Conclusions

Ca\textsuperscript{2+} influx can trigger the opening of RyR2 and subsequent electric impulse is conducted into the cell interior, where well-developed network of ventricular T-tubules ensures that ing junctional sarcoplasmic reticulum membrane (SR). A-release units, the ryanodine receptors (RyR2s) on the oppos-
tional in determining the electric and mechanical properties of cardiac muscle.\textsuperscript{1} In adult ventricular myocytes, LTCCs are predominantly located in the transverse tubules (T-tubules),\textsuperscript{2} where they form dyadic complexes with calcium-sensing and release units, the ryanodine receptors (RyR2s) on the opposing junctional sarcoplasmic reticulum membrane (SR). A well-developed network of ventricular T-tubules ensures that the electric impulse is conducted into the cell interior, where Ca\textsuperscript{2+} influx can trigger the opening of RyR2 and subsequent release of SR Ca\textsuperscript{2+} stores. Atrial myocytes are believed to lack an elaborate T-tubule network\textsuperscript{3,5} and their Ca\textsuperscript{2+} signaling is substantially different from that in ventricular myocytes.\textsuperscript{5,8} Lack of a regular T-tubular system has been thought to affect the distribution of LTCCs and give rise to the unique Ca\textsuperscript{2+} signaling in atrial myocytes.\textsuperscript{6,9,10}

Clinical Perspective on p 2384

A number of important LTCC subpopulations have been identified in ventricular myocytes that associate with unique
macromolecular signaling complexes and scaffolding proteins, which enables the modulation of Ca$^{2+}$ signaling. Although the main population of LTCCs is localized to dyadic junctions, extradyadic channels are also associated with the surface membrane. Caveolin-3 (Cav3)-rich signaling microdomains are found to harbor specific LTCCs that may play an important role in modulation of Ca$^{2+}$ signaling, particularly in cells lacking organized T-tubules such as atrial and neonatal myocytes. However, until recently, it was difficult to test this hypothesis because of the lack of appropriate experimental approaches.

The spatial compartmentation of Ca$^{2+}$-signaling complexes was first assessed by immunofluorescence microscopy, but imaging does not provide information on the functionality of channel proteins within a subcellular domain. Recent methodological advances have made it possible to image the topography of a live cardiomyocyte and study the clustering of functional ion channels within specific microdomains. Here, we used a super-resolution scanning patch-clamp technique to study the distribution of functional LTCCs in different structural microdomains of the sarcolemma of rat and human atrial myocytes. We provide direct evidence for 2 distinct subpopulations of atrial LTCCs: one localized in the T-tubules and another linked to caveolae structures. Our findings demonstrate that LTCCs, which are located in caveolae, critically contribute to atrial Ca$^{2+}$ signaling, particularly in cardiomyocytes lacking an organized T-tubule network. These different LTCC subpopulations may underlie the regional heterogeneity of Ca$^{2+}$ signaling and susceptibility to spontaneous Ca$^{2+}$ release events in the atrial. Using a rat model of heart failure (HF), we highlight the relevance of the concept of microdomain-specific remodeling of LTCCs: a disruption in the delicate balance of dynamic interactions between dyadic LTCCs and their cellular microenvironment can alter Ca$^{2+}$ signaling and lead to pathological changes in cellular physiology.

Materials and Methods

Detailed methods are provided in the online-only Data Supplement Methods.

All studies complied with the United Kingdom Home Office regulation governing the care and use of laboratory animals and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Myocyte Isolation, T-Tubule Characterization, and Animal Models

Single atrial myocytes were isolated separately from both left (LA) and right (RA) atrial of control and 16-week post–myocardial infarction rats (online-only Data Supplement Table I). The subcellular T-tubule system was visualized by confocal imaging of Di-8-ANEPPS–stained cells. Surface topography was characterized by scanning ion conductance microscopy which uses a glass nanopipette as a sensitive probe.

Super-resolution Scanning Patch-Clamp With Pipette Clipping Modification

After generating a topographical image of the cell surface by scanning ion conductance microscopy, the tip diameter of the pipette was widened by clipping to increase the area of attachment. The pipette was then lowered to a specific location until it touched the membrane, and a high-resistance seal was established. Recordings were then performed in a cell-attached mode. Controlled widening of the scanning nanopipette tip is described in detail in the online-only Data Supplement Methods. Macroscopic calcium currents were recorded by using the whole-cell patch-clamp technique.

Optical Mapping and Data Analysis

Optical mapping of cells loaded with the Ca$^{2+}$-sensitive fluorescent dye Fluo-4 AM via a complementary metal-oxide semiconductor camera ULTIMA-L (SciMedia, USA Ltd, Costa Mesa, CA) was used to monitor localized changes in [Ca$^{2+}$].

Statistical Analysis

All graphs and statistical analysis were performed by using either GraphPad prism 5 or Origin version 6.1. The average values were calculated throughout all cells studied within the groups and then compared between the groups. Normality was tested using the Kolmogorov-Smirnov test. In cases where data failed the normality test, the nonparametric Mann-Whitney test was used instead of the unpaired Student $t$ test, and the nonparametric Kruskal-Wallis test was used instead of analysis of variance. Statistical differences were assessed with analysis of variance, Student $t$ test, Mann-Whitney test, Kruskal-Wallis test, $\chi^2$, and Fisher exact test as appropriate. All data are expressed as mean±standard error of the mean. A value of $P<0.05$ was considered statistically significant.

Results

In Situ T-Tubule Imaging in Isolated Rat Atrial Preparations

To characterize the atrial T-tubular network, we performed in situ T-tubule imaging on intact rat atrial using whole-mount fluorescence labeling with glycophilic lectin wheat germ agglutinin. We found significant region-dependent heterogeneity in T-tubule structure throughout the atrial. Although the LA myocardium predominantly consisted of cardiomyocytes with T-tubules (Figure 1A, Right), in the RA, we observed 3 groups of cardiomyocytes: cells (1) with organized T-tubules, (2) with disorganized T-tubules, and (3) with absent T-tubules (Figure 1A, Left).

Subcellular T-Tubule System in Rat Atrial Cardiomyocytes

Confocal imaging of Di-8-ANEPPS–stained cardiomyocytes isolated separately from the LA and RA revealed that, although about one-third of cells do not have T-tubules (=39% in RA; 34% in LA), other cardiomyocytes possess a T-tubular network of some sort. We also found cells with organized T-tubular networks similar to those found in ventricular myocytes, and those with disorganized T-tubules (Figure 1B). The majority of atrial myocytes with organized T-tubules were isolated from the LA (=40% versus 2% in LA versus RA). Conversely myocytes isolated from the RA were more likely to have disorganized T-tubule network (26% versus 59% in LA versus RA) (online-only Data Supplement Figure I). In ventricular rat myocytes, T-tubules are distributed regularly at ~2-μm intervals, as demonstrated by Fourier transform of binarized images of T-tubules (Figure 1C). In contrast, the atrial T-tubular network is less dense and less regular than in ventricular myocytes. Atrial myocytes with organized T-tubules showed a smaller, but still distinct peak in the Fourier transform plot (3.1±0.6 AU versus 11.3±0.9 AU in atrial (n=31 cells from 4 rats) versus ventricular myocytes (n=45 cells from 3 rats), $P<0.001$). On the other hand, atrial cells with
Figure 1. Spatial heterogeneity of the atrial T-tubular system: in situ and in vitro measurements. A, In situ confocal imaging of T-tubules (TTs) in intact rat atrial preparation stained with wheat germ agglutinin. In the middle, the schematic outlines of the isolated rat atrial preparation showing the main anatomic features. The enlarged images from the endocardium of the right (RAA) and left (LAA) atrial appendages demonstrate typical atrial myocytes with organized TTs (white arrows), disorganized TTs (red arrows), or mixture of both types. AVN indicates atrioventricular node; CT, crista terminalis; IAS, interatrial septum; IVC, inferior vena cava; SAN, sinoatrial node; SVC, superior vena cava; and TRAB, trabeculae. B, Di-8-ANEPPS membrane staining showing a TT network in ventricular myocytes and in atrial myocytes with organized, disorganized, and absent TTs. Below the confocal images, enlarged areas of 40×5 µm are shown that were binarized and used in TT density and regularity measurements. For structured atrial myocytes, 3D reconstructions of the TTs obtained from confocal stack images are shown (see also online-only Data Supplement Movies I and II). C, Representative distribution of power of the predominant frequency retrieved from 2D Fourier transformation of confocal images (B insets). D, Average width of cells with different
disorganized or absent T-tubules (both in LA and RA) did not show a dominant peak on the Fourier transform plot (Figure 1C). Organization of the atrial T-tubular network correlates with cell width: cells showing organized T-tubular networks were larger than cells with disorganized or absent T-tubules (Figure 1D and online-only Data Supplement Figure II).

**Surface Structures in Rat Atrial Myocytes**

Our previous scanning ion conductance microscopy imaging of rat ventricular myocytes has clearly showed the surface topography to be structured with T-tubule openings arranged along Z-grooves and the domed crests located in between them. To characterize cardiomyocyte topography, we have introduced the Z-groove index: a ratio of the observed Z-groove length to the total extrapolated Z-groove length (as if they were present throughout the entire surface). We applied this analysis to characterize topography of atrial myocytes. We found that the larger an atrial myocyte, the more regular its surface topography. This is consistent with the more organized T-tubule network noted in these larger myocytes. Atrial myocytes with organized surface structures and apparent T-tubule openings, similar to those seen in ventricular myocytes, were wider than those with patchy nonstructured areas on their surface, which in turn were wider than atrial myocytes that entirely lacked surface structures (Figure 1E). Similarly, the Z-groove index was significantly higher in LA versus RA myocytes (0.66±0.03 versus 0.50±0.03 in LA versus RA, respectively, P<0.001), confirming the presence of more structured myocytes in the LA.

**Rat Atrial Myocyte Ca\(^{2+}\) Signaling**

Atrial myocytes differ strikingly from ventricular myocytes in shape, magnitude, and kinetics of subcellular Ca\(^{2+}\) transients and in the dynamic of spontaneous Ca\(^{2+}\) release events, as well.\(^{14}\) These differences were hypothesized to be largely attributed to the distinct structure of atrial T-tubules and to altered distribution of LTCCs and their coupling to RyR, as well.\(^{10}\) Optical mapping of spontaneous Ca\(^{2+}\) activity revealed that atrial myocytes have different Ca\(^{2+}\) cycling than ventricular myocytes. Among all spontaneous Ca\(^{2+}\) release events, Ca\(^{2+}\) waves propagating throughout the entire cell (Figure 2A) were distinguished from nonpropagating Ca\(^{2+}\) release events (Figure 2B). Although we did not distinguish Ca\(^{2+}\) sparks from Ca\(^{2+}\) puffs,\(^{22,23}\) we observed 2 groups of nonpropagating Ca\(^{2+}\) events: one with a smaller amplitude (23±1% of the amplitude of the corresponding paced Ca\(^{2+}\) transients, 60% of all nonpropagating Ca\(^{2+}\) events) and another with a higher amplitude (60±4% of the paced Ca\(^{2+}\) transient amplitude, 40% of all nonpropagating Ca\(^{2+}\) events), which could be attributed to Ca\(^{2+}\) puffs and Ca\(^{2+}\) sparks, respectively.\(^{22}\) In comparison with ventricular myocytes, atrial cells showed a significantly higher frequency of nonpropagating Ca\(^{2+}\) release events following a period of high-frequency stimulation (Figures 2C and 2D and online-only Data Supplement Figure III).

We found that wider rat atrial myocytes exhibited a significantly higher number of spontaneous Ca\(^{2+}\) release events (Figure 2E). No spontaneous Ca\(^{2+}\) release events were observed in cells thinner than 11.0±0.4 \(\mu\)m (\(P=0.002\) versus cells with events, Figure 2E). In addition, nonpropagating Ca\(^{2+}\) release events were observed in atrial myocytes 13.2±0.6 \(\mu\)m wide, whereas propagating Ca\(^{2+}\) waves were found in cells ≥14.9±1.1 \(\mu\)m wide (data not shown). Cardiomyocytes isolated from the LA demonstrated a higher number of Ca\(^{2+}\) release events than RA myocytes (Figures 2F and 2G). In atrial myocytes that exhibited spontaneous Ca\(^{2+}\) release events, the number of events was found to be proportional to cell width, as shown in Figure 2H.

**Spatial Localization of Functional LTCCs**

In adult rat ventricular myocytes, functional LTCCs are predominantly localized in the T-tubules.\(^{2}\) Here, we applied the same super-resolution scanning patch-clamp method\(^{2}\) (Figures 3A through 3C) and found that, in contrast to ventricular myocytes, in rat atrial cells LTCCs were recorded with similar frequency from T-tubules (T-LTCC), crests (C-LTCC), and nonstructured areas (Figure 3D).

Although no difference in LTCC open probability was found in different locations (\(P\) of open) at –6.7mV: 0.060±0.006 for T-LTCCs versus 0.067±0.013 for C-LTCCs, not significant [NS]; and versus 0.053±0.006 in nonstructured area, NS), T-LTCCs possessed 40% higher amplitude at negative voltages in comparison with the other 2 groups of channels (Figure 3E). Like many other types of channels, LTCCs show multiple subconductance levels in addition to the largest and the main open state of the channel.\(^{24,25}\) Examples of these substates are given in Figure 3F, Top, and online-only Data Supplement Figure IV. As summarized in Figure 3F, Bottom, C-LTCCs exhibited low-amplitude subconductance states more frequently than T-LTCCs: open probability of low-amplitude subconductance states was 0.027±0.005 and 0.005±0.002 for C-LTCCs (n=4) and T-LTCCs (n=5), respectively, \(P<0.01\).

In addition, we explored microdomain-specific distribution of functional LTCCs in several human RA samples (Figure 4A). On average, human atrial myocytes had a Z-groove index of 0.57±0.02 (n=27), which is similar to that measured in rat RA. Similar to rat, human LTCCs were recorded with similar frequency from T-tubules and crest (Figure 4B). We did not observe any difference in the voltage-current characteristics between the areas (Figure 4C). However, in contrast to rat, human T-LTCCs had significantly higher open probability than C-LTCCs: \(P\) of open at –6.7mV: 0.03±0.002 for T-LTCCs versus 0.017±0.001 for C-LTCCs, \(P<0.001\).

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**Figure 1 Continued.** TT structure isolated from left ventricle (LV, n=45 cells from 3 rats) and LA and RA (n=29/2, 26/35, and 22/38 cells from 6 rats for organized, disorganized, and absent TTs for cells from LA/RA, respectively). \(^*\) \(P<0.001\) versus atrial myocytes; \(**\) \(P<0.001\) versus other cell groups within the atrium by unpaired Student \(t\) test. E, Correlation between surface structure and cell size. Optical images and topography scans (zoomed areas) of a ventricular myocyte and atrial myocytes with various degrees of organization of surface structures are shown. TTs, crests, and nonstructured areas are indicated by arrows. Note that the cell shown in the right-most panel does not possess any organized surface structures.
Caveolae as a Source of Extradyadic LTCCs

It has been demonstrated in ventricular myocytes that some LTCCs could be housed in caveolae.\textsuperscript{12,13} We hypothesized that atrial extratubular LTCCs recorded from the crests and nonstructured areas might be localized to caveolae. To address this hypothesis, we used 2 different approaches to disrupt caveolae: treatment with methyl-\textbeta-cyclodextrin (M\textbeta-CD) and direct LTCC inhibition in Cav3-containing membranes using a specific peptide inhibitor Rem.
Incubation of atrial myocytes with 10 mM MβCD for 30 minutes at room temperature resulted in ~60% depletion of caveolae (Figure 5A), with no changes in cell topography and T-tubule density (online-only Data Supplement Figure V). While MβCD did not affect LTCCs occurrence in the T-tubules (28% versus 28%, before and after MβCD, NS), it completely abolished the occurrence of extratubular LTCCs (30.1% versus 28%, before and after MβCD, NS). In contrast, MβCD did not affect LTCCs occurrence in the T-tubules and T-tubule density (online-only Data Supplement Figure V).

**Ignition of Ca^{2+} Sparks Through Caveolae**

Along with changes in LTCC distribution, MβCD significantly suppressed the occurrence of spontaneous Ca^{2+} release events in rat atrial myocytes (online-only Data Supplement Figure VIII). MβCD treatment significantly decreased the number of spontaneous Ca^{2+} sparks, but not waves (Figure 5E) and reduced the number of cells featuring spontaneous Ca^{2+} release events (Figure 5F). Moreover, removing caveolae via MβCD treatment also reduced the amplitude of Ca^{2+} sparks in atrial myocytes (Figure 5G), as has been previously demonstrated in neonatal ventricular myocytes and arterial smooth muscle cells.17

**Caveolae-Targeted LTCC Antagonist Eliminates Occurrence of Extradyadic LTCC Current**

To confirm that extratubular LTCCs are localized to Cav3-associated caveolae structures, rather than lipid rafts, we used a Cav3-targeted LTCC-blocking agent, Rem peptide.22 The caveolae-targeted LTCC blocker (Rem1-265-Cav) was generated by molecular modification of Rem, a member of the RGK GTPase family that is known to potently inhibit LTCCs.23 Makarewich
et al. have demonstrated that in rat ventricular myocytes Rem Cav localizes to plasma membrane specifically within caveolin-containing lipid rafts, rather than lipid rafts in general, and does not displace molecules normally found in caveolae.

With 60% to 65% infection efficiency, expressing Rem in rat atrial myocytes resulted in a significant decrease in the occurrence of the single LTCC on the crest area of sarcolemma (Figure 6A) without affecting LTCC biophysical properties (Figure 6B). Similarly, Rem Cav decreased spontaneous Ca release events in infected cells (Figure 6C). Truncation of the membrane-docking domain in the Rem Cav peptide (Rem) resulted in the inability of Rem to affect the occurrence of the single LTCC current on the crest and to change spontaneous Ca events (Figure 6). No changes in the single channel open probability were observed.

**HF Model and Structural Changes in the Atrial**

To study disease-associated atrial remodeling, we used the 16 weeks of post–myocardial infarction rat model of HF. This model recapitulates many features of chronic HF in patients including adverse remodeling of the organ, characterized by left ventricle dilatation, reduced ejection fraction, and raised filling pressures (online-only Data Supplement Table I). Atrial cellular hypertrophy was noted with an increase in planar width (Figure 7A).

Similar to control, in HF we also observed 3 groups of cardiomyocytes, although cells with organized T-tubules were found exclusively in the LA (Figure 7B). Despite cell hypertrophy, HF led to a profound degradation of the T-tubule system in all groups of cells, decreasing the proportion of structured myocytes and reducing the T-tubule density in both LA and RA (Figure 7C). Similarly, HF resulted in the loss of surface structures and a reduction in Z-groove index (0.57±0.01 versus 0.45±0.02 in control versus HF, P<0.001).

**Functional Atrial Remodeling in HF**

Along with the degradation of the atrial T-tubular system, HF also caused altered Ca cycling. We found a ≈30% increase in spontaneous Ca spark frequency and a 3-fold increase in wave frequency (Figure 8A and 8B). The average amplitude of nonpropagated Ca events was increased, perhaps suggesting a greater proportion of high-amplitude events (Ca events), as opposed to low-amplitude events (Ca puffs) (23/77 versus 60/40 for Ca puffs/sparks in HF versus control, respectively). In addition, failing atrial myocytes were associated with slower and wider Ca waves (Figure 8D and online-only Data Supplement Figure IX), which may result from T-tubule degradation and increase time required for dyad-to-dyad Ca propagation.

**Microdomain-Specific Remodeling of Atrial LTCCs in HF**

When characterized by the super-resolution scanning patch clamp, LTCCs in HF cells demonstrate the same equal distribution between T-tubules and crests as observed in control cells (34.3% and 30.5% versus 28% and 30.1% for T-LTCCs and C-LTCCs in HF versus control, respectively, NS). This corresponds with the similar caveolae density in HF and control myocytes (3.7±0.5 caveolae/µm in control versus 4.6±0.4 caveolae/µm in HF, P=0.167). Although we did not observe any significant changes in open probability for T-LTCCs and C-LTCCs in HF, we found that T-LTCCs had ≈25% smaller amplitude in HF in comparison with control T-LTCCs (Figure 8E). No changes in C-LTCC amplitude were revealed in HF. The decrease in T-LTCC amplitude in HF was associated with a change in the accessibility of the channel subconductance states (Figure 8F). In HF, the occupancy of low-amplitude substates becomes more accessible than high-amplitude substates in comparison with control (0.018±0.006 and 0.006±0.002 versus 0.006±0.002 and 0.075±0.005 for low- and high-amplitude subconductance states in HF versus control, respectively).

**Discussion**

**Functional Anatomy of Atrial Myocardium**

Atrial myocytes have long been perceived as having no or very few T-tubules. However, recent experimental evidence...
Glukhov et al  LTCC Distribution in Atrial Myocytes

Figure 5. Cholesterol depletion removes caveolae, abolishes the occurrence of extratubular LTCCs decreasing whole-cell $I_{Ca,L}$ and suppressing spontaneous Ca$^{2+}$ sparks. A, Ultrastructural changes in rat atrial myocytes after methyl-$\beta$-cyclodextrin incubation. Electron micrographs of representative control (Left) and 10 mM methyl-$\beta$-cyclodextrin (M$\beta$CD)-treated rat atrial (Right) myocytes are shown. Caveolae are marked by arrowheads. Right, Caveolae per micrometer in the cellular membrane before and after M$\beta$CD treatment (n=16 for control and n=10 for M$\beta$CD group, n=3 rats per group). *** $P<0.001$ by unpaired Student t test. B, Typical 10×10 µm topographical scans of control (Left) and M$\beta$CD-treated (Right) rat atrial myocytes. Below are single-channel recordings obtained from the T-tubule (TT) and the crest of sarcolemma (Crest). C, Percentage of LTCC current occurrence in the T-tubules and crests. *** $P<0.001$ for Crest M$\beta$CD LTCC versus other groups, by Fisher exact test. D, Whole-cell $I_{Ca,L}$ density (n=9 for control and n=12 for M$\beta$CD groups) before and after M$\beta$CD treatment. * $P<0.05$ by unpaired Student t test. E, Along with changes in $I_{Ca,L}$, M$\beta$CD significantly suppressed the occurrence of spontaneous Ca$^{2+}$ sparks. *** $P<0.001$ by Mann-Whitney test. The proportion of cells with spontaneous Ca$^{2+}$ events (F) and Ca$^{2+}$ spark amplitude (G) were also decreased following the treatment. Ca$^{2+}$ spark amplitude was calculated as a percentage from the amplitude of electrically induced Ca$^{2+}$ transient measured during 4-Hz pacing. n=82 cells from 12 rats and n=99 cells from 7 rats for control and M$\beta$CD groups, respectively. * $P<0.05$, ** $P<0.01$ by unpaired Student t test. LTCC indicates L-type calcium channel.

demonstrates that atrial myocytes from certain species, such as sheep, cows, horses, humans, and even rodents do possess an organized T-tubule network. In general, atrial T-tubules are sparse and less regular when compared with those in ventricular myocytes as assessed both in situ and in vitro. Our observations of T-tubules in rat atrial myocytes are consistent with those previously observed using electron microscopy and fluorescent membrane labeling. In the present study, we found significant heterogeneity of T-tubule organization between LA and RA, which might correlate with the arrangement of pectinate muscle bundles within the atrial appendages. It has been reported in rabbit atrial that myocytes isolated from the crista terminalis were significantly larger than those from the pectinate muscles, whereas the shape (the ratio of the length to the width) was found to be similar in the 2 types of cells.

Anatomic heterogeneity of the T-tubular system within the atrial has been proposed to underlie heterogeneous calcium
current measured within the right atrium, in addition to different expression of LTCCs. In healthy dogs, Ca2+ influx through LTCCs was found to be largest in crista terminalis cells, intermediate in cells from the appendage and pectinate muscles, and smallest in atrioventricular ring cells. In contrast, in rabbit right atrial myocytes isolated from different areas, whole-cell clamp recordings showed no definite variation in the density of the voltage-dependent LTCCs. Frisk and colleagues used both isolated atrial cells and tissue to demonstrate that in pig and rat atrial there was a high variability in the distribution of T-tubules and L_\text{Ca} among cells, with a steep dependence of L_\text{Ca} on atrial myocyte capacitance and T-tubule density. The authors observed more T-tubules in the epicardium than in the endocardium, which may contribute to the synchronization of contraction across the atrial wall. Thus, anatomic heterogeneity of the T-tubule network and L_\text{Ca} may explain complex features of atrial electric and mechanical activity including highly anisotropic physiological activation patterns, action potential duration distribution, and contractile function. In addition, disease-associated loss of T-tubules found in HF and atrial fibrillation may have an impact on calcium cycling and promotes the development of triggers of arrhythmia. One might hypothesize that distinct anatomic regions within the atrial will differentially respond to the stress and thus promote the propagation of arrhythmia triggers at specific locations aroused from superposition of areas of significant T-tubular degradation with those of profound molecular remodeling. The mechanisms that result in the development of such trigger “hot spots” require special investigation and will be the focus of our follow-up studies.

**Role of Caveolae Structures in Atrial Ca2+ Signaling**

It has been proposed that, because of the lack of a regular T-tubule system in atrial myocytes, differential spatial distribution of LTCCs with regard to their coupling to RyR2s may underlie a unique atrial myocyte Ca2+-signaling process. In the present study, we uncovered for the first time the distinct distribution of functional atrial LTCCs in the sarcolemma where they appear at a similar frequency both in the T-tubules and the crests, in contrast to ventricular myocytes where LTCCs were primarily clustered in the T-tubules. We demonstrated the importance of the extradyadic channels, which are predominantly located in caveolae, in the regulation of Ca2+ signaling, particularly in cardiomyocytes lacking an organized T-tubule network. The function of LTCCs localized in caveolae remains open to question. It has been proposed that some LTCCs housed in Cav3-rich microdomains, could play an important role in the modulation of Ca2+ signaling. Indeed, local spontaneous Ca2+ release events are plentiful and, in contrast to ventricular myocytes, seem to be normally present in healthy atrial.

Walden and colleagues demonstrated important differences in Ca2+-handling mechanisms between ventricular and atrial myocytes: atrial myocytes were found to have a more robust and abundant Ca2+ uptake mechanism and a higher SR Ca2+ content than ventricular myocytes. Higher SR Ca2+ content in atrial myocytes has been proposed to lead to Ca2+ overload and thus increase the sensitivity of RyR2s to cytosolic [Ca2+]i. This means that 1 Ca2+ spark is more likely to trigger another Ca2+ spark in atrial myocytes but remains a rare event in normal ventricular myocytes. Therefore, the benefit of the elevated SR Ca2+ content is that it should improve the synchrony of the atrial [Ca2+] transient when T-tubules are disorganized or absent. The downside is that in atria the elevated SR Ca2+ content and enhanced [Ca2+]SR lead to the increased sensitivity of the Ca2+-induced Ca2+ release process. For thin cells, or thick cells with organized T-tubules, the Ca2+ signal propagation is likely to be relatively stable. However, larger myocytes with disorganized T-tubules may have an increased propensity toward subcellular Ca2+ alternans and thus appear to be more prone to Ca2+ sparks as demonstrated in our study (Figure 2). Uncontrolled
Along with elevated diastolic [Ca^{2+}], complexes such as diastolic [Ca^{2+}] alternans, will activate the Na/Ca exchanger and thereby generate Na/Ca exchanger current (I_{Na,Ca}) between action potentials with subsequent induction of delayed afterdepolarizations. Thus, the high SR Ca^{2+} content in atrial myocytes may be proarrhythmic. Such arrhythmogenic tendencies are unmasked in HF as cells undergo hypertrophy and the T-tubule system degrades (Figures 7 and 8). Slower and wider Ca^{2+} waves observed in failing cells in the present study would lead to prolonged depolarization time required for Na/Ca exchanger activation and thus they are more likely to result in the formation of delayed afterdepolarizations observed in failing atrial. Along with elevated diastolic [Ca^{2+}], SR Ca^{2+} overload, observed in HF, it would result in the formation of foci of triggered ectopic activity located within areas of significant T-tubular degradation.

**Unique Atrial Myocyte Ca^{2+} Signaling**

It is possible that the mechanism behind the localized spontaneous Ca^{2+} release events in atrial differs from that in ventricles. In rat ventricular myocytes, 85% of all Ca^{2+} sparks evoked by electric stimulation occur within 0.5 µm of a T-tubule, and formamide-induced detubulation significantly reduces Ca^{2+} sparks in rat ventricular myocytes, suggesting an important role for T-tubules in Ca^{2+} spark initiation. Despite the broad distribution of RyRs in atrial myocytes principally along the Z-lines, most Ca^{2+} sparks occur within 1 µm of the sarcolemma. In contrast to ventricular myocytes, where the close (≈12 nm) proximity of RyRs and LTCCs in dyadic junctions of T-tubules facilitates Ca^{2+} release from the SR, atrial myocytes have an additional, functionally separated nonjunctional Ca^{2+} release site in the central SR not associated with T-tubules. Based on immunochemical experiments, these 2 kinds of atrial Ca^{2+} release sites were proposed to differ in their probability of initiating a Ca^{2+} spark, with some being designated as eager sites, whereas others normally fail to spark. As demonstrated by Woo et al in rat atrial myocytes, although the frequency of spontaneous unitary Ca^{2+} sparks was significantly higher in the dyads, the compound sparks, that is, localized Ca^{2+} release composed of several unitary events occurring synchronously and occupying >2 sarcomeres, appeared more prevalent in nonjunctional sites. These findings support the idea that the retarded dissipation of unitary nonjunctional focal Ca^{2+} releases may facilitate the activation of neighboring release sites, leading to recruitment of a larger number of units that which in turn improves synchronicity of the atrial [Ca^{2+}] transients despite their less organized T-tubule network.

Nonjunctional Ca^{2+} events might be attributed to inositol-1,4,5-trisphosphate (IP_{3})-dependent activation of nonjunctional RyRs. Recently, Horn et al have shown that IP_{3} can effectively modulate RyR openings and Ca^{2+} spark probability. Direct interaction between Cav3 and IP_{3}-associated G_{q}-protein-coupled receptor-signaling pathway has been demonstrated in canine ventricular myocytes which can link IP_{3}-dependent nonjunctional Ca^{2+} events to caveolae and explain a significant decrease in occurrence of spontaneous Ca^{2+} sparks observed in atrial myocytes treated with MjBCD (Figures 5E through 5G). Interestingly, a similar reduction in Ca^{2+} sparks via direct inhibition of caveolae-housed LTCCs by Rem protein (Figure 6) highlights the importance of these channels in the initiation of Ca^{2+} sparks. Alternatively, it is possible that MjBCD- or Rem265-Cav3-induced reduction in the whole-cell I_{Ca,L} decreases the steady-state SR Ca^{2+} load and thus suppresses the occurrence of spontaneous Ca^{2+} transients observed in our experiments.

**Microdomain-Specific Remodeling of Atrial LTCCs in HF**

Decrease in atrial I_{Ca,L} in HF has been shown in both animal models and patients with congestive HF. Taking into account the T-tubule degradation, one would expect a reduction of the number of functional channels to be mainly responsible...
for the reduction in $I_{\text{Ca,L}}$. However, as we demonstrated in the present study, an additional mechanism responsible for the reduction in $I_{\text{Ca,L}}$ in failing atrial might be represented by the reduction in the amplitude of T-LTCCs. Despite the decreased $I_{\text{Ca,L}}$, an increase in SR Ca\(^{2+}\) load (caffeine-induced [Ca\(^{2+}\)]\(_{\text{i}}\) release) has been observed in failing atrial.\(^4^4\)\(^4^9\) In addition to the increased SR Ca\(^{2+}\) loading, a significant reduction in calsequestrin expression has been found in failing atrial,\(^4^4\) and this has been linked to increase in SR Ca\(^{2+}\) leak and atrial arrhythmogenesis, perhaps as a result of decreased SR Ca\(^{2+}\) buffering.\(^3^0\) Both SR Ca\(^{2+}\) leak and elevated diastolic [Ca\(^{2+}\)]\(_{\text{i}}\) may affect T-LTCC current, causing a reduction in amplitude, either through Ca\(^{2+}\)-dependent inactivation or phosphorylation (Figures 8E and 8F). Therefore, a disruption in the delicate balance of dynamic interactions between dyadic LTCCs and their microenvironment may alter Ca\(^{2+}\) signaling and can lead to pathological changes in cellular physiology. This extends beyond the classical concept of electric remodeling, stressing that alterations of spatial compartmentation of ion channels and receptors are responsible for pathology, in addition
to classically appreciated changes in protein expression and posttranslational modifications.

**Limitations**

In the present study, we used MβCD to disrupt caveolar structures that might have some potential side effects on cellular electrophysiology. However, it is the only available approach to physically destroy caveolae at the moment and it is widely used by many groups, without significant effects on T-tubule structure. Accordingly, in our study, after 30 minutes treatment with 10 mM MβCD, no changes in cell topology and T-tubule density were found (online-only Data Supplement Figure V). In addition, MβCD treatment did not affect T-LTCC biophysical properties; neither T-LTCC occurrence nor their open probability and current-voltage characteristics were altered. Moreover, similar results on LTCC distribution and Ca2+ activity were obtained with use of a specific Cav3-targeted LTCC-blocking agent, Rem peptide, suggesting no direct effect of MβCD on LTCC. Therefore, the influence on the use of MβCD in this study should be limited, if there is any, and will not affect the result and the deducting conclusion regarding the LTCC distribution and functioning.

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**Disclosures**

None.

**References**

1. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Anna Rev Physiol*. 2008;70:23–49. doi: 10.1146/annurev.physiol.70.113006.100455.

2. Bhargava A, Lin X, Novak P, Mehta K, Korchev Y, Delmar M, Gorelik J. Unique atrial myocyte Ca2+ signaling. *J Mol Cell Cardiol*. 2009;46:448–451. doi: 10.1016/j.yjmcc.2008.12.004.

3. Glukhov et al. LTCC Distribution in Atrial Myocytes 2383

4. Kirk MM, Izu LT, Chen-Izu Y, McCulle SL, Wier WG, Balke CW, Trafford AW, Richards MA, Clarke JD, Saravanan P, Voigt N, Dobrev D, Eisner DA, Balijepalli RC, Foell JD, Hall DD, Hell JW, Kamp TJ, Louch WE, Dirksen RT, Beam KG. Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca2+ channels expressed in dyogenic myotubes. *Proc Natl Acad Sci U S A*. 1998;95:1903–1908.

5. Richards MA, Clarke JD, Saravanan P, Voigt N, Dobrev D, Eisner DA, Trafford AW, Dibb KM, Clarke JD, Eisner DA, Trafford AW. Functional role for transverse (t-) tubules in the atria. *J Mol Cell Cardiol*. 2013:58:84–91. doi: 10.1016/j.yjmcc.2012.11.001.

6. Frisk M, Koivumäki JT, Norseng PA, Maleckar MM, Sejersted OM, Löhn M, Fürstenau M, Sagach V, Elger M, Schulze W, Luft FC, Haller H, Gollasch M. Ignition of calcium sparks in arterial and cardiac muscle through caveolae. *Circ Res*. 2000;87:1034–1039.

7. Grabner M,Dirksen RT,Bean KG. Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca2+ channels expressed in dyogenic myotubes. *Proc Natl Acad Sci U S A*. 1998;95:1903–1908.

8. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Reretta RM, Rizzo V, Molkentin JD, House SR. A caveole-targeted L-type Ca2+ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. *Circ Res*. 2012;110:669–674. doi: 10.1161/CIRCRESAHA.111.264428.

9. Balijepalli RC, Foell JD, Hall DD, Hell JW, Kamp TJ. Localization of LTCC and Ca2+ channels in a caveolar macromolecular signaling complex is required for beta(-2)-adrenergic regulation. *Proc Natl Acad Sci U S A*. 2006:103:7500–7505. doi: 10.1073/pnas.0503465103.

10. Brette F, Despa S, Bers DM, Orchard CH. Spatiotemporal characteristics of SR Ca2+ uptake and release in detubulated rat ventricular myocytes. *J Mol Cell Cardiol*. 2005;39:804–812. doi: 10.1016/j.yjmcc.2005.08.005.

11. Dobrev D, Teos LY, Lederer WJ. Unusual atrial myocyte Ca2+ signaling. *J Mol Cell Cardiol*. 2009;46:448–451. doi: 10.1016/j.yjmcc.2008.12.004.
Heart failure is one of the most important causes of arrhythmic atrial remodeling. The overall risk of atrial arrhythmias increases almost 6-fold when heart failure is present. Despite these troubling statistics, the mechanisms of atrial remodeling in the settings of heart failure are still poorly understood. In this study, we introduced a novel concept of electrophysiological remodeling resulting from changes in the subcellular compartmentation of calcium-signaling complexes, accompanied by a disruption of cellular structure. We provide direct evidence for 2 distinct subpopulations of functional L-type calcium channels, and their posttranslational modifications. We anticipate that our assay will be a development platform for more sophisticated therapeutic approaches for atrial-remodeling treatments based on the subcellular distribution of their targets.