The structure and function of cardiac t-tubules in health and disease

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The transverse tubules (t-tubules) are invaginations of the cell membrane rich in several ion channels and other proteins devoted to the critical task of excitation–contraction coupling in cardiac muscle cells (cardiomyocytes). They are thought to promote the synchronous activation of the whole depth of the cell despite the fact that the signal to contract is relayed across the external membrane. However, recent work has shown that t-tubule structure and function are complex and tightly regulated in healthy cardiomyocytes. In this review, we outline the rapidly accumulating knowledge of its novel roles and discuss the emerging evidence of t-tubule dysfunction in cardiac disease, especially heart failure. Controversy surrounds the t-tubules’ regulatory elements, and we draw attention to work that is defining these elements from the genetic and the physiological levels. More generally, this field illustrates the challenges in the dissection of the complex relationship between cellular structure and function.

Keywords: t-tubule; heart failure; structure–function; cell membrane

1. HISTORICAL PERSPECTIVES

The transverse tubules (t-tubules) are invaginations of the external membrane of skeletal and cardiac muscle cells (figure 1), which are rich in ion channels that are important for excitation–contraction coupling [2] (figure 2). The transverse t-tubule network of mammalian ventricular cardiomyocytes was first discovered by Linder, using electron microscopy (EM), in 1956 [3]. The first report of axial t-tubules was in 1971 [4].

EM studies (e.g. [5,6]) confirmed that t-tubules were invaginations of the external membrane (the sarcolemma), and described their transverse and axial radiations, which paralleled findings in skeletal muscle. These studies described in ventricular cells a less regular but much larger t-tubule system. The finding that the external membrane penetrated the cell’s centre was used to explain the earlier finding that it took less than 40 ms for excitation to travel from the external membrane to the centre of the cell, a distance of some 50 µm [7]. This also explained the observation that cells with well-demarcated invaginations contracted more rapidly than those without such structures [8].

Ryanodine, a drug that inhibits contraction in both cardiac and skeletal muscle, was found to bind only to those elements of the sarcoplasmic reticulum (SR) in proximity to the t-tubule system [9], in structures termed triads [5]. Functional studies identified the ryanodine receptor (RyR2) as the ion channel responsible for SR Ca²⁺ release in cardiac muscle [10] and defined its Ca²⁺ dependence [11–15], culminating in the theory of Ca²⁺-induced Ca²⁺ release (CICR) [14].

Soeller & Cannell’s [1] two-photon live-imaging study avoided the technical flaws of the early EM studies, which involved traumatic fixation and limited three-dimensional insight. Their work elaborated the exquisite complexity of the t-tubule system (figure 1), which runs deep into cardiomyocytes and varies in its diameter. Recent work has reconstructed a three-dimensional model of the network and provided a new level of detail using computational approaches [16]. It confirms that the cardiac t-tubules are not transverse only, but have protrusions in many directions and a diameter that varies from 20 to 450 nm. Skeletal muscle t-tubules are much smaller, with a diameter between 20 and 40 nm [17,18].

2. PHYSIOLOGICAL T-TUBULE FUNCTIONS

In cardiomyocytes, contraction is initiated by depolarization-mediated Ca²⁺ entry via sarcolemmal L-type Ca²⁺ channels (LTCCs), which triggers Ca²⁺ release from the SR via RyR2 [19]. The juxtaposition of sarcolemmal LTCCs and the RyR2 forms a dyad (figure 2) [20]. This close association (approx. 12 nm) is critically dependent on normal t-tubule structure and guarantees that the Ca²⁺ trigger results in adequate Ca²⁺ release from the intracellular stores. This process of CICR is most effective in ventricular cells where the t-tubules ensure that Ca²⁺ is released in close proximity to all sarcomeres, regardless of how deep they lie within the cell [21]. The t-tubule network is responsible for just one-third of the capacitance of the membrane, but most of the influx of Ca²⁺ that triggers the release of intracellular SR Ca²⁺ enters across the t-tubular fraction. This is because there is a different complement of ion channels in the surface and t-tubular fractions, with a threefold to ninefold higher number of LTCCs in the t-tubule fraction than in the surface sarcolemma [22].
The myofilaments represent 45 to 60 per cent of the cardiomyocytes’ volume [19]. These contractile complexes are organized into sarcomeres, with only a minority of these units close to the surface sarcolemma. In cells without t-tubules, the wave of Ca\(^{2+}\) propagates from the periphery of the cell into the centre [23] either by simple diffusion or by a wave of propagated CICR. Such a system would first activate the peripheral sarcomeres and then the deeper sarcomeres, resulting in sub-maximal force production. A system where current is simultaneously relayed to the core of the cell would mean a larger instantaneous force is produced, which is more equally shared between sarcomeres. The t-tubules make this possible by triggering SR Ca\(^{2+}\) release near to all sarcomeres simultaneously.

This role for t-tubules in cardiac muscle is to a certain extent supported by comparative biology studies. Although there is little evidence that differences in t-tubule density are related to cell size, there appears to be a correlation with species (which reveals a possible association with heart rate). For example, the mouse, which has a heart rate of approximately 600–800 beats per minute (bpm) at rest, has a denser t-tubule network than the pig, whose heart rate is less than 100 bpm [24]. This trend suggests that the t-tubules are more important in hearts where the rapid cycling of Ca\(^{2+}\) is necessary in order to cope with high heart rates. Nevertheless, there can be little doubt that t-tubules promote the synchronous and efficient activation of the cell, and that these capacities fail when the t-tubules are disrupted; indeed, the Ca\(^{2+}\) transient is less synchronous in atrial cells that have a less-developed t-tubule system.

The t-tubules restrict diffusion of the extracellular fluid, creating a microdomain of ions of a concentration that is relatively stable in comparison with the wider extracellular space. This may be a mechanism to prevent rapid changes in the extracellular fluid from adversely affecting CICR. However, investigators have argued that it is theoretically possible that as more Ca\(^{2+}\) is drawn out of the t-tubule cleft, it may become Ca\(^{2+}\)-depleted [2]. Savio-Galimberti et al. [16] have visualized single t-tubules at very high resolution, and their analysis suggests, that as the cardiomyocyte contracts, the t-tubules are squeezed and this ‘pumps’ the microdomain contents into the extracellular space. It is not yet clear whether such a mechanism is quantitatively important in replenishing the contents of the t-tubular microdomain, and more studies are needed. However, recent computational studies, which have modelled the t-tubular microdomain as containing ion concentrations either fixed at those of the extracellular space or altering by diffusion, suggest that the accumulation and depletion of ions in the t-tubule may have a significant effect on membrane currents [25,26]. Whether this scenario is prevented by t-tubule pumping or other mechanisms, such as Ca\(^{2+}\) extrusion at the t-tubules by the Na\(^{+}–\)Ca\(^{2+}\) exchanger (NCX) [27], is a matter to be established by experimental approaches.

The density of LTCCs is higher in t-tubules than in the surface sarcolemma [28,29] (figure 3). Brette et al. [21] estimated that approximately 75 to 80 per cent of the L-type current flows across the t-tubules, indicating that they are the main source of the Ca\(^{2+}\) trigger. The Ca\(^{2+}\) current of the t-tubules is more readily inactivated by Ca\(^{2+}\) flow than the same current at the surface, indicating that Ca\(^{2+}\) released from the SR may have a greater auto-regulatory role on the Ca\(^{2+}\) current in the t-tubules, further supporting their special role in CICR [21,30].

3. NON-Ca\(^{2+}\)-INDUCED CA\(^{2+}\) RELEASE ROLES FOR THE T-TUBULE
(a) Ca\(^{2+}\) handling
The t-tubules are not only a site for Ca\(^{2+}\) influx, but also for Ca\(^{2+}\) efflux. NCX also appears more highly concentrated in the t-tubule [27]. T-tubular NCX appears to have privileged access to the Ca\(^{2+}\) released from the SR [31] and may occur in a specialized zone in the t-tubule. The SR Ca\(^{2+}\)-ATPase (SERCA) is preferentially expressed close to the t-tubules [32], indicating that the major Ca\(^{2+}\) extrusion pathways are near to the influx pathways at the t-tubule. Recent evidence suggests that Ca\(^{2+}\) efflux through the sarcolemmal Ca\(^{2+}\)-ATPase occurs only in the t-tubule, and Ca\(^{2+}\) efflux through NCX occurs predominantly through the t-tubule [33]. Just as the major influx pathways in the t-tubules allow for rapid contraction throughout the cell, so the extrusion pathways allow for rapid relaxation throughout the cell and further implicate the t-tubule in Ca\(^{2+}\) homeostasis [2,28]. These data suggest the hypothesis that the t-tubules may...
have a specialized role in the extrusion of intracellular Ca\textsuperscript{2+}, but this requires further experimental studies.

**Signalling functions**

The \(\beta\)-adrenergic system is known to modulate the function of a number of key Ca\textsuperscript{2+} channels and contractile proteins. Indeed, a number of key proteins in this signalling pathway (e.g. stimulatory G-protein, Gs) are localized at the t-tubule [34]. Reports suggest that the \(\beta_2\)-adrenoceptor (AR) system is more tightly coupled with the modulation of the LTCCs at the t-tubule than at the surface sarcolemma, as \(\beta\)-adrenergic stimulation causes a greater increase in the Ca\textsuperscript{2+} current in normal cells compared with detubulated cells [35]. This may have a structural basis, as a subpopulation of L-type Ca\textsuperscript{2+} channels resides in caveolae associated with the t-tubule network, and may perform signalling functions [36,37]. These LTCCs co-localize with a \(\beta_2\)-AR/G-protein macromolecular signalling complex raising the possibility that they process small Ca\textsuperscript{2+} signals that could be important in inducing hypertrophy and controlling contractility. There are conflicting reports as to the effect of caveolae disruption on \(\beta_2\)-AR stimulation of the L-type Ca\textsuperscript{2+} current, with some studies stating that caveolae are necessary [37] and some to the contrary [38]; however, it is clear that caveolae-based signalling complexes modulate Ca\textsuperscript{2+} signalling in cardiomyocytes. Interestingly, it is known that mice with a deficiency of caveolin-3 (the major structural protein of caveolae) develop heart failure [39]. Recent evidence shows that this protein is responsible not only for t-tubule formation in cardiac muscle, but also for shuttling of the L-type Ca\textsuperscript{2+} channel to be functionally homologous across the external and the t-tubule membrane, and are not spatially localized to any specific site. However, the \(\beta_2\)-ARs are spatially localized to the t-tubule in normal cells. In heart failure, this spatial localization is lost, with a relative redistribution to the cell surface. The normally tight spatial localization of the \(\beta_2\)-AR response depends on its co-localization with protein kinase A (PKA), which limits the spread of the cAMP response. The normally striated pattern of PKA is lost in heart failure. This ‘globalization’ of the \(\beta_2\)-AR response may partly mediate the loss of its cardioprotective effects and its role in driving the disease process in heart failure. The full implications of this remain to be tested (figure 4).

4. T-TUBULE REGULATION

The t-tubule network is extremely dynamic; it appears after birth (e.g. [41]), when the ventricular pressures rise, and disappears when cardiomyocytes are placed in culture [42], or in heart failure (see later). T-tubules are also absent in stem cells and in neonatal cardiomyocytes. The exact mechanisms responsible for these changes remain unknown. However, they appear to be regulated by both biochemical (table 1) and biophysical (e.g. load and heart rate) factors. Both these factors appear to interact under both physiological and disease conditions, and govern the t-tubules’ structure and function.

Among the biochemical factors, amphiphysin 2 (BIN1) has a fundamental role in t-tubule formation [43]. When ectopically expressed in non-muscle cells, it results in tubular formation, and is concentrated at the sites of developing membrane striations in muscle. Total knockdown of BIN1 is perinatal lethal, with a dilated cardiomyopathy phenotype. Recent evidence shows that this protein is responsible not only for t-tubule formation in cardiac muscle, but also for shuttling of the L-type Ca\textsuperscript{2+} channel.
The major consequence of detubulation in diseased cardiac myocytes is a loss or uncoupling of LTCCs, and changes to BIN1 expression may play a role in mediating changes to both gross t-tubule structure and correct protein localization. Juncophilin 2 has an important role in promoting junction formation between the sarcoplasmic membrane and the t-tubule. It has recently been reported to be downregulated proportionally during the progression from hypertrophy to heart failure. This may partly mediate the uncoupling observed between the t-tubule and SR in cardiac muscle. Recent evidence using conditional knockdown of juncophilin 2 suggests that its reduction is sufficient for dyadic disruption and deranged local CICR, which is associated with a dilated cardiomyopathy and premature mortality. These changes may arise owing to juncophilin 2’s membrane-binding role or owing to a possible direct binding with RyR2.

Tcap was identified as a part of the stretch-sensitive complex in myocardium in previous studies. The effect of Tcap mutations on t-tubule structure in cardiac muscle is unknown; disruption of Tcap in zebrafish produces a form of muscular dystrophy, suggesting that it is important in force production. Lack of Tcap is

Table 1. Molecules involved in t-tubule regulation.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function and Characteristics</th>
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<tr>
<td>amphiphysin 2</td>
<td>Involved in tubule formation; mutations in BIN1 (encodes amphiphysin 2) in myopathies and drosophila orthologue regulates structure of EC coupling machinery; shuttles L-type Ca(^{2+}) channel to the t-tubule.</td>
</tr>
<tr>
<td>myotubularin</td>
<td>Mutations are associated with X-linked myotubular myopathy and t-tubule disruption. There is a putative amphiphysin–myotubularin pathway regulating t-tubule biogenesis. Overexpression causes accumulation of membrane saccules. May also directly modulate RyR function and alters the expression of DHPR and RyR.</td>
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<tr>
<td>tropomyosin</td>
<td>Absence of certain isoforms of tropomyosin (an actin-binding protein) disrupts t-tubules, suggesting the myofilaments may be involved in maintenance of t-tubule system.</td>
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<tr>
<td>Tcap</td>
<td>Mutations cause muscular dystrophy-like phenotype with associated t-tubule disruption. Forms part of stretch-sensitive complex that is defective in some cardiomyopathies (e.g. HOCM). Essential for load-dependent formation of t-tubules in skeletal muscle.</td>
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<tr>
<td>juncophilins</td>
<td>Critical for accurate association of t-tubule and SR membrane; downregulated in heart failure; knockdown in culture causes disrupted t-tubule structure. Conditional knockdown results in cardiomyopathy, impaired CICR, reduced co-localization of LTCC; possibly owing to a direct interaction between RyR and JPH2.</td>
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<tr>
<td>mitsugumin</td>
<td>Mice lacking mitsugumin-29 have t-tubule disruptions but only have limited myopathy.</td>
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<tr>
<td>triadin</td>
<td>Structural proteins that bind RyR. Triadin null mice have preserved contractility but reduced Ca(^{2+}) transient amplitude and disrupted t-tubule structure in skeletal muscle.</td>
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<tr>
<td>obscurin</td>
<td>Depletions in zebrafish cause profound disruption to the t-tubules.</td>
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Figure 4. Beta-adrenergic receptor distribution is altered in heart failure. (a) Normally, β2-adrenoceptors (ARs) are concentrated in the t-tubules, whereas β1-ARs are spread across both membrane fractions. (b) In heart failure, t-tubule abnormalities are associated with a reversal of the normal β2-AR distribution. Scanning ion conductance microscopy (SICM) images from Nikolaev et al. [40].
associated with disrupted t-tubule development and abnormal muscle function. Tcap expression is increased by stretch and appears to correlate with the level of t-tubule development, suggesting the novel possibility that Tcap promotes t-tubule formation in response to increased stretch. Tcap mutations can cause dilated cardiomyopathy (DCM) or hypertrophic cardiomyopathy (HOCM), which could be related to the differential response of the myocardium to stretch [50]. In a small group of patients with DCM or HOCM, Hayashi et al. [50] found that DCM-associated Tcap mutations impaired the interaction of Tcap with its partners in the stretch-signalling complex, whereas HOCM-associated mutations promoted the interaction of these molecules. They suggest that these different Tcap mutations were consistent with an impaired stretch-response in DCM patients, and a hyper-reactive stretch response in HOCM patients, possibly affecting the clinical manifestation of the cardiomyopathy. The recent findings from the zebrafish discussed above raises the possibility for a role in t-tubule structural disruption in the mechanism of this subset of rare cardiomyopathies, but this remains to be tested.

Based on the data available, we propose a model of t-tubule regulation that occurs at three levels. First, biochemical factors (e.g. amphiphysin 2) result in the formation of tubular invaginations of the cell membrane and recruitment of the appropriate ion channels [43,64]. Subsequently, a second class of regulatory molecules sensitive to biomechanical factors triggers appropriate membrane–membrane interactions with the SR and its RyR2 (e.g. junctophilin 2 [53,54]). This level of regulation appears to be required chronically. A third level of biomechanically sensitive regulatory molecules are responsible for the dynamic regulation of the t-tubule system when conditions are changed physiologically (e.g. the speculative role of Tcap [50,52,65]). Changes to the biomechanical conditions of the heart may trigger inappropriate reductions or alterations in the regulatory components at any level, and therefore they may all be important under disease conditions.

5. T-TUBULE DYSREGULATION IN HEART FAILURE

(a) Animal models

The presence of t-tubular changes in heart failure has been demonstrated in both human studies and animal models (e.g. [66–73]). In animal models, heart failure is associated with t-tubule disarray, which appears to be caused by a number of myocardial insults, including sustained tachycardia [68], spontaneous hypertension [74] and myocardial infarction [75]. T-tubule changes appear to occur early in the transition from hypertrophy to heart failure [62], suggesting they occur early in the pathogenic mechanism.

Work using a pig post-ischaemic cardiomyopathy model suggests that t-tubule dysfunction is causally related to the contractile dysfunction of the failing heart [75]. The impaired contractility of the failing heart was associated with a reduced Ca\(^{2+}\) release synchronicity, a longer time to peak Ca\(^{2+}\) release and a lower peak concentration of Ca\(^{2+}\). The authors of this study documented a significant reduction in t-tubule density, while the L-type Ca\(^{2+}\) current and SR Ca\(^{2+}\) content were not deleteriously affected. They suggest that the flaw is therefore in the gain of the CICR process, which implicates the structural dysregulation of the t-tubules in the pathophysiology of the failing cardiomyocyte. We also recently showed that failing rat cardiomyocytes have an increased Ca\(^{2+}\) spark frequency, which is consistent with an uncoupling of the Ca\(^{2+}\) release machinery [73] mediated by structural disruption to the t-tubules. The mechanisms mediating this increased Ca\(^{2+}\) spark frequency are likely to be multi-faceted, including changes to the regulation of the RyR2. Further work suggests that the level of detubulation correlates with the degree of heart failure, and regions of delayed Ca\(^{2+}\) release are spatially localized to regions of disrupted t-tubular structure [24,66,67,75]. Structural uncoupling of the Ca\(^{2+}\) release machinery may be a final common pathway in heart failure, as it appears to be a mechanism common to diabetic cardiomyopathy [76], hypertensive cardiomyopathy [63,74,76] and ischaemic heart failure [75].

The possible causal role of t-tubule dysregulation in the impaired contractility of heart failure is supported by evidence from experiments where the t-tubules are artificially disrupted. Detubulation by culture or osmotic shock mimics some of the changes in Ca\(^{2+}\) handling observed in heart failure, with a reduced Ca\(^{2+}\) transient synchronicity leading to a slow Ca\(^{2+}\) transient, associated with diminished but prolonged contraction [30,77–80]. The degree of transverse t-tubule loss that can be tolerated without functional changes is not clear. Action potentials may access a similar portion of the cell via an axial or transverse t-tubule, possibly providing a safety mechanism. Other compensatory mechanisms may initially mitigate the loss of t-tubules, including an increased SR Ca\(^{2+}\) content. These questions require further investigation.

(b) Human studies

Loss of and disruptions to the regularity of t-tubules occur in human cardiac cells from patients with heart failure, irrespective of the aetiology [73]. We have shown that patients with HOCM, DCM and ischaemic heart failure all had profound t-tubule damage, with a reduction in the t-tubule density and parameters of cell surface regularity (figure 5). This mirrors the findings concerning t-tubule disruption in animal models of heart failure from an ischaemic, hypertensive, tachycardic or obstructive phenotype described above, and suggests that t-tubule disruption is a common pathway in heart failure. Failing human cardiomyocytes have t-tubules that run more on the longitudinal axis, and are often dilated and bifurcated rather than aligned normally along the radial axis of the cardiomyocyte [81]. Recent work has described the detailed structural remodelling of the t-tubules that occurs in human heart failure [82].

6. T-TUBULE DYSREGULATION IN ATRIAL FIBRILLATION

Although atrial cells lack the complex t-tubule structure of ventricular cells, some reports suggest that there is a functional uncoupling of the L-type Ca\(^{2+}\) channel and RyR2 leading to asynchronous, chaotic Ca\(^{2+}\) release, which may be implicated in the poor contractility and arrhythmia of experimental models of atrial fibrillation. In larger animals, a more extensive atrial t-tubule network has been reported, with evidence that the t-tubules of atrial cells are disrupted in models of heart failure [72]. The consequences of the loss of the network of t-tubules in atrial versus ventricular
Figure 5. Heart failure, regardless of the aetiology, disrupts the t-tubule network. SICM images from the surface of cardiomyocytes from (a) non-failing and (b) failing human hearts. The black line is a one-dimensional surface map from (c) non-failing and (e) failing human cardiomyocytes. Confocal images after staining with di-8-ANNEPPS in (d) non-failing and (f) failing cardiomyocytes. (g) T-tubule and (h) Z-groove ratios in cardiomyocytes isolated from patients with DCM (dilated cardiomyopathy), HF secondary to ischaemic heart disease (IHD) or HOCM. NF, non-failing. (i) Prolonged TTP and relaxation times (R50 and R90) in human failing cardiomyocytes (black bars, n = 12) compared with non-failing human cardiomyocytes (white bars, n = 6). **p < 0.01 versus non-failing. From Lyon et al. [73].

Figure 6. Mechanical load alters t-tubule structure. Both mechanical overload and unloading impair CICR and alter t-tubule structure. We have shown that t-tubular abnormalities in heart failure are reversible by mechanical unloading [85]. We propose that the t-tubule system is sensitive to the degree of chronic load, and that normal t-tubule structure depends on normal myocardial load.
cardiomyocytes appear to have different effects on Ca\(^{2+}\) handling [83]. Detubulation of atrial myocytes appears to have significantly less effect on cellular Ca\(^{2+}\) handling than in ventricular cells. Nevertheless, in atrial cells where t-tubules are present, they have a major impact on the Ca\(^{2+}\) transient morphology. Whether t-tubular changes play a major role in the pathological remodelling of atrial myocytes, given their paucity under physiological conditions, is a major question for future work.

7. REVERSIBILITY OF T-TUBULE DYSREGULATION

A recent report by Stolen et al. [84] using an animal model of diabetic cardiomyopathy demonstrated disrupted t-tubular structure in cardiomyocytes and that interval training can induce reverse remodelling. This is an important first report of changes to the t-tubule network that are associated with improved function.

We recently showed that chronic mechanical unloading alters the t-tubule structure (figure 6) [65] of normal hearts. We also recently reported that mechanical unloading of failing hearts reversed the remodelling of the t-tubule system and improved CICR [85]. This further shows the dynamic regulation of the t-tubules by biomechanical factors.

8. CONCLUSION AND FUTURE DIRECTIONS

There have been major breakthroughs in understanding how the t-tubule system is implicated in the function of the normal and diseased heart. However, there remain major unanswered questions, especially relating to the t-tubules’ regulation and whether this is involved in compensatory responses of the heart to physical stressors, and how it becomes dysregulated in disease. Ultimately, research must focus on testing whether t-tubule dysfunction is caused in the mechanisms of disease. This must involve the characterization of the molecular details of its regulation, which may lead to therapeutic targets.

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