Variable t-tubule organization and Ca^{2+} homeostasis across the atria

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Submitted 2 May 2014; accepted in final form 15 June 2014

Frisk M, Koivumäki JT, Norseng PA, Maleckar MM, Sejersted OM, Louch WE. Variable t-tubule organization and Ca^{2+} homeostasis across the atria. Am J Physiol Heart Circ Physiol 307: H609–H620, 2014. First published June 20, 2014; doi:10.1152/ajpheart.00295.2014.—Although t-tubules have traditionally been thought to be absent in atrial cardiomyocytes, recent studies have suggested that t-tubules exist in the atria of large mammals. However, it is unclear whether regional differences in t-tubule organization exist that define cardiomyocyte function across the atria. We sought to investigate regional t-tubule density in pig and rat atria and the consequences for cardiomyocyte Ca^{2+} homeostasis. We observed t-tubules in approximately one-third of rat atrial cardiomyocytes, in both tissue cryosections and isolated cardiomyocytes. In a minority (~10%) of atrial cardiomyocytes, the t-tubular network was well organized, with a transverse structure resembling that of ventricular cardiomyocytes. In both rat and pig atrial tissue, we observed higher t-tubule density in the epicardium than in the endocardium. Consistent with high variability in the distribution of t-tubules and Ca^{2+} channels among cells, L-type Ca^{2+} current amplitude was also highly variable and steeply dependent on capacitance and t-tubule density. Accordingly, Ca^{2+} transients showed great variability in Ca^{2+} release synchrony. Simultaneous imaging of the cell membrane and Ca^{2+} transients confirmed t-tubule functionality. Results from mathematical modeling indicated that a transmural gradient in t-tubule organization and Ca^{2+} release kinetics supports synchronization of contraction across the atrial wall and may underlie transmural differences in the refractory period. In conclusion, our results indicate that t-tubule density is highly variable across the atria. We propose that higher t-tubule density in cells localized in the epicardium may promote synchronization of contraction across the atrial wall.

excitation-contraction coupling; confocal microscopy; three-dimensional reconstruction; Ca^{2+} handling; membrane structure

IN CARDIAC MYOCYTES, contraction is elicited by a transient rise in cytosolic Ca^{2+} concentration during the action potential. This Ca^{2+} transient is triggered by Ca^{2+} influx through L-type Ca^{2+} channels, which, in turn, triggers Ca^{2+} release from ryanodine receptors (RyRs) in the sarcoplasmic reticulum, a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (3). In ventricular cardiomyocytes, efficient CICR is enabled by the presence of t-tubules. This tortuous network of deep invaginations of the sarcomemal membrane places Ca^{2+} channels in close proximity to RyRs at functional elements called dyads (27). A dense, well-organized t-tubule network thus ensures synchronous and rapid Ca^{2+} release across the cell and powerful contraction of the heart (27). Experimental detubulation by osmotic shock results in “U-shaped” Ca^{2+} transients when visualized by confocal linescans, as Ca^{2+} is first released at the sarcolemma and then propagates into the cell interior as a wave of CICR (6). Furthermore, loss and/or disorganization of t-tubules during heart failure results in “orphaned” RyRs, which are activated only after diffusion of Ca^{2+} from intact dyads (16, 25, 26, 38). The resulting slow, dysynchronous Ca^{2+} transients have been linked to slow cardiomyocyte contraction and reduced strength of the heartbeat in this condition (27).

While an important role of t-tubules in ventricular cardiomyocytes is well established, their role in atrial cells is less clear. Indeed, t-tubules were traditionally believed to be absent from atrial myocytes (4, 7, 17, 42), and U-shaped Ca^{2+} transients have often been reported (21, 29). However, recent studies have contradicted this view, as larger mammals, including humans, have been demonstrated to exhibit an extensive t-tubular network in atrial cardiomyocytes (11, 23, 34). Moreover, these t-tubules have been shown to participate in CICR and contribute to the Ca^{2+} transient (11, 23). While the discrepancy between these results and earlier work remains unexplained, it may result from differences in the regions of the atria examined if there are gradients in t-tubule density. We therefore presently sought to conduct detailed mapping of t-tubule organization and functionality across the atria. In pig atrial myocytes, we observed regional variation in t-tubule configuration in both atria, with t-tubule density notably higher in the epicardium than in the endocardium. A similar distribution was observed in rat atrial cells, despite the widely held belief that t-tubules are sparse in the atria of small rodents. The resulting improved synchrony of Ca^{2+} release in the epicardium was demonstrated to aid synchronization of contraction across the atrial wall.

METHODS

Ethical approval and tissue preparation. This study was approved by the Norwegian National Committee for Animal Welfare under the Norwegian Animal Welfare Act and conforming with Directive 2010/63/EU of the European Parliament. Experimental protocols were approved by the Ethics Committee of the University of Oslo.

Pigs (Sus scrofa) aged 12–16 wk were premedicated with ketamine (30 mg/kg) intramuscularly and anesthetized by propofol (bolus 5 mg/kg and 15 mg·kg^{-1}·h^{-1} iv) and fentanyl (bolus 10 μg/kg and 35 μg·kg^{-1}·h^{-1} iv). Anesthetized pigs were euthanized by a KCl overdose. The right and left atria were immediately excised and frozen in liquid nitrogen.

Wistar rats (10 wk old) were anesthetized (2% isoflurane and 98% O_{2}) and euthanized by cervical dislocation. Hearts were then excised and placed in cool, oxygenated buffer solution containing (in mmol/l) 130 NaCl, 25 HEPES, 5.4 KCl, 0.5 MgCl_{2}, 0.4 NaH_{2}PO_{4}, and 5.5 D-glucose (pH 7.4). These hearts were then either mounted on a
Langendorff setup for cell isolation (described below) or the atria were removed and embedded in Tissue-Tek OCT compound, with subsequent freezing in liquid nitrogen-cooled isopentane. Both pig and rat tissue were stored at −80°C until use.

Cryosections. Using a cryostat, pig and rat atrial tissue was sliced into 20-μm-thick sections at −20°C, collected on Poly-prep Slides (Sigma-Aldrich), and fixed in 4% paraformaldehyde for 30 min at room temperature. As shown in Fig. 1, A and B, four sections were excised from the porcine atria at regular intervals across the main chamber and one section was excised from the appendage. Due to the smaller size of the rat atria, imaging of the complete atria was possible by assembling low-magnification images (Fig. 2A). Endocardial and epicardial layers were defined as the innermost and outermost 300 μm of tissue, respectively.

In porcine atrial tissue, t-tubules were stained with wheat germ agglutinin (WGA) conjugated to Alexa fluor 488 (Molecular Probes, Eugene, OR) prepared in PBS containing (in mmol/l) 137 NaCl, 2.7 KCl, 10 Na2HPO4, and 2 KH2PO4 at 20 μg WGA/ml PBS for 30 min (34). We found that WGA was unsuitable for staining t-tubules in rat atrial tissue and instead stained for dystrophin, as this protein has previously been shown to be an effective t-tubule label (14, 20, 22). Sections were stained with dystrophin polyclonal antibody (catalog no. PA1–21011, Affinity BioReagents, 1:100 PBS with 1% BSA) for 1 h at room temperature followed by secondary antibody conjugated to Alexa fluor 480 (1:200 PBS with 0.5% BSA) for an additional 1 h at room temperature. T-tubules and cellular membranes were visualized using a confocal scanning system (LSM 710, Zeiss, Jena, Germany). Images collected at ×60 magnification were deconvolved.
using Huygens Essential software (Scientific Volume Imaging, Hilversum, The Netherlands), and, for each cell, t-tubule density \( t\)-index \((15)\) was determined by thresholding image intensity of the entire cell. To avoid bias, this threshold value was determined by the Otsu method \((32)\) using an automated algorithm in ImageJ (National Institutes of Health). The \( t\)-index was then calculated for the interior of the cell, which was defined as the above-threshold area divided by the cross-sectional area. Only transversely oriented cells were included in these analyses, and unspecific staining of the nucleus was excluded from tubule density measurements. Cells exhibiting a \( t\)-index of \( \geq 2 \) were defined as being tubulated, which roughly corresponds to the level detectable by eye. In some cells, the shortest distance from each point in the cell to the nearest t-tubule or cellular membrane was calculated from binarized images to create “distance maps” using custom-made software. This was accomplished by analyzing two-dimensional images taken at the center of the vertical center of \( z\)-stacks with the “nearestNeighbor” function in MatLab (The Mathworks, Natick, MA), which uses Delaunay triangulation to locate the suprathreshold point closest to a given location. The results were then plotted in image files where distances were indicated by a color scale. In addition, t-tubule organization was assessed by fast Fourier transform (FFT) analyses in MatLab, and the mean power spectrum was plotted as a function of spatial frequency in the longitudinal direction of the cell. The amplitude of the peaks in the power spectrum reflects the uniformity of transverse staining at the \( z\)-lines, whereas the power between peaks represents the amount of staining at the level of the A-band \((39)\).

**Cardiomyocyte isolation.** Freshly excised rat hearts were mounted on a Langendorff setup and perfused retrogradely through the aorta with the buffer solution described above \((37^\circ C)\) containing 200 U/ml collagenase type II (Worthington Biochemical, Lakewood, NJ) and 0.04 mM Ca\(^{2+}\). After 20 min of perfusion, the right and left atria were carefully excised to exclude ventricular tissue and stored in cold buffer for further digestion. The left ventricle was cut down, minced,
and then gently tritured with a cutoff Pasteur pipette for ~1 min in collagenase-free buffer containing 1% BSA and 0.02 U/ml deoxyribonuclease I (Worthington Biochemical). The cell-containing solution was then filtered through 200-μm nylon mesh. After sedimentation, cells were washed three times in buffer solution with 0.5% BSA, and the Ca²⁺ concentration was increased progressively (0.1, 0.2, and 0.5 mM). After Langendorff perfusion, atria were diced into chunks (~1 mm³) and mechanically agitated for 25 min in a solution containing 

and the Ca²⁺ was replaced with a similar solution but without deoxyribonuclease, in a centrifuge (Labfuge 400, Heraeus Instruments, Frankfurt, Germany) at 900 rpm for 5 min. After samples had been spun, the buffer was replaced with a similar solution but without deoxyribonuclease, and the Ca²⁺ concentration was increased as for ventricular tissue. The right and left atria were isolated separately. Isolated ventricular and atrial cells were stored at room temperature until use. 

Patch-clamp experiments. For patch-clamp experiments, cells were plated on laminin-covered coverslips, mounted on the stage of an inverted microscope, and superperfused with buffer solution containing (in mmol/l) 20 CsCl, 1 MgCl₂, 135 NaCl, 10 HEPES, 10 d-glucose, 4 4-aminopyridine, and 1 CaCl₂ (pH 7.4) at 37°C. L-type Ca²⁺ currents (I_L) were examined in whole cell voltage-clamp configuration using glass pipettes with a resistance of 1–2 MΩ filled with a solution containing (in mmol/l) 133 CsCl, 0.33 MgCl₂, 4 ATP Mg salt, 0.06 EGTA, 10 HEPES, and 20 tetraethylammonium chloride (pH 7.2). Discontinuous voltage-clamp experiments were performed using an Axoclamp 2B amplifier (Axon Instruments, Inverness, Scotland) with a switching frequency of 8–10 Hz and recorded by

pCLAMP software (Axon Instruments). I_L was elicited by a train of 10 conditioning pulses (50 ms) from −70 to 0 mV followed by 210-ms depolarizing voltage steps from −40 to +50 mV in 10-mV increments. Cell capacitance was estimated by integrating the capacitive current elicited by a 10-mV hyperpolarizing step from a holding potential of −70 mV. Myocyte volume was derived from the central plane of the imaged cell and calculated as follows: volume = (π × cell length × width × depth)/4, where the cell depth was assumed to be one-third of the width (5).

Immunolabeling. Immunolabeling was performed as previously described by Swift et al. (39). In brief, isolated cells were plated in laminin-coated wells and allowed to precipitate for 1 h before fixation in 4% paraformaldehyde. After 10 min of being quenched in 100 mmol/l glycine, cells were permeabilized for 10 min in 0.03% Triton X-100 and blocked for 2 h in high-blocking solution containing 150 mmol/l NaCl, 17.5 mmol/l Na₃citrate, 0.02 mmol/l Na₂PO₄, 5% goat serum, and 3% BSA. Cells were incubated overnight at 20°C with primary antibody (anti-Ca₁.2, catalog no. ACC-003, Alomone Labs, and anti-RyR, catalog no. MAI-83782, Thermo Scientific) in low-blocking solution containing 150 mmol/l NaCl, 17.5 mmol/l Na₃citrate, 0.02 mmol/l Na₂PO₄, 2% goat serum, and 1% BSA followed by 2-h incubation with secondary antibody (Alexa 488- and Alexa 543-conjugated IgG, 1:200, Molecular Probes). Labeled cardiomyocytes were scanned by confocal microscopy, and collected images were deconvolved and analyzed by FFTs, as described above for t-tubule images.

Ca²⁺ transients. In some experiments, Ca²⁺ transients were recorded in cardiomyocytes loaded with 20 μM fluo-4 AM (Molecular Probes) for 10 min before being mounted in the perfusion chamber. Cells were superfused with HEPES-Tyrode solution containing (in mmol/l) 140 NaCl, 1 CaCl₂, 0.5 MgCl₂, 5.0 HEPES, 5.5 glucose, 0.4 NaH₂PO₄, and 5.4 KCl (pH 7.4, 37°C) and were field stimulated at 1 Hz through two platinum electrodes. Ca²⁺ transients were imaged by a 7. Live confocal scanning system (Zeiss) in line-scan mode with the 1.024-pixel scan line placed along the longitudinal axis of the cell, avoiding nuclei (pixel width: 0.32 μm). Cells were only examined if it was possible to focus on the entire length of the cell in a single plane. Myocytes were scanned every 1.5 ms, and sequential scans were downloaded to create two-dimensional images with time in the x-axis. Dyssynchrony of Ca²⁺ transients (dyssynchrony index) was subsequently quantified as previously described (26). In brief, using ImageJ, Ca²⁺ transients were thresholded to half-maximal fluorescence (F₅₀) values and outlined. The dyssynchrony index was then calculated as the SD of the time to F₅₀. In some experiments, after the measurement of Ca²⁺ transients, cardiomyocytes were subsequently stained with 10 μM RH-237 (Invitrogen) for 5 min to investigate the relationship between Ca²⁺ release synchrony and t-tubule structure. RH-237 was excited at 543 nm, and light emission was collected above 600 nm.

Mathematical modeling. Functional consequences of transmural gradients in t-tubule density and Ca²⁺ homeostasis were examined by simulating isometric tension development. Representative recordings of Ca²⁺ transients were calibrated to Ca²⁺ concentration as previously described by Cheng et al. (9), assuming a resting Ca²⁺ concentration of 100 nM, and incorporated in a well-established model of cooperative activation and cross-bridge cycling in cardiac muscle, as previously described by Rice et al. (33). The value of the species-dependent cross-bridge cycling rate parameter (κm) was set to 4/3, based on the assumption that the rate is slightly faster in the atria compared with the ventricles (κm ≈ 5/3, 2/3).

Statistics. Statistical difference was tested with t-tests or, when applicable, ANOVA followed by the Bonferroni multiple-comparison procedures using Sigmaplot software (Systat Software, Chicago, IL). P values of ≤0.05 were considered statistically significant. All data are presented as means ± SE.

RESULTS

T-tubule organization in the intact atria. T-tubule density was systematically examined across intact right and left pig atria with confocal imaging of WGA-stained cryosections from predefined locations, as shown in Fig. 1, A and B, left. Representative images are shown for cryosections from the epicardium (Fig. 1, A and B, middle) and endocardium (Fig. 1, A and B, right) in both transverse and longitudinal orientations. In total, 34% of pig atrial cells exhibited a detectable t-tubular network, and the proportion of right atrial cells with t-tubules exceeded the proportion of tubulated left atrial cells (40.7% vs. 28.5%, P < 0.05). Cardiomyocytes from the right atrium were also observed to be larger than their counterparts in the left atrium (Fig. 1C) and to exhibit a more extensive t-tubule network (Fig. 1D). Interestingly, compared with cells located on the endocardial side of the atrial myocardiun, epicardial cells were 14% larger (cross-sectional area: 177 ± 4 vs. 153 ± 3 μm², P < 0.05; Fig. 1C) and had 45% more t-tubules overall.
(t-index: 1.72 ± 0.06% vs. 1.18 ± 0.05%, P < 0.05). This transmural gradient in t-tubule density held true in both right and left atria of all hearts investigated (Fig. 1, C and D) and when data were separately analyzed from the main chamber and atrial appendage (data not shown).

A more extensive t-tubule network would be expected to allow the propagating action potential to reach more regions of the cell interior. Calculated distances from all points across the cell to the nearest t-tubule or surface membrane were used to create colored distance maps, as shown in Fig. 1, A and B. Distances to the nearest membrane were lower in the pig epicardium compared with the pig endocardium (mean: 1.04 ± 0.04 vs. 1.21 ± 0.04 μm, P < 0.05), indicating that the higher t-tubule density in these cells compensated for their larger size (Fig. 1, E and F).

Similar analyses of t-tubule density and distance maps were performed on cryosections from the rat atria. Due to the small size of rat atria, imaging of complete, dystrophin-stained atria was possible by assembling a number of confocal images taken at low (×10) magnification (Fig. 2A). The red boxes in Fig. 2A show areas where regions were imaged at higher magnification (×60), as shown in Fig. 2A, a–h (both transversely and longitudinally oriented, with distance maps shown on the right). We observed significant t-tubule staining in 30% of right atrial cells and 32% of left atrial cells (defined as t-index > 2; see METHODS). However, there were marked differences in cardiomyocyte size and t-tubule density between these chambers, which were opposite to those observed in the pig. Cardiomyocyte cross-sectional area and t-tubule density were 33% and 12% higher, respectively, in the left rat atrium than in the right atrium. T-tubule density was also lower in the atrial appendage compared with the main chamber (1.12 ± 0.01% and 1.33 ± 0.04%, P < 0.05). As in pig atria, cardiomyocytes located epicardially had more t-tubules (29% higher t-index) than endocardial myocytes, and this was true in both right and left atria in all hearts and in the main chamber and appendage (data not shown). Indeed, some epicardial cells showed a surprisingly high density of very organized t-tubules (for example, see Fig. 2A,g). This was reflected by FFT analyses, which showed higher peak power at a frequency near 0.6 μm⁻¹, which is indicative of an organized, predominantly transverse oriented t-tubule network (Fig. 2D). Representative FFTs are also shown for a myocyte containing t-tubules that were not well organized and for an unubulated myocyte. The smaller size and higher t-tubule density of epicardial cells (Fig. 2, B and C) resulted in a shorter distance to the nearest membrane compared with endocardial cells (mean: 1.19 ± 0.02 vs. 1.30 ± 0.06 μm, P = 0.05; see distance maps in Fig. 2A,a–h). Therefore, we hypothesized that, in both pig and rat atria, higher t-tubule density in the epicardium augments action potential propagation into cardiomyocytes, synchronizing Ca²⁺ homeostasis.

T-tubule structure determines atrial cardiomyocyte Ca²⁺ homeostasis. More detailed analyses of t-tubular structure and its consequences for function were performed in isolated rat atrial cardiomyocytes, with comparisons made with ventricular cells. All examined ventricular cells exhibited a well-developed t-tube network organized in a primarily transverse direction, as shown by the di-8 ANEPPS stains and distance maps shown in Fig. 3, A and B. We observed three populations of t-tubule patterns in isolated atrial cardiomyocytes. As in intact atria, a significant proportion of atrial cells (58%) was devoid of t-tubules (“untubulated atrial cells” in Fig. 3C), whereas 32% of myocytes exhibited a significant, albeit disorganized, t-tubule network (“tubulated atrial cells”). A small fraction (≈10%) of atrial cells was observed to exhibit a t-tubule density equivalent to ventricular cells (Fig. 3D), and the t-tubule network appeared to be well organized (“organized tubulated atrial cells”). This latter cell population resembled cells observed in the epicardial layer of the intact atrium. Quantification of t-tubule organization by FFT analyses showed robust peaks in ventricular cells (Fig. 3F). FFTs did not reveal peaks in “tubulated” atrial cells, but small peaks were observed in “organized tubulated” cells, similar to observations in the intact epicardium. Thus, although t-tubule density was remarkably high in this subpopulation of cells, organization, as assessed by FFT, remained below the level observed in ventricular myocytes.

We next investigated how variability in the t-tubule network of atrial cells influenced Ca²⁺ homeostasis. Antibody labeling of L-type Ca²⁺ channels showed a well-organized distribution along z-lines in ventricular cells (Fig. 4A). Atrial myocytes exhibited variable staining of the Ca²⁺ channel (Fig. 4B), in agreement with variable t-tubule organization. Although many cells did not exhibit significant Ca²⁺ channel labeling in the cell interior, a subset showed significant staining, and a minority exhibited Ca²⁺ channels with a regular, striated organization. The resulting irregularity in FFT analyses of Ca²⁺ channel stains thus resembled those obtained for t-tubule organization (Fig. 3F). Measurements of I_{Ca} were in agreement with Ca²⁺ channel localization data. I_{Ca} measurements in atrial cells varied considerably in amplitude and were, on the average, markedly smaller than those in ventricular cells (Fig. 4, C and D). Membrane capacitance, an estimate of cellular surface area, was positively correlated with I_{Ca} magnitude in both atrial and ventricular cardiomyocytes (atrial cardiomyocytes: r² = 0.50, P < 0.05; ventricular cardiomyocytes: r² = 0.80, P < 0.05; Fig. 4E). The surface-to-volume ratio was also positively correlated with I_{Ca} in atrial cells (r² = 0.38, P < 0.05), indicating that cells with more t-tubules exhibited larger I_{Ca} (Fig. 4F). No such correlation was observed in ventricular cardiomyocytes (r² = 0.03), where consistent t-tubule organization resulted in little cell-to-cell variation in surface-to-volume ratios. Together, these data indicate that t-tubules in atrial cardiomyocytes contain functional Ca²⁺ channels and that both t-tubule density and I_{Ca} vary considerably between cells. Regular, striated labeling of RyRs was observed across both ventricular and atrial cardiomyocytes (Fig. 4, A and B). Thus, the pattern of Ca²⁺ release during the action potential would be expected to predominantly result from t-tubule rather than RyR organization.

Line-scan images of Ca²⁺ transients showed that ventricular cardiomyocytes exhibited rapid and synchronous Ca²⁺ release across the cell (Fig. 5A). As with t-tubule and Ca²⁺ channel stains (Figs. 3 and 4), Ca²⁺ release patterns in atrial cardiomyocytes could be divided into three groups. One population of cells exhibited U-shaped Ca²⁺ release, another exhibited dysynchronous Ca²⁺ release, and a third exhibited very synchronous Ca²⁺ release (Fig. 5, B–D). Thus, dysynchronous index values were larger and more variable in atrial cells than in ventricular cells (Fig. 5E). Corresponding to the fraction of atrial cells with t-tubules, 40% of dysynchronous index mea-

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00295.2014 • www.ajpheart.org
measurements in atrial cells fell within the range of ventricular cell values. Since the synchrony of Ca\(^{2+}\) release influences the rate of Ca\(^{2+}\) rise and decline (28), atrial cells exhibited more variable kinetics of Ca\(^{2+}\) transients than ventricular cells (time to peak, Ca\(^{2+}\) transient magnitude, and time to half-relaxation; Fig. 5, F–H). To confirm that differences in Ca\(^{2+}\) release kinetics among cells resulted from variability in t-tubule organization and L-type Ca\(^{2+}\) channels, we simultaneously imaged t-tubules (RH-237) and Ca\(^{2+}\) (fluo-4). Figure 6, A–D, shows t-tubule stains with scan-line positions indicated (left), distance maps (middle), and line-scan images with distance to the nearest membrane measurements overlaid (right). In both ventricular and atrial cells, we observed that when the line scan was drawn close to a cell membrane or t-tubule, Ca\(^{2+}\) release occurred early. Cells with the highest tubule density were observed to have the most synchronous Ca\(^{2+}\) release. Thus, gradient t-tubule organization across the atria results in variable cardiomyocyte Ca\(^{2+}\) homeostasis.

Mathematical modeling. Using mathematical modeling, we investigated the functional consequences of a transmural gradient in atrial t-tubule organization and Ca\(^{2+}\) homeostasis. Based on cells simultaneously stained for t-tubules and Ca\(^{2+}\), Ca\(^{2+}\) transients were selected from cells with t-tubule density representative of the epicardial and endocardial layer (Fig. 6E). These Ca\(^{2+}\) transients were incorporated in a modified model of cooperative activation and cross-bridge cycling in cardiac muscle (33) to predict isometric force generation. The smaller and slower Ca\(^{2+}\) transient from the endocardial cell (low
t-tubule density) triggered reduced magnitude and kinetics of force development compared with the epicardial cell (time to peak in endocardial cells: 92.9 ms vs. time to peak in epicardial cells: 63.7 ms; Fig. 6). Since electrical activation of the atrium is reported to spread considerably faster in the endocardium than in the epicardium (10, 35), lower t-tubule density and slower force development in the endocardium may serve to synchronize transmural contraction.

**DISCUSSION**

The present study demonstrated that an appreciable t-tubule network is present in a large fraction of pig and rat atrial cardiomyocytes. In both species, t-tubular structures are, to a large extent, localized in the epicardial layer rather than the endocardial layer. We also observed differences in t-tubule density between the right and left atrium. Although a few
Fig. 5. Ca\textsuperscript{2+} transient characteristics of rat ventricular and atrial cardiomyocytes. A, left: ventricular cells exhibited uniform Ca\textsuperscript{2+} transients in confocal line-scan images, with similar characteristics of local transients (indicated by arrows; right) at various locations across the cell. Consistent with variable t-tubule density in atrial cells, distinct patterns of Ca\textsuperscript{2+} release were observed. These were characterized as cells exhibiting U-shaped Ca\textsuperscript{2+} release, consistent with the absence of t-tubules (B), dyssynchronous Ca\textsuperscript{2+} release characterized by multiple release sites (C), or synchronous Ca\textsuperscript{2+} release as expected of cells with high t-tubule density (D). Consistent with variable patterns of Ca\textsuperscript{2+} release, atrial cells also exhibited great variability in Ca\textsuperscript{2+} transient characteristics compared with ventricular cells (E–H). n = 12 ventricular cells and 45 atrial cells from 9 hearts.
Fig. 6. T-tubule density determines Ca\textsuperscript{2+} release synchrony and the rate of force development. Atrial myocytes were simultaneous imaged for t-tubules (RH-237) and Ca\textsuperscript{2+} transients (fluo-4). Left: confocal images of t-tubules. Middle: diagrams of the shortest distance to a cellular membrane or t-tubule across the cell. Right: line-scan images of Ca\textsuperscript{2+} transients recorded at the line shown in the left and middle images, with plotted distance to the nearest membrane. A–D: in ventricular cells (A) and the three distinct types of atrial cells (B–D), scanned positions near membranes always exhibited early Ca\textsuperscript{2+} release. E: compared with epicardial cells, lower t-tubule density in endocardial cells produced slow Ca\textsuperscript{2+} transients and slow modeled force development.
studies have reported rudimentary t-tubules in a proportion of rat atrial myocytes (18, 19, 37), it remains generally believed that t-tubules are absent or very sparsely distributed in this tissue (13, 40). In apparent contrast to this view, we observed a subpopulation (≈10%) of cells with well-organized t-tubules at densities similar to those found in ventricular cells. Variable t-tubule density and organization between cells were associated with variable L-type Ca\textsuperscript{2+} channel localization, I_{Ca}, synchrony of Ca\textsuperscript{2+} release, and force development.

It has previously been demonstrated that t-tubule density and cell size are correlated in atrial cardiomyocytes (40). This finding suggests that larger cells may require a more extensive t-tubule network for effective propagation of action potentials to the cell interior. To some extent, our findings are consistent with this notion. For example, we observed that in the pig, cardiomyocytes from the right atrium were larger and more tubulated than those from the left atrium (Fig. 1, C and D). In the rat, larger, more tubulated cells were found in the left atrium (Fig. 2, B and C). However, closer examination of cell populations revealed distinct exceptions to the “cell size determines t-index” paradigm. For example, in rat atria, cells in the epicardial layer were both smaller and had higher t-tubule density than those in the endocardium, resulting in markedly shorter distances to the nearest cell surface membrane or t-tubule. Furthermore, when the organized tubulated cell population was selected out, we observed that these cells were, in fact, of very similar size to untubulated cells (Fig. 3E). Indeed, we found no overall correlation between t-tubule density and cell area in either pig or rat atria (data not shown) or between median distance to the nearest t-tubule/membrane and cell area (Fig. 1E). Collectively, these data suggest that size is not the only determinant of atrial t-tubule density. Instead, our data suggest that t-tubule density may define particular functional roles of different cell populations. Excitation of the epicardial and endocardial myocardium is reported to be discordant in dog atria, with faster endocardial conduction observed due to specialized structures such as crista terminals and pectinate muscles (10, 36). Thus, higher t-tubule density, and more rapid kinetics of Ca\textsuperscript{2+} release and force development in epicardial cells (Fig. 6, E–F), may compensate for the delayed arrival of the action potential, allowing contraction to be synchronized with the endocardial layer. Further experiments, perhaps examining contractility in muscle strips from epicardial and endocardial layers, would be required to verify this hypothesis. We suggest that such experiments would be most easily carried out in large animals such as pigs, where the atrial wall is thick enough to allow dissection into endocardial and epicardial layers.

Transmural gradients in t-tubule density may also contribute to known differences in effective refractory period (ERP). Despite the fact that cells with low t-tubule density exhibit very small-magnitude I_{Ca}, total action potential duration is prolonged due to small inward rectifier K\textsuperscript{+} currents (24). Thus, regions of the atria with lower t-tubule density would be expected to have a longer refractory period. Indeed, it has been shown that ERP is ≈20 ms longer in the endocardium than in the epicardium (12). Thus, while t-tubules likely help synchronize transmural contraction of the atrium, another equally important function may be the synchronization of ERP across the atrial wall. Regional variation in t-tubule density across the atria may have important consequences for arrhythmias, especially since the atrial t-tubule network has been reported to be disrupted in heart failure (11) and atrial fibrillation (23, 41). Based on our above discussion of the effect of t-tubules on ERP, we expect that regions of the atria with fewer t-tubules would be less prone to early reactivation. However, action potentials are expected to propagate more rapidly when t-tubule density is lower since the tubules serve as an “electrical sink.” Thus, the net effect of t-tubule density on the generation of reentrant arrhythmias remains unclear. Also unclear is the effect of t-tubules on RyR activity (31). Some studies have suggested that loss of junctophilin and t-tubules may promote RyR sensitization (2) and Ca\textsuperscript{2+} waves (8). Conversely, our own data indicate that RyR activity is reduced at orphaned RyRs (25). In the event that spontaneous SR Ca\textsuperscript{2+} release does occur, generation of delayed afterdepolarizations is consequent on removal of released Ca\textsuperscript{2+} via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Thus, cells lacking t-tubules would be expected to be less prone to afterdepolarizations and triggered activity resulting from Ca\textsuperscript{2+} waves (40). Clearly, additional work is required to address these controversies.

This study supports growing evidence for an extensive t-tubule network in the atria across a range of mammalian species. Previous publications reported that a well-developed atrial t-tubule system exists in larger mammals, including sheep (11, 23), dogs (41), horses, cows, and humans (34). Our present data indicate that pig atria can be added to this list. Perhaps more surprising is that we also observed a substantial t-tubule network in rat atria, with overall densities similar to those found in the pig, and a subpopulation of cells with very well organized t-tubules. This finding runs counter to several previous studies that have reported sparse t-tubule networks in rat atrial cardiomyocyte (1, 37, 42). A possible explanation for this discrepancy is that most of this previous work has examined isolated cardiomyocytes rather than intact tissue (37, 42). We observed that t-tubules in rat atria appear to be quite thin, suggesting that they might easily seal off during suboptimal isolation, preventing access of membrane stains. Indeed, in our experience, atrial cell isolation is more technically challenging than ventricular cell isolation. With the presently observed regional differences in t-tubule density, tubulated myocytes may also have been missed in previous studies if a nonrepresentative population of cells was isolated. Since we observed quite similar t-tubule densities and organization in intact tissue and isolated cells, we believe that our own cell isolation technique yields cells that are representative of the atria in situ, with intact and accessible t-tubules.

In conclusion, we have shown that a substantial proportion of cardiomyocytes are tubulated in rat and pig atria and that there is marked regional variability in t-tubule organization. T-tubules are functional in these cells, resulting in a spectrum of patterns of Ca\textsuperscript{2+} release. Of note, we observed higher t-tubule density in cells localized in the epicardium compared with in the endocardium, which we propose promotes synchronization of contraction across the atrial wall. Regional differences in t-tubule density are also likely to influence refractory period and arrhythmogenesis, which is of particular interest in conditions such as heart failure and atrial fibrillation, where t-tubules are lost.
ACKNOWLEDGMENTS

The authors thank the Section of Comparative Medicine, Oslo University Hospital Ullevål (Oslo, Norway), for animal care.

GRANTS

This work was supported by the South-Eastern Norway Regional Health Authority, The Research Council of Norway, Anders Jahre’s Fund for the Promotion of Medical Research at Oslo University Hospital, University of Oslo, and European Union Project Grant FP7-HEALTH-2010.2.4.2-4 (“MEDIA-Metabolic Road to Diastolic Heart Failure”).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

34. Sabouri S, Matene E, Vinet A, Richer LP, Cardinell R, Armour JA, Page P, Kus T, Jacques M. Simultaneous epicardial and noncontact...


