conduction slowing.

- Solution 2 – Reduced Perinexal width: Effects of $[\text{Na}^+]_o$ and $[\text{K}^+]_o$

WT: To determine the relative effects of varying ionic composition in preparations with smaller W_p , Solution 2C was used as a control since it had similar $[\text{Na}^+]_o$ and $[\text{K}^+]_o$ to Solution 1. In WT hearts (Figure 6, Left Panels), decreasing $[\text{Na}^+]_o$ (Solutions 2B), increasing $[\text{K}^+]_o$ (Solution 2A) and both increasing $[\text{Na}^+]_o$ and decreasing $[\text{K}^+]_o$ (Solution 2) did not significantly change CV_L , CV_T , or AR relative to Solution 2C.

HZ: In HZ hearts (Figure 6, Right Panel), decreasing $[\text{Na}^+]_o$ alone (Solution 2B) or increasing $[\text{K}^+]_o$ alone (Solution 2A) did not affect CV_L , CV_T or AR relative to Solution 2C. However, the combined effect of decreasing $[\text{Na}^+]_o$ and increasing $[\text{K}^+]_o$ (Solution 2) reduced CV_T relative to Solution 2C without significantly altering CV_L or AR. In summary, altering $[\text{Na}^+]_o$ and/or $[\text{K}^+]_o$ does not significantly affect CV in WT hearts with narrow perinexi, but both decreasing $[\text{Na}^+]_o$ and increasing $[\text{K}^+]_o$ in HZ hearts with narrow perinexi can significantly slow CV.

DISCUSSION

The purpose of this study was to determine how varying extracellular sodium, potassium and W_p modulates of the CV-GJ relationship in the Gja1 heterozygous null mouse. It has been previously demonstrated that all three factors can individually modulate CV [20,37,33,32]. Interestingly, we demonstrate that modest variations of these parameters, which individually might not produce a response, can in combination significantly affect conduction. Furthermore, the ionic concentrations in perfusates used in this study mostly lie within reported physiological values for mice. Specifically, mouse serum sodium level ranges from 140–160 mM and potassium ranges from 5–7.5 mM [22]. Together, these results suggest that combinatorial and physiologic variations in ionic concentration and W_p can significantly modify the CV-GJ relationship.

Conduction and the Morley and Eloff et al. Solutions

The results of the present study reproduce the CV-GJ relationship reported by Morley et al, where Solution 1 did not produce conduction slowing in Cx43 HZ hearts [19]. However, our results are only partially consistent with those of Eloff and co-workers. In brief, Eloff et al.

reported significant CV_L and CV_T slowing in HZ animals relative to WT [10]. In our experiments, Solution 2 did not produce significant CV slowing in HZ hearts relative to WT. However, when comparing Solution 2 in WT and HZ hearts, we demonstrate that Solution 2 slowed CV_T more in HZ than in WT hearts. These data provide evidence that CV_T sensitivity to Cx43 level is greater with the Eloff et al. perfusate. Experimental differences such as multiple and serial perfusions in this study may underlie the lack of 1:1 agreement with the Eloff et al. study. Yet, the Eloff et al. perfusate was not the only solution to reveal decreased CV in the Cx43 HZ mouse. Specifically, the modified Morley Solution 1A, slowed CV_T significantly in HZ animals relative to WT animals, again demonstrating that differences between WT and HZ animals can be elicited by varying perfusate composition. Therefore, we provide further evidence that conduction slowing secondary to a 50% loss of Cx43 *can* be unmasked by perfusate composition.

Effect of the perinexal width

We have previously demonstrated that bulk V_{IS} confounds the CV-GJ relationship [33,32]. More recently, it was demonstrated that intercellular separation within the intercalated disk at the perinexus correlates well with CV changes [32]. With the support of computational modeling, we proposed that ephaptic coupling – the generation of electric fields in restricted spaces between myocytes – may mechanistically modulate the CV-GJ relationship. In the present study, it is demonstrated that Solutions 1 and 1C produced wider perinexi than Solutions 2 and 2C. When we controlled for similar $[Na^+]_o$ and $[K^+]_o$, WT and HZ preparations with wider perinexi exhibited greater transverse conduction slowing than preparations with narrower perinexi – consistent with our previous results.

One important ionic difference between the solutions that could underlie a change in perinexal spacing is $[Ca^{2+}]_o$. The intercalated disk is composed of many junctional proteins which require extracellular calcium to form and maintain cell-to-cell adhesion.[35,7] Solution 2, with the highest $[Ca^{2+}]_o$ produced the narrowest W_P consistent with the hypothesis that W_P can be modulated by $[Ca^{2+}]_o$.

Effect of Extracellular Sodium ion concentration

Hyponatremia has been associated with slowed conduction in the heart [37], presumably by reducing cellular excitability [2]. Reducing cellular excitability could affect the rate of extracellular potential change in the perinexus and thereby weaken ephaptic coupling between myocytes. Despite the relatively small change in $[Na^+]_o$ (~5%) in this study, the finding that altered $[Na^+]_o$ can modulate CV in HZ hearts is consistent with a previous report [37]. Yet, the extent of CV modulation appears also to depend on $[K^+]_o$ and W_P . More specifically, CV modulation by varying $[Na^+]_o$ was evident in *hypokalemic*-Cx43 HZ preparations with wide perinexi, and *hyperkalemic*-Cx43 HZ preparations with narrow perinexi. Under both conditions, GJ coupling is likely reduced in HZ hearts, presumably increasing conduction dependence on ephaptic coupling. However, ephaptic cell-to-cell transmission of action potential is probably reduced in the first instance by wider perinexi and in the second instance by reduced excitability.

Effect of Extracellular Potassium ion concentration

The relationship between $[K^+]_o$ and CV is biphasic. Small increases in $[K^+]_o$ raise the resting membrane potential closer to the threshold of voltage gated sodium channel activation and could result in supernormal conduction [20]. Further increasing $[K^+]_o$ can slow conduction by inactivating voltage gated sodium channels and thereby reducing excitability [36,12]. Relative to Solution 1, Solution 2 had 34% more $[K^+]_o$, which would alter the potassium reversal potential by approximately 10.9 mV. In preparations with wide perinexi, this degree of increased $[K^+]_o$ slowed CV in both WT and HZ mice hearts perfused with Solution 1 combinations (Solution 1B and 1C), presumably due to reduced excitability. On the other hand, in hearts with narrower perinexi and a stronger ephaptic contribution to conduction, CV is less sensitive to $[K^+]_o$. These findings are consistent with previous data from our group demonstrating that CV is more sensitive to sodium channel availability during loss of gap junctional coupling and/or increased W_p . Based on computational models, the proposed mechanism for differential CV sensitivity to sodium channel availability is related to the rate and amplitude of extracellular potential change in the intercalated disk [32,17]. In short, GJ uncoupling may increase CV dependence on an ephaptic mechanism. It is important to note that while the Morley et al. solution produced the widest W_p , the higher $[Na^+]_o$ and lower $[K^+]_o$ masked the effects of increased W_p . Likewise, while the Eloff et. solution produced the narrowest W_p , the lower $[Na^+]_o$ and higher $[K^+]_o$ may have relatively reduced ephaptic coupling despite the narrow perinexal space.

A final important finding in this study is that the CV – $[K^+]_o$ relationship may be modulated by W_p . Specifically, hearts with smaller perinexi were the most resistant to changes in $[K^+]_o$ possibly due to compensation by stronger ephaptic coupling.

Perfusates and the CV-Cx43 Mouse

The present study only analyzed the CV modulation by two ions, associated with cellular excitability, in detail from two different studies. However, other independent groups have analyzed the CV-Cx43 relationship with a variety of perfusates. When comparing the Tyrode solutions of only adult mouse studies with an approximate 50% Cx43 reduction, we find close agreement with our results. For example, the study by Van Rijen et al. also reported no change in CV in an inducible Cx43 knock-out mouse model.[31] Importantly, the Van Rijen et al. study utilized a solution with relatively low $[K^+]_o$ (4.5mM), low $[Na^+]_o$ (109.2 mM), and high $[Ca^{2+}]_o$. In short, our Solution 1 is closest to their perfusate composition, and this perfusate did not produce significant conduction slowing in WT or HZ hearts. In contrast to our results, a study by Guerrero et al.[14] reported that a solution identical to the Eloff et al. solution, and similar to Solution 2 used in this manuscript, slowed conduction by nearly 50 % in transgenic Cx43 HZ hearts.

Limitations

The use of buffers for superfusion and perfusion is a foundational tool for studying biological processes *ex vivo*. Further, *in vivo* studies report a range of physiologic normal serum ionic composition. It is important to note that not all sodium, potassium, and particularly calcium quantifications are related to ionized concentrations in experimental

buffers, plasma, or blood. Therefore, it may be difficult at this point to suggest that a superior perfusate exists which will maximally enhance the CV-GJ relationship.

With respect to calcium, previous studies have demonstrated that elevated intracellular calcium can slow CV by uncoupling gap junctions.[18,15] However, in this study, when we controlled for sodium and potassium (Solution 2 versus 1C), we found that higher extracellular calcium was associated with faster CV, arguing against calcium-induced inhibition of Cx43. This further illustrates that the relationship between ionic concentrations, W_P , CV and Cx43 is complex and requires further study.

Perinexal width measurements were made in glutaraldehyde-fixed tissue. Although most fixation protocols have been associated with alterations in the tissue structure we previously demonstrated that glutaraldehyde fixation is a relatively robust approach to measure extracellular volumes [33]. Furthermore, this and our previous study [32] demonstrate that significant trends in W_P due to solution composition are measurable after glutaraldehyde fixation. This being said, developing new approaches to dynamically measure microdomain structural changes is important given the emerging importance of ephaptic conduction.

Increasing perfusate osmolarity has been associated with larger V_{IS} , cell size reduction and slow CV [33]. In this study we measured V_{IS} and W_P , and determined that all changes in extracellular volumes did not correlate with osmolarity. Additionally, in contrast with our previous study, we report here that the relatively higher osmolarity perfusates are associated with increased CV (increasing $[Na^+]_o$). However, we did not quantify changes in cell size. It is important to note that the relationship between CV and cell size is controversial as well, with computational models predicting that either increasing or reducing cell size can slow CV.[26,25,29,33]. While the primary results here fit well with the theory that alterations in the CV-GJ relationship are modulated by W_P and perfusate composition, we cannot exclude the possible role of perfusates altering cell size and the CV-GJ relationship.

Summary and Conclusions

The literature indicates that GJ uncoupling is a common factor associated with several cardiac diseases [6,21,5]. However, there is debate over the nature of the relationship with some studies suggesting a relatively close correlation, whereas others indicate a more non-linear relationship with a threshold in GJ coupling below which conduction slows or fails. The present study provides data that may go some way to resolving the controversy, indicating that perfusate composition *can* exacerbate gap junctional uncoupling leading to slowed conduction and increased arrhythmia susceptibility.

Further, dehiscence of the intercalated disk has also been observed under pro-arrhythmic cardiac conditions such as hypocalcemia [13,11] and sepsis [6]. Like GJ uncoupling, modest separation at the perinexus does not always correlate with conduction slowing, but there is now mounting evidence that W_P can modulate the CV-GJ relationship under specific conditions by altering ephaptic coupling (EpC). Our new results suggest that physiologic ionic differences also modulate the CV-GJ-EpC relationship. In conclusion, future studies and therapies designed to address conduction slowing secondary to loss of functional gap

junctions may consider extracellular ionic composition as a confounding modulator of arrhythmogenic conduction slowing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Modulation of Interstitial Volume by Perfusates

Images of H&E stained tissue from WT (a) and HZ (b) hearts perfused with Solutions 1 and 2 show V_{IS} as white space, myocytes in gray and nuclei as black spots. Percent V_{IS} (c) is similar in WT hearts. In HZ hearts, a larger V_{IS} was observed during perfusion of Solution 1 relative to 2. **Statistics:** Unpaired, two-tailed, equal variance and sample size Student's t-test with Bonferonni correction (2 comparisons/dataset). *, $p < 0.05$.

Fig. 2. Modulation of the Perinexus by Perfusates

The perinexus (highlighted in yellow) is modulated by the base solutions (1 and 2) and the modified solutions (1C and 2C). Solution 1 increases W_p in both WT and HZ hearts compared to Solution 2 (a) and Solution 1C has wider W_p compared to Solution 2C (b). Combined W_p for WT and HZ are summarized as a function of distance from edge of the GJ plaque (c). **Statistics:** Single Factor ANOVA Post hoc test – Unpaired, two-tailed, equal variance and sample size Student's t-test. *, $p < 0.05$.

Fig. 3. Modulation of Conduction by Perfusates

Representative activation maps from WT and HZ mice hearts (a). Crowding of isochrones lines along with summary CV_L (b), CV_T (c) and Anisotropic Ratio (d) demonstrate that Solution 2 slows conduction in HZ hearts. $\square$ indicates pacing site. **Statistics:** Paired, one-tailed, equal variance and sample size Student's t-tests with Bonferonni correction. *, $p < 0.05$.

Fig. 4. Conduction Velocity – Perinexal width Relationship

Solution 2 and 1C have similar $[Na^+]_o$ and $[K^+]_o$ but produced different W_P (Solution 2 – Small W_P , Solution 1C – Large W_P) and different CV_T in the same heart. There was no change in CV_L during either solution perfusion (a) but Solution 2 was associated with faster CV_T (b). No change in AR (c) was observed. CV_T slowing is greater in HZ hearts relative to WT during Solution 1C perfusion (d). **Statistics:** Paired, one-tailed, equal variance and sample size Student's t-tests. *, $p < 0.05$.

Fig. 5. Modulation of Conduction Velocity by altering Ionic Composition of Solution 1

Relative change in longitudinal conduction velocity (a), Transverse conduction velocity (b) and anisotropic ratio with wide W_P (c). Varying sodium modulated CV_T only in HZ hearts, whereas varying potassium altered CV in WT and HZ hearts. **Statistics:** * denotes $p < 0.05$ detected by paired, two-tailed, equal variance and sample Student's t-tests with Bonferroni correction performed on percent changes relative to zero. # denotes $p < 0.05$ between WT and HZ hearts determined by unpaired, two-tailed, equal variance and sample size Student's t-tests.

Fig. 6. Modulation of Conduction Velocity by altering Ionic Composition of Solution 2
Relative change in longitudinal conduction velocity (a), Transverse conduction velocity (b) and anisotropic ratio (c) with narrow WP. Significant changes were observed only when both sodium was reduced and potassium was increased. **Statistics:** * denotes $p < 0.05$ detected by paired, two-tailed, equal variance and sample size Student's t-tests with Bonferonni correction performed on percent changes relative to zero.

Table 1

Perfusate Composition used in this study

Tyrode Composition (in mM)									
	1	1A	1B	1C	2	2A	2B	2C	
NaCl	130	130	130	130	118.3	126.2	118.3	126.2	
NaHCO3	24	17.3	24	17.3	29	29	29	29	
NaH2PO4	1.2		1.2						
Total [Na ⁺]	155.2	147.3	155.2	147.3	147.3	155.2	147.3	155.2	
KCl	4	4	4	4	4.7	4.7	3	3	
KH2PO4			2.1	2.1	1.4	1.4	1	1	
Total [K ⁺]	4	4	6.1	6.1	6.1	6.1	4	4	
MgCl2	1	1	1	1					
MgSO4					1	1	1	1	
Glucose	5.6	5.6	5.6	5.6	10	10	10	10	
CaCl2	1.8	1.8	1.8	1.8	3.4	3.4	3.4	3.4	
BDM	15	15	15	15	10	10	10	10	
Osmolarity (mOs m)	318.8 ± 0.9	304.8 ± 1.1	318.4 ± 1.3	307.8 ± 0.7	310.4 ± 1.3	328 ± 0.8	308.2 ± 0.7	323.6 ± 0.8	

Solution 1 is the published Morley et al. solution [19] and Solution 2 is the published Eloff et al. solution [10]. Osmolarity values are reported as Mean ± SD.