

Role of the transverse-axial tubule system in generating calcium sparks and calcium transients in rat atrial myocytes

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Cardiac atrial cells lack a regular system of transverse tubules like that in cardiac ventricular cells. Nevertheless, many atrial cells do possess an irregular internal transverse-axial tubular system (TATS). To investigate the possible role of the TATS in excitation–contraction coupling in atrial myocytes, we visualized the TATS (labelled with the fluorescent indicator, Di-8-ANEPPS) simultaneously with Ca²⁺ transients and/or Ca²⁺ sparks (fluo-4). In confocal transverse linescan images of field-stimulated cells, whole-cell Ca²⁺ transients had two morphologies: ‘U-shaped’ transients and irregular or ‘W-shaped’ transients with a varying number of points of origin of the Ca²⁺ transient. About half (54%, *n* = 289 cells, 13 animals) of the cells had a TATS. Cells with TATS had a larger mean diameter (13.2 ± 2.8 μm) than cells without TATS (11.7 ± 2.0 μm) and were more common in the left atrium (*n* = 206 cells; left atrium: 76 with TATS, 30 without TATS; right atrium: 42 with TATS, 58 without TATS). Simultaneous measurement of Ca²⁺ sparks and sarcolemmal structures showed that cells without TATS had U-shaped transients that started at the cell periphery, and cells with TATS had W-shaped transients that began simultaneously at the cell periphery and the TATS. Most (82 out of 102 from 31 cells) ‘spontaneous’ (non-depolarized) Ca²⁺ sparks occurred within 1 μm of a sarcolemmal structure (cell periphery or TATS), and 33% occurred within 1 pixel (0.125 μm). We conclude that the presence of a sarcolemmal membrane either at the cell periphery or in the TATS in close apposition to the sarcoplasmic reticulum is required for the initiation of an evoked Ca²⁺ transient and for spontaneous Ca²⁺ sparks.

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In mammalian cardiac cells, the opening of voltage-gated L-type Ca²⁺ channels causes the opening of closely opposed sarcoplasmic reticulum (SR) Ca²⁺ release channels (ryanodine receptors; RyRs) through the process of Ca²⁺-induced Ca²⁺ release (CICR; Fabiato, 1983; Stern, 1992). In ventricular cells, the well-developed, homogenous network of transverse tubules (T-tubules) uniformly spreads depolarization into the cell, resulting in a nearly synchronous SR Ca²⁺ release throughout the entire cell (Cheng *et al.* 1994; Wier *et al.* 1995). Atrial cells lack T-tubules. Thus, depolarization occurs only at the outer cell membrane, resulting in a whole-cell Ca²⁺ transient that begins at the edges of the atrial cell and arrives at the centre of the cell after a significant delay (about 30–80 ms) (Berlin, 1995; Hüser *et al.* 1996; Mackenzie *et al.* 2001). Similarly, the loss of T-tubules in guinea-pig ventricular myocytes, which occurs over time in cell culture, is paralleled by the replacement of the spatially uniform whole-cell Ca²⁺ transient with a Ca²⁺ transient exhibiting marked spatial inhomogeneities (Lipp

et al. 1996). Since the RyRs are uniformly distributed along the z-lines in both cell types (atrial and ventricular cells; Lewis Carl *et al.* 1995; Mackenzie *et al.* 2001), these differences in the spatial pattern of excitation result from the distribution of dihydropyridine receptors (DHPRs) in the sarcolemma and T-tubules.

Calcium sparks are localized, subcellular releases of calcium, which occur either spontaneously (Cheng *et al.* 1993), or can be evoked by membrane depolarization (López-López *et al.* 1994, 1995; Cannell *et al.* 1995). Regardless of their method of initiation, Ca²⁺ sparks represent the opening of a cluster of RyRs (Blatter *et al.* 1997; Cannell & Soeller, 1999; Bridge *et al.* 1999; Lukyanenko *et al.* 2000; Izu *et al.* 2001). ‘Spontaneous’ Ca²⁺ sparks, those that occur without cell depolarization, are thought to result from the random opening of a cluster of RyRs. In ventricular cells, spontaneous Ca²⁺ sparks occur at the z-line (Shacklock *et al.* 1995; Parker *et al.* 1996), where RyRs exist at the junctional SR (Lewis Carl

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al. 1996). However, spontaneous Ca^{2+} sparks have been observed only rarely in the interior of cat atrial myocytes (Hüser *et al.* 1996), and only at the edges of canine Purkinje myocytes (Cordeiro *et al.* 2001), despite RyRs being distributed throughout the cell interior. Since these cell types lack T-tubules, these results suggest that spontaneous Ca^{2+} sparks may not be due to the opening of any cluster of RyRs but only clusters of RyRs that are in close apposition to the sarcolemmal membrane.

Rat atrial myocytes provide a useful system in which to study the role of sarcolemma–junctional SR interaction in excitation–contraction coupling. Most rat atrial myocytes have a sarcolemmal tubule system, which is intermediate between the mammalian ventricular cell (T-tubules at all the z-lines) and the typical mammalian atrial or Purkinje cell (no internal sarcolemmal system). This transverse-axial tubule system (TATS) has a predominantly longitudinal orientation, is poorly developed when compared to the T-tubules in ventricular cells, and has been observed in variable frequencies (Hibbs & Ferrans, 1969; Forssmann & Girardier, 1970; Leeson, 1980; Yamasaki *et al.* 1997).

In this study, we exploit the TATS to investigate the physiological significance of the sarcolemma in atrial excitation–contraction coupling and the generation of spontaneous Ca^{2+} sparks. We demonstrate that evoked Ca^{2+} transients and spontaneous Ca^{2+} sparks occur at sites where the SR is in close apposition to the sarcolemmal membrane, either at the cell surface or in the TATS.

METHODS

All rats used in the present study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine and the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services Publication No. (NIH) 85–23, revised 1985).

Cell isolation and solutions

Two-month-old Sprague-Dawley rats (200–300 g body weight) were anaesthetized with sodium pentobarbitone (170 mg kg^{-1} injected i.p.). Single atrial cells were obtained using a modification of an enzymatic technique for obtaining isolated ventricular cells described in detail previously (López-López *et al.* 1995). Briefly, the hearts were removed from the animals via mid-line thoracotomy, and perfused with a nominally calcium-free physiological salt solution (PSS) containing (mM): NaCl 135, KCl 4, MgSO_4 1, NaH_2PO_4 0.33, glucose 10, Hepes 10, pH 7.25, at room temperature (21–23 °C), for 3 min. The heart was then perfused with the same solution supplemented with 1 mg ml^{-1} collagenase (Boehringer Mannheim Type B, Indianapolis, IN, USA) and 0.02 mg ml^{-1} protease (Type XIV, Sigma, St Louis, MO, USA) for 3 min. The atria were removed, minced with microdissecting scissors and shaken in PSS supplemented with 5 mg ml^{-1} collagenase (Boehringer Mannheim Type B) and 1.5 mg ml^{-1} protease (Type XIV, Sigma) for 7–10 min. The tissue chunks were removed and gently triturated with a large bore

Pasteur pipette. The supernatants from the enzyme digestion and from the trituration were then gently centrifuged at low speed for 5 min. The pellet was resuspended in PSS with 0.25 mM Ca^{2+} . The cells were successively washed and resuspended in PSS containing 0.5 and 1.0 mM Ca^{2+} . The only cells chosen for analysis were those that had the typical fusiform shape of isolated atrial cells, were relatively straight and qualitatively uniform in width, and had visible striations.

Fluorescence recording and analysis

Cells were loaded with the Ca^{2+} indicator, fluo-4, acetoxymethyl ester (fluo-4 AM, Molecular Probes, Eugene, OR, USA), by incubation for 30 min at 25 °C in PSS containing 1.0 mM Ca^{2+} , fluo-4 AM (5 μM) and pluronic acid (2.5 μM). The sarcolemma and tubule membranes were labelled by incubation of the cells for 5 min in PSS containing 1.0 mM Ca^{2+} to which Di-8-ANEPPS (10 μM , Molecular Probes) had been added. All cells were studied within 1 h of loading. The leakage of Di-8-ANEPPS into intracellular structures was minimized by the short exposure times, as was observed in previous studies on ventricular cells (Shacklock *et al.* 1995).

The cells were studied using an Axiovert 100 inverted microscope (Carl Zeiss, Germany) equipped with a $\times 40$ water immersion lens (numerical aperture = 1.2) and attached to a confocal laser scanning unit (LSM 410, Carl Zeiss). Fluo-4 fluorescence was excited by 488 nm light from a krypton/argon laser and emitted fluorescence was filtered with a 515 nm long-pass filter and collected with a Hamamatsu R3896 photomultiplier tube (Bridgewater, NJ, USA). For double-label experiments, a 560 nm dichroic mirror split the emitted light. The Di-8-ANEPPS signal was filtered with a 590 nm long-pass filter. The fluo-4 signal was passed through a 515–540 nm band-pass filter to a second photomultiplier tube. A 64 μm line was scanned and divided into 512 pixels of 0.125 μm each. This line was repeatedly scanned 512 times at a frequency of 0.717 or 0.478 lines per millisecond to make one linescan image. Two to sixteen consecutive images were collected at each scan location. The lateral resolution of the system is 0.26 μm and the theoretical z-resolution is 1.1 μm . Field stimulation was delivered between parallel platinum electrodes at the edges of the tissue bath, at a voltage at least twice the stimulation threshold. Cell width was measured on the screen of the Zeiss LSM 410 microscope using optical calipers, at the widest point of the cell in the x–y plane, excluding branch points. In order to separate Ca^{2+} release triggered by the TATS deep within the cell from that triggered by peripheral couplings at the cell periphery, the microscope's focal plane was placed at the central plane of the cell. To accomplish this, the bottom and top of the cell were identified and the focal plane chosen at the point equidistant from each edge. Although we did not routinely record the thickness of the cell, cells were rejected that were clearly flattened, i.e. cell thickness \ll cell width. We occasionally observed folds in the membrane (Fig. 2D, upper left, for example), which were distinguished from the TATS in z-sections by their much larger width and their pair-wise appearance in the x–t confocal linescans. Linescan positions were selected to not include these folds. Data were analysed using custom routines written in IDL (Research Systems, Inc., Boulder, CO, USA).

Measurement of cell width and thickness

Cells widths were determined with optical calipers that are part of the Zeiss software package. In double-labelled cells, the boundaries of the cell were determined from the Di-8-ANEPPS fluorescence or from the fluo-4 fluorescence. Cells chosen for study were relatively straight and qualitatively uniform in width as

judged by eye. When there was some visible difference in width along the length of the cell, the average width was measured.

In some cells, both cell width and thickness were measured from y - z sections of Di-8-ANEPPS-labelled cells using optical cursors. The step size in the axial direction was 0.25 μm . Cells chosen for these measurements satisfied our criteria for analysis but otherwise were chosen at random. For this analysis, cells were *not* rejected if they were flattened.

Visualization of three-dimensional structure of TATS

The sarcolemma was labelled with Di-8-ANEPPS as above. After the cells had settled on the coverglass, the solution was carefully suctioned out and replaced with paraformaldehyde (4%) to fix the cells. Fixation was needed to prevent cell movement while stacks of x - y confocal images of the cell were collected. For these experiments, we used a BioRad Radiance confocal microscope with $\times 60$ Nikon 1.2 NA water immersion objective. The microscope was focused near the bottom of the cell and x - y confocal images were obtained at successively higher focal planes. The distance between focal planes was 0.25 μm , which was controlled by the LaserSharp software and the focus motor. The three-dimensional (3-D) structure of the TATS is visualized as surfaces of equal fluorescence (Fig. 1C). To obtain these isofluorescence surfaces, each x - y section was averaged using a 3×3 smoothing kernel, then all pixels with values less than 60 fluorescence units were eliminated. Deconvolution of the optical distortions of the microscope's objective was not carried out. Isofluorescence surfaces with values of 70 fluorescence units were found using IDL routines.

RESULTS

Three-dimensional organization of TATS

Figure 1A shows an x - y section of an atrial cell labelled with the membrane marker Di-8-ANEPPS. As expected the surface sarcolemma was heavily labelled. Additionally, what appear to be fine tube-like structures with a reticular pattern could be seen within the cell. These structures do not have a regular pattern but have strong longitudinal and transverse components. This reticular pattern is consistent with the transverse-axial tubule system, or TATS, previously described in rat atrial cells (Forssmann & Girardier, 1970; Leeson, 1980). Figure 1B shows the TATS at successive focal planes (top to bottom, left to right) of the region of Fig. 1A enclosed in the continuous box. The first image was taken at 7.25 μm from the cell bottom and the focal plane for each image was separated by 0.25 μm .

In order to obtain a clearer picture of the structure of the TATS, we made a 3-D reconstruction of the TATS from optical z -sections of the continuous box region that extended from 5.50 to 11 μm from the bottom of the atrial cell separated by 0.25 μm . In this section, the cell was 12.75 μm thick so the TATS is at least 1.75 μm from the surface. The 3-D reconstruction of the TATS viewed at different angles is shown in Fig. 1C. The axes in each of the views provide scale and orientation. The full-axis is along the microscope's axis and is 5.5 μm ; the half-axes are along the x and y directions and are 1 μm long. Note the axes are near the compact TATS structure near the left-hand of the

images in Fig. 1C. Figure 1Ca and b shows views from slightly off the microscope's axis. Figure 1Cc shows a similar view to that in Fig. 1Ca but viewed from the bottom. The reticulated pattern that is suggested in some of the single z -slices is clearer in the 3-D-reconstructed images. Volumes that extend beyond the region used in the reconstruction appear truncated and lightly coloured in Fig. 1C and 1D.

A connection between the surface sarcolemma and the internal TATS is indicated by the green arrow in the region in the dashed box in Fig. 1A. A 3-D reconstruction of that region is shown at two viewing angles in Fig. 1D.

Relationship between ultrastructure and the spatial pattern of Ca²⁺ release

Excitation-contraction coupling was examined by obtaining linescan images oriented transversely to the long axis of field-stimulated atrial myocytes (Fig. 2A and B). Two types of Ca²⁺ transients were observed: (1) U-shaped transients (Fig. 2B) like those observed in cat atrial myocytes (Hüser *et al.* 1996), and (2) irregular or W-shaped transients (Fig. 2A) with varying numbers of points of origin. Both of these differed from Ca²⁺ transients recorded in rat ventricular cells (Fig. 2C), which appear as straight horizontal bands indicating near-simultaneous release of Ca²⁺ throughout the cell.

Sample full-frame (x - y) images from rat atrial cells treated with Di-8-ANEPPS to label sarcolemmal membrane structures are shown in Fig. 2D and E. Two hundred and eighty-nine cells from 13 animals were studied. Labelled cells were classified into three categories based on their appearance: those with TATS, those without TATS, and mixed. Cells with TATS had a fine reticular pattern of tubules throughout the cell (Fig. 2D). Cells classified as without TATS had no tubules or, more commonly, only a few short tubules (Fig. 2E). Cells were classified as mixed when they had a reticular network at one end of the cell but not the other, or when they had one or two isolated tubules running the length of the cell. Although there was a continuum of tubule density, about 90% of cells could be classified as having either a TATS or no TATS by the aforementioned criteria. Of these cells, 54% had a TATS. In all cases, the pattern of sarcolemmal labelling was in sharp contrast to the regular pattern (1.8 μm spacing) of transverse tubules observed in ventricular cells (Fig. 2F; and see Shacklock *et al.* 1995).

To evaluate whether the TATS seen in some rat atrial cells participates in excitation-contraction coupling, 34 cells were double-labelled with Di-8-ANEPPS and fluo-4 (Fig. 3). Linescan images oriented transverse to the long axis of the cell were collected during field stimulation (0.3–0.5 Hz). Cells without TATS had only U-shaped Ca²⁺ transients (18 cells; Fig. 3A). Cells with TATS (16 cells) usually had W-shaped Ca²⁺ transients with the points of

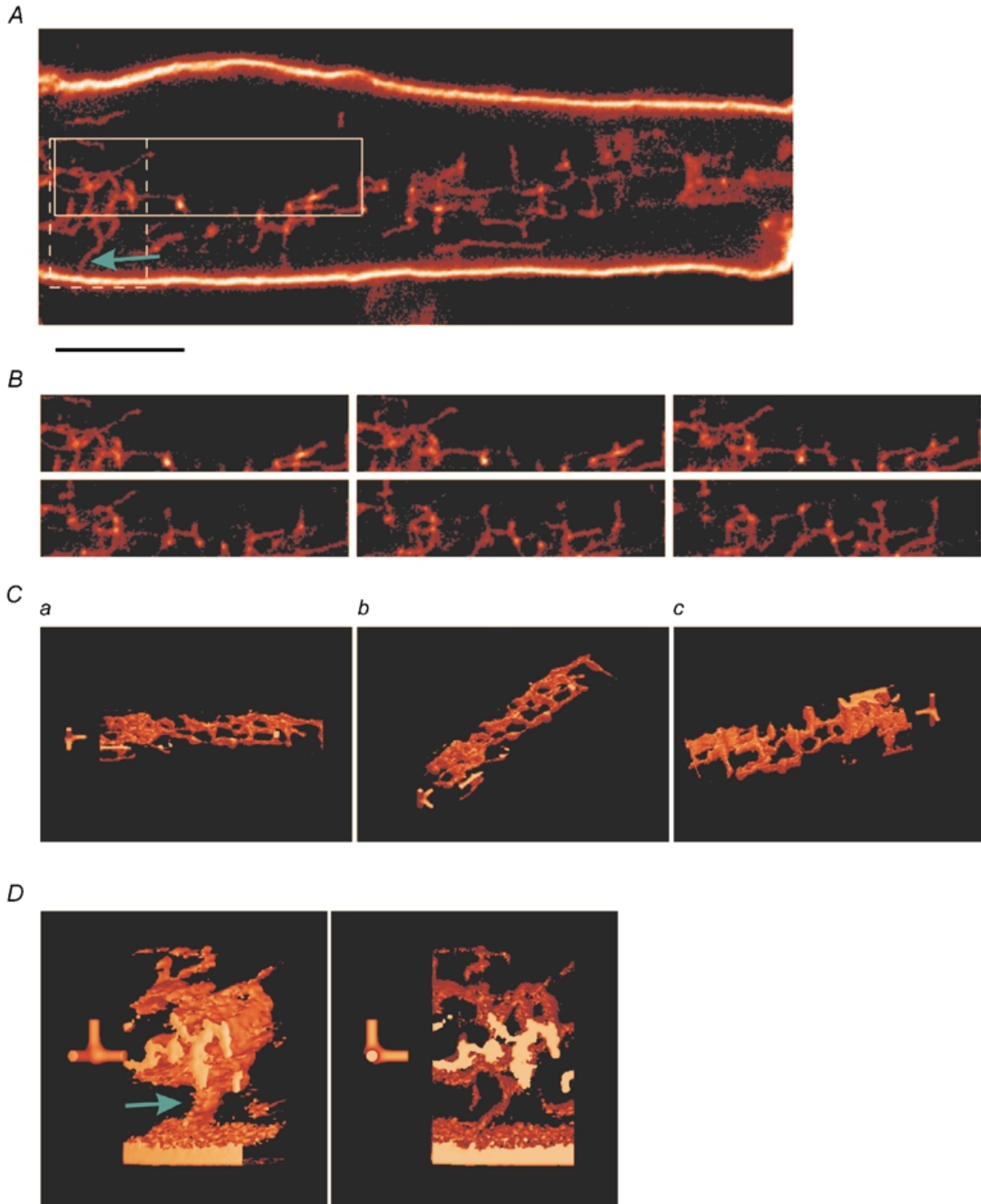


Figure 1. Three-dimensional structure of TATS

A shows an optical section of a Di-8-ANEPPS-labelled atrial cell. The surface sarcolemma is heavily labelled as are finer internal membrane structures that are consistent with the previously described transverse-axial tubular system (TATS). The focal plane lies $7.25\ \mu\text{m}$ from the bottom of the cell. Scale bar is $5\ \mu\text{m}$. B shows the region enclosed by the continuous box in A at different focal planes. The focal plane is $7.25\ \mu\text{m}$ from the bottom of the cell (top left) and increases by $0.25\ \mu\text{m}$ increments (left to right, top to bottom). C shows a 3-D reconstruction of the $5.5\ \mu\text{m}$ thick section of the region in B. D shows a reconstruction of the $4.25\ \mu\text{m}$ thick region in the dashed box in A. The green arrows in A and D show the connection between the surface sarcolemma and the TATS. The full-axis is along the axial direction and the half-axes are along longitudinal and transverse axes of the cell. In both C and D, the short axes are $1\ \mu\text{m}$ long; the full-axis is $5.5\ \mu\text{m}$ long in C and $4.25\ \mu\text{m}$ in D.

the W corresponding to the location of the tubules as marked by the Di-8-ANEPPS signal (Fig. 3B and C; 14 cells). These results indicate that initiation of SR Ca²⁺ release in response to field stimulation is located at the TATS as well as the peripheral sarcolemmal membrane in rat atrial cells.

In mammalian ventricular cells, the T-tubule system serves to transmit sarcolemmal depolarization throughout the cell, ensuring synchronous contraction (Cheng *et al.* 1994; Shacklock *et al.* 1995). If the TATS serves a similar function in atrial cells, then there should be a correlation between the presence of a TATS and cell size. Although there was substantial overlap, cells with TATS had larger mean diameters than cells without TATS (13.2 ± 2.8 vs. 11.7 ± 2.0 μm , $n = 289$ cells from 13 animals, $P < 0.0001$, Mann-Whitney U test for comparison of non-normally

distributed variable; Fig. 4A). We observed a TATS more frequently in left atrial cells than in right atrial cells (Fig. 4B; 72% vs. 42%; $n = 206$ cells from 4 animals; left atrium: 76 with TATS, 30 without TATS; right atrium: 42 with TATS and 58 without TATS). The Fisher exact test applied to this categorical data (presence vs. absence of TATS, left vs. right atrium) confirmed a statistically greater proportion of TATS in left atrial cells ($P < 0.0001$).

Spontaneous Ca²⁺ sparks (i.e. those observed in quiescent cells) occur only in association with a closely apposed sarcolemmal membrane

If Ca²⁺ sparks in quiescent atrial cells are due to the opening of a random cluster of RyRs in the SR, then they should be distributed throughout the cell, as are the RyRs (Lewis Carl *et al.* 1995; Mackenzie *et al.* 2001). To test this hypothesis, 102 Ca²⁺ sparks (31 cells; 11 animals) were

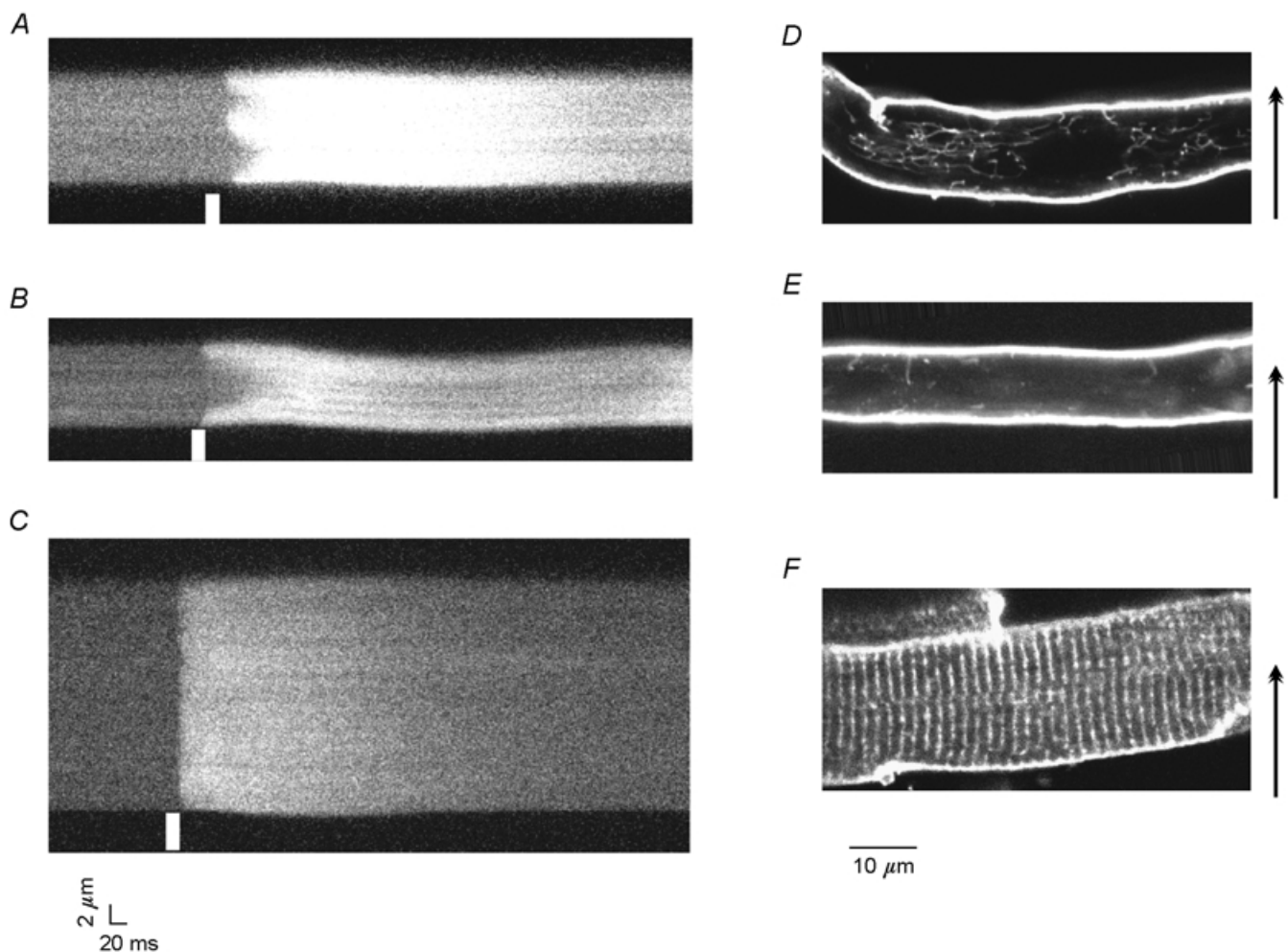


Figure 2. Relationship between TATS and the shape of the Ca²⁺ transient

Linescan images of Ca²⁺ transients in atrial (A and B) and ventricular (C) cells were taken along a direction transverse to the longitudinal axis of the cells (arrows). Ca²⁺ transients in atrial cells fell into two categories: those with numerous initiation points termed W-shaped (A) and U-shaped transients (B). Ventricular cells (C) exhibited nearly simultaneous activation across the cell. White bars denote the time of the field stimulation pulse. D–F show confocal images of Di-8-ANEPPS labelling of sarcolemmal membranes of atrial cells (D and E) and a ventricular cell (F). D shows a cell with a TATS with a prominent longitudinal component while the cell in E is almost devoid of TATS. The ventricular cell (F) shows a regular pattern of Di-8-ANEPPS labelling of T-tubules that extend from the surface and project radially into the cell.

recorded from quiescent, double-labelled rat atrial cells. Linescans were obtained transverse to the long axis of the cell. Scan lines were chosen so as to cross the maximal number of defined tubules. Cells with Ca^{2+} sparks in the middle were selected for scanning, resulting in an enriched population of Ca^{2+} sparks occurring away from the edge of the cell and in a cell population consisting exclusively of cells with TATS. The scan line was positioned so as to intersect two or three discrete tubules if possible. In cells without TATS, Ca^{2+} sparks occurred only at the periphery of the cell. In cells with TATS, Ca^{2+} sparks arose at the periphery and at the tubules, as shown in Fig. 5A and B. The distribution of distances between the location of the peak of the Ca^{2+} spark to the nearest membrane structure (i.e. tubule system or sarcolemma) is shown in Fig. 5C. Thirty-three per cent of the Ca^{2+} sparks were within 1 pixel ($0.125 \mu\text{m}$) of the nearest sarcolemmal structure, and 80% were within $1 \mu\text{m}$. When a Ca^{2+} spark was not clearly associated with a tubule in the plane of the linescan, a manual search along the z-axis usually identified a tubule within $1 \mu\text{m}$ of the Ca^{2+} spark. In rare cases (2 cells), no tubules were visible in the plane of the scan. The mean cell

width was $13.4 \pm 2.7 \mu\text{m}$ (31 cells) and the mean number of tubules per scan line was 2.6 ± 1.4 (31 cells).

Localized Ca^{2+} release vs. regenerative Ca^{2+} waves

Berlin (1995) and Hüser *et al.* (1996) both observed that, in some cells, the rise in Ca^{2+} concentration following electrical depolarization did not decrease from periphery to centre as would be expected if Ca^{2+} release were localized to only the cell periphery and Ca^{2+} passively diffused to the cell interior. Instead, the maximal Ca^{2+} concentration was similar at the periphery and centre of the cell suggesting regenerative SR Ca^{2+} release.

A method for distinguishing localized from regenerative Ca^{2+} release is to plot the time to reach a particular concentration vs. the distance from the cell edge. When Ca^{2+} release occurs only at the cell periphery and Ca^{2+} spreads by passive diffusion, the time-to-target curve is concave-up, indicating that it takes increasingly longer to reach the target value the greater the distance from the periphery. If Ca^{2+} release is triggered at the cell periphery and spreads as a regenerative Ca^{2+} wave to the centre of the cell, the time-to-target plot is initially linear and then

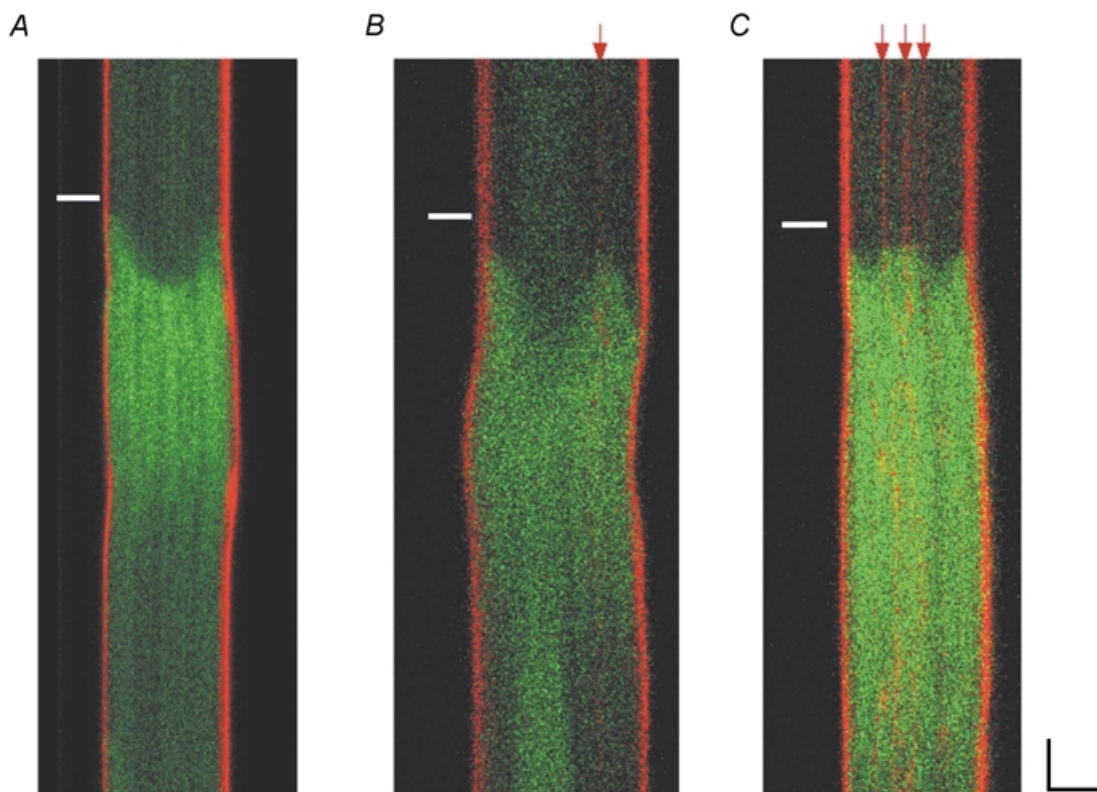


Figure 3. The presence and location of sarcolemmal tubules determines the shape of the whole-cell Ca^{2+} transient

Cells were double-labelled with fluo-4 (green signal) and Di-8-ANEPPS (red signal) and scanned transversely. Red arrows highlight the positions of the sarcolemmal tubules. A, cell without TATS showing U-shaped transient. All cells without TATS had U-shaped transients (18 cells). B and C, cells with TATS usually (14 of 16 cells) had irregular, or W-shaped, transients and the points of the W corresponded to the locations of the tubules as marked by the Di-8-ANEPPS signal. White bars denote the time of the field stimulation pulse. Scale bars are $5 \mu\text{m}$ and 50 ms.

becomes concave-down. Both types of time-to-target plots can be seen in field-stimulated rat atrial myocytes. Figure 6A shows a linescan image of a rat atrial cell during field stimulation. The corresponding time-to-target plot (Fig. 6B) is concave-up indicating that the myofibrils at the centre of the cell were activated by Ca²⁺ passively diffusing from release sites at the cell periphery. A linescan image from a different atrial cell during field stimulation is shown in Fig. 6C. The time-to-target plot from this cell (Fig. 6D) is linear, consistent with activation by a regenerative Ca²⁺ wave. Thus, both diffusion and regenerative Ca²⁺ waves are seen in these cells.

Atrial cells are not flattened

If isolated atrial cells are very thin (in the *z*-direction) then Ca²⁺ released from SR near the cell periphery would be sufficient to fully activate myofibrils near the centre. To ascertain the thickness of the isolated cells, we measured the width and thickness in cells labelled with Di-8-ANEPPS. The mean cell width was only slightly larger than the thickness (14.02 ± 3.54 vs. 11.81 ± 2.47 μm , $n = 20$, $P = 0.03$, *t* test). Since the confocal microscope used here samples an area about 1 μm thick and the focal plane chosen for the experiments was at the centre of the cell, the linescan images were several microns away from either the top or bottom of the cell.

DISCUSSION

Contraction in cardiac cells requires the coordinated release of Ca²⁺ from the SR. In mammalian ventricular cells, the extensive T-tubular system conducts electrical depolarization rapidly into the cell allowing for near synchronous SR Ca²⁺ release throughout the cell. Atrial

cells lack an extensive T-tubular network so coordinated SR Ca²⁺ release and contraction in atrial cells must depend on an entirely different cellular process(es). The data presented here demonstrate that in rat atrial cells a TATS exists and that it has a functional role in excitation–contraction coupling. In addition, the unique structural aspects of these cells allow us to demonstrate that spontaneous Ca²⁺ sparks do not occur uniformly throughout the cell as would be expected by the distribution of RyRs or Ca²⁺ release sites. Instead, spontaneous Ca²⁺ sparks require very specific conditions to exist. They occur almost exclusively where there is close apposition of the sarcolemma and the SR.

Calcium transient shape

In ventricular cells, the near synchronous SR Ca²⁺ release throughout the cell results in a horizontal band of fluorescence in a linescan image transverse to the longitudinal axis of the cell (Fig. 2C). In contrast, the rise in [Ca²⁺]_i is asynchronous in atrial cells. In guinea-pig (Berlin, 1995) and cat (Hüser *et al.* 1996) atrial cells, Ca²⁺ release is *initiated* at the cell periphery and there is substantial delay in the rise of Ca²⁺ near the centre of the cell (about 30–80 ms). This asynchronous rise in [Ca²⁺]_i results in a U-shaped Ca²⁺ transient in the confocal image. Our results confirm these findings in rat atrial cells that lack a TATS (Fig. 2B). However, Ca²⁺ release can also be initiated at the TATS giving rise to complex patterns of Ca²⁺ transients (Figs 2A, and 3B and C).

Distribution of TATS in atrial cells and its functional implications

Previous studies of atrial ultrastructure have generated conflicting results. Several investigators have reported the presence of a TATS in rat atrial cells (Forssmann &

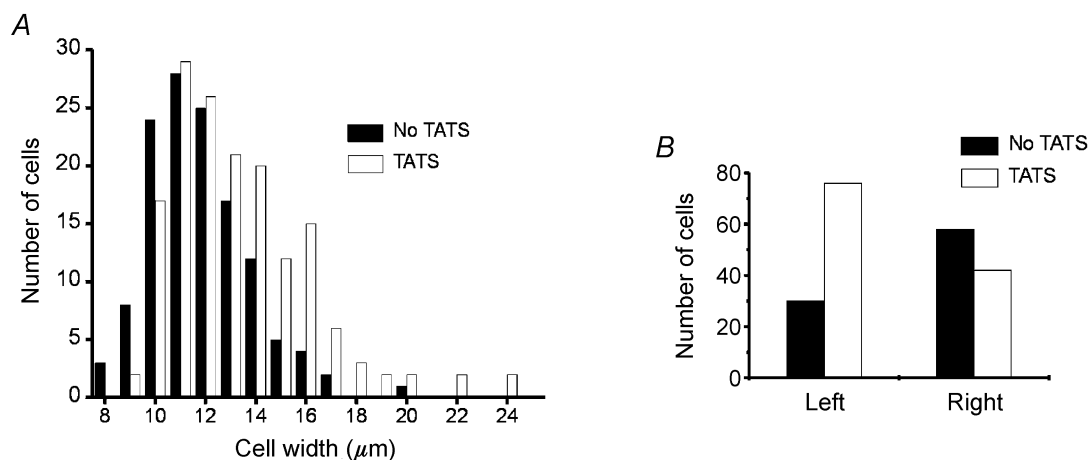


Figure 4. Cells with TATS are larger and the left atrium has more cells with TATS

A, histogram of cell widths with (\square) and without (\blacksquare) TATS (289 cells from 13 animals). Cells with TATS had a slightly larger mean width than cells without TATS (13.2 vs. 11.7 μm , $P < 0.0001$, Mann-Whitney *U* test). B, a large majority of left atrial cells had a TATS (76 of 106 cells (72%) had TATS, 30 (28%) did not have TATS) but there was a slight preponderance of cells without TATS in the right atrium (42 of 100 cells (42%) had TATS, 58 (58%) did not have TATS). There is a statistically greater proportion of TATS in left atrial cells than in right atrial cells ($P < 0.0001$ by the Fisher exact test).

Girardier, 1970; Leeson, 1980; Forbes *et al.* 1984). Others however, have not observed any intracellular structures consistent with these sarcolemmal invaginations (Hibbs & Ferrans, 1969; Yamasaki *et al.* 1997). It is interesting to note that in all studies where the sarcolemmal membranes were labelled or stained, a TATS was always identified. Our results confirm this observation. A TATS was observed in more than half of the rat atrial cells stained with the membrane indicator Di-8-ANEPPS.

In ventricular cells, the T-tubules ensure rapid and coordinated contraction throughout the cell. The TATS may serve a similar function in atrial cells, leading to a more rapid, coordinated, and forceful contraction. In support of this conclusion is the observation that atrial cells with TATS had a larger mean diameter than cells without TATS (13.2 ± 2.8 vs. $11.7 \pm 2.0 \mu\text{m}$, $P < 0.0001$), although there is substantial overlap between the groups (Fig. 4A). In addition, the TATS was more prevalent in

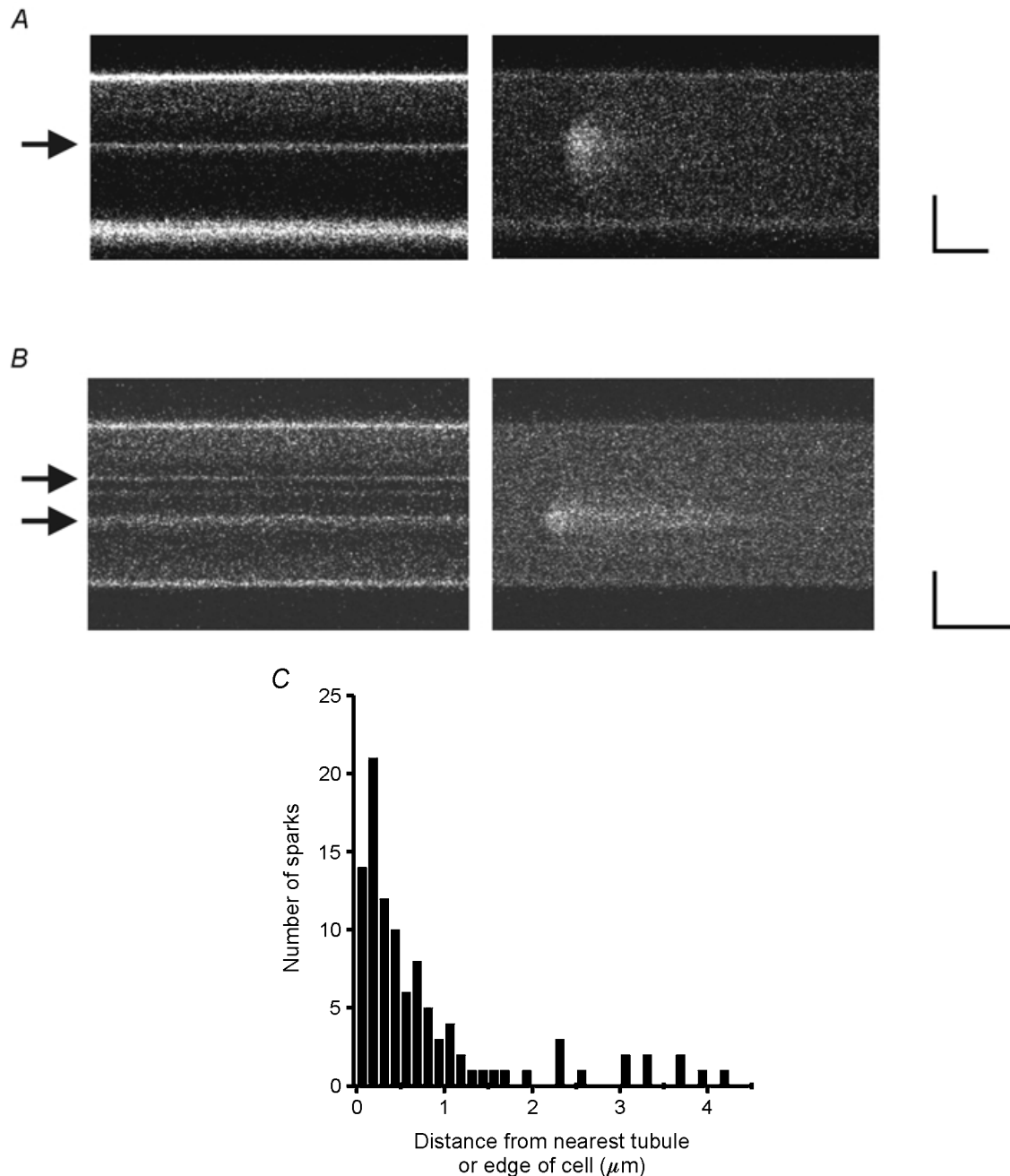


Figure 5. Spontaneous Ca²⁺ sparks occur on TATS

A and B show examples of spontaneous Ca²⁺ sparks in rat atrial cells double-labelled with Di-8-ANEPPS (left) and fluo-4 (right). Arrows highlight the location of the sarcolemmal tubules. Scale bars are 5 μm and 50 ms. C, histogram of distances between the position of the peak of the Ca²⁺ spark and the nearest membrane structure. Thirty-three per cent of the Ca²⁺ sparks were within 0.125 μm (1 pixel) of the nearest sarcolemmal structure and 80 % were within 1 μm .

cells from the left atria (Fig. 4B), which generate higher pressures than the right atria. Similar results are observed in mice where there is a higher incidence of interior junctional SR (Forbes *et al.* 1990) and TATS (Forbes *et al.* 1984) in left atrial cells than in right atrial cells.

Spontaneous Ca²⁺ sparks do not represent the random opening of Ca²⁺ release units (RyRs) in the sarcoplasmic reticulum

Despite the broad distribution of RyRs in atrial cells, principally on the z-lines (Lewis Carl *et al.* 1996), Ca²⁺ sparks that occur without cell depolarization occur predominantly where there is close apposition of RyRs with the sarcolemmal membrane. In cat atrial cells, which lack T-tubules, Hüser *et al.* (1996) found that spontaneous Ca²⁺ sparks occur mainly at the edge of cell at the surface sarcolemma. Similarly, spontaneous Ca²⁺ sparks occurred at the cell periphery in rabbit Purkinje and neonatal rat myocytes, both of which lack T-tubules (Löhn *et al.* 2000; Cordeiro *et al.* 2001). Our experiments extend these observations. In the vast majority of quiescent cells studied (31 of 33 from 11 animals), most Ca²⁺ sparks occurred within 1 μm of either the cell edge or a TATS. In fact, 33 % of the Ca²⁺ sparks occurred within 1 pixel (0.125 μm) of the cell edge or a TATS (Fig. 5).

Ca²⁺ sparks distant from a sarcolemmal structure were very rare in quiescent cells. We observed spontaneous Ca²⁺

sparks away from a visible tubule or cell edge in only 2 of 33 quiescent cells. It is possible that spontaneous Ca²⁺ sparks never occur without an adjacent sarcolemmal membrane and that in these two cells a TATS was present near the observed Ca²⁺ spark but was out of the plane of the linescan image. Similarly, Hüser *et al.* (1996) saw Ca²⁺ sparks at the centre of cat atrial cells in only 2 of 12 cells even under conditions that cause Ca²⁺ overload of the SR (10 mM bath Ca²⁺).

In ventricular cells, Ca²⁺ sparks are observed even in the absence of extracellular Ca²⁺ (Cheng *et al.* 1993) and also in permeabilized cardiac cells (Lukyanenko & Györke, 1999) where voltage-dependent Ca²⁺ entry through surface membrane Ca²⁺ channels cannot occur. The fact that there is an association between Ca²⁺ sparks in quiescent atrial cells and the sarcolemma suggest several interesting possible mechanisms. There could be a direct coupling of the SR Ca²⁺ release elements to a surface membrane protein, involvement of a secondary protein that is only distributed in the SR at sites where there is close apposition to the sarcolemma (e.g. IP₃ receptors; Mackenzie *et al.* 2002), an alteration in the microenvironment of RyRs when they are in apposition to the sarcolemmal membrane, a differential Ca²⁺ loading of the SR at these sites, or different properties of the RyRs at these sites. Distinguishing between these possibilities will require further studies.

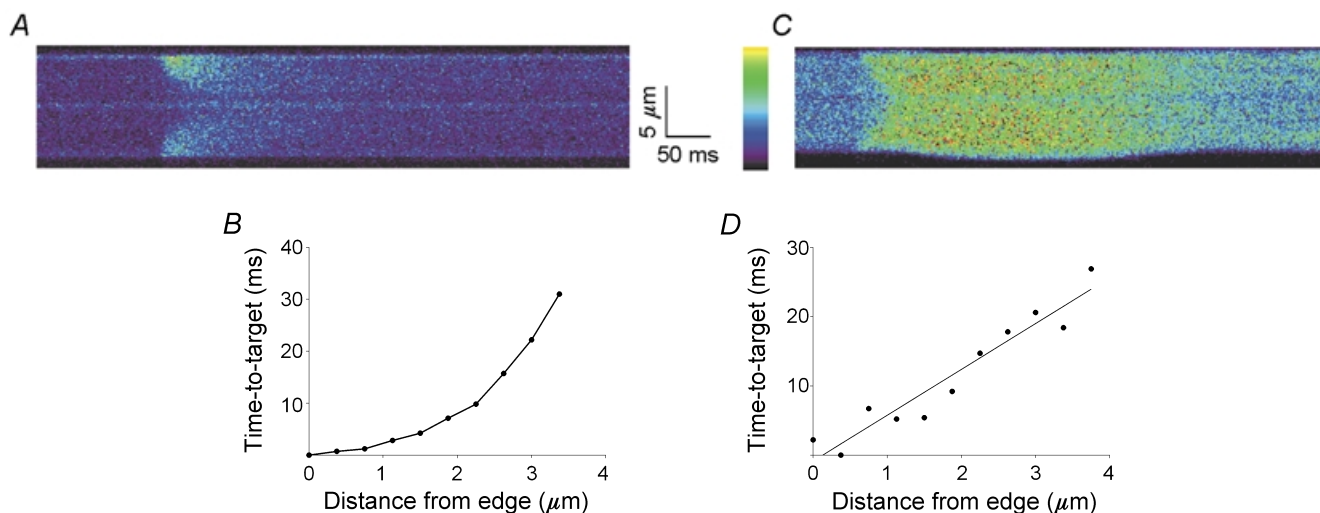


Figure 6. Time-to-target plots distinguish between non-propagating and propagating Ca²⁺ release

A, transverse linescan image of a rat atrial cell. B, corresponding plot of the time to reach a target fluorescence level as a function of distance from the cell's lower edge (time-to-target plot). The concave-up time-to-target plot is consistent with non-propagating Ca²⁺ release from the edge of the cell and the passive diffusion of Ca²⁺ to the centre of the cell. C shows a linescan image from another cell and D its corresponding time-to-target plot. The linear time-to-target plot suggests that Ca²⁺ spreads to the centre of the cell as a propagated Ca²⁺ wave. The target fluorescence value is usually chosen to be at the midpoint of the baseline and peak fluorescence values. In cases where the fluorescence decreases rapidly from the surface (e.g. A), a lower target fluorescence value (~20% of peak fluorescence) is used to obtain more data points allowing a better determination of the shape of the time-to-target plot. Using both simulated and experimental linescans, we found that the target value does not change either the shape of the time-to-target plot or the calculated Ca²⁺ wave velocity. Colour scale bar ranges from 0 to 255 fluorescence units from black to yellow.

Physiological significance of TATS and Ca²⁺ waves

When Ca²⁺ release is confined to the periphery of the cell, the time for the myofilaments to be activated increases *disproportionately* with distance from the surface. This slowing of activation with distance is seen in the concave-up shape of the time-to-target plots of Fig. 6B. A possible physiological function of regenerative Ca²⁺ waves and Ca²⁺ release from the TATS is to reduce the time lag between myofilament activation near the surface and centre of the cell. The concave-up time-to-target plot demonstrates that it takes a progressively longer time to increase the Ca²⁺ concentration the greater the distance from the edge of the cell or Ca²⁺ release site. When Ca²⁺ release at the edge of the cell initiates a Ca²⁺ wave, the Ca²⁺ concentration at the centre of the cell will rise more quickly. By allowing Ca²⁺ release at sites within the cell, the TATS also increases the speed at which the interior of the atrial cell is activated. The fact that the TATS is seen more frequently in larger cells is consistent with this concept.

Conversely, narrow or thin cells could be rapidly activated even when Ca²⁺ release is localized to the cell periphery. Two factors account for this. First, geometry demands that a large fraction of the cell's volume be located close to the cell periphery. Second, while diffusive transport is slow over large distances it is very rapid over short distances, as seen in the time-to-target plot in Fig. 6B. In thin or narrow cells, Ca²⁺ released from the SR close to the cell periphery would rapidly diffuse in a thin annular region that nevertheless activates a significant fraction of the myofibrils. Therefore, the TATS or Ca²⁺ waves may not play a significant role in hastening contraction in thin cells.

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Supplementary material

The online version of this paper can be found at:

<http://www.jphysiol.org/cgi/content/full/547/2/441>

and contains supplementary material comprising a figure and a movie.

The figure shows an atrial cell labelled with Di-8-ANEPPS. The white box delineates the x - y region used for the 3-D reconstruction shown in the accompanying movie. The scale bar is 5 μm .

The movie shows a 3-D reconstruction of the Di-8-ANEPPS-labelled membrane; the rotation is around the longitudinal axis of the cell. The continuous walls are the surface sarcolemma. The fine reticular structures comprise the TATS. This 3-D image was created from 27 z -sections spaced 0.25 μm apart. The reconstruction encompasses the 6.75 μm thick volume 5.5 μm from the bottom of the cell to 0.5 μm to the top surface of the cell.