Original research

The zebrafish as a novel animal model to study the molecular mechanisms of mechano-electrical feedback in the heart

Andreas A. Werdich a,*, Anna Brzezinski a, Darwin Jeyaraj a, M. Khaled Sabeh c, Eckhard Ficker a, Xiaoping Wan a, Brian M. McDermott Jr b, Calum A. MacRae c, David S. Rosenbaum a

a The Heart and Vascular Research Center, MetroHealth Campus, Case Western Reserve University, 2500 MetroHealth Drive, R657 Cleveland, OH 44109, USA
b Department of Otolaryngology-Head and Neck Surgery, Case Western Reserve University, School of Medicine, Cleveland, OH 44106, USA
c Cardiovascular Division, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115, USA

1. Introduction

1.1. Stretch-induced ventricular electrical remodeling in the heart

Ventricular electrical remodeling (VER) is a persistent change in the electrophysiological properties of myocardium in response to a change in heart rate or activation sequence (Jeyaraj et al., 2010). Abnormal ventricular activation is commonly associated with a variety of cardiac pathologies including conduction system disease, myocardial infarction, hypertrophy and heart failure and is induced by ventricular pacing. The alteration of ventricular activation by pacing causes an inversion of the T wave of the electrocardiogram which was first described by Chatterjee et al. (1969).

This T wave abnormality was later termed “T-wave memory” by Rosenbaum et al. (1982), because it persists long after the cessation of pacing and dissipates at a rate that depends on the history of prior pacing (Wecke et al., 2005). Importantly, T-wave memory exhibits accumulation, i.e. the duration and the degree of the T wave abnormality observed is dependent on the duration and degree of abnormal activation (del Balzo and Rosen, 1992). Because electrical excitation and mechanical contraction are tightly coupled (Bers, 2002), any alteration of electrical activation changes the temporal and spatial properties of contraction, leading to mechanical dysynchrony (Bank et al., 2011; Gjesdal et al., 2011). Therefore, it was hypothesized that an alteration of myocardial mechanics is the underlying cause of cardiac memory. Sosunov et al. (2008) examined this possibility in a Langendorff-perfused rabbit heart. They reported that stretching just a single point on the epicardium induced a T-wave alteration which was similar to that observed in cardiac memory. This result clearly supports the
hypothesis that altered mechanical activity, rather than the direction of current flow per se, is a key determinant of cardiac memory. Recently, using an in vivo dog model of pacing-induced VER, we found that periods of mechanical stretch are in fact a mechanism for triggering VER and T wave memory (Jeyaraj et al., 2007). Importantly, action potential durations were markedly increased in high-strain regions and unchanged in low-strain regions, accounting for the inversion of the T wave.

Taken together, these reports suggest that tissue stretch is a prerequisite for T-wave memory and that long-term alteration of ventricular activation causes persistent electrical remodeling via a mechano-electrical feedback mechanism (Marrus and Nerbonne, 2008). It is important to point out that in this context, VER produces persistent electrophysiological changes, such as action potential prolongation, which outlast the period of applied stretch. This should be distinguished from transient mechano-electrical effects which have been studied extensively and are only present as long as cardiac muscle is actively stretched (Iribe et al., 2009; Kohl et al., 2006; Nishimura et al., 2006, 2008).

1.2. Clinical consequences of ventricular electrical remodeling

Results from both randomized clinical trials and smaller mechanistic studies have suggested that long-term ventricular pacing has significant adverse effects, ranging from impaired mechanical function (Tantengco et al., 2001) to increased risk of hospitalization, new or worsened heart failure, and death (Sweeney et al., 2003; Thambo et al., 2004). The DAVID trial showed that patients who received continuous dual-chamber pacing had significantly worse outcomes than patients with back-up, i.e. less frequent, ventricular pacing, presented by worsening of heart failure and increased mortality (Wilkoff et al., 2002). This result was confirmed by the MADIT II trial, which demonstrated that ventricular pacing was linked to worsening of heart failure (Moss et al., 2002). Some reports suggested that there is a direct relationship between ventricular pacing, cardiac memory, mechanical dysfunction and increased susceptibility to arrhythmias (Alessandrini et al., 1997; Medina-Ravell et al., 2003), thus highlighting the need to further investigate the effects of ventricular pacing. This is particularly paramount for pacing therapy in children with congenital or acquired atrioventricular (AV) block because in these patients, pacing has been shown to be highly beneficial (van Geldorp et al., 2011).

When the normal pattern of activation is disturbed by ventricular pacing, impulse propagation occurs between ventricular muscle cells, rather than through the fast His-Purkinje network, causing slowing of conduction (Scher and Young, 1955; Scher et al., 1953; Vassallo et al., 1984) and mechanical dysynchrony (Fang et al., 2010; Rosenbush et al., 1982). In a study of 93 patients with sinus node dysfunction who had been paced for at least 6 months, Fang et al. (2010) found that half had developed significant mechanical dysynchrony. Those patients who developed dysynchrony had significantly larger ventricles and lower ejection fraction than those who did not develop dysynchrony. Furthermore, in another report, Zhang et al. (2008a) showed that, of 79 patients who were paced from the right ventricle more than 90% of the time, 26% developed systolic heart failure within the 8-year follow-up period. Right-ventricular pacing created left-ventricular mechanical dysfunction and accelerated the progression of heart failure (Tse and Lau, 1997; Zhang et al., 2008a). Conversely, clinical studies aimed at synchronizing electrical activation and mechanical contraction by bi-ventricular pacing improved survival (Abraham et al., 2002; Bristow et al., 2004; Tu et al., 2011). In a recent review, Bank et al. (2011) have summarized many of these clinical observations.

1.3. Molecular mechanisms of ventricular electrical remodeling

The aforementioned studies established clear correlations between pacing-induced mechanical dysynchrony and electrical dysfunction. Therefore, a number of investigations have been aimed at understanding the molecular mechanisms that link abnormal mechanical stretch with adverse electrical remodeling in the heart. Recent reports have been focused on the G protein-coupled mechanoreceptor pathway as a possible mechanism (Patel et al., 2010; Sharif-Naeini et al., 2010; Storch et al., 2012). This pathway is an ideal candidate to explain stretch-induced VER because it couples a pressure sensor (for example, the angiotensin II type 1 receptor, AT1R) to a depolarizing, calcium-permeable ion channel such as the transient receptor potential potential canonical, TRPC, channel (Rowell et al., 2010) via a Gq protein-coupled signaling cascade. There is substantial evidence that Gq protein-mediated signaling is involved in the maladaptive hypertrophic response of the heart to pressure overload (Akhter et al., 1998; Wettchureck et al., 2001) that causes cellular Ca2+ influx and hypertrophy via activation of the NFAT-calcineurin pathway (Bush et al., 2006; Kuwahara et al., 2006; Nakayama et al., 2006; Onohara et al., 2006; Seth et al., 2009). Moreover, expression of the protein ‘regulator of G-protein signaling 2’ (RGS2), an endogenous inhibitor of Gq function, was reduced in ventricular myocardium from hypertensive patients (Semplicini et al., 2006), from end-stage heart failure patients with left-ventricular assist devices (Takeishi et al., 2000), and in a dog model of dysynchronous heart failure (Chakir et al., 2009). Consistent with this, mice lacking RGS2 responded more rapidly to pressure overload than their wild-type counterparts because of Gq hyper-activation, leading to hypertrophy, heart failure and early death (Takimoto et al., 2009). Activation of AT1R (Bkaily et al., 2003; Gusev et al., 2009), down-regulation of RGS2 (Calo et al., 2008; Klaiber et al., 2010; Semplicini et al., 2006) and membrane stretch (Malhotra et al., 1999; Onohara et al., 2006) have been shown to independently activate Gq signaling and Ca2+ influx through TRPC channels.

Other signaling pathways are known to transduce mechanical signals into electrophysiological and structural remodeling events, and these pathways include: protein kinase A, protein kinase C, protein kinase G, calmodulin–activated kinase, calcineurin, mitogen-activated protein kinase, integrin and tyrosine kinase (Heineke and Molkentin, 2006; Romer et al., 2006; Sadoshima and Izumo, 1997). They activate a number of key transcription factors (for example, MEF2, GATA4, etc.) that regulate myocyte growth and function. Some mechanosensitive ion channels (MSC) that are present in the sarcolemmal membrane and in the extracellular matrix/cytoskeleton have been shown to be directly gated by stretch, thereby affecting excitation-contraction coupling on a beat-to-beat basis (Iribe et al., 2009; Kohl et al., 2006). In the beating heart, it is likely that the final common pathway linking mechanical signals to electrophysiological and structural remodeling is not represented by a single signaling pathway, but rather a combination of these mechanisms.

1.4. Limitations of current animal models for studying mechano-electrical feedback mechanisms in the heart

Studies in intact hearts from large animals (for example dog, sheep or lamb) with physiologies that are close to human have provided some important insights into the VER phenotypes which are clinically relevant and would have otherwise been difficult to obtain from patients (Dixon and Spinale, 2009). Although these large-animal experiments at the intact-organ level are informative because of their high level of complexity, they allow only limited control of tissue parameters. For example, perfusion of whole-organ
preparations with drugs affecting excitation-contraction coupling causes highly non-uniform electrophysiological effects due to significant drug concentration gradients (Lacroix et al., 1999). Moreover, tissue stretch by pacing or pressure is difficult to control in magnitude and direction in a large 3-dimensional intact heart preparation because mechanical load is distributed heterogeneously across the endocardial, epicardial and transmural walls, causing regional structural and electrical remodeling that is difficult to localize or access experimentally (Spragg et al., 2003; van Oosterhout et al., 1998). For the same reasons, gene manipulation studies at the whole-organ level aimed at investigating the influences of altered protein expression on arrhythmia susceptibility have proven difficult (Rapti et al., 2011). Simplified cell culture models have been developed that circumvent some of the complexities inherent to intact tissue models and provide in vitro preparations that can be tested under controlled conditions, including high-precision stretch (Zhang et al., 2008b). Presently available cardiac cell culture models are derived from neonatal rat, neonatal mouse or embryonic chick (Tung and Zhang, 2006); although more recently, other cell culture models have become available that are derived from human stem cell sources (Arbel et al., 2010). These models significantly contributed to our understanding of electrical remodeling in response to stretch, but they lack important structural and electrical properties of adult, in vivo, cardiac tissue, which define cardiac electrical activity and are needed for an accurate representation of arrhythmia susceptibility. Among those properties are cell shapes, cell architecture (e.g. fiber structures), and cellular composition of ion channels and junction proteins, as reviewed by Kleber and Rudy (2004). Recent advances in tissue engineering have allowed the reproduction of some aspects of cellular architecture (Bian et al., 2012; McCain and Parker, 2011), but while electrically functional, these artificial tissues have been re-assembled from dissociated cardiac myocytes and are therefore fundamentally different from naturally grown heart tissue.

1.5. The zebrafish as a new animal model to study mechano-electrical feedback in the heart

Over the past decade the zebrafish (danio rerio) has emerged in the field of cardiovascular research as an attractive model organism. The popularity of this animal model is largely a result of its fecundity, diploid genome, and the transparency of its embryos, making it possible to study organ development in vivo (Haffter and Unspleiss-Vollhard, 1996). Sequencing of the zebrafish genome by the Sanger Institute transformed this species into a major genetic model organism (Vogel, 2000). Any gene in the zebrafish genome is readily manipulated using antisense morpholinos (Nasevicius and Ekker, 2000), making high-throughput, phenotype-driven genetic and chemical screens possible. Recently, stable gene knockouts have become feasible using zinc-finger nuclease technologies (Meng et al., 2008; Urmov et al., 2010). Zebrafish grow to about 3 cm in length, but during embryonic and larval stages, they are approximately 1–2 mm long. During these stages, zebrafish can live for several days in standard well plates, surviving on nutrients that are provided by their yolk sacs. Zebrafish are inexpensive to raise, and a pair of adults can routinely lay hundreds of fertilized eggs in a single morning. Therefore, even a small fish facility can produce thousands of embryos in a day making large-scale screens feasible. The zebrafish is ideal for phenotype-driven screens because cardiac phenotypes can be observed in vivo using a simple dissection microscope (Anderson and Ingham, 2003; Grunwald and Eisen, 2002; Patton and Zor, 2001). Several of the mutants identified in these screens exhibited cardiac arrhythmias (Stainier et al., 1996). Closer examinations of the hearts from these mutants led to the discovery of many zebrafish cardiac ion channels (Chen et al., 1996; Novak et al., 2006; Warren et al., 2001) and calcium-handling proteins (Zhang et al., 2010), which are known to play important roles in cardiac excitation-contraction coupling in the human heart. These and other reports show that there are striking similarities in ion channel expression and function between the human and the zebrafish heart and indicate that the electrophysiological mechanisms governing the generation of the human and zebrafish ventricular action potentials are similar (Baker et al., 1997; Milan and Macrae, 2008; Shin and Fishman, 2002; Warren and Fishman, 1998). Thus, for many electrophysiological phenomena the zebrafish should prove to be a suitable model.

For the electrophysiologist, one particularly interesting feature of the zebrafish is that severe cardiac defects can be studied that would be embryonic lethal in other animal models. The zebrafish embryo is small enough so that it can survive for several days by diffusion alone and in complete absence of blood circulation (Burggren and Pinder, 1991; Pelster and Burggren, 1996; Stainier et al., 1996). Compared with other animal models, this is an important advantage because mechano-electrical feedback resulting from physiological cardiac contraction can be reduced or completely eliminated. This was first demonstrated by the silent heart (silh) mutant, which was found to result from a mutation in the tnt2 gene encoding the thin-filament contractile protein cardiac troponin T. Mutations in this gene cause complete cessation of cardiac contraction (Sehert et al., 2002). Dosage of a morpholino targeted against this gene allowed a controlled reduction of the wild-type transcript, thereby creating morphant zebrafish with hearts that showed reduced contractility (Becker et al., 2011). These tnt2 morphants reproduced many of the cellular and transcriptional responses seen in patients with sarcomeric protein mutations that cause contractile dysfunction. A similar noncontractile phenotype is caused by a mutation in the unc-45b gene, which encodes a chaperone required for myofibril assembly (Etard et al., 2007). Interestingly, hearts from both noncontractile tnt2 and unc-45 morphants generated action potentials, but APDs, conduction velocities and calcium transients were significantly disturbed (Becker et al., 2011; Panakova et al., 2010). These reports show that mechano-electrical feedback mechanisms from cardiac contraction are important for the development of normal cardiac electric activity and calcium signaling in the heart.

In summary, the zebrafish embryo possesses a number of important advantages compared with other animal models that make it attractive for studying mechano-electrical feedback mechanisms in the heart: (1) a diploid genome, with almost every gene identified to have a human ortholog with a comparable function, (2) powerful genetic tools (e.g. morpholino oligos) that allow efficient knockdown of any gene of interest, (3) a large number of identified cardiac ion channels and calcium-handling proteins that are known to play important roles in excitation–contraction coupling in the human heart, (4) heart rates and ventricular action potentials that are comparable to those in humans, and (5) a small (<0.5 mm) heart that allows precise experimental control of the experimental conditions, e.g. extracellular ion concentrations, or as described here, the magnitude and direction of mechanical load using the carbon fiber method. To test the hypothesis that the zebrafish is a suitable animal model for studying mechano-electrical feedback mechanisms in the heart, we established a zebrafish heart stretch system that combines efficient genetic manipulation with high-precision stretch and high-resolution electrophysiology.

2. Materials and methods

2.1. Heart isolation

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Institutional
Animal Care and Use Committee at Case Western Reserve University. Hearts were isolated from zebrafish embryos 72 h post-fertilization (h.p.f.) and placed in Tyrode’s solution which contained (in mM) Na\(^+\) (136), K\(^+\) (5.4), Mg\(^{2+}\) (1.0), PO\(_4^{3-}\) (0.3), Ca\(^{2+}\) (1.8), glucose (5.0) and HEPES (10.0) at pH 7.4. For stretch experiments and fluorescence recordings, hearts were placed into an experimental perfusion chamber with integrated field stimulation electrodes (RC27NE, Warner Instruments, LLC, CT, USA) that contained Tyrode’s solution. For fluorescence measurements, the Tyrode’s solution was supplemented with the excitation-contraction uncoupler blebbistatin (10 \(\mu\)M, 203390, EMD Chemicals Inc., Philadelphia, PA) to eliminate contractions. The perfusion chamber was mounted onto the stage of an inverted microscope (Axiovert-100, Zeiss, Germany), as shown in Fig. 1A. All experiments were carried out at 21 °C.

2.2. Experimental system for the application of controlled stretch to zebrafish embryo hearts

We developed a system for the precise application of controlled stretch to the ventricles of isolated zebrafish embryo hearts based on a carbon fiber method that was previously applied to isolated cardiac myocytes (Iribe et al., 2007). Briefly, carbon fibers (Tsukuba Materials, Ltd., Tsukuba, Japan), 10 \(\mu\)m in diameter, were cut into 1 mm long pieces and glued into glass capillaries that were pulled from 2 mm-outlet diameter, 1.12 mm-inner diameter glass pipettes (1B200F-6 World Precision Instruments, FL, USA) using a cyanoacrylate-based adhesive (Fig. 1B, C). The final section (~2 mm long) of the glass capillary containing the fiber was bent by approximately 25° to allow near-parallel alignment with the bottom of the perfusion chamber (Fig. 1C). Each pipette was mounted on a high-precision motorized micro-manipulator (MP-285, Sutter Instruments, CA, USA). Before each experiment, fibers were aligned near horizontal and near parallel to each other. They were brought into contact with the ventricle of an isolated heart and firmly attached via electrostatic forces as described by Yasuda et al. (2001). Position control was accomplished using the motorized micromanipulators. Mechanical load was applied by moving the micromanipulators in opposite directions causing the fibers to deflect and the ventricular tissue to stretch (Fig. 1D and Fig. 2A).

The positions of the micromanipulator and the carbon fiber tips were monitored in real time during the experiment to control the amount of stretch. Tip distances were recorded at a frame rate of 30 s\(^{-1}\) using a high-speed CCD camera (PentaMAX, Princeton Instruments, NJ, USA) that was mounted on the microscope. Fiber positions were measured using custom-designed edge detection software written in Matlab (R2011, MathWorks, MA, USA, Fig. 2B). Slippage, i.e. sliding of the fibers relative to the heart surface, was apparent by a gradual increase of the stretch transient baseline and was used as an exclusion criterion. Hearts were left contracting in sinus rhythm against the mechanical load of the carbon fibers for 90 min. Then, they were separated from the fibers and stained with the transmembrane potential-sensitive dye Di-8-ANEPSS (D3167, Invitrogen, NY) for the measurements of action potential durations and conduction velocities.

2.3. High-resolution optical mapping of isolated zebrafish embryo hearts

We previously described in detail the measurement of action potentials and the estimation of conduction velocities in isolated zebrafish embryo hearts (Panakova et al., 2010). Briefly, hearts were stained for 20 min with 84 \(\mu\)M of Di-8-ANEPPS. They were then thoroughly rinsed with Tyrode’s solution to remove all extracellular

---

Fig. 1. Experimental system for the application of controlled stretch to zebrafish embryo hearts. The zebrafish heart stretch system was designed using a conventional inverted fluorescence microscope with attached micromanipulators (panel A). A high-speed CCD camera was used for measuring action potentials and conduction velocities. Two carbon fibers were attached to the ventricle of an isolated zebrafish embryo heart (panel B). The terminal 2 mm of a glass pipette was bent by 25° and a 1 mm long section of carbon fiber was glued into each pipette using a cyanoacrylate-based adhesive (panel C). Zebrafish hearts were stretched from the force-free position (panel D, left) to the stretched position (panel D, right) using the micromanipulators.
dye. Subsequently, individual hearts were placed into the perfusion chamber which contained Tyrode’s solution supplemented with the excitation-contraction uncoupler blebbistatin (10 μM, 203390, EMD Chemicals Inc., PA) to ensure that all measurements were done in mechanically unloaded hearts. This drug has previously been shown to effectively uncouple the excitation-contraction process in the zebrafish embryonic heart without significantly altering the action potential morphology (Jou et al., 2010). The perfusion chamber was then re-positioned onto the stage of the inverted microscope for fluorescence analysis. All fluorescence measurements reported in the figures were carried out approximately 40 min after the end of stretch. This time was required for the careful detachment, staining, rinsing and re-positioning of the hearts onto the microscope stage. Fluorescence light emission was recorded using the attached CCD camera at a frame rate of 200 s⁻¹. Using a magnification of 40×, the pixel-to-pixel distance was 5.5 μm. Two fluorescence recordings were carried out in sequence for each heart: the first recording was done during sinus-node activation and in the absence of any field stimulation to estimate conduction velocities, and the second recording was done during steady-state field stimulation at a rate of 60 beats-per-minute (bpm) to measure action potential durations (APDs). Stimulation voltages were set at approximately 1.2 times the activation threshold causing simultaneous excitation of the entire heart independent of the field direction. At least 30 beats were counted before each APD measurement. Conduction velocity vector fields were estimated using an established algorithm (Bayly et al., 1998), which we previously applied to action potential measurements from 72-h.p.f-old embryonic (Panakova et al., 2010) and adult (Kikuchi et al., 2010; Wang et al., 2011) zebrafish. Briefly, fluorescence images were exported as 16 bit TIFF stacks and processed spatially using a 2 × 2-pixel averaging filter. Activation times were calculated for each pixel as the time from baseline to 50% depolarization. In computer simulations of two-dimensional cardiac tissue, this criterion for electrical activation was found to correlate well with the time of maximum depolarizing sodium inward current (Fast and Kleber, 1995). Isochronal maps that represent the positions of the wavefront in 5 ms time intervals were generated from the activation times using the contour plotting function provided by Matlab (Fig. 3A). Ventricles and atria were each divided into 3 non-overlapping regions-of-interest (ROIs), 6 × 6 pixels in size (33 × 33 μm² of tissue area) covering the inner curvature (IC), mid-ventricular myocardium (MV) and outer curvature (OC) as shown in Fig. 3A. Taking into account the activation time and spatial coordinates of each pixel, conduction velocity vector maps were estimated in each ROI (Fig. 3A, B) using an established algorithm described by Bayly et al. (1998). The action potential duration (APD) was defined as the time from 20% depolarization to 80% repolarization and measured during steady-state field pacing at 60 bpm (Fig. 3C). All measurements of APD and conduction

Fig. 2. Carbon fiber technique for applying controlled stretch to zebrafish embryo hearts. Time-dependent carbon fiber deflections and tip displacements were monitored during the experiment via the attached CCD camera. Increasing the mechanical load by moving the micromanipulators apart caused an increase in fiber deflection and a near-proportional increase in % tissue stretch (panel A). Mechanical loading of the zebrafish ventricle caused an immediate, but transient, elevation of the contractile force and rate of contraction (panel B). Contraction rates were the same at the end of the stretch period (panel C). “Small” and “large” stretch amounts were defined as relative (panel D) and absolute (panel E) increases in tissue length between the fiber tips. Stretch directions were defined corresponding to the zebrafish heart anatomy (panel F): hearts were stretched along an axial (Ax) direction by attaching one fiber near the outer curvature (OC) and the other one near the outflow tract (OFT) of the ventricle. The transversal (Tr) stretch direction was defined perpendicular to the axial direction and across the atrium (panel F, G).
velocity were averaged across the three ROIs in the ventricle (and atrium) to obtain overall measures of ventricular (and atrial) APDs and conduction velocities.

3. Results

3.1. Electrophysiological properties of the zebrafish embryo heart

Ventricular action potentials were characterized by a fast upstroke and a pronounced plateau phase, similar in shape and duration to the human ventricular action potential. Ventricular action potentials shortened with increasing heart rates (Fig. 3C), an observation that is well known from the mammalian heart as APD restitution (Pastore et al., 2006). The maximum APD differences within a broad range of heart rates (60 bpm to 120 bpm) were smaller than 20 ms. Importantly, this is far less than the APD changes that were associated with stretch-induced VER, reassuring that any spontaneous variations in APD due to heart rate fluctuations do not obscure the reliable detection of VER responses in this model. However, to completely avoid any rate dependencies, all APD measurements were carried out during field stimulation at 60 bpm. Mean conduction velocities during sinus activation were higher in the ventricle than in the atrium, with slow conduction zones emerging at the sino-atrial node (SAN), the atrio-ventricular junction (AVJ) and the outflow tract (Fig. 3A), as we have previously described (Panakova et al., 2010).

3.2. Time course study to determine the optimal time point for stretch experiments in the developing embryo heart

An important consideration for using embryonic, rather than adult, zebrafish was the possibility of combining ventricular stretch with efficient genetic manipulation using morpholino antisense oligos. These compounds are most effective in embryos that are less than 90 h.p.f. old (Nasevicius and Ekker, 2000). Therefore, our goal was to test whether there was a time interval during zebrafish heart development that was late enough so that developmental influences on the stretch phenotype were small but early enough so that morpholinos could still be used effectively. We measured APDs and conduction velocities in the ventricles from developing zebrafish embryo hearts in 24-h intervals, i.e. from 24 h.p.f. to 120 h.p.f. (Fig. 4). We found that APDs shortened and ventricular conduction velocities increased markedly within the first 48 h of development, but became very stable after 72 h.p.f. In fact, mean APDs and conduction velocities were not significantly different between 72 h.p.f. and 120 h.p.f., suggesting that during this time electrophysiological changes caused by cardiac development are negligible. Given the relative stability of these properties at 72 h.p.f., this was an optimal time point of development to apply mechanical stretch interventions.

3.3. Validation of the carbon fiber technique for applying controlled stretch to zebrafish embryo hearts

The carbon fibers attached reliably to the ventricles of isolated 72 h.p.f zebrafish embryo hearts upon touch as previously described for isolated cardiac myocytes (Iribe et al., 2007; Yasuda et al., 2001). The magnitude of stretch could be controlled via the attached micromanipulators. Moving the fiber tips apart increased the ventricular mechanical load as evident from the changes in the fiber deflection (Fig. 2A) which is in first approximation proportional to the restoring force (Iribe et al., 2007; Nishimura et al., 2004). The fiber deflection was calculated as the difference between the manipulator and the fiber tip displacements. Because hearts contracted in sinus rhythm, changes in contractile force and frequency could readily be observed. The zebrafish ventricle responded to the increase in mechanical load with an immediate increase in the amplitude and rate of contraction (Fig. 2B). This effect has been described previously for larger mammalian heart preparations (Bollensdorff et al., 2011). However, contraction rates decreased shortly after the stretch was applied and eventually became independent of the stretch amount at the end of the stretch period (Fig. 2C). It was possible to stretch ventricles by up to 300% of their unloaded length, before detachment occurred (Fig. 2D). Despite these large stretch amounts, contractions remained periodic and stable during the stretch experiment. “Small” and “large” stretch, respectively, were defined as relative increases in tissue length of (25 ± 3)% and (230 ± 50)% and measured as the relative increase in fiber-tip distance (Fig. 2D, E). Small stretch was comparable in magnitude with relative stretch amounts that were previously reported in isolated rat, guinea pig and frog cardiac myocytes (Iribe and Kohl, 2008; Iribe et al., 2009; Riemer and Tung, 2003). Large stretch was chosen to be on the same order of magnitude as the relative increase in circumferential strain that we
previously measured in paced dog hearts using tagged MRI imaging (Jeyaraj et al., 2007). This was done to assure that the magnitudes of stretch that we tested fell within physiologically reasonable ranges. To study the influence of stretch direction on the stretch response, we stretched ventricles across an outer curvature-outward axis (axial stretch, Ax), and along a transversal axis (transversal stretch, Tr), which was perpendicular to the axial direction (Fig. 2F, G).

3.4 Demonstration of stretch-induced ventricular electrical remodeling in the zebrafish embryo heart

In the zebrafish ventricle, 90 min of axial ventricular stretch caused a profound, more than two-fold increase in the ventricular action potential duration (APD) compared with unstretched control hearts (Fig. 5A, C). The APD was unchanged in the atrium of the post-stretch hearts (Fig. 5B, C), demonstrating that, as expected, the stretch response was localized to the cardiac chamber that was subjected to stretch, i.e. the ventricle. This electrophysiological stretch response was maintained 40 min after the end of stretch and in mechanically unloaded hearts. To test if the remodeled phenotype persisted even longer than 40 min, we measured APDs in four hearts 90 min after the end of stretch. We found that in these hearts, APD prolongation was still significant, i.e. 357 ± 30 ms in post-stretch hearts vs. 216 ± 8 ms in unstretched control hearts, demonstrating that the electrophysiological change outlasted the period of applied stretch. We then examined the dependence of APD prolongation on the magnitude and direction of stretch. We found that small axial stretch caused the same increase in APD as large axial stretch, indicating that over a large range of stretch amounts, the APD phenotype was independent of the mechanical load. However, changing the direction of stretch, as defined in Fig. 2, from axial stretch to transversal stretch, attenuated the stretch response (Fig. 5D): Large transversal stretch resulted in a smaller, not significant increase of the mean APD compared with unstretched control hearts, indicating that the stretch response was dependent on the direction of applied stretch. Within the ventricles of the unstretched control hearts, APDs were distributed homogeneously (Fig. 5E). However, it is possible that the carbon fiber method caused heterogeneous ventricular stretch, with the largest mechanical loads occurring between the fibers, resulting in a heterogeneous response. We therefore tested if stretch increased the spatial heterogeneity of APD, i.e. the dispersion of repolarization, as this may have occurred in the heart shown in Fig. 5E. We calculated the dispersion of repolarization as the maximum absolute differences in APD between the three ROIs that are shown for a representative unstretched heart in Fig. 3A. Interestingly, ventricular stretch did not significantly increase the dispersion of repolarization in the ventricle, suggesting that the stretch response was fairly uniform throughout the ventricle (Fig. 5C).

We then compared the conduction velocities in stretched and unstretched hearts (Fig. 6). Large ventricular axial stretch significantly slowed the conduction velocities in the ventricle but not in the atrium (Fig. 6A–C). This decrease in the conduction velocity was not significant for small axial and large transversal stretch (Fig. 6D), suggesting that conduction slowing was less sensitive to stretch than APD prolongation. Taken together, our data demonstrate that the response of the zebrafish ventricle to stretch is dependent on the amount and the direction of stretch and that large axial stretch causes the most severe electrophysiological phenotype.

4. Discussion

The goal of this study was to examine the possibility of using the zebrafish model to investigate mechano-electrical feedback mechanisms in the heart. The power of this approach resides in the possibility to rapidly test the overlapping functions of genes that are involved in the response of the intact vertebrate heart to stretch. The similarities that exist between the zebrafish and the human heart, in particular the documented high conservation of gene expression and function, should facilitate translation at every level to other model systems or to human (Shin and Fishman, 2002).

4.1. Electrical stability of the developing zebrafish heart during assessment of ventricular electrical remodeling

One of the anticipated challenges in assessing stretch responses in a model that is undergoing cardiac development is that it might be difficult to determine if any given electrophysiological change was attributable to cardiac development or to the stretch perturbation itself. We found that 72 h.p.f. was a suitable time point for our stretch experiments, because in the vicinity of that time, developmental changes in APD and conduction velocity are small (Fig. 4) and morpholino antisense strategies are still very effective (Ekker, 2000). In a recent time course study, Leong et al. (2010) demonstrated that expression levels of the zebrafish kcnh2 gene encoding the α-subunit of the rapid delayed rectifier current (I\textsubscript{Kr}) were stable after 72 h.p.f. These results suggest that developmental influences on the electrophysiological stretch response are small—yet they may still exist and contribute to the stretch phenotype, especially for longer stretch durations. As in every embryonic model, these developmental changes may not be completely eliminated but could possibly be controlled by carefully characterizing the model and keeping the experiment durations short.

4.2. Ventricular stretch caused ventricular electrical remodeling in the isolated zebrafish embryo heart

Zebrafish ventricles responded with profound and persistent electrophysiological changes to stretch. Only 90 min of ventricular stretch caused a significant prolongation of the ventricular action...
potential duration (Fig. 5) and a decrease of the ventricular conduction velocity (Fig. 6). When stretch was applied, the zebrafish heart responded with an immediate increase in the contraction rate (Fig. 2B), suggesting that there was some acute influence of ventricular stretch on the sino-atrial node. However, this effect was transient because contraction rates decreased within the first minute of stretch and eventually became independent of the stretch amount (Fig. 2C). Moreover, there was no effect of ventricular stretch on APDs and conduction velocities in the atria of post-stretch hearts, reaffirming that it was mechanical stretch that induced the persistent electrophysiological response. This result is consistent with previously published data from mammalian VER models. The APD was prolonged in regions of high circumferential strain that were induced by ventricular pacing in our dog model (Jeyaraj et al., 2007). In a similar model, Patel et al. (2001) reported reduced expression of the gap junction protein connexin 43 and conduction slowing after induction of T-wave memory. However, as in most of the published electrophysiological studies about cardiac memory to date, strain patterns were not recorded.

In our zebrafish model, the electrophysiological stretch response was maintained for at least 90 min after the end of stretch and in the absence of any mechanical load. In contrast to this, activation of mechanosensitive ion channels that mediate electrophysiological changes on a beat-to-beat basis are fully reversible when the mechanical load is removed (Bollensdorff et al., 2011; Iribe et al., 2009). Because the response outlasted the duration of stretch, we define the zebrafish stretch response here as VER in accordance with our previous definition (Jeyaraj et al., 2010, 2007). These observations raise an important question: how could only 90 min of stretch result in a subsequent maintained effect? A possible mechanism may be the post-transcriptional modification of ion channels, pumps and exchangers, for example via phosphorylation. Phosphorylation of contractile proteins and activation of the Na\(^+\)/H\(^+\) exchanger were found in acutely stretched rabbit (Monasky et al., 2010) and rat (Alvarez et al., 1999; Calaghan and White, 2004; Perez et al., 2011) heart muscle preparations, respectively, and have been suggested to be important contributors to the instantaneous slow-force response to a change in muscle length (Luers et al., 2005). Another recent study identified several post-translational modifications of focal adhesion proteins in uniaxially stretched fibroblasts (Hoffman et al., 2012), demonstrating that stretch-induced protein modifications can occur in a variety of cell types independently from transcription or translation. It is not known, however, how long such protein changes would persist in the zebrafish heart.

It is further conceivable that transcriptional changes are responsible for the zebrafish stretch response. Studies in cultured neonatal rat myocytes demonstrated that brief periods of stretch, lasting less than 60 min, induced features of the hypertrophic response, including changes in contractile protein expression and activation of signal transduction pathways (Komuro et al., 1990, 1991, 1996; Sadoshima and Izumo, 1993). One study concluded that just 5 min of stretch were sufficient to induce rapid secretion of VEGF and increased expression of both VEGF and VEGF receptor
mRNA in cultured cardiac myocytes (Seko et al., 1999). More recent data in a cell culture model indicated that 60 min of pulsatile stretch were sufficient to cause substantial upregulation of intercellular gap junction proteins likely leading to the significant conduction velocity increase that was observed (Zhuang et al., 2000). These and other experimental data on the effects of mechanical forces on the remodeling of gap junctions were reviewed in detail by Jeffrey Safitz and Kléber (2004). Taken together, the results of these studies demonstrate that short periods of stretch, lasting from several minutes to hours, can produce electrical remodeling. Clearly, additional expression studies and direct exploration of candidate pathways are necessary to understand the molecular mechanism of stretch-induced VER in the zebrafish.

It is possible that tissue damage contributed to the conduction slowing observed in post-stretch hearts. To test this hypothesis, we used a significantly smaller stretch amount (Fig. 2D, E) corresponding to a smaller loading force (Fig. 2A). A similar stretch amount had previously been applied using the same method to isolated rat, guinea pig and frog cardiac myocytes without causing damage (Iribe and Kohl, 2008; Iribe et al., 2009; Riemer and Tung, 2003). We found that in the zebrafish ventricle, the much smaller axial stretch amount did not cause significant conduction slowing (Fig. 6D), suggesting that it preserved normal cell-to-cell coupling. However, small axial stretch caused the same APD prolongation as large axial stretch (Fig. 5D). This result indicates that the stretch-induced APD prolongation was independent of any mechanical uncoupling or tissue damage that might have contributed to the conduction slowing. Additionally, we obtained consistently high signal-to-noise ratios (>10) from stretched ventricles, even from those areas that were located right between the fibers and subjected to the largest stretch amounts. This indicates that the stretched ventricular myocytes were viable and generated action potentials. Our observations are supported by previous studies showing that fish are able to increase stroke volumes by up to 300% to increase cardiac output, unlike mammals who accomplish this by accelerating heart rate (Patrick et al., 2011; Shiels et al., 2006). Although we cannot rule out structural changes, our optical

Fig. 6. Demonstration of persistent conduction slowing in stretched zebrafish ventricles. Ventricular stretch decreased the ventricular conduction velocities as evident from representative 5 ms isochronal maps of an unstretched control heart (panel A) and a post-stretch heart (panel B). Ventricular conduction velocities were significantly decreased in post-stretch hearts (n = 6, panel C) compared with unstretched control hearts (n = 5, panel C). Ventricular stretch did not influence the atrial conduction velocities. Only large axial Ax stretch (n = 6) significantly reduced the ventricular conduction velocities compared with unstretched control hearts (n = 5, panel D). Neither small axial Ax stretch (n = 5) nor large transversal Tr stretch (n = 4) caused significant conduction slowing compared with the unstretched control hearts (panel D). NS = not significant.
mapping data indicate that stretch-induced APD prolongation occurs independent from electrical uncoupling.

We also found that the stretch-induced VER phenotype was dependent on the direction of stretch. Axial stretch increased the APD (Fig. 5D) and slowed ventricular conduction (Fig. 6D), while transversal stretch resulted in a significantly attenuated response. Previous reports showed in rat neonatal cell cultures that the electrophysiological stretch response depended on how stretch was applied. Heterogeneous shear stress increased the APD and decreased the conduction velocity, while uniform hydrostatic pressure did not cause any statistically significant changes (Zhang et al., 2008b). Cyclic linear axial stretch in a similar model increased the conduction velocity, likely due to an upregulation of gap junction proteins (Zhuang et al., 2000). Static stretch produced significantly smaller changes than pulsatile stretch. Anisotropic biaxial stretch of micropatterned cells grown as linear strands showed upregulation of connexin 43 and N-cadherin with transversal stretch, but not with axial stretch (Gopalan et al., 2003).

Last, some components of the heart stretch response may be species-dependent. For example, short-term (seconds to minutes) stretch by pressure overload caused APD prolongation in the intact rabbit heart (Sung et al., 2003), while APD shortening occurred in an open-chest lamb model (Chen et al., 2004). Decreased mechanical load by intravenous infusion of sodium nitroprusside, a potent vasodilator, increased left ventricular APD in the pig heart linearly with decreased systolic ventricular pressure (Dean and Lab, 1989). This variability in the stretch response might be due to subtle electrophysiological differences in ion channel expression and function that exist between species leading to very different cellular behaviors, as illustrated in a recent simulation study by O'Hara and Rudy (2011), thus leaving the possibility that the molecular mechanisms underlying the electrophysiological stretch response are universal.

5. Conclusions

VER represents a serious clinical problem that affects a large number of adults and children with cardiac conduction system defects or ventricular pacing. Recent data from patients and animal models suggest that VER is triggered by a mechanoelectrical feedback mechanism, but the molecular pathways that link mechanical stretch and electrical remodeling are not known. Our results indicate that the zebrafish is a suitable animal model for studying the molecular mechanisms that cause stretch-induced VER because it reproduces the key electrophysiological phenotypes that have been described in mammalian VER models, and yet, allows efficient genetic manipulation and precise experimental control. In the future, the zebrafish VER model could provide important insights into the complex molecular processes that drive detrimental electrical remodeling in the heart response to stretch.

Editors' note

Please see also related communications in this issue by Takahashi and Naruse (2012) and McNary et al. (2012).

Acknowledgments

This project was supported by a grant from the National Institutes of Health (RO1-HL0054807, David S Rosenbaum) and a post-doctoral fellowship award from the American Heart Association (10POST4150110, Andreas A Werdich). We thank the members of the McDermott laboratory in the Department of Otolaryngology-Head and Neck Surgery at Case Western Reserve University for expert help with zebrafish.

References


del Balzo, U., Rosen, M.R., 1992. T wave changes persisting after ventricular pacing in canine heart are altered by 4-aminopyridine but not by lidocaine.
A.A. Verdich et al. / Progress in Biophysics and Molecular Biology 110 (2012) 154–165

Implications with respect to phenomenon of cardiac ‘memory’. Circulation 85, 1464–1472.


blunts the slow force response to myocardial stretch. J. Appl. Physiol. 111, 874–880.


