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Spatial variability in T-tubule and electrical remodeling of left ventricular epicardium in mouse hearts with transgenic Gαq overexpression-induced pathological hypertrophy

Wen Tao^a, Jianjian Shi^a, Gerald W. Dorn II^b, Lei Wei^{a,c}, and Michael Rubart^{a,*}

^aRiley Heart Research Center, Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA

^bDepartment of Medicine, Washington University, St. Louis, MO, USA

^cDepartment of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN, USA

Abstract

Pathological left ventricular hypertrophy (LVH) is consistently associated with prolongation of the ventricular action potentials. A number of previous studies, employing various experimental models of hypertrophy, have revealed marked differences in the effects of hypertrophy on action potential duration (APD) between myocytes from endocardial and epicardial layers of the LV free wall. It is not known, however, whether pathological LVH is also accompanied by redistribution of APD among myocytes from the same layer in the LV free wall. In the experiments here, LV epicardial action potential remodeling was examined in a mouse model of decompensated LVH, produced by cardiac-restricted transgenic Gαq overexpression. Confocal linescanning-based optical recordings of propagated action potentials from individual *in situ* cardiomyocytes across the outer layer of the anterior LV epicardium demonstrated spatially non-uniform action potential prolongation in transgenic hearts, giving rise to alterations in spatial dispersion of epicardial repolarization. Local density and distribution of anti-Cx43 immune reactivity in Gαq hearts were unchanged compared to wild-type hearts, suggesting preservation of intercellular coupling. Confocal microscopy also revealed heterogeneous disorganization of T-tubules in epicardial cardiomyocytes *in situ*. These data provide evidence of the existence of significant electrical and structural heterogeneity within the LV epicardial layer of hearts with transgenic Gαq overexpression-induced hypertrophy, and further support the notion that a small portion of electrically well connected LV tissue can maintain dispersion of action potential duration through heterogeneity in the activities of sarcolemmal ionic currents that control repolarization. It remains to be examined whether other experimental models of pathological LVH, including pressure overload LVH, similarly exhibit alterations in T-tubule organization and/or dispersion of repolarization within distinct layers of LV myocardium.

Keywords

Pathological cardiac hypertrophy; T-tubules; Electrical remodeling; Voltage-sensitive dye; *In situ* confocal microscopy

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*Corresponding author at: Riley Heart Research Center, Wells Center for Pediatric Research, Indiana University School of Medicine, 1044 West Walnut Street, Indianapolis, IN 46202-5225, USA. Tel.: +1 3172781538; fax: +1 3172789298. mrubartv@iupui.edu.

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1. Introduction

Pathological left ventricular hypertrophy (LVH) is typically associated with prolongation of ventricular action potentials and alterations in the spatial dispersion of repolarization, both of which increase the susceptibility to arrhythmias [1,2]. Several studies have examined the effects of hypertrophy on the transmural distribution of action potential duration (APD) in rodent models of pathological LVH. These investigations concordantly found a loss of the native gradient in APD across the LV free wall, resulting from a more pronounced action potential (AP) prolongation in epicardial *versus* endocardial LV cardiomyocytes [1,3,4]. A previous study by Gomez et al. suggests the possibility that spatial reorganization of repolarization in the hypertrophied heart may extend beyond elimination of the transmural APD gradient [5]. Specifically, the authors observed a marked increase, rather than a decrease, in APD variability among single cardiomyocytes isolated from the LV free wall of rats with pressure-overload LVH, with maximal differences in APD exceeding two orders of magnitude. These seemingly conflicting data can be reconciled by a model wherein hypertrophy on average decreases APD differences between epicardial and endocardial layers, but at the same time increases APD variability within a given layer of the left ventricular wall. To date, such alterations in spatial dispersion of APD have not been demonstrated in the intact heart with pathological hypertrophy, but proof of its existence would be critical in understanding arrhythmias associated with this condition.

In the experiments here, LV epicardial AP remodeling was examined in a mouse model of decompensated LVH, produced by cardiac-restricted transgenic Gαq overexpression [6]. This model has previously been shown to recapitulate many molecular, functional, and structural alterations typically associated with pressure overload-induced pathological LVH [6–9]. We used confocal linescanning to optically monitor transmembrane action potentials from Langendorff-perfused hearts loaded with the membrane staining, voltage-sensitive dye ANNINE-6plus. Recordings of propagated APs from individual *in situ* cardiomyocytes distributed across the anterior LV epicardium demonstrated spatially non-uniform AP prolongation in transgenic hearts, giving rise to large intra-epicardial variability in repolarization. Local density and distribution of anti-connexin43 immune reactivity in Gαq hearts were unchanged compared to wild-type hearts. *In situ* confocal microscopy also revealed heterogeneous alterations of the transverse-axial tubular system (TATS) in LV epicardial cardiomyocytes, including patchy loss of tubules and growth of axial tubular elements. These results provide, to the best of our knowledge, the first direct experimental evidence of the existence of substantial APD heterogeneity within the same layer of the LV free wall in a rodent model of pathological LV hypertrophy. Because the increase in spatial APD dispersion was not associated with changes in cell-to-cell coupling, these data support the notion that a small region of electrically well connected ventricular myocardium can maintain spatial heterogeneity in APD through differences in intrinsic repolarization properties of cardiomyocytes. It remains to be seen whether the cardiac remodeling processes described here for the Gαq model similarly occur in the pressure overload LVH model, and/or other models of pathological LVH.

2. Materials and methods

2.1. Transgenic mice

Transgenic mice with cardiac myocyte-specific overexpression of Gαq (Gαq mice, 25-copy line, FVB/N, provided by G. W. Dorn II, Washington University, St. Louis) and wild-type (FVB/N) littermates were used. Our previous studies have demonstrated that adult Gαq-25 transgenic mice (>3 months of age) consistently develop progressive cardiomyopathy manifested as cardiac hypertrophy, LV chamber dilation, and systolic and diastolic

dysfunction [6–9]. For the current experiments, animals were studied at 5 to 7 months of age when cardiomyopathy should be present. Transgene presence was confirmed using polymerase chain reaction of genomic DNA isolated from tail tissue.

2.2. Optical action potential recording from Langendorff-perfused hearts

Heart preparation, dye loading, confocal imaging, and image processing were performed as previously described [10]. For imaging we used a laser scanning upright microscope (Zeiss LSM510 Meta; Carl Zeiss Thornwood, NY) with Ar-ion laser (488 nm). The heart was imaged through a Zeiss C-Apo 40× 1.2 NA water immersion lens equipped with an adjustable collar. The detection pinhole radius was set to 957 nm (3.8 Airy units), providing an axial (z) resolution of 0.82 μm and lateral (x – y) resolution of 0.25 μm , as measured at full width at half maximum of calculated point spread functions (Scientific Volume Imaging, Hilversum, Netherlands). To optically monitor cardiomyocyte APs, repetitive line-scans (1042 lines/s) were obtained from end-to-end membrane junctions between two adjacent *in situ* cardiomyocytes or across multiple T-tubular membranes in parallel to the long axes of individual *in situ* cardiomyocytes during continuous electrical pacing (3 Hz) at the lateral right ventricular base (see Supplemental Fig. 1A). Each line-scan image is composed of 10,000 lines, encompassing about 27 consecutive APs (Supplemental Fig. 1B). Line-scan images were obtained from within 225×225 μm^2 confocal image frames. These confocal image frames (which will be referred to as regions hereafter) were randomly distributed across the anterior left ventricular epicardium at midwall level. APs were recorded from cardiomyocytes located within the three outermost layers. By averaging all pixels in each line of a line-scan image in the vertical direction, the time course of the spatially averaged fluorescence was obtained. Fractional ANNINE-6plus fluorescence changes relative to baseline ($\Delta F/F_0$) were determined and normalized such that 0 represents the baseline fluorescence intensity and 1 represents the peak fluorescence intensity (see Supplemental Fig. 1C). Optical APs shown in this study reflect the ensemble average of all consecutive cardiac cycles in a line-scan image at 3 Hz steady-state pacing. Only recordings with S/N ratio exceeding 12 were included in the analysis. Average S/N ratios of the entire data sets were (mean±SD) 54.1±18.1 and 55.6±21.4 in wild-type and transgenic hearts, respectively. Comparisons of APDs recorded from end-to-end junctional membranes with those recorded from T-tubular membranes in each of the two flanking cardiomyocytes revealed no significant differences in hearts of either genotype (Supplemental Fig. 1D). We thus pooled APD values for the tubular and surface membranes for comparison between genotypes.

All images were acquired in the presence of 50 μM cytochalasin D and ryanodine. Exposure of hearts to cytochalasin D alone or in combination with blebbistatin, EGTA–AM, or BAPTA–AM resulted in only insufficient suppression of motion artifacts during confocal linescanning. Ryanodine at 1 μM concentration eliminated AP-related increases in free cytosolic calcium concentration within 30 min after onset of exposure in both wild-type and Gαq hearts (see Supplemental Data and Supplemental Fig. 2). Thus, a 30-min pre-incubation period with cytochalasin D and ryanodine preceded the AP measurements.

2.3. Intracellular microelectrode recordings of transmembrane action potentials

Glass pipettes filled with 3-M KCl solution (10–20 MΩ) were mounted on a micromanipulator and used to impale cardiomyocytes in the anterior midsection of the LV epicardium of Langendorff-perfused hearts. The electrical signal recorded with the microelectrode was amplified (Dagan Instruments, Minneapolis, MN), and the amplifier's signal output was fed to a 16-bit analog to digital converter (Molecular Devices, Sunnyvale, CA), sampled at 25 kHz, and analyzed using the Clampfit function of the pClamp software (Molecular Devices). All recordings were performed in the presence of 50 μM cytochalasin

D and 1 μM ryanodine in the perfusate. To enable direct comparisons with optical measurements, data acquisition was started after a 30-min pre-incubation period with both drugs.

2.4. Measurements of cardiomyocyte dimensions

To assess the degree of cardiomyocyte hypertrophy, we measured minimal Feret's diameters of individual *in situ* cardiomyocytes [11,12]. High-resolution (40 $\times$) confocal *XY* scans (50,625 μm^2) were acquired from ANNINE-6plus-loaded hearts. Cardiomyocytes could be readily identified by the presence of T-tubules. Boundaries of individual cardiomyocytes were outlined manually and minimal cardiomyocyte diameters were determined using ImageJ (NIH) software. Histograms were produced from data for each genotype to determine the distribution of cardiomyocyte minimal diameter.

To assess alterations in the T-tubular network, high-magnification *XY* images were obtained. Images of fluorescent subresolution spheres (Molecular Probes) were recorded under the same conditions to calculate the point spread function. Deconvolution of single frame images was performed with Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands).

2.5. Histology, immunofluorescence labeling and quantification of Cx43

Hearts were immersion-fixed for >24 h with a mixture of 1.08% cacodylic acid, 1% paraformaldehyde, and 0.67% NaCl and cryoprotected for 12 h with 30% sucrose in PBS. Epicardial tissue wedges ($\sim 2 \times 2 \times 1$ mm) were removed from the midsection of the anterior wall of the left ventricle, embedded in Tissue-Tek OCT compound (Triangle Biomedical Sciences, Durham, NC), frozen at -80°C , and cryosectioned at 10 μm parallel to the epicardium. Only sections located within a distance of 30 μm below the epicardial surface were used for immunofluorescence assays. Sections were stained for histology with Masson's trichrome. Adjacent sections were used for immunohistochemistry. Sections were stained with a polyclonal rabbit anti-Cx43 antibody (Millipore, Billerica, MA; 1:100) and a monoclonal mouse anti- α -actinin antibody (Sigma-Aldrich, St. Louis, MO; 1:200) overnight before the following secondary antibodies were applied for 2 h: fluorescein-conjugated goat anti-rabbit IgG (Millipore, 1:100) and Alexa 555-conjugated rat anti-mouse IgG (Life Technologies, Grand Island, NY; 1:200). The sections were mounted with antifade medium (Vectashield; Vector Laboratories, Burlingame, CA) and examined by scanning laser microscopy (Olympus FV1000) using a 20×0.95 NA water immersion objective. Images were comprised of 1024×1024 pixels ($634 \times 634 \mu\text{m}^2$) and were obtained by sequential illumination with 405-nm, 488-nm and 559-nm laser light while fluorescence was collected in the blue (425–475 nm), green (500–545 nm) and red (576–675 nm) range. Series of confocal sections were taken through the depth of the tissue sample at 0.5 μm axial steps. To improve signal-to-noise ratio, each image from the series was signal-averaged. Image stacks were obtained from 6 fields of view distributed among two hearts per genotype. To quantitate the amount of Cx43 expression with respect to α -actinin, we used ImageJ software. The red and green color images were thresholded using ImageJ's AutoThreshold function. The density of Cx43 was expressed as the percent of α -actinin positive label occupied by Cx43 positive label.

2.6. Statistical analyses

In all cases, data distribution was tested using Shapiro–Wilk's normality test. The *t*-test was used for comparison of normally distributed data. The Mann–Whitney's *U*-test, or where multiple groups were considered, Kruskal–Wallis test (followed by Dunn's *post-hoc* test), was used for comparison of not-normally distributed data. Values are presented as either mean $\pm$ SEM, or median with interquartile range. The degree of APD variability was

estimated by calculating all ($n!/(2*(n-2)!)$) pairwise absolute differences in APD, where n denotes the number of cardiomyocytes within a $225 \times 225 \mu\text{m}^2$ confocal image region (intra-regional variability) or the number of image regions within the same heart (inter-regional variability). SigmaPlot (Systat Software, Inc., San Jose, CA) was used for all statistical analyses.

3. Results

3.1. Structural remodeling of *in situ* Gαq cardiomyocytes

To assess minimal cardiomyocyte diameter and structure of the TATS of *in situ* cardiomyocytes, confocal *XY* scans were obtained from the midportion of the anterior LV epicardial layer of ANNINE-6plus-loaded hearts. Representative examples are shown in Fig. 1. *In situ* wild-type cardiomyocytes generally exhibited an elongated rectangular shape and a regular striated ANNINE-6plus staining pattern, reflecting T-tubular membranes (Fig. 1Aa). In contrast, confocal *XY* scans from Gαq hearts revealed heterogeneous alterations in cardiomyocyte geometry and TATS organization, as illustrated in panels Ab through Ad of Fig. 1. The images shown in panels Ab and Ac were taken from two randomly selected regions within the same Gαq heart. Cardiomyocytes shown in panel Ad and also some myocytes in panel Ac were markedly shorter and wider compared to their counterparts in panel Ab, and their TATS appeared to be more disorganized. To examine the type of TATS disorganization in more detail, we also acquired high-magnification *XY* scans from *in situ* cardiomyocytes. Representative images are presented in Fig. 1B. Wild-type cardiomyocytes (Figs. 1Ba and Bb) exhibited a regular striated ANNINE-6plus staining pattern, reflecting a predominately transverse orientation of the tubular membranes, although some longitudinal tubules were also observed (Fig. 1Bb). In contrast, pronounced spatial disorganization of the tubular network was observed in Gαq cardiomyocytes, characterized by loss of tubular membranes resulting in irregular gaps between adjacent T-tubules (Bc and Bd), a gain in the longitudinal elements giving rise to a mesh-like appearance of the TATS (Be), or loss of a striated pattern with disordered staining in both the longitudinal and transverse direction (Bf). Other Gαq cardiomyocytes retained a striated ANNINE-6plus staining pattern indistinguishable from that seen in wild-type cardiomyocytes (Fig. 1Bg), indicating that TATS remodeling evolved in a spatially non-uniform pattern in the LV epicardial layer of Gαq overexpressing hearts.

To assess the extent of cardiomyocyte hypertrophy, we measured minimal diameters of *in situ* cardiomyocyte cross sections in confocal *X-Y* images from hearts of either genotype. The results are summarized in the histograms shown in Fig. 1C. The distributions were non-gaussian with median values of 32.6 and 40.4 μm in wild-type and Gαq hearts, respectively ($P < 0.001$). Median values for minimal cardiomyocyte diameter were not significantly different among individual Gαq hearts ($P = 0.383$ by Kruskal–Wallis one way analysis of variance), suggesting similar degrees of cardiomyocyte hypertrophy.

3.2. Marked increase in spatial APD dispersion within the LV epicardial layer of Gαq hearts

Optical action potentials were recorded in line-scan mode from end-to-end junctional membranes or across multiple T-tubules (see Supplemental Fig. 1). Comparisons of APDs measured at the junctional membranes with those measured at the T-tubular membranes in cardiomyocytes on both sides of the junction showed no significant differences in hearts of either genotype (see Supplemental Fig. 1D). We thus pooled APD values for the tubular and surface membranes for comparison between genotypes. As shown in Table 1, both early and late repolarization was markedly delayed in Gαq cardiomyocytes compared to wild-type cells. Further, the interquartile ranges for each APD were larger for transgenic hearts than wild-type hearts, indicating increased APD variability in the Gαq group.

We next examined whether an increase in spatial dispersion of repolarization contributed to the increase in APD variability seen in *Gαq* hearts. We initially compared APs that were recorded from different cardiomyocytes within the same randomly selected $225 \times 225 \mu\text{m}^2$ regions. Representative examples are depicted in Fig. 2. High-magnification images from areas centered around the respective AP scan lines demonstrate orderly T-tubule arrangements in the wild-type myocytes, whereas *Gαq*-overexpressing cells exhibit spatially non-uniform T-tubular disorganization with patchy loss or growth of T-tubules (Figs. 2B, E and H). Shape and time course of repolarization appeared to exhibit only small variations among individual wild-type and *Gαq* cardiomyocytes located within the same region (Figs. 2C, F and I). To quantitatively compare the degree of intra-regional cell-to-cell variability in APD between wild-type and transgenic hearts, we calculated all pairwise absolute APD differences ($|\Delta\text{APD}|$) between individual *in situ* cardiomyocytes within each region. The results are summarized in Fig. 2J. For each time point of repolarization, the median values for $|\Delta\text{APD}|$ were small (~ 3 ms and ~ 6 ms in the wild-type and *Gαq* hearts, respectively), but were significantly larger in *Gαq* compared to wild-type hearts, suggesting a slight increase in microscopic, i.e. intra-regional, dispersion of repolarization within the anterior LV epicardial layer of *Gαq* hearts.

Because the increase in intra-regional APD heterogeneity was of small magnitude and thus could not entirely account for the larger variability in repolarization in the *Gαq* group, we next assessed the degree of APD dispersion on a macroscopic scale, i.e. between individual imaged regions. Fig. 3 shows end-to-end junctional and T-tubular membrane APs that were recorded from two separate $225 \times 225 \mu\text{m}^2$ imaged regions located at midwall level within the anterior LV epicardial layer of the same *Gαq* heart. Low-magnification *XY* scans (Figs. 3A and D) show different degrees of changes in cardiomyocyte dimensions, and high-magnification images (Figs. 3B and E) illustrate severe T-tubular remodeling characterized by pronounced increases in longitudinal elements. Periodic sharp bands of reduced ANNINE-6plus fluorescence intensity (arrows) reflect AP-related transient changes in dye emission at a stimulation rate of 3 Hz. Optical APs recorded from the regions demarked by green lines in Fig. 3A were markedly prolonged both at early and late instants of repolarization compared to those recorded from the second region shown in Fig. 3D (Figs. 3C and F). Dot plots shown in Fig. 4A depict the complete comparison of the APDs at 30, 50, and 70% repolarization from all $225 \times 225 \mu\text{m}^2$ regions grouped according to heart and genotype. It is apparent that there are larger inter-regional APD differences in *Gαq* *versus* wild-type ventricles. To quantitatively compare the degree of spatial APD dispersion between the wild-type and *Gαq* ventricles, we calculated all pairwise absolute differences between the regional APD medians for each heart. The results are summarized in the box and whisker plots shown in Fig. 4B. For all three time points of repolarization, the median values for $|\Delta\text{APD}|$ were ~ 3 - to ~ 9 -fold larger compared to those calculated for the wild-type group ($P < 0.001$).

To confirm our optical measurements, we next used standard microelectrode techniques to monitor transmembrane APs within small regions ($\sim 500 \mu\text{m}$ in diameter) that were randomly distributed across the anterior LV epicardial layer of Langendorff-perfused wild-type and *Gαq* hearts. Impalements were obtained from multiple cells within each region in the presence of $50 \mu\text{M}$ cytochalasin D and $1 \mu\text{M}$ ryanodine, enabling comparisons with the optical data. Regions were at least 2 mm apart from each other. Representative *in situ* transmembrane APs from three different regions each within the same wild-type and *Gαq* heart are illustrated in the Supplemental Fig. 3. AP properties are summarized in Table 2. Overall, electrical measurements revealed similar resting membrane potentials and peak AP amplitudes, but significantly smaller phase 0 maximal upstroke velocities and prolonged APD at all three instants of repolarization in *Gαq* *versus* wild-type hearts. Superimposition of normalized APs from each region suggested the presence of more pronounced APD

variability both intra- and inter-regionally in the Gαq heart compared to the wild-type heart (Figs. 5A and B). Dot plots shown in Fig. 5C depict the complete comparison of the APDs at 30, 50, and 70% repolarization from all regions grouped according to heart and genotype. To quantify the degree of APD variability, we used the same approach as outlined above for the optical recordings. The results are summarized in Figs. 5D and E. Both intra- and inter-regional APD heterogeneity were more pronounced in Gαq hearts, in complete agreement with the optical measurements. Also, the magnitudes of both intra- and inter-regional APD differences were very similar to those obtained optically (compare Figs. 2J and 5D for intra-regional, and Figs. 4B and 5E for inter-regional APD variability).

Because dispersion of repolarization has previously been reported to depend on pacing site [13], we examined whether varying the site of pacing across the lateral right ventricular base influenced spatial variability in APD under the experimental conditions employed. These experiments showed no pacing site-dependency of dispersion of repolarization in hearts of either genotype (data not shown).

Collectively, these results demonstrate that increases in both intra- and inter-regional variability in APD contributed to the more pronounced heterogeneity of repolarization within the outer epicardial layer of Gαq hearts compared to that seen in wild-type hearts.

3.3. Expression of Cx43 in LV epicardial sections

Gap junction-mediated electrotonic coupling is an important mechanism to attenuate repolarization heterogeneities between cardiomyocytes. Accordingly, we next examined Cx43 expression in the anterior LV epicardium. Fig. 6A represents typical examples of Cx43/α-actinin double immunofluorescence staining in epicardial sections from a wild-type and Gαq heart. Cx43 was predominately localized along end-to-end junctions in either genotype, although some Cx43 was found along side-to-side membrane junctions (arrows in Fig. 6B). The average Cx43 density was not statistically different between wild-type and Gαq overexpressing sections (Fig. 6C). Further, there was no histological evidence for increased interstitial collagen deposition in Gαq tissue (Fig. 6D).

4. Discussion

Our study revealed that remodeling of the LV epicardial layer of Gαq overexpressing hearts with decompensated hypertrophy results in heterogeneous alterations of TATS micro-architecture and spatially non-uniform AP prolongation in the LV epicardium, increasing APD dispersion on a microscopic and macroscopic scale. Density and distribution of anti-Cx43 immune reactivity in LV epicardial layers of Gαq hearts were unchanged compared to wild-type hearts, suggesting preservation of intercellular electrical coupling. These results support the notion that a small region of electrically well connected ventricular myocardium can sustain spatial heterogeneity in APD through differences in the intrinsic repolarization of cardiomyocytes.

4.1. Structural remodeling of in situ cardiomyocytes

Alterations in the structure of the TATS seen in the Gαq hearts, including the appearance of local gaps in the network, gain of longitudinal tubular elements, or loss of transverse tubular regularity, have been reported previously in single cardiomyocytes isolated from spontaneously hypertensive rats with heart failure [14] and in *in situ* epicardial cardiomyocytes from rat hearts exposed to chronic pressure overload [15]. Similar TATS restructuring has also been described for other animal models heart failure [16–21] and for failing human hearts [22]. The type and severity of TATS disorganization in our study were found to exhibit marked variability across the LV epicardial layer of individual hearts,

suggesting that the processes underlying TATS remodeling are not evenly distributed among cardiomyocytes from a given region of the left ventricle of and are not spatially coordinated in $G\alpha_q$ transgenic hearts.

In theory, TATS restructuring can contribute to electrical remodeling in $G\alpha_q$ hearts *via* several mechanisms. First, proliferation of longitudinal tubules and/or an increase in the depth of T-tubular invaginations due to increases in cardiomyocyte diameter can cause a mismatch between the number of T-tubular openings at the surface and the T-tubular volume, restricting the diffusional exchange between the fluid contained in the T-tubular lumen and the bulk extracellular fluid [23]. Accentuated ion depletion/accumulation within the deeper portions of the T-tubular network could then give rise to altered activities of ion channels and electrogenic ion transporters residing along the T-tubular membranes, modulating cardiomyocyte repolarization. Second, expression and/or distribution of ion handling proteins and/or their respective modulators within the TATS can change, giving rise to reorganization of protein function within the tubular network [24]. Third, the efficacy of ryanodine receptor-mediated Ca^{2+} release in regulating the activities of Ca^{2+} -sensitive ion conductances in the tubular membranes can be altered, due to misalignment of SR and tubular membranes [14]. If occurring in a spatially heterogeneous manner, TATS remodeling in $G\alpha_q$ hearts could then contribute indirectly, *via* differential modulation of Ca^{2+} -sensitive repolarizing currents, to alterations in epicardial dispersion of APD. Future studies will need to address these possibilities.

4.2. Electrical remodeling

AP prolongation is a well-recognized hallmark of cardiac hypertrophy [1,2]. In this study, in a transgenic model of decompensated cardiac hypertrophy we observed prolongation of APD through the outer epicardial layer of the anterior LV midwall. When compared to wild-type hearts, $G\alpha_q$ hearts also exhibited a more pronounced spatial heterogeneity of repolarization over most of the AP duration, which was evident in a significantly increased APD variability on a microscopic scale and, to a larger extent, on a macroscopic scale.

We found no significant differences in average APD measured at the surface and T-tubular membranes of *in situ* $G\alpha_q$ cardiomyocytes, suggesting tight electrical coupling between these two membrane compartments. We cannot, however, entirely discard the existence of subcellular repolarization gradients for two reasons. First, exclusion of data with signal-to-noise ratio <12 may have caused a selection bias against poorly stained membrane regions. Second, only small portions of the sarcolemma within individual cardiomyocytes were monitored for action potential measurement. Future use of confocal imaging systems equipped with high-rate frame acquisition mode in combination with further improved voltage sensors may resolve subcellular gradients in transmembrane potential.

Our optical and electrical measurements concordantly demonstrate the existence of small APD gradients within the anterior LV epicardium of wild-type hearts. Because APD values were obtained by averaging 20–27 consecutive cardiac cycles, a major contribution of beat-to-beat variability in APD to dispersion of repolarization is unlikely. Previous optical voltage mapping studies in Langendorff-perfused wild-type mouse hearts similarly revealed APD heterogeneity along the anterior LV epicardium, with the average APD_{75} being 10 ms and 5 ms shorter at the apex than at the base in the absence and presence of cytochalasin D, respectively [25]. Our values for $|\Delta APD_{70}|$ in wild-type mouse hearts are well within this range. Experimental data have also demonstrated small regional differences in early stages of repolarization in wild-type mouse hearts [26], in agreement with our observation of milliseconds-long differences for APD_{30} and APD_{50} across the LV epicardial layer.

Cardiac-restricted Gα_q overexpression heterogeneously prolonged APD within as well as between microscopic regions of the LV epicardial layer compared to wild-type hearts (Figs. 2J, 4B, 5D and E). Marked increases in spatial APD dispersion has been previously observed in mouse hearts lacking the slow component of the transient outward current, $I_{to,s}$, and in hearts lacking both the slow and fast component of I_{to} , with average apex-to-base APD₇₅ differences being 16 ms and 14 ms, respectively [27]. Intriguingly, $I_{to,s}$ ablation caused a more pronounced APD prolongation in the center of the anterior LV epicardium compared with the contiguous epicardium. These results suggest that augmented spatial variability in the activity of sarcolemmal ion currents that control cardiomyocyte repolarization underlies the increase in APD heterogeneity seen in the epicardial layer of Gα_q hearts.

In addition to the possible electrical consequences of TATS restructuring mentioned above, additional ionic mechanisms responsible for local inhomogeneities of AP prolongation include increased cell-to-cell variability in the densities of I_{to} and/or the inwardly rectifying K^+ current, I_{K1} , based on previous investigations in ventricular cardiomyocytes isolated from Gα_q hearts [28,29].

Electrotonic interactions through intercellular coupling are thought to markedly attenuate manifestation of APD heterogeneity in cardiac tissue, as compared to isolated cells [13,30,31]. In this study, we found that both density and distribution of anti-Cx43 immune reactivity were unchanged in epicardial sections from Gα_q hearts compared to wild type heart. Moreover, there was no evidence for fibrosis-induced disruption of cell-to-cell connectivity in the epicardium of Gα_q hearts. These data support the notion that small portions of electrically well connected cardiac tissue can maintain significant APD gradients, most likely through heterogeneity in the intrinsic repolarization properties of individual cardiomyocytes. This interpretation is also consistent with a previous finding demonstrating regional APD differences in hypertrophied rat cardiac muscle in the absence of significant changes in the profile of spatial electrotonic voltage spread [32]. Moreover, Efimov's group recently demonstrated in non-failing human hearts the presence of midmyocardial islands of cardiomyocytes with distinctly long APD₈₀ and steep APD₈₀ gradient (27 ms/mm) compared with the neighboring myocardium without evidence for changes in local Cx43 density [33]. Collectively, our data and those by others strongly support the concept that heterogeneity in activities of repolarizing currents alone, in the absence of significant changes in intercellular coupling, can produce pronounced spatial heterogeneity in APD in short distances. We cannot, however, entirely discard the possibility that increased intercellular resistivity due to changes in the phosphorylation state of Cx43 proteins, as previously reported for the aortic constriction rat heart failure model [34], contributed to manifestation of epicardial APD heterogeneity in our experiments.

We and others have demonstrated that cardiac-restricted Gα_q overexpression recapitulates many molecular, functional and structural alterations typically associated with pressure overload-induced pathological LVH [6–9]. However, it will be important to determine whether alterations in epicardial dispersion of repolarization observed in the present study similarly occur in response to chronically augmented LV afterload. Intriguingly, Gomez and co-workers reported an increase in intraregional APD variability in LV cardiomyocytes isolated from adult rat hearts after aortic banding [5]. Further, it was previously demonstrated that accumulation of mRNAs encoding fetal isoforms of contractile proteins is neither spatially nor temporally synchronized among myocytes from a given region of the LV after transaortic constriction in the adult rat [35]. These results suggest that the response to and/or the effects of hemodynamic overload are non-evenly distributed within distinct LV cell layers and further suggest the appropriateness of the mouse Gα_q model for the study of

electrical remodeling associated with LVH. The mechanism linking pressure overload to heterogeneous action potential prolongation has yet to be identified.

Previous studies have demonstrated marked differences in the effect of hypertrophy on epicardial *versus* endocardial K^+ currents, which were thought to reflect transmural gradients in wall tension [1,3,4]. It remains to be determined whether and to what extent changes in APD seen in the LV epicardium of hearts with LVH apply to deeper layers of the LV wall, and whether mechanical factors (e.g. regional variations in strain) play a role in the remodeling process [36]. The combined use of imaging techniques with improved penetration depth (e.g. two-photon fluorescence microscopy [37]) and speckle-tracking based strain imaging [38] may provide important insights into this matter.

4.3. Implications

Dispersion of repolarization is a known arrhythmogenic factor. Given that dispersion of refractoriness parallels dispersion of repolarization, spatial heterogeneity of APD may give rise to highly non-uniform recovery of excitability, facilitating local conduction slowing and/or block during premature beats or rapid activation. Ensuing wavebreaks would then favor local reentry phenomena, initiating and/or maintaining arrhythmias. Our findings suggest that characterization of arrhythmia mechanisms associated with cardiac hypertrophy should incorporate microscopic aspects of repolarization and conduction.

Recent computer simulations have raised questions about the utility of the mouse heart for studying mechanisms of arrhythmias in humans, particularly for understanding the consequences of spatial heterogeneity of repolarization [39]. Our data support the notion that the mouse heart is capable of sustaining substantial heterogeneity of repolarization within small regions of the electrically well connected mouse myocardium, in accordance with previous experimental results by others [40], and thus is suitable for the study of arrhythmia mechanisms involving spatial heterogeneity of repolarization. Consistent with this interpretation, increased or reduced dispersion of repolarization, resulting from altered expression of cardiac K^+ currents, has previously been shown to promote and prevent, respectively, arrhythmias in mouse hearts [27]. Importantly, it appears likely that this connection between arrhythmia susceptibility and variability in repolarization also applies to large mammals, including humans [41].

Electrophysiological consequences of hypertrophy are typically assessed in a limited number of cardiomyocytes isolated from defined anatomic regions of the heart. While this method is informative and well established, the enzymatic isolation procedure can lead to cardiomyocyte damage with unforeseeable electrophysiological consequences and selectively isolate a subpopulation of cells. The approach presented here allows fast screening of both electrical and morphological features of a large number of cardiomyocytes while still in their natural habitat, complementing enzymatic isolation.

4.4. Limitations

The experiments here utilized cytochalasin D and ryanodine to suppress motion during imaging. Optical voltage mapping studies in Langendorff-perfused wild-type mouse hearts demonstrated a slight reduction in the apex-to-base APD gradient in the presence of cytochalasin D [25], suggesting that the accentuation in epicardial APD variability seen in $G\alpha_q$ hearts is unlikely to result from electrophysiological effects of the drug.

As discussed above, TATS restructuring could result in misalignment of tubular and SR membranes, altering the efficacy of ryanodine receptor-mediated Ca^{2+} release in regulating the activities of sarcolemmal, Ca^{2+} -sensitive ion conductances and/or their respective modulators. Consequently, spatial variability in type and/or extent of T-tubular remodeling

would be expected to heterogeneously modulate Ca^{2+} -sensitive currents, causing local variations in APD. Thus, pharmacological disruption of ryanodine receptor-mediated Ca^{2+} signaling to Ca^{2+} -sensitive effector proteins in the tubular membranes could obscure the contribution of heterogeneous alterations in Ca^{2+} -regulated currents to alterations in spatial dispersion of repolarization that we observed in Ga_q hearts. Future studies will need to address the impact of TATS restructuring and concomitant changes in Ca^{2+} signaling on electrical remodeling in hypertrophic hearts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Structural remodeling of *in situ* Gαq cardiomyocytes. A: confocal XY scans from ANNINE-6plus-loaded hearts. Thick lines of high fluorescence intensity (arrows) represent the outer sarcolemma of cardiomyocytes, whereas periodic lines of lower fluorescence intensity running perpendicular to the outer membrane correspond to transverse tubular membranes. Right images in each panel show zoom-in views of areas in the corresponding left images. Images in panels b and c are from the same Gαq heart. Scale bar, 20 μm. B: high-magnification confocal cross-sections from T-tubular membranes of *in situ* cardiomyocytes. Scale bars, 5 μm. C: distributions of minimal diameter of *in situ* cardiomyocyte cross-sections. Values were calculated from 186 wild type and 124 Gαq cardiomyocytes distributed among 7 hearts per genotype.

Fig. 2.

Small increases in intra-regional APD variability within the LV epicardial layer of Gαq hearts. A, D and G: full-frame mode images obtained from a wild-type and two Gαq hearts loaded with ANNINE-6plus. Numbered white lines denote positions of line-scan mode data acquisitions for AP recording. B, E and H: high-zoom XY scans of areas centered in the correspondingly numbered line-scan positions in A, D and G. Each image is composed of a square-shaped region with side length equal to the length of the respective AP scan line, which is bisecting the high-magnification image horizontally (green lines). White scale bars, 5 μm. C, F and I: overlays of normalized optical APs recorded along the green lines in B, E and H. Numbers above the traces refer to the numbers of the scan lines in B, E and H. Stimulation rate was 3 Hz. Lower traces show overlays of 200-ms repolarization segments starting from the peak. Traces were filtered using moving average. J: box and whisker plots of absolute APD differences ($|\Delta\text{APD}|$) between individual cardiomyocytes within the same 225×225 μm² acquired image frames. Values were calculated from 290 and 185 measurements in 7 wild-type and 7 Gαq hearts, respectively. * P 0.001 versus wild-type by Mann–Whitney’s rank sum test.

Fig. 3.

Heterogeneity of TATS morphology and repolarization across the anterior LV epicardium of $G\alpha_q$ hearts. A and D: confocal cross-sections taken from two different regions within the same ANNINE-6plus-loaded $G\alpha_q$ heart. Green lines denote positions of line-scan mode data acquisitions for AP recording. Green E and T denote end-to-end and tubular membranes, respectively. Red scale bar, 20 μm . B and E: high-zoom XY scans of epicardial areas centered in the correspondingly numbered line-scan positions in A and D. Each image encompasses a square-shaped region with side length equal to the length of the respective AP scan line, which is bisecting the high-magnification image (green lines). Periodic sharp decreases in ANNINE-6plus fluorescence intensity (arrows) result from AP-induced transient reductions in dye emission. Red scale bars, 5 μm . C and F, Tracings of normalized optical APs that were recorded along the green scan lines in the corresponding images in B and E. Stimulation rate was 3 Hz.

Fig. 4.

Enhanced inter-regional APD dispersion across the anterior LV epicardial layer of $G\alpha q$ hearts. A: dot plots of APD₃₀, APD₅₀, and APD₇₀ in wild-type and $G\alpha q$ hearts. Each dot represents the median of 4 to 8 AP measurements within a single $225 \times 225 \mu\text{m}^2$ image location. Vertical bars denote inter-quartile range. Numbers on the x -axis mark individual hearts. B: box and whisker plots of absolute APD differences ($|\Delta APD|$) between epicardial regions within the same hearts. For each heart, all $(n!/(2*(n-2)!))$ pairwise differences of regional APD medians were calculated, where n denotes the number of randomly selected $225 \times 225 \mu\text{m}^2$ image frames per heart from which AP measurements were obtained. Values were calculated from 19 and 22 measurements distributed among 7 wild-type and 5 MHC- $G\alpha q$ hearts, respectively. * $P < 0.001$ versus wild-type by Mann-Whitney's rank sum test.

Fig. 5. Spatial dispersion of electrically measured APD in the LV epicardial layer. A and B: representative transmembrane APs recorded with intracellular microelectrodes from different locations across the anterior LV epicardial layer of Langendorff-perfused mouse hearts during steady-state stimulation at 3 Hz. Recordings were obtained from the same wild-type and Gαq heart, respectively, in the presence of 50 μM cytochalasin D and 1 μM ryanodine. Each tracing represents the ensemble average of 25 consecutive APs. In each panel, APs recorded from different cells within the same microscopic region (~500 μm in diameter) were normalized to their respective peaks, color encoded, and superimposed. The different panels show results from individual regions that were at least 2 mm apart from each other. C: dot plots of electrically measured APD₃₀, APD₅₀, and APD₇₀ in 2 wild-type and 2 Gαq hearts. Each dot represents the median of 5 to 6 measurements within a microscopic region. Vertical bars denote inter-quartile range and numbers on the x-axis indicate individual hearts. D: absolute APD differences (|ΔAPD|) between individual cardiomyocytes within the same microscopic location. Values were calculated from 117 and 148 measurements distributed among 2 wild-type and 2 Gαq hearts, respectively. * $P < 0.005$ versus wild-type by Mann–Whitney’s rank sum test. E: absolute APD differences (|ΔAPD|) between epicardial regions within the same hearts. Values were calculated from 11 and 20 measurements distributed among 2 wild-type and 2 Gαq hearts, respectively. * $P < 0.001$ versus wild-type by Mann–Whitney’s rank sum test.

Fig. 6. Similar distribution and density of anti-connexin43 immune reactivity in wild-type and $G\alpha q$ hearts. A and B: representative confocal images from the anterior LV epicardium taken from a wild-type and a $G\alpha q$ heart. Green, Cx43; red, α -actinin; blue, nuclei. Scale bar, 50 μm . B: magnified views of the boxed regions in A. Scale bar, 20 μm . C: average Cx43 densities in the subepicardium. Six randomly selected fields of view ($634 \times 634 \mu m^2$) distributed among two hearts per genotype were analyzed. For each image, the ratio of Cx43 to α -actinin was calculated. $P=n.s.$ D: trichrome staining of sections from the subepicardium taken from a wild-type and a $G\alpha q$ heart. Arrow denotes collagen (blue signal). Scale bars, 50 μm .

Table 1

Action potential durations of *in situ* left ventricular cardiomyocytes from wild-type and Gαq hearts.

Genotype	APD ₃₀ , ms	APD ₅₀ , ms	APD ₇₀ , ms
<i>Wild-type</i> N=7	5.8	10.6	25.0
n=191	(4.8, 7.7)	(8.6, 13.4)	(19.2, 31.7)
<i>Gαq</i> N=7	9.6 *	22.1 *	66.2 *
n=167	(6.7, 15.4)	(12.5, 33.6)	(47.0, 86.4)

Values are medians. Numbers in parentheses indicate interquartile ranges. N = number of hearts; n = number of action potential recordings.

* $P < 0.001$ versus wild-type by Mann–Whitney's rank sum test.

Table 2

Properties of electrically measured transmembrane action potentials of *in situ* left ventricular cardiomyocytes from wild-type and *Gαq* hearts.

Genotype	RMP, mV	APA, mV	dV/dt _{max} , V/s	APD ₃₀ , ms	APD ₅₀ , ms	APD ₇₀ , ms
<i>Wild-type</i>						
N=2	-77.1	79.5	57	3.2	6.2	14.4
n=45	(-79.2, -75.3)	(75.6, 84.1)	(47, 71)	(2.7, 3.8)	(5.0, 7.1)	(9.5, 18.1)
<i>Gαq</i>						
N=2	-76.0	68.7 *	25 *	7.8 *	16.9 *	92.1 *
n=61	(-78.1, -74.2)	(62.3, 75.0)	(22, 36)	(5.8, 11.9)	(10.9, 27.2)	(55.8, 112.8)

Values are medians. Numbers in parentheses indicate interquartile ranges. N = number of hearts; n = number of action potential recordings. RMP = resting membrane potential; APA, phase 0 action potential amplitude; dV/dt_{max}=maximal phase 0 upstroke velocity.

* $P < 0.001$ versus wild-type by Mann-Whitney's rank sum test.