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Regional Distribution of T-tubule Density in Left and Right Atria in Dogs

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Abstract

Background—The peculiarities of T-tubule morphology and distribution in the atrium – and how they contribute to excitation-contraction coupling – are just beginning to be understood.

Objective—To determine T-tubule density in the intact, live right and left atria in a large animal and to determine intra-regional differences in T-tubule organization within each atrium.

Methods—Using confocal microscopy, T-tubules were imaged in both atria in intact, Langendorff-perfused normal dog hearts loaded with di-4-ANEPPS. T-tubules were imaged in large populations of myocytes from the endocardial surface of each atrium. Computerized data analysis was performed using a new MatLab routine, AutoTT.

Results—There was a large percentage of myocytes that had no T-tubules in both atria with a higher percentage in the right atrium (25.1%) than in the left (12.5%, $p < 0.02$). The density of transverse and longitudinal T-tubule elements was very low in cells that did contain T-tubules but there were no significant differences in density between left atrial appendage, the pulmonary vein – posterior left atrium, right atrial appendage and right atrial free wall. In contrast, there were significant differences in sarcomere spacing and cell width between different regions of the atria.

Conclusion—There is a sparse T-tubule network in atrial myocytes throughout both dog atria, with significant numbers of myocytes in both atria – the right atrium more so than the left - having no T-tubules at all. These regional differences in T-tubule distribution, along with differences in cell width and sarcomere spacing, may have implications for the emergence of substrate for atrial fibrillation.

Keywords

t-tubules; atrium; left atrial appendage; posterior left atrium

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INTRODUCTION

Excitation-contraction (E-C) coupling is driven by myocyte depolarization causing opening of L-type voltage-operated Ca^{2+} channels (LTCCs) on the sarcolemma, with the released Ca^{2+} activating ryanodine release channels (RyRs) to release sarcoplasmic reticulum Ca^{2+} stores. Alteration of this Ca^{2+} -induced Ca^{2+} release (CICR)¹⁻³ can create conditions for triggered activity as well as reentry²⁻⁴.

Much of our current understanding of E-C coupling has been obtained from ventricular myocytes. Recent years have seen a significant interest in understanding E-C coupling in the atrium, in light of studies suggesting a role for abnormal E-C coupling in the genesis of atrial fibrillation (AF)^{5, 6}, the most common heart rhythm disorder. Recent studies indicate significant differences in atrial versus ventricular Ca^{2+} transients^{7, 8}. These differences are thought to at least partially reflect differences in transverse tubule (T-tubule) organization in atria compared to ventricles⁷. In ventricular myocytes, CICR is driven by an extensive network of T-tubules which are narrow (~200 nm) inward projections of the sarcolemma^{2, 3, 8}. In contrast, adult atrial myocytes appear to lack well-developed T-tubules^{9, 10}. Earlier studies indicated that T tubules are almost entirely absent in atrial myocytes¹¹, with E-C coupling in atrial myocytes postulated to originate at LTCCs at the cell periphery and locally amplified by junctional RyRs in the subsarcolemma. More recent studies suggest that adult atrial myocytes possess at least a rudimentary transverse-axial tubular network¹²⁻¹⁴. The case for functional T-tubules is stronger for large animal atria^{7, 12, 14, 15}. Regardless, even in large animals, T-tubule distribution and morphology demonstrate significant heterogeneity between studies^{7, 11, 12, 14, 16}. This is likely in part because T-tubules have thus far largely been studied in fixed tissue sections by electron microscopy or more recently by fluorescent probes in isolated myocytes. Unfortunately, T tubules are extremely labile and can undergo considerable change in shape and morphology in response to tissue fixation and cell culture^{17, 18}. Understandably, T-tubules should ideally be visualized in their native environment in the intact, live atrium. Indeed, Chen et al have recently reported that single photon confocal imaging can be used to visualize the cardiomyocyte T-tubule system in Langendorff-perfused hearts¹⁷. We have previously reported use of the same technique to assess E-C coupling and T-tubule organization in the intact rat ventricle^{19, 20}. Using a similar technique, we now report a systematic examination of the T-tubule network in the intact, Langendorff-perfused right and left canine atrium. We also correlated T-tubule distribution with sarcomere spacing and cell width in different regions of the two atria.

METHODS

See Data Supplement for Methods

RESULTS

Use of AutoTT in T-tubule analysis in atrial and ventricular myocytes

Typical recordings of myocytes in intact heart are shown in Figure 1. Figure 1A shows a typical image obtained in a rat left ventricle, providing a basis for comparison with subsequent images obtained in dog atrium. The ventricle shows a T-tubule network that is uniformly well-organized in all myocytes in the image, consistent with previous work^{21–23}.

In contrast, the T-tubule system in endocardial myocytes of intact right atrium (RA) is sparse and, in many myocytes absent entirely (figure 1B). In contrast to the ventricle, T-tubules are observed as small clusters of short tubules that never traverse the entire cell width. Figure 1C shows an expanded image of the myocyte with the greatest distribution of T-tubules from Figure 1B. Some T-tubules appear as finger-like projections from the sarcolemma because the orientation of the image successfully captures the fusion of the tubule with the sarcolemma. In addition, there are also clustered arrays that appear as individual points which are in fact arising from sarcolemma that is out-of-focus in the image. Note that surrounding myocytes have small clusters of usually less than 10 tubules and often as little as only isolated 1–2 tubules.

The automated analysis of two representative RAA myocytes is shown in Figure 2. Myocyte 1 (left column) has several clusters of T-tubules which were successfully identified by AutoTT. The top image shows the outline of the cell excluding all sarcolemma. The image in the center shows the discrimination of sarcolemma (green) and cell interior (pink) by AutoTT and indicates the total cell area used by the software to calculate tubule densities. Finally, the bottom image shows the skeletonized image with the T-tubules discriminated after thresholding.

Myocyte 2 was selected because it had no obvious T-tubules, although there were several bright spots indicating isolated T-tubule projections into the current plane of focus. After exclusion of the sarcolemma (top image) and identification of the region of interest for analysis (middle), the bottom image shows that there are several tubules remaining that were then included in the analysis.

The table at the bottom of Figure 2 summarizes the results from the analyses of these two myocytes. Myocyte 1 had transverse tubules that comprise 0.83% of its total internal area and longitudinal tubules in 0.08% of total area. Average sarcomere spacing in the transverse tubules was 2.07 μm . In contrast, Myocyte 2 had virtually no tubules, with transverse and longitudinal elements accounting for 0.01% and 0.02% of cell area, respectively. Maximal cell width was measured independently and was nearly the same for both myocytes.

Figure 3 shows a similar analysis for a rat ventricular myocyte. The top image shows the 2-D image of the cell and the middle image shows the skeletonized image in which a very robust and highly organized T-tubule network is apparent. When T-tubule organization was analyzed in this myocyte using AutoTT (table at bottom of Figure 3) the results show a nearly 10-fold higher T-tubule density compared to atrium with only a doubling in longitudinal tubule density, indicating that not only is there a much greater density of T-

tubules in ventricle but that nearly all tubules reside in the transverse orientation, as would be expected for normal ventricle. In all ventricular myocytes ($n = 41$ myocytes from 4 rats), average T-tubule density was $7.2 \pm 0.10\%$ and average longitudinal tubule density was $2.0 \pm 0.11\%$. These values demonstrate the validity of using this program to analyze tubule density in ventricle and demonstrate the difference between ventricle and atrium in terms of T-tubule density and organization.

We also assessed T-tubule density in a small number of isolated atrial myocytes. As shown in Supplemental Figure 1, T-tubule density in isolated atrial myocytes was very consistent with that seen in the intact atrium, with T-tubule density being extremely small ($< 2\%$ of the total cell volume) in most atrial myocytes.

Regional differences in t-tubule organization in left and right atria

We then used AutoTT to measure t-tubule organization in large populations of myocytes from different regions of the left and right atria. One of the most striking results was the identification of a significant number of myocytes in which there were no T-tubules at all. As shown in Table 1 (see Data Supplement), the percentage with myocytes with no T-tubules was highest in the RA free wall, followed by the RAA, LAA and PV-PLA. 25% of all myocytes from the RA and 12.5% of myocytes from the left atrium (LA) were found to be entirely without any measureable T-tubules.

We then analyzed T-element density and sarcomere spacing in all myocytes demonstrating T-tubules from each region (Figures 4–5). Only T-element density is shown (left column) since there were virtually no longitudinal tubules in any atrial myocytes. Figure 4 shows the summary data for LA myocytes. The events histograms show data from all myocytes from the LAA, PV-PLA and combined average of all LA myocytes. T-element density was equivalent in both regions of the LA (about 0.4% of total cell area). Sarcomere spacing was about 2.2 μm in both regions and consequently throughout the LA. Sarcomere spacing could only be obtained in myocytes that demonstrated at least a partial T-tubule system (and not in myocytes that completely lacked T-tubules). Finally, cell width was somewhat larger in PV-PLA than in the LAA, with a wider distribution in the PV-PLA accounting for the greater average width.

In the RA (Figure 5) there was a slightly higher T-element density in the RAA than in the RA free wall. Sarcomere spacing was the same in both regions of the RA and cell width was greater in the RAA than in the free wall.

Interestingly, when the different regions of the two atria were compared, we found no significant differences in T-element density (Figure 6A). Even when myocytes without T-tubules are included, there was no difference in overall T-element density (Figure 6B). When myocytes were grouped into RA and LA only, there was no difference in T-element density when T-tubule containing (Figure 6C) or all myocytes (Figure 6D) were compared. Thus, the major difference in T-tubule distribution between LA and RA is the fact that about twice the number of myocytes in the RA are completely devoid of T-tubules.

Regional differences in cell width and sarcomere spacing

There were significant differences in cell width between regions (Figure 7A). Myocytes were smaller in the RAFW than in the RAA and PV-PLA. LAA myocytes were smaller than those in the RAA and PV-PLA. Overall, myocytes in the RA were wider than those in the LA (Figure 7B).

Interestingly, sarcomere spacing was greater in the LAA than in both regions of the RA (figure 7C). Spacing in the PV-PLA was not significantly different from any of the RA regions or compared to the LAA. Overall though, sarcomere spacing was greater in the LA than in the RA (figure 7D).

DISCUSSION

A systematic examination of T-tubule density and organization in the intact canine atrium reveals that while T tubules are present in the majority of canine atrial myocytes, the density of these T tubules is quite sparse. More importantly, a significant number of cells in both atria demonstrated a complete absence of T-tubules. Among the cells that did demonstrate a T-tubule network, T-tubules density did not differ among different regions of the atria. Lastly, we discovered significant differences in sarcomere spacing and cell width in different regions of the atria.

T-tubule expression in the atria – comparison with prior studies

Lately, some investigators have begun to examine T-tubule morphology and distribution in normal atria as well as in HF and AF. Smyrnias et al²⁴ reported that tubular structures were present in a significant minority of rat adult atrial myocytes, and were unlike ventricular T-tubules. Frisk et al¹¹ observed T-tubules in approximately one-third of rat atrial cardiomyocytes, in both tissue cryosections and isolated myocytes. In $\approx 10\%$ of atrial myocytes, the T-tubular network was well organized, with a transverse structure resembling that of ventricular myocytes. Both rat and pig atrial tissue demonstrated a higher T-tubule density in the epicardium than endocardium. In pigs, a greater percentage of atrial myocytes (34%) demonstrated a functional T-tubule network. Furthermore, RA myocytes were larger than LA myocytes and exhibited a more extensive T-tubule network. Dibb et al¹³ showed a substantial T-tubule network in isolated sheep atrial myocytes as well as in wheat germ agglutinin labelled atrial tissue sections from horse, cow, and human atria¹⁴. Taken together, these studies demonstrate a detectable T-tubule network in varying numbers of atrial myocytes in large animals and to an extent in small animals (rats).

Our study in the live canine intact atrium confirms several of the above findings, with evidence of at least a partial T-tubule network in several atrial myocytes. Unlike prior studies, we found at least a partial T-tubule network in a majority of right and left atrial myocytes (75% of RA and 87.5% of LA myocytes); this number is significantly higher than reported by Frisk et al¹¹ in pig and rat atria. Some of these differences may be species related or related to localization in endocardium compared to epicardium. Since T-tubules can change morphology in response to culture conditions, acute stretch, HF and AF^{17, 25, 26}, the smaller percentage of cells exhibiting T-tubules in prior studies may be an artifact of

tissue fixation and/or cell isolation. Perhaps most importantly, our study shows that 25% of RA myocytes and 12.5% of LA myocytes completely lack any T-tubules whatsoever. This finding may have important physiological and perhaps pathological significance, as discussed below.

Effect of T-tubule distribution on E-C coupling in atrial myocytes

In normal ventricular myocytes, the rise of systolic Ca^{2+} is uniform from the sarcolemma to the cell interior, owing to a well-developed T-tubule network. On the other hand, atrial myocytes – both from small and large animals – demonstrate a less homogeneous spread of the transient, with the initial rise of the Ca^{2+} transient occurring at the cell periphery and then Ca^{2+} being released sequentially as a wave of CICR propagating towards the cell center²⁷ (producing a characteristic delayed central Ca^{2+} transient observed as a ‘U’- or ‘V’-shaped Ca^{2+} profile⁹). The absence of T-tubules, increased Ca^{2+} buffering capacity of atrial cells²⁸ and the subsarcolemmal Ca^{2+} diffusion barrier of the mitochondria and SERCA pumps²⁹ are all thought to contribute to the delayed atrial Ca^{2+} transient. Frisk et al¹¹ reported a high variability in the distribution of T-tubules and Ca^{2+} channels in pig and rat atrial myocytes; this was paralleled by a similar variability in L-type Ca^{2+} current amplitude that was dependent on capacitance and T-tubule density. Furthermore, cells with the highest tubule density had the most synchronous Ca^{2+} release. The authors concluded that gradients in T-tubule organization across the atria result in variable cardiomyocyte Ca^{2+} homeostasis.

In a related study, Smyrniak et al²⁴ found that tubular structures were present in a significant minority of adult atrial myocytes and significantly altered the onset, amplitude, homogeneity and recovery of Ca^{2+} transients. Although we have not reported Ca^{2+} cycling data here (see Limitations), we have noted a similar U or V shaped pattern to the Ca^{2+} transient in canine atrial myocytes (unpublished data).

Taken together, the atrial T-tubule system – even if poorly developed – appears to contribute significantly to E-C coupling in atrial myocytes. These findings assume greater importance in light of our findings, where a significant percentage of right and left atrial myocytes completely lacked any T-tubules. Nonetheless, the precise physiological significance of a sparser T-tubule network in atrial myocytes is not known. Unlike in the ventricle, the relatively sparse and irregularly distributed T-tubules in atrial myocytes appear to have a more subtle effect on E-C coupling, both in the absence and presence of neurohormonal agonists²⁴. Since some studies demonstrate a correlation between atrial cell width and T-tubule expression^{14, 30}, it has been suggested that the physiological significance of T-tubules – and therefore E-C coupling – in normal atria is to promote synchrony of contraction and recovery. Other studies though do not indicate a clear relationship between atrial cell width and T-tubule expression¹¹. It is therefore possible that T-tubules and downstream E-C coupling may have a rather limited role in atrial contractile function. Regardless of their role in atrial contractility, the fact that cells without T-tubules have significantly less synchronous Ca^{2+} release compared to tubulated myocytes and the existence of T-tubule gradients in the atria – including the presence of myocytes altogether lacking T-tubules in each atrium – is likely to set up E-C coupling gradients within the atria. These gradients are likely amplified in pathological states that result in further loss of T tubules, such as HF and AF^{31, 32}. Below

we discuss how changes in E-C coupling resulting from T-tubule loss likely contribute to substrate for arrhythmogenesis.

Effect of T-tubules on AF substrate formation

Studies in ventricular myocytes have shown that T-tubules are plastic structures that undergo remodeling during HF³³. Lately, investigations in atrial myocytes reveal a similar loss of T-tubules in HF¹³ or AF³⁴. The consequences of this T-tubule loss on the systolic Ca^{2+} transient are dramatic with the Ca^{2+} transient restricted to the cell periphery¹³.

The increased prevalence of T-tubules in atria of large mammals – who are much more likely to develop AF than species with smaller atria - may be of pathophysiological importance in AF. Indeed, we have reported that normal canine atrial myocytes – unlike normal ventricular myocytes – are highly prone to develop triggered Ca^{2+} waves during systole when subjected to rapid pacing³⁵. However, the precise role of T-tubules in creation of these triggered Ca^{2+} waves – and therefore an arrhythmogenic substrate - is far from clear. Frisk et al¹¹, using mathematical modeling, suggested that transmural T-tubule gradients may be at least partially contributing to transmural ERP differences in the atrium. Since both LTCCs and I_{NCX} are heavily concentrated in and around T-tubules, a loss of T-tubules is expected to significantly alter the effects of these currents on membrane depolarization. Indeed, Lenaerts et al³⁴ demonstrated that in a sheep model of AF, reduction in $I_{\text{Ca,L}}$ correlates with a loss of atrial T-tubules. Louch et al²³ showed that cells with low T-tubule density, in spite of exhibiting very small-magnitude $I_{\text{Ca,L}}$, demonstrate prolongation of the action potential duration, likely due to reduced potassium currents (I_{K1} , I_{Kur} , I_{Kr} , I_{KAch})^{4, 36}. Regardless of whether T-tubule loss contributes to shortening or prolongation of the atrial ERP, it is likely that altered T-tubule gradients due to a loss of T-tubules will affect atrial repolarization. Whether these altered T-tubule gradients actually contribute to the AF disease state is not clear. Further studies are needed to systematically assess the role of T-tubule loss on creation of the AF disease state.

Since AF triggers are thought to arise in the PVs in several patients, future studies also need to systematically examine T-tubule anatomy and related Ca^{2+} cycling in this region. Even though we did not detect any significant difference in T-tubule density between the PVs and the rest of the left or right atrium, a key finding of our study is that 25% of all RA myocytes and 12.5% of all LA myocytes completely lacked T-tubules. Furthermore, cell width was greater in the PVs than in the LAA or RA free wall, with sarcomere spacing also trending to be greater in the PVs than in the RA. The larger size of PV myocytes may be at least partially contributing to the greater number of tabulated cells in the LA versus the RA. It is tempting to speculate that peculiarities in T-tubule density/distribution, sarcomere spacing and cell width may be contributing, at least in part, to the unique electrophysiological characteristics of the PVs and PLA. Indeed, it is well known that electrophysiological remodeling in AF differs between the right and left atrium^{37, 38}. It is also known that the PVs have more inhomogeneous conduction and repolarization than the rest of the LA, with differences in fiber orientation, autonomic innervation and differential expression of ion channels⁴ postulated to play a part. In light of the current study, future studies are needed to

examine if inter-atrial and intra-atrial differences in T-tubule distribution contribute to the propensity of the PVs and adjoining PLA for the emergence of AF triggers.

Limitations of current study

Our study did not assess E-C coupling and how it relates to the differences in T-tubule distribution in the RA versus the LA. In ongoing studies, we are systematically assessing E-C coupling in isolated atrial myocytes and attempting to correlate E-C coupling with T-tubule structure/distribution in isolated canine atrial myocytes. Another limitation is that we only examined endocardial atrial myocytes; since prior work indicates the presence of significant epicardial-endocardial gradients in T-tubule distribution in fixed tissue slices of the atrium¹¹, these gradients need to be systematically examined in the live, intact atrium as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Examples of T-tubule staining in intact rat left ventricle (A), dog right atrium (B) and in dog right atrium at high magnification (C).

Figure 2. Representative examples of dog right atrial myocytes showing one cell with extensive T-tubules (left panels) and another with no discernible T-tubules (right panels). The top panel shows the cell outline with the cell membrane as presented by AutoTT. The middle panels show the region actually analyzed (magenta) and the cell membrane excluded (green). The bottom panels show the thresholded, skeletonized T-tubules after image processing. The table shows some of the measurements made by AutoTT for these myocytes. T-ED – percent cell volume for transverse tubules; L-ED – same for longitudinal tubules; Spacing measures mean distance between T-tubules; TTi refers to total percentage of cell volume for all tubules.

	T-ED (%)	L-ED (%)	Spacing (μm)	TTi	Cell Width (μm)
Myocyte	8.0589	1.6539	2.2335	2.6447	51.94

Figure 3. Staining of T-tubules in an intact rat LV for purposes of comparison to dog atrium in Figure 2. Top panel shows original image and middle panel shows outlined and thresholded image from AutoTT. Table summarizes T-tubule characteristics in this myocyte.

Figure 4.

Summary of T-tubule density as percent of total cell volume (left column), sarcomere spacing (middle column) and cell width (right column) in myocytes from LAA, PV-PLA and total in whole left atrium. Graphs show events histograms summarizing results from many cells in all 5 dog atria.

Figure 5.

Summary of T-tubule density as percent of total cell volume (left column), sarcomere spacing (middle column) and cell width (right column) in myocytes from RAA, RA free wall and total in whole right atrium. Graphs show events histograms summarizing results from many cells in all dog 5 atria.

Figure 6. Summary of regional differences in T-tubule density (panels A–B) and in right vs. left atrium in T-tubule containing cells only (panel C) and in all myocytes from RA and LA (panel D).

Figure 7.

Summary of regional differences in cell width (panels A) and in RA and LA (panel B) and regional differences in sarcomere spacing (panel C) and in all myocytes from RA and LA (panel D).