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Review Article

There Goes the Neighborhood: Pathological Alterations in T-Tubule Morphology and Consequences for Cardiomyocyte Ca^{2+} Handling

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T-tubules are invaginations of the cardiomyocyte membrane into the cell interior which form a tortuous network. T-tubules provide proximity between the electrically excitable cell membrane and the sarcoplasmic reticulum, the main intracellular Ca^{2+} store. Tight coupling between the rapidly spreading action potential and Ca^{2+} release units in the SR membrane ensures synchronous Ca^{2+} release throughout the cardiomyocyte. This is a requirement for rapid and powerful contraction. In recent years, it has become clear that T-tubule structure and composition are altered in several pathological states which may importantly contribute to contractile defects in these conditions. In this review, we describe the "neighborhood" of proteins in the dyadic cleft which locally controls cardiomyocyte Ca^{2+} homeostasis and how alterations in T-tubule structure and composition may alter this neighborhood during heart failure, atrial fibrillation, and diabetic cardiomyopathy. Based on this evidence, we propose that T-tubules have the potential to serve as novel therapeutic targets.

1. Structure and Role of T-Tubules

1.1. Morphology. The plasma membrane of ventricular cardiomyocytes is comprised of both the surface sarcolemma and a branching network of T-tubules which project into the cell interior. These invaginations were so named since they were initially observed as *transverse* elements, which occur near the Z lines at regular intervals along the cell [1]. However, detailed imaging has shown that the T-tubule network is actually quite complex, and contains numerous longitudinal components which run from one Z line to the next [2, 3]. While T-tubule diameter varies between 20 and 450 nm throughout the cardiomyocyte, more than 50% of tubules have a diameter between 180 and 280 nm [3]. Their total volume has been estimated to be 0.8–3.6% of the cardiomyocytes volume [4, 5]. Estimates of the fraction of the total sarcolemma in the T-tubules (versus surface membrane) range from 21–64% [4], although in a recent review employing computer modeling, it was suggested that the true fraction is close to 50% [6]. The large variability in these estimates likely reflects the different methodologies used for calculation and differences between species, but might also be due to the considerable plasticity of the T-tubules. Indeed, T-tubules are absent in the neonatal heart [7, 8] and develop progressively after birth [9, 10]. Also, as we will discuss in this review, there is an important remodeling of the T-tubules during pathological conditions.

1.2. The Dyadic "Neighborhood". During the cardiac action potential, contraction is triggered in myocytes by a process known as excitation-contraction (EC) coupling [4]. During this process, electrical excitation of the cell membrane triggers a transient rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]$), which results in myocyte contraction as Ca^{2+} binds to the myofilaments. T-tubules play a pivotal role in EC coupling by allowing the action potential to propagate into the cell interior, and by providing proximity between the

excitable cell membrane and the sarcoplasmic reticulum (SR), the main intracellular Ca^{2+} store. The SR membrane is apposed to the T-tubule membrane in highly specialized junctional microdomains (Figure 1). Here L-type Ca^{2+} channels face SR Ca^{2+} release channels, known as ryanodine receptors (RyRs), with a stoichiometry of 4–10 RyRs per L-type Ca^{2+} channel [11]. The two adjacent membranes are separated by a gap of 10–15 nm called the dyadic cleft. Clusters of RyRs and L-type Ca^{2+} channels and the dyadic cleft which separates them together constitute a functional unit called a couplon, or dyad [12] (Figure 1). The number of RyRs in a single dyad is still a matter of debate; analysis from electron micrographs reports numbers from 30 to 270, depending on species [13]. A recent study using a combination of confocal imaging and image processing suggested that the true number is in the upper range (120 to 260 RyRs) in rat [14]. However, a new electron microscopy tomography study in mouse demonstrated that there is a large variability in the size of the dyad [5]. The authors also showed that most dyads are significantly smaller than previously estimated; more than one-third of dyads are equal or smaller than the size necessary to hold ~15 RyRs, and the average dyad holds only 7.7 RyR tetramers.

When one or several L-type Ca^{2+} channels are open, Ca^{2+} release is triggered from RyRs in that couplon by an amplification system known as Ca^{2+}-induced Ca^{2+} release (CICR) [15]. This can be observed as evoked Ca^{2+} sparks in unstimulated myocytes [16, 17], although Ca^{2+} sparks can also result from spontaneous RyR openings in the absence of L-type Ca^{2+} current [18]. When an action potential travels through the cardiomyocyte, thousands of individual Ca^{2+} sparks are triggered, and their spatiotemporal summation constitutes the Ca^{2+} transient [4]. The extent of CICR and magnitude of the Ca^{2+} transient are critically dependent on the SR Ca^{2+} content [19], which in turn is determined by the balance between SR refilling and release. However, efficient coupling between Ca^{2+} influx and SR Ca^{2+} release also requires the precise positioning of Ca^{2+} channels, RyRs, and other proteins within the dyadic "neighborhood", as will be discussed below.

Following release, Ca^{2+} is recycled into the SR by the SR/ER Ca^{2+} ATPase (SERCA) and extruded from the cell by the Na^+/Ca^{2+}-exchanger (NCX) and the plasma membrane Ca^{2+} ATPase (PMCA). The PMCA is believed to be a quite minor contributor to overall Ca^{2+} removal due to its relatively slow kinetics [20]. In addition, it has not been demonstrated that the PMCA is present in the T-tubules, which is a requirement for an efficient Ca^{2+} extrusion pathway. On the other hand, NCX plays an important role in Ca^{2+} extrusion. It is localized both in the surface membrane and in the T-tubular membrane, but with a three times higher density in the T-tubules [21] (Figure 1). The contribution of NCX to total Ca^{2+} removal from the cytosol varies across species from ~30% in the rabbit to ~7% in the rat [22, 23]. The NCX is electrogenic since it exchanges 3 Na^+ for 1 Ca^{2+}, which means that its transport rate is dependent on both the membrane potential and transmembrane concentration gradients for Na^+ and Ca^{2+}. Since ionic concentrations may vary considerably in different submembrane spaces or ion pockets, NCX activity will very much depend on its localization as well as the localization of other nearby Na^+ and Ca^{2+} handling proteins. For example, we have observed that the Na^+/K^+-ATPase α_2 isoform is preferentially localized in the T-tubules where it regulates an Na^+ pool which is shared by NCX [24]. Data from several other studies also support a close relationship between Na^+/K^+-ATPase and NCX function [25–28]. Since NCX and SERCA compete for Ca^{2+}, this local control of Na^+ homeostasis can importantly regulate SR Ca^{2+} load and the magnitude of the Ca^{2+} transient [29]. Thus, alterations in T-tubule structure or more subtle alterations in the dyadic neighborhood would be expected to have important functional consequences.

Although the NCX functions predominantly to extrude Ca^{2+} from the cell (forward mode), it can also function in reverse mode to facilitate Ca^{2+} entry. We have recently shown that NCX-mediated Ca^{2+} influx contributes to an early phase of Ca^{2+} entry which actually precedes that from Ca^{2+} channels [30]. Ca^{2+} influx via reverse-mode NCX can even trigger SR Ca^{2+} release, albeit with low efficiency [31]. We have calculated that such a role requires the presence of an Na^+ channel in the dyad to locally elevate intracellular $[Na^+]$ [30] (Figure 1). However, the localization of both NCX and the Na^+ channel in the dyad has been disputed [32]. Further study of the localization of these proteins is therefore required, both in normal cardiomyocytes and in disease states where the efficiency of NCX-mediated CICR may be altered.

1.3. T-tubule Density/Organization: Control of Ca^{2+} Release Synchrony. The vast majority of studies of T-tubular structure and function have been conducted on ventricular myocytes. Interestingly, other cardiac cell types are often stated to lack T-tubules. In reality, however, T-tubules have been observed in Purkinje cells [33] and their presence has been clearly documented in atrial myocytes from a number of species [34–42], albeit at a lower density than that observed in ventricular cells. To our knowledge, T-tubules have not yet been examined in human atrial cells, but are very likely to be present since large mammals such as sheep ([40, 41] see Figure 2(b), left panel) and dog [42] exhibit a surprisingly high T-tubule density.

The extent and organization of the T-tubule network is an important determinant of the spatial homogeneity of SR Ca^{2+} release throughout the cardiomyocyte. A dense, well-organized T-tubule network, such as that observed in mouse and rat ventricle myocytes, (Figures 2(c) and 2(d), left panels) allows for very synchronized CICR in these cells (Figure 3(a)) [43, 44, 47]. Heinzel et al. [48] demonstrated that a somewhat lower T-tubule density in pig ventricular myocytes was associated with less synchronous Ca^{2+} release (Figure 3(a)). In cat atrial cells, which have a very low T-tubule density, there is a wave-like propagation of the Ca^{2+} transient from the sarcolemma to the cell interior [49] (Figure 3(a)). Thus, in all of these cell types, Ca^{2+} release is initially triggered following influx of Ca^{2+} at the surface sarcolemma and at locations where T-tubules (and Ca^{2+}

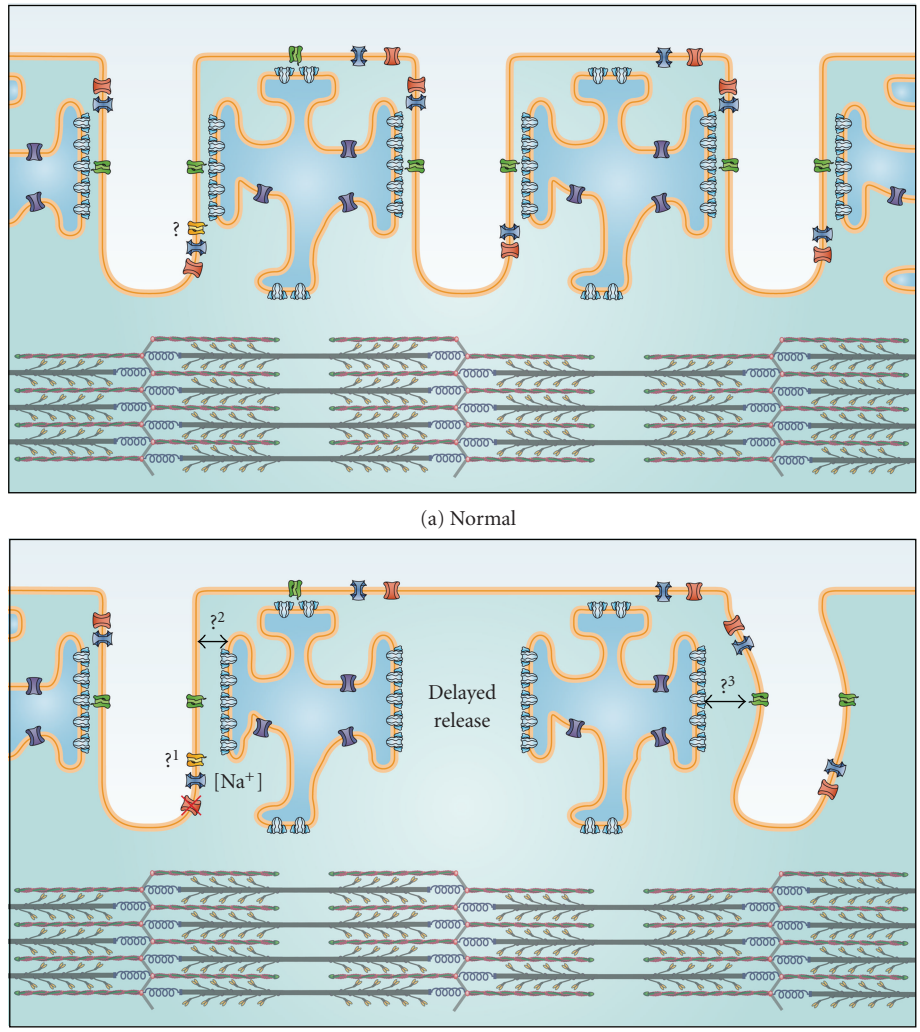
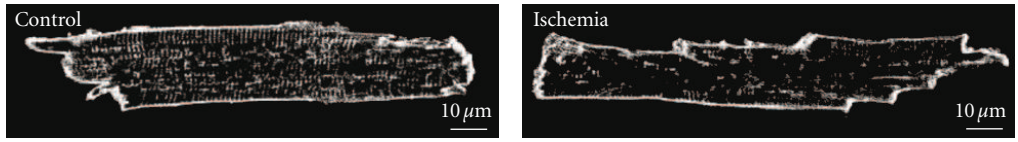
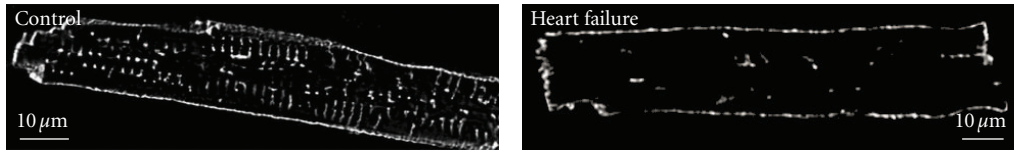


Figure 1: Schematic representation of the dyadic neighborhood in normal and failing cells. (a) Excitation-contraction coupling occurs at functional junctions between Ca^{2+} channels in the T-tubules and ryanodine receptors in the SR. Depending on their localization, other proteins in the dyadic neighborhood such as SR Ca^{2+} ATPase (SERCA), NCX, NKA, and Na^{+} channels can also regulate Ca^{2+} homeostasis. Question mark: The positioning of the Na^{+} channel at the dyad is still controversial. (b) During heart failure, T-tubule loss and/or disorganization occurs leading to the formation of orphaned ryanodine receptors, which do not have apposing Ca^{2+} channels. Ca^{2+} release in these regions is delayed leading to slower and weaker contractions. Other putative alterations in the dyadic neighborhood are indicated by the question marks: (1) it is unclear whether the Na^{+} channel is present in the dyad of failing cardiomyocytes. Some experimental evidence suggests that the distance between the SR and T-tubule is increased in heart failure (2), while T-tubule disorganization may lead to dyadic clefts with variable width (3).

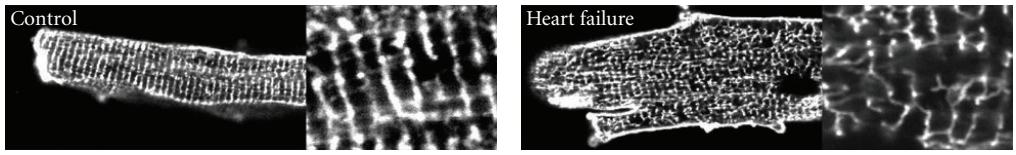
channels) are present, followed by propagation of released Ca^{2+} into regions where T-tubules are absent. Since there is a uniform distribution of RyRs across myocytes [47, 48, 50], this diffusing Ca^{2+} may then trigger SR Ca^{2+} release. Meethal et al. [51] observed spontaneous Ca^{2+} sparks at sites of irregular gaps occurring between adjacent T-tubules in dog cardiomyocytes. This shows the presence of functional RyRs and suggests that a propagating Ca^{2+} wave could trigger CICR at these locations. In atrial myocytes, the extent of CICR propagation into the interior of the cell has been



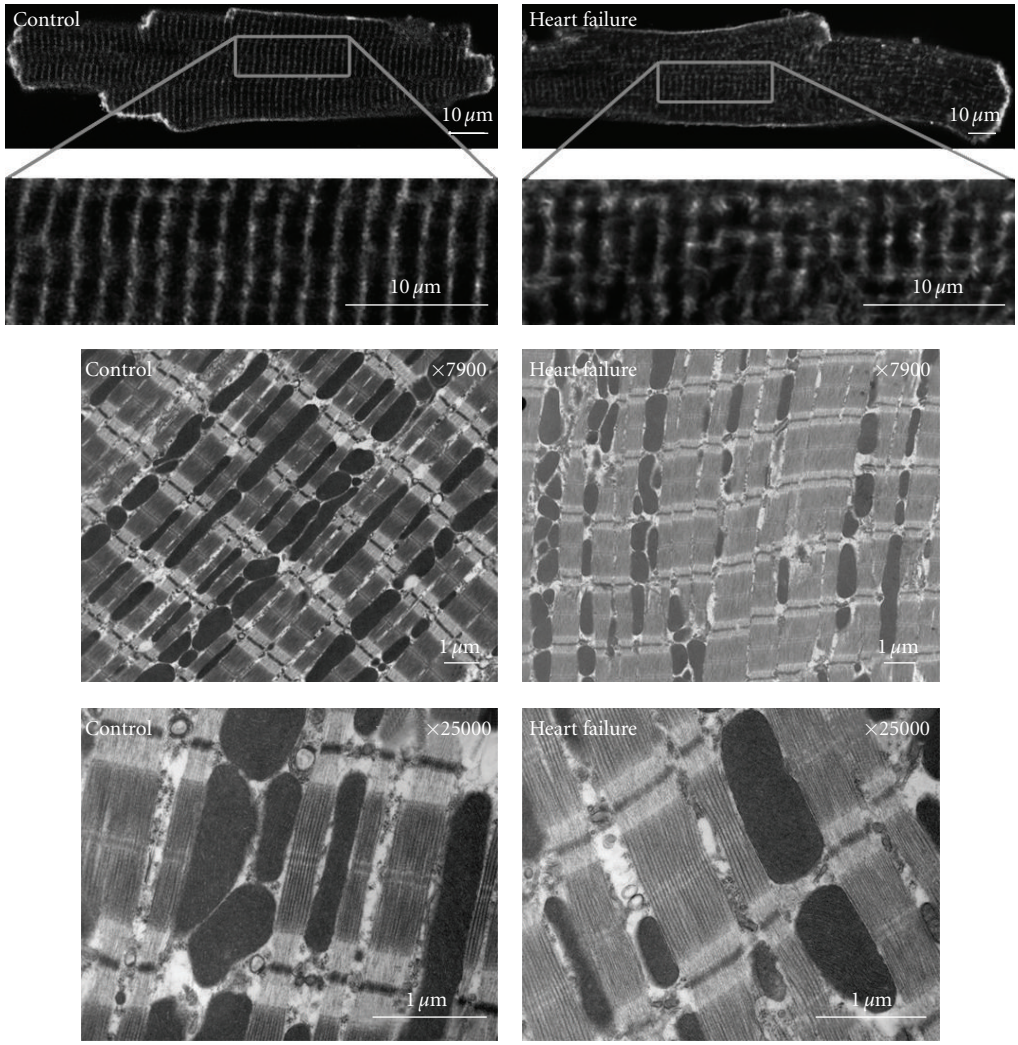
(a) Chronically ischemic myocardium (pig)



(b) Failing atrial cardiomyocytes (sheep)

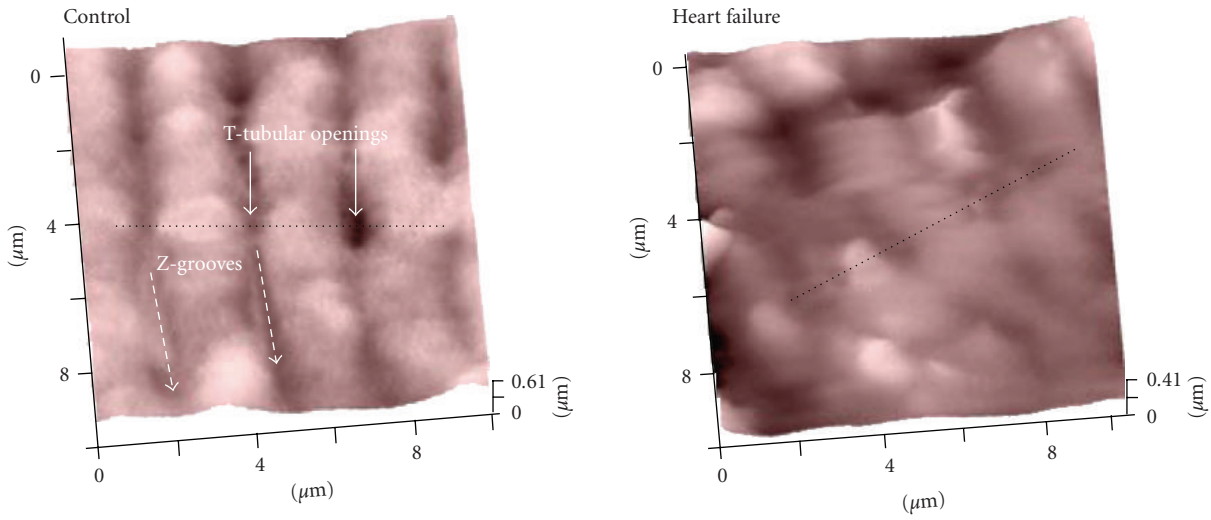


(c) Postinfarction heart failure (mouse)



(d) Postinfarction heart failure (rat)

Figure 2: Continued.



(e) Human heart failure

Figure 2: Examples of T-tubule alterations in various pathological states. Left panels show images from controls; right panels show images from (a) chronically ischemic pig myocardium (from [43]), (b) failing atrial ovine cardiomyocytes (from [40]), and post-infarction heart failure models in (c) mouse (from [44]) and (d) rat (from [45]). Panel (d) shows representative electron micrographs from control and failing rat hearts showing T-tubule disruption. (e) Scanning ion conductance microscope images from the surface of human nonfailing (control) and failing cardiomyocytes showing loss of T-tubular openings (from [46]). All figures are reproduced with permission.

shown to depend on both SR content and β-adrenergic tone [50]. Thus, control of Ca^{2+} release synchrony does not solely rely on T-tubular structure, but also on cardiomyocyte status. These considerations may have particular significance in pathological conditions, as will be described in the following chapters. This discussion will initially and most thoroughly address T-tubule alterations in heart failure, where there are now considerable data available, followed by a briefer discussion of more preliminary data from atrial fibrillation and diabetes.

2. Heart Failure

Heart failure is a progressive and chronic disease, characterized by an impaired ability of the heart to pump blood. This condition is on the rise in the western world, and diagnosis carries an alarmingly high mortality rate of more than 60% after 5 years [54]. Depressed contractility in heart failure is widely believed to result, at least in part, from reduced magnitude of the cardiomyocyte Ca^{2+} transient [29]. A number of studies have indicated that decreased SR Ca^{2+} release in this condition results from lowered SR Ca^{2+} content [55, 56], due to reduced SERCA function and/or greater Ca^{2+} leak from the SR [29]. In addition, the ability of the Ca^{2+} current to trigger CICR, the so-called "gain of Ca^{2+} release", is reduced in failing cells [43, 57–60]. Slowing of SR Ca^{2+} release also occurs in heart failure, which slows contraction and additionally reduces the power of the heartbeat [46, 61–69].

2.1. T-tubule Loss/Disorganization. A growing body of evidence indicates that impaired Ca^{2+} homeostasis in failing myocytes may involve alterations in T-tubular structure. Kamp and colleagues [70, 71] observed loss of T-tubules in ventricular cardiomyocytes in a dog model of tachycardia-induced heart failure. This finding has since been confirmed in other animal models of heart failure including post-infarction rabbit [72] and rat [46], and pigs with chronic ischemia [43] (Figure 2). Similar observations have also been reported in failing human ventricular myocytes [46, 73]. A recent investigation of atrial T-tubules in heart failure also showed dramatic T-tubule loss in sheep following atrial pacing [40] (Figure 2).

Others have not observed decreased T-tubule density in failing myocytes, but rather a structural disorganization. We reported that heart failure progression in mice and rats following myocardial infarction was associated with loss of the uniform, transverse T-tubule pattern, with a greater proportion of tubules present in the longitudinal direction ([44, 45], Figure 2). We additionally observed the appearance of irregular gaps between adjacent T-tubules in failing cells. Similar T-tubular disorganization has been observed in myocytes from failing spontaneously hypertensive rats [47] and in failing human left ventricle [74]. Dilated T-tubules have also been reported in human heart failure [53, 73, 75]. Thus, there is now compelling evidence supporting altered T-tubular structure in heart failure, although it is unclear under what conditions this may be manifested as T-tubule loss or as reorganization.

2.2. Slowed, Dyssynchronous Ca^{2+} Release. The consequences of T-tubule alterations for EC-coupling were initially investigated by experimentally promoting T-tubule loss by either cell culture [53, 76] or detubulation [52, 77]. In both cases,

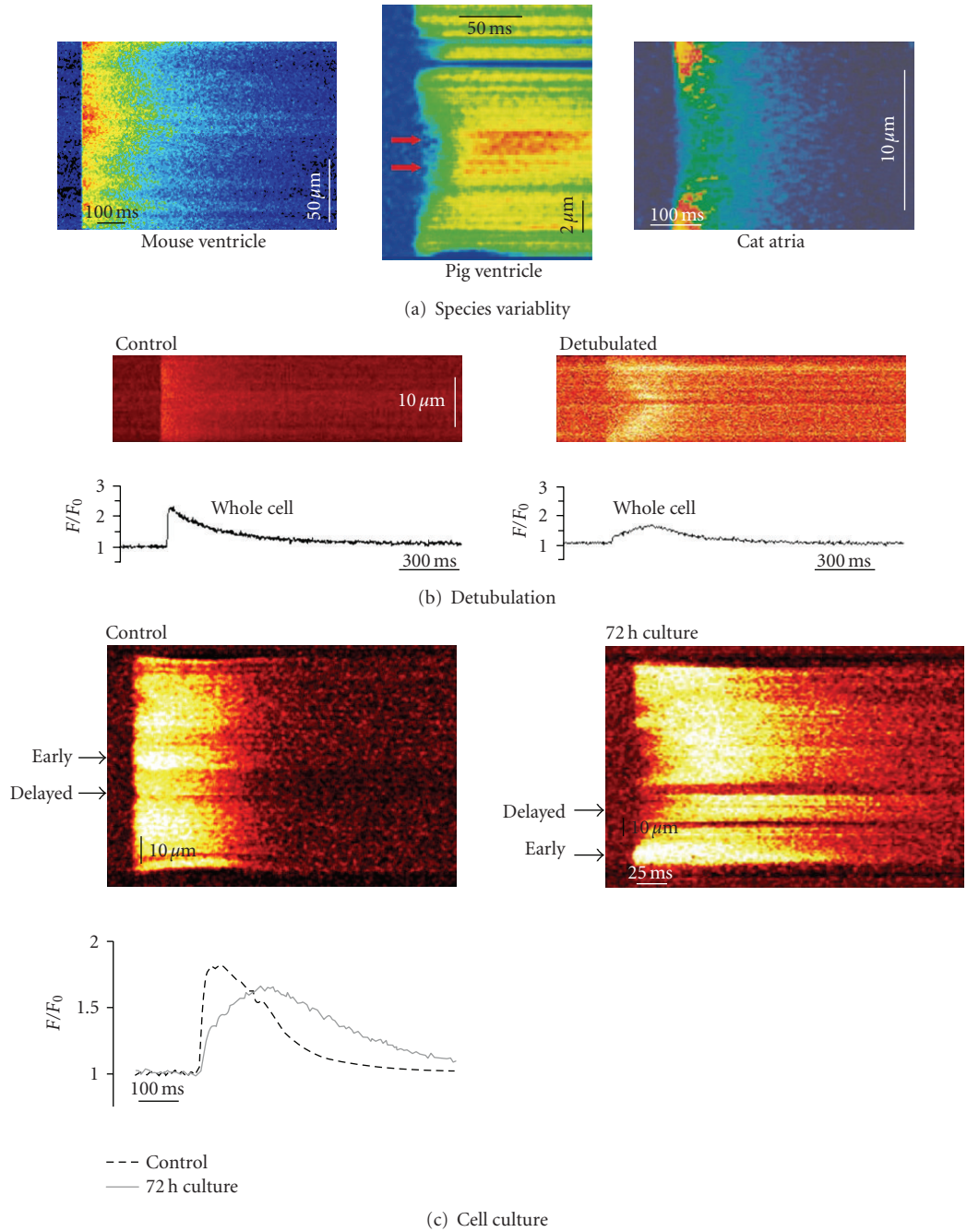


Figure 3: T-tubule density affects Ca^{2+} release synchrony. (a) Variable amount of T-tubules in different cell types affects the homogeneity of the Ca^{2+} transient, as illustrated in confocal line scans of myocytes from mouse ventricle ((a), unpublished data), pig ventricle ((b), from [43]), and cat atria ((c), from [49]). (b) Experimental loss of T-tubules using the detubulation technique causes delayed Ca^{2+} release in the centre of the cardiomyocyte (from [52]). (c) T-tubules are lost when cardiomyocytes are kept in culture resulting in dyssynchronous slowing of the Ca^{2+} transient (from [53]). All figures are reproduced with permission.

T-tubule loss was associated with desynchronization of Ca^{2+} release across the cell. When cells were detubulated, wave-like propagation of the Ca^{2+} transient from the sarcolemma to the cell interior was observed [52, 77, 78] (Figure 3(b)), which resembled the pattern of Ca^{2+} release reported in other cells with very low T-tubule density [8, 49, 79, 80] (Figure 3(a)). With less dramatic T-tubule reduction during cell culture, a fragmented Ca^{2+} release pattern was observed [53] (Figure 3(c)). This indicated that SR Ca^{2+} release was initially triggered at sites where T-tubules were present, followed by propagation into regions devoid of T-tubules [53]. This situation is similar then to that observed in normal

myocytes which have a moderate T-tubular density, such as pig ventricular myocytes ([43], Figure 3(a)), and sheep atrial myocytes [40]. Spatially summating Ca^{2+} release in cells which had lost T-tubules showed that the overall Ca^{2+} transient became slowed and reduced in magnitude [52, 53] (Figures 3(b) and 3(c)). Therefore, such experiments served as proof of principle that large reductions in T-tubule density could reproduce reported alterations in the failing Ca^{2+} transient.

Although dyssynchronous Ca^{2+} release was first observed in failing myocytes by Litwin et al. [81], it was not until later studies by Louch et al. [44] and Song et al. [47] that such alterations were directly linked to alterations in T-tubular structure. In both studies, small regions of delayed Ca^{2+} release were observed to occur at irregular gaps between adjacent T-tubules following T-tubule disorganization (Figure 4). We demonstrated this phenomenon by simultaneously visualizing the T-tubule network and intracellular $[Ca^{2+}]$ (Figure 4(a)). Song et al. [47] employed an alternative approach, and demonstrated that when intracellular Ca^{2+} was highly buffered, regions of delayed Ca^{2+} release did not occur, as Ca^{2+} diffusion into the gaps between T-tubules was prevented. They observed, however, that the ryanodine receptor distribution remained intact in failing myocytes, suggesting that T-tubule disorganization resulted in some ryanodine receptors becoming "orphaned", without opposing Ca^{2+} channels. More recent work has similarly linked T-tubule loss in failing ventricular [43, 46] and atrial myocytes [40] to reduced Ca^{2+} release synchrony. Importantly, as in studies with experimental loss of T-tubules [52, 53, 76, 78], reduced Ca^{2+} release synchrony in failing cells has been shown by a number of investigators to promote slowing and broadening of the overall Ca^{2+} transient [43, 44, 46, 47], a hallmark of the failing condition. Results from studies with experimental reduction in T-tubules (discussed above) suggest that reduced T-tubular organization and/or density also likely contributes to the reduction in Ca^{2+} transient amplitude in heart failure.

2.3. Ca^{2+} Current and Gain of Ca^{2+} Release. The concept of orphaned ryanodine receptors, a spatial mismatching of Ca^{2+} channels and ryanodine receptors (Figure 1(b)), was initially proposed by Gómez et al. [82] to account for reduced gain of Ca^{2+} release in failing myocytes. Recent direct observation of divergent T-tubule and ryanodine receptor localization supports this hypothesis [43, 47]. However, reduced efficiency of the Ca^{2+} release trigger may also have other underlying mechanisms. There is a general consensus that L-type Ca^{2+} current density is unchanged in heart failure when measured during voltage-clamp steps (for review, see [29]). However, prolongation of the action potential and loss of an early repolarization notch in failing cells reduce the driving force for Ca^{2+} entry, resulting in decreased peak Ca^{2+} current [65, 83, 84]. In addition, the time course of Ca^{2+} entry is prolonged during the failing action potential, which reduces efficiency for triggering Ca^{2+} release [65, 84]. Such alterations in Ca^{2+} current desynchronize Ca^{2+} release, as has been demonstrated by switching a voltage-clamped action potential stimulus from a normal human action potential to a failing human action potential [83, 84]. Importantly, this dyssynchronous Ca^{2+} release pattern is variable from beat to beat [83], which distinguishes it from the consistent pattern maintained across beats caused by alterations in T-tubule structure [44, 47]. We believe that slowed, dyssynchronous Ca^{2+} release in failing cells likely results from a combination of alterations in T-tubules and action potentials.

An alterative proposal put forward to explain reduced gain of Ca^{2+} release in failing myocytes is an expansion of the dyadic cleft, resulting in a greater distance between Ca^{2+} channels and ryanodine receptors [60, 82]. To our knowledge, there is not yet direct evidence based on imaging to support this hypothesis. However, we have observed that failing myocytes exhibit a longer delay between the upstroke of the action potential and the upstroke of the Ca^{2+} transient [44], which is consistent with this notion. Importantly, this delay was observed at all locations across the cell, not simply at those locations where large gaps between neighboring T-tubules had led to the formation of orphaned ryanodine receptors. We have proposed that a more subtle drift of T-tubules may occur throughout the cell, which less dramatically increases the T-tubule to SR distance (Figure 1(b)), yet is sufficient to delay CICR and reduce gain [44]. This hypothesis is supported by the findings of Xu et al. [60], who also observed that failing cells exhibited a slowed response of RyRs to the opening of a single Ca^{2+} channel. This was associated with reduced CICR efficiency, since there was a greater chance that RyRs would not open, and Ca^{2+} release which was desynchronized across the cell. Further work employing high resolution imaging and detailed examination of local control of EC coupling is required to confirm the hypothesis that expansion of the dyadic cleft impairs cross-talk between L-type Ca^{2+} channels and RyRs.

While L-type Ca^{2+} current density is generally observed to be unchanged in heart failure [29], the number of Ca^{2+} channels is reportedly reduced [70, 85]. However, increased single channel activity appears to maintain normal Ca^{2+} current density [85, 86], and this may result from increased phosphorylation of the Ca^{2+} channel by protein kinase A and/or CaMKII [86, 87]. Since Ca^{2+} channels are concentrated in the T-tubules, a decrease in Ca^{2+} channel number would, in fact, be expected in failing myocytes if T-tubules are lost. Indeed, experimentally promoting loss of T-tubules results in decreased Ca^{2+} current density [53, 88]. The consequence of reduced Ca^{2+} channel number for EC-coupling in failing cells is unknown. Litwin et al. [81] observed that reduced Ca^{2+} current density in cardiomyocytes isolated from the border zone of post-infarction rabbit promoted dyssynchronous Ca^{2+} transients. It is unclear whether loss of Ca^{2+} channels in failing cardiomyocytes from non-infarcted myocardium might also promote dyssynchrony, but this almost certainly depends on the localization of remaining channels. Are they redistributed between T-tubules and the surface sarcolemma? Some dyads are clearly disrupted by T-tubule reorganization (Figure 1(b)), but do those which remain intact have a normal composition of Ca^{2+} channels and ryanodine receptors? This issue could be addressed by

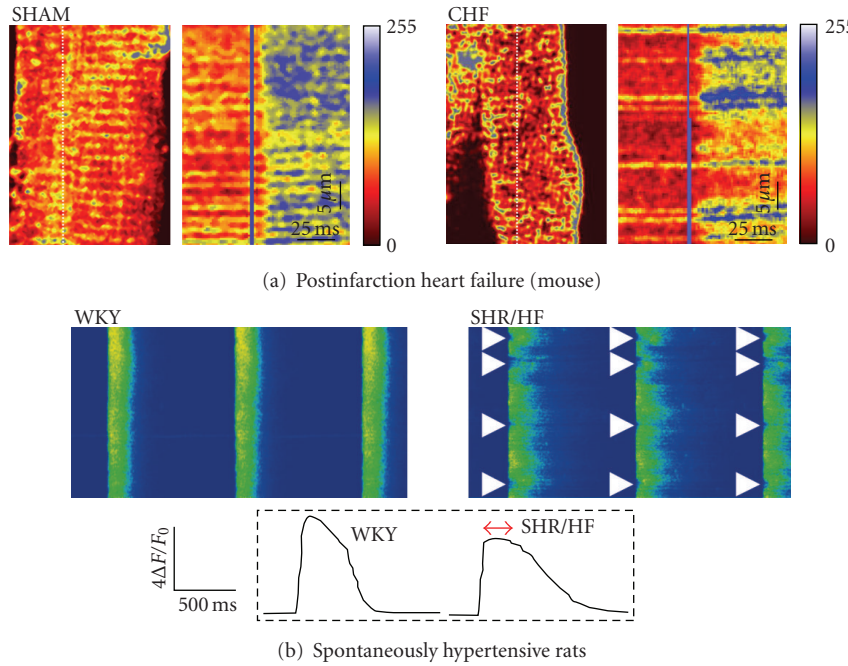


FIGURE 4: Dyssynchronous Ca^{2+} transients in failing cardiomyocytes. (a) Simultaneous imaging of T-tubules stained with di-8-ANEPPS and Ca^{2+} transients in fluo-4-AM loaded myocytes using confocal line scans. The position of the line scan is indicated as a vertical dotted line in T-tubule images, and T-tubules appear as horizontal lines in line-scan images. In myocytes from mice with congestive heart failure (CHF) following myocardial infarction, Ca^{2+} release was delayed in regions lacking T-tubules (right panel), but was synchronous in sham-operated controls (left panel), from [44]. (b) Regions of delayed Ca^{2+} release were also observed in cardiomyocytes from spontaneously hypertensive rats with heart failure (SHR/HF), but not in controls (Wistar-Kyoto (WKY) rats). From [47] Copyright (2006) National Academy of Sciences, USA. All figures are reproduced with permission.

the technique of Hayashi et al. [5], who recently employed electron tomography to estimate the number of RyRs in each dyad based on the calculated dyadic volume. In addition, the number of Ca^{2+} channels present in a couplon could be calculated by detailed analysis of Ca^{2+} sparks [89, 90]. To date, more rudimentary analyses of Ca^{2+} sparks have indicated that sparks in failing myocytes have similar characteristics to those from normal cells [57, 60].

Even without alterations in T-tubular structure, L-type Ca^{2+} current characteristics would be expected to be altered in heart failure. The reduced amplitude and slowed time course of Ca^{2+} release would be predicted to reduce Ca^{2+}-dependent inactivation. However, alterations in Ca^{2+} current kinetics have often not been observed in failing myocytes [87, 91]. Bito et al. [87] have suggested that this apparent discrepancy may result from superimposition of NCX current over Ca^{2+} currents, which impairs detection of altered Ca^{2+} current kinetics. On the other hand, at least at high stimulation frequencies, greater Ca^{2+} current inactivation might be expected in failing myocytes, as lowered SERCA function promotes cytosolic Ca^{2+} accumulation [87, 92–94]. Such alterations may contribute to the negative force-frequency response which is a characteristic of human heart failure [56]. Finally, facilitation of Ca^{2+} current might be altered since this phenomenon is tightly regulated by SR-derived Ca^{2+} release [95], which is reduced in heart failure, and CaMKII [96] which is upregulated [97]. Importantly, loss of T-tubules may reduce facilitation, since Ca^{2+} current is preferentially modulated by SR Ca^{2+} release at the T-tubules rather than at the surface sarcolemma [88]. Indeed, studies conducted to date have shown decreased facilitation in failing human atrial and ventricular myocytes [88, 98, 99].

2.4. Ryanodine Receptor Function. There is growing evidence that ryanodine receptor function is importantly altered in heart failure. In isolated bilayer experiments, Marx et al. observed greater RyR activity [69] and suggested that greater SR Ca^{2+} leak via RyRs contributes to reduced SR Ca^{2+} content. This finding has since been confirmed in intact myocytes [100, 101]. The Marks group proposed that increased leak results from "hyper-phosphorylation" of RyR by PKA, which causes dissociation of FKBP12.6 from RyR [69]. This destabilizes the RyR complex, and causes functional uncoupling of neighboring RyRs [102]. Thus, while arrays of RyRs normally tend to open and close together, RyR uncoupling in failing myocytes is proposed to result in a greater open probability during diastole. However, these findings remain controversial as other groups have not been able to document dissociation of FKBP12.6 from RyR in either healthy or diseased myocytes [103–105]. Instead, recent work has suggested that greater RyR activity in heart failure may result from phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II [106] or

increased relative expression of RyR regulatory proteins triadin and junctin [100]. Regardless of the underlying mechanism, increased SR Ca^{2+} leak is believed to be detrimental in heart failure since reduced SR content impairs contractility. In addition, increased Ca^{2+} leak is thought to be pro-arrhythmic since spontaneously released Ca^{2+} is extruded from the cell by NCX, resulting in afterdepolarizations which may trigger an extra action potential [29].

It is unclear if and how alterations in RyR function in heart failure are related to T-tubular changes. However, Meethal et al. recently reported that in failing cells, spontaneous Ca^{2+} sparks occur more frequently at the irregular gaps between T-tubules created by T-tubule loss [51]. Resting Ca^{2+} levels were also higher at these locations. These observations suggest that orphaned ryanodine receptors exhibit greater activity than those in intact dyads, and that this overactivity may importantly contribute to increased SR leak and arrhythmic potential in failing cells. Are groups of orphaned RyRs also functionally uncoupled? Perhaps, and if so, such alterations might theoretically exacerbate Ca^{2+} release dyssynchrony by slowing propagation of CICR into regions where T-tubules are absent. Similarly, altered SR content and β-adrenergic tone might also influence synchrony by affecting wave propagation speed [77].

2.5. Other Alterations in the Dyadic "Neighborhood". The neighborhood of proteins controlling local Ca^{2+} concentration in the dyad is complex, and includes many more proteins besides L-type Ca^{2+} channels and ryanodine receptors. As described in the introduction, Ca^{2+} regulation is closely linked to the activity of Na^+-handling proteins. NCX expression and activity are increased in heart failure, and reverse-mode function is enhanced due to action potential prolongation, a reduced Ca^{2+} transient, and elevation of intracellular $[Na^+]$ (for review see [107]). Depending on the precise arrangement of the dyadic neighborhood, greater NCX-mediated Ca^{2+} entry might play an increased role in triggering Ca^{2+} release in heart failure. However, such a role depends on the relative proximity of NCX, ryanodine receptors, and the Na^+ channel [30], and these measurements have not yet been reported in failing myocytes. Regardless, increased NCX-mediated Ca^{2+} entry is believed to support the Ca^{2+} transient in heart failure, partially counteracting reductions in SR content resulting from SERCA downregulation [107]. However, Fowler et al. [108] showed that Na^+ accumulation was not translated into SR loading when cells were detubulated, and that this resulted in the development of a negative force-frequency relationship. Therefore, loss of NCX molecules along with T-tubules in failing myocytes may have significant consequences, especially at physiological frequencies. Confirmation of this hypothesis awaits a detailed examination of NCX distribution in failing cells.

Increased Na^+ levels in heart failure [109–111] appear to result, at least in part, from reduced expression and activity of the Na^+-K^+ ATPase [28, 45]. We have observed that the Na^+-K^+ ATPase α_2 isoform is preferentially localized in the T-tubules, where it regulates local $[Na^+]$ near NCX [24]. Therefore, down-regulation of α_2 isoform in heart failure impairs crosstalk between the Na^+–K^+ ATPase and NCX, which may locally elevate $[Na^+]$ and impair NCX-mediated Ca^{2+} extrusion [28, 45]. There is also growing evidence that increased late Na^+ current [112–114] and increased Na^+-H^+ exchanger activity [111, 115] additionally contribute to Na^+ accumulation in failing cells.

Interestingly, although T-type Ca^{2+} channels are normally expressed at very low density in ventricular cells, a number of studies have reported that their expression is up-regulated in hypertrophy and heart failure (for review see [116]). In myocytes from rats with pressure overload, Martínez et al. [117] observed that T-type Ca^{2+} channel entry was one-third that carried by L-type channels, and thus a significant contributor to overall Ca^{2+} influx. It is unknown if T-type channels are expressed in the dyad or T-tubules of failing cells, but if so it is possible that they may importantly modulate local $[Ca^{2+}]$, or even serve as triggers for Ca^{2+} release. However, in possible contradiction to this notion, a recent study employing T-type channel overexpression showed preferential targeting of these channels to the surface sarcolemma [118]. Nevertheless, increased T-type Ca^{2+} channel expression is not without consequence, as it has been shown to play an important role in triggering of pathological cellular hypertrophy [119], and may be pro-arrhythmic [120].

Although the PMCA is believed to be only a minor contributor to Ca^{2+} removal during the decline of the Ca^{2+} transient [20], data from the NCX knockout mouse suggest that this Ca^{2+} pump can have a surprisingly large capacity to extrude Ca^{2+} when challenged [121]. Similarly, we have suggested that PMCA up-regulation might help maintain diastolic function following SERCA knockout [28, 122]. Therefore, with reduced SERCA function and impaired NCX-mediated Ca^{2+} extrusion (due to $[Na^+]_i$ accumulation) in failing cells, the PMCA may theoretically become a more important Ca^{2+} extrusion pathway. However, a recent study by Mackiewicz et al. [123] showed that PMCA function is, in fact, progressively reduced during heart failure progression in rats following myocardial infarction. To our knowledge, no other data on PMCA function in heart failure are currently available, and protein localization is unknown. Based on available information, it appears that PMCA function in heart failure is most importantly involved with signaling pathways underlying cellular hypertrophy [124, 125] and not maintenance of the Ca^{2+} transient.

Another Ca^{2+} transporting protein which has recently come into focus in ventricular cardiomyocytes is the inositol 1,4,5-triphosphate receptor (IP_3R). Initially thought to play only a minor role in ventricular cells since it is markedly outnumbered by the RyRs (50 : 1) [126], recent evidence shows that there is an important upregulation of IP_3Rs in the junctional SR during hypertrophy and heart failure [127]. The authors showed that although Ca^{2+} fluxes through IP_3Rs are smaller than through RyRs [128], enhanced Ca^{2+} release through IP_3Rs in hypertrophic cardiomyocytes increases Ca^{2+} transient magnitude. Although such an effect might be thought beneficial, increased IP_3R-mediated Ca^{2+} release is arrhythmogenic since the close proximity of IP_3Rs and RyRs leads to RyR sensitization [127]. The presence of NCX

nearby IP_3Rs would promote early afterdepolarizations and thus extra-systolic Ca^{2+} transients. However, it remains to be determined how loss/disorganization of the T-tubules affects the distance between NCX and IP_3Rs during heart failure. Ankyrin-B may be importantly involved, since this protein coordinates NCX, NKA and IP_3R in a cardiac T-tubule/SR microdomain [129].

The above discussion illustrates that there are complex modifications in the dyadic neighborhood in heart failure, involving RyR, the L-type Ca^{2+} channel, and Na^+ handling proteins such as the Na^+-K^+ ATPase and NCX. To fully understand the heart failure phenotype, it is critical that alterations in the distribution and function of these proteins are elucidated. This investigation will require a detailed analysis of T-tubule composition, and not simply overt changes in T-tubular structure as has largely been conducted to date.

3. Atrial Fibrillation

Atrial fibrillation is a common type of arrhythmia which is caused by local reentry circuits. This prevents coordinated atrial contraction, which compromises the heart's blood pumping capacity [130]. There is also an increased risk of stroke in this condition since blood pools and clots in the dysfunctional atria [131]. Recent evidence suggests that decreased contractile force of the atrial muscle [132] may, at least partly, result from pathological alteration of T-tubular structure. Lenaerts et al. [41] observed a 45% reduction in T-tubule density in right atrial cardiomyocytes following persistent atrial fibrillation. As in reports from failing myocytes described above, T-tubule loss during atrial fibrillation was associated with spatially dyssynchronous Ca^{2+} release but an intact ryanodine receptor distribution [41]. Therefore, the authors suggested that a reduced efficiency of EC coupling in this condition results from fewer Ca^{2+} channel-RyR couplings (more orphaned ryanodine receptors), and that this effect contributes to reduced Ca^{2+} transient and contraction magnitude.

While some myocyte alterations following atrial fibrillation may resemble those that occur in failing myocytes, there are also important differences. Unlike in heart failure, atrial myocytes following persistent fibrillation are widely reported to exhibit reduced L-type current density [41, 133–136]. Interestingly, L-type Ca^{2+} channel expression is reported to be reduced in both conditions [41, 70, 85, 133], while single channel function is increased [85, 86, 137]. It is unclear why this increase in function is sufficient to maintain normal current density in heart failure but not atrial fibrillation. Another important difference between the two conditions is that SR content is widely reported to be reduced in heart failure [55, 56] but may be normal in atrial fibrillation [41]. This might suggest that loss of T-tubules and associated Ca^{2+} channels is a primary defect in Ca^{2+} homeostasis in atrial fibrillation, but only one component of more complex alterations in heart failure. In both conditions, impaired function of the contractile machinery has also been proposed [138, 139].

Lenaerts et al. [41] were the first to examine T-tubule alterations in atrial fibrillation, but more investigation is required. For example, the importance of slowing of Ca^{2+} release has not yet been demonstrated in this condition, but would be expected to reduce contractile power. As discussed above, important pathological alterations in the dyadic neighborhood may include modified targeting and regulation of Ca^{2+}- and Na^+-handling proteins, and this issue also remains largely unexplored in atrial fibrillation.

4. Diabetic Cardiomyopathy

Many diabetic patients exhibit cardiac dysfunction [140]. In type-2 diabetes, reduced *in vivo* and cardiomyocyte contractile functions are modeled in the db/db insulin-resistant "diabetic" mouse [141–144]. Decreased contractility in these mice is associated with greater SR Ca^{2+} leak, which reduces SR Ca^{2+} content and the magnitude of the Ca^{2+} transient [142, 144]. In addition, the magnitude of the L-type Ca^{2+} current is reduced while single channel activity is increased [141]. This suggests that there are a decreased number of Ca^{2+} channels, similar to that reported in heart failure and atrial fibrillation. In a recent study, Stølen et al. [144] showed that cardiomyocytes from sedentary db/db mice exhibit reduced T-tubule density, which may account for Ca^{2+} channel loss. As expected, T-tubule loss was associated with dyssynchronous SR Ca^{2+} release and reduced Ca^{2+} transient amplitude. Although Ca^{2+} release kinetics were not reported in this study, an expected reduced rate of rise of the Ca^{2+} transient could contribute to the slowed contraction observed *in vivo* [142]. Another interesting observation in myocytes from db/db mice is a longer delay between the stimulus and the upstroke of the Ca^{2+} transient [144]. This is in agreement with our finding in failing post-infarction cardiomyocytes [44], which we have hypothesized reflects an expansion of the dyadic cleft (Figure 1(b)). Although such alterations together with T-tubule loss would be expected to reduce CICR efficiency, Pereira et al. [141] reported unaltered gain of EC coupling in db/db cardiomyocytes. The reason for this apparent discrepancy is unclear, but it may result from other unknown alterations in the dyadic neighborhood.

T-tubule alterations have also been reported in GK/Jcl and SDT/Jcl rat models of type II diabetes [145]. Although T-tubule loss was not observed in these models, the authors showed a disorganization of T-tubules and a decrease in the number of complete SR/T-tubule junctions. Ca^{2+} homeostasis was not investigated in this study, but dyssynchronous Ca^{2+} release would be expected. However, dyssynchronous Ca^{2+} transients have been reported in cardiomyocytes isolated from rats with streptozotocin-induced type-1 diabetes [146]. However, in this study dyssynchrony was attributed to a subpopulation of RyRs (37% of total RyR number) which were unresponsive to Ca^{2+}. They proposed that some of these inactive RyRs may be localized in the dyad, preventing Ca^{2+} influx through L-type Ca^{2+} channels from triggering SR Ca^{2+} release. We suggest that Ca^{2+} channels in these inactive dyads could be described as "functionally orphaned", a reversal

of the situation that arises when ryanodine receptors are orphaned by removal of dyadic Ca^{2+} channels following T-tubule disruption. Interestingly, Shao et al. [146] observed that functional RyRs remaining in diabetic myocytes were hyperphosphorylated, which resulted in greater activity, increased SR Ca^{2+} leak, and reduced SR content [146]. Both Ca^{2+} transients and contractions were reduced in magnitude and exhibited a slowed rate of rise, as expected when SR content is reduced and SR Ca^{2+} release desynchronized. If and how modifications in T-tubular structure contribute to altered Ca^{2+} homeostasis in type-1 diabetes remains to be determined.

5. Mechanisms Controlling T-tubular Structure and Growth

Our discussion thus far has described T-tubules and the dyadic neighborhood in health and disease, but not the mechanisms controlling their formation and maintenance. T-tubules are absent at birth, and develop thereafter by progressive invagination from the surface sarcolemma [2, 10]. Caveolae, which are small bulbous pockets at the cell surface, and the associated protein caveolin are believed to play an essential role in this process [147]. However, RyRs are present in the SR and aligned at the Z-disc very early in development [148]. The formation of dyads is therefore dependent on the later development of T-tubules. Ziman et al. [9] recently showed that dyad formation occurs as junctophilin-2 arrives with the maturing T-tubules. This protein spans the dyadic cleft, and anchors the T-tubular and SR membranes [149]. This role is believed critical for dyad formation, as junctophilin arrival at this location establishes efficient and spatially synchronous EC coupling [9].

Junctophilin alterations may promote loss of dyadic integrity in pathophysiological conditions. The interaction between junctophilin and caveolin-3 is down-regulated in cardiomyopathy [150]. In addition, junctophilin mutations are reported in cardiomyopathy patients [151, 152]. We hypothesize that abnormal junctophilin expression or function may promote drift of L-type Ca^{2+} channels from RyRs as the dyad becomes unanchored, resulting in reduced CICR gain. Indeed, decreased junctophilin expression in the triadin knockout mouse was recently reported to be associated with decreased colocalization of Ca^{2+} channels and RyRs [153].

A tubule-forming protein called amphiphysin-2 may also be involved in T-tubule maintenance. In skeletal muscle, this protein has been shown to be highly concentrated at the T-tubules [154], and Lee et al. [155] reported that expressing amphiphysin 2 in nonmuscle cells induces T-tubule-like invaginations in the plasma membrane. In addition, amphiphysin 2 mutation results in disorganized T-tubular structure in skeletal muscle [156]. Since this protein links the plasma membrane with submembranous cytosolic scaffolds [157], this finding suggests that unanchoring of the T-tubules from the cytoskeleton may lead to T-tubule drift. Although a role of amphiphysin 2 in T-tubule formation and maintenance is not yet established in cardiomyocytes, connections between the sarcolemma and cytoskeleton are well known [158]. Interestingly, failing cardiomyocytes show disorganization of cytoskeleton structure [158], and loss of T-tubules during cell culture has been linked to changes in actin [159]. Therefore, while T-tubule drift may occur if cytoskeletal anchors are disrupted, alterations in the structure of the cytoskeleton itself might also contribute to T-tubule changes in disease states.

During normal myocyte maintenance, lysosomes are known to degrade cellular organelles by autophagy [160, 161]. Interestingly, Meethal et al. [51] recently reported that in both normal and failing cardiomyocytes, lysosomes were present at gaps between T-tubules, suggesting that T-tubule degradation was occurring at these locations. They also observed that T-tubule loss in failing myocytes was associated with increased density of lysosomes. It is as yet unclear whether greater lysosome activity is the initiating event responsible for abnormal T-tubule degradation. Insight into this issue might be provided by an investigation of T-tubules in lysosomal storage diseases, although we are unaware of any such study to date.

The finding that heart failure, atrial fibrillation, and type-2 diabetes are all associated with T-tubule disruption, suggests that this is may be a common outcome triggered by hypertrophy. Interestingly, physiological hypertrophy resulting from training is not associated with T-tubule disruption [144]. This suggests that T-tubule disruption may result from activation of signaling pathways which are specifically involved in pathological hypertrophy.

6. Treatment/Future Perspectives

Based on what is now extensive evidence that altered T-tubular structure may contribute to the pathophysiology of several disease states, it is clear that T-tubules have the potential to serve as therapeutic targets. Preventing T-tubule loss and/or disorganization would be expected to improve Ca^{2+} release synchrony, leading to a more rapid rise of the Ca^{2+} transient and greater contractile power. To our knowledge, the only such intervention reported to date is exercise training. Stølen et al. [144] observed that db/db diabetic mice which underwent aerobic interval training avoided the T-tubule loss and Ca^{2+} release dyssynchrony present in sedentary mice. It is unknown which pathways activated by exercise training afforded this protection.

Development of more targeted treatment strategies will almost certainly rely on an improved understanding of the mechanisms underlying T-tubular maintenance as discussed above. Molecular targets may include components of the cytoskeleton and signaling molecules involved in triggering hypertrophy. Theoretically, promoting growth of new T-tubules could also be beneficial. For example, proliferation of longitudinal tubules, as has been observed in pathological conditions might provide greater opportunity for Ca^{2+} cycling across the sarcolemma, and also SR Ca^{2+} release if dyads form at these new tubules. Synchrony of Ca^{2+} release also might be increased by reducing the occurrence and size of delayed release regions by increasing the velocity at which Ca^{2+} propagates. Although the precise mechanisms

controlling Ca^{2+} wave speed are only beginning to be elucidated, it appears that increasing SR Ca^{2+} content and β-adrenergic tone may promote greater Ca^{2+} release synchrony [77]. Altering T-tubule composition also has therapeutic potential. Strategies aimed at increasing expression of the L-type Ca^{2+} channel and NCX may be beneficial, as we have recently observed that enhanced transsarcolemmal Ca^{2+} cycling can remarkably compensate for impaired SR function [28, 122]. Finally, it may be possible to optimize the role of NCX as a Ca^{2+} release trigger in failing cardiomyocytes by precisely localizing other Na^+ handling proteins in the dyad, or by direct molecular enhancement of NCX activity.

7. Summary

In this review, we have described the dyadic neighborhood established by the close proximity and functional coupling of the T-tubular and SR membranes. This neighborhood allows for tight local control of $[Ca^{2+}]$. It includes Ca^{2+} channels and RyRs, as well as proteins involved with Na^+ homeostasis and maintenance of dyadic integrity. We have summarized data indicating that T-tubule loss and/or disorganization occurs in heart failure, atrial fibrillation, and diabetic cardiomyopathy. Resulting alterations in the dyadic neighborhood are associated with reduced Ca^{2+} release synchrony and impaired efficiency of CICR. Other modifications in EC coupling proteins which may be shared between these disease states include greater RyR leak, decreased Ca^{2+} channel number, and increased single Ca^{2+} channel activity. While the mechanisms responsible for disruption of the dyadic neighborhood remain largely unknown, recent evidence indicates that anchoring of the dyad may be importantly compromised. We suggest that further investigation of these mechanisms will reveal novel therapeutic targets for improving EC coupling in pathophysiological conditions.

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