The spatial pattern of atrial cardiomyocyte calcium signalling modulates contraction

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Summary
We examined the regulation of calcium signalling in atrial cardiomyocytes during excitation-contraction coupling, and how changes in the distribution of calcium impacts on contractility. Under control conditions, calcium transients originated in subsarcolemmal locations and showed local regeneration through activation of calcium-induced calcium release from ryanodine receptors. Despite functional ryanodine receptors being expressed at regular (~2 μm) intervals throughout atrial myocytes, the subsarcolemmal calcium signal did not spread in a fully regenerative manner through the interior of a cell. Rather, there was a diminishing centripetal propagation of calcium. The lack of regeneration was due to mitochondria and SERCA pumps preventing the inward movement of calcium. Inhibiting these calcium buffering mechanisms allowed the globalisation of action potential-evoked responses. In addition, physiological positive inotropic agents, such as endothelin-1 and β-adrenergic agonists, as well as enhanced calcium current, calcium store loading and inositol 1,4,5-trisphosphate infusion also led to regenerative global responses. The consequence of globalising calcium signals was a significant increase in cellular contraction. These data indicate how calcium signals and their consequences are determined by the interplay of multiple subcellular calcium management systems.

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Introduction
Decoding incoming extracellular information is a crucial process in cell biology. In many instances, the effects of extracellular signals are transduced by intracellular messengers, amongst which Ca2+ ions play a central role (Berridge et al., 2003; Petersen et al., 1994; Pozzan et al., 1994). Ca2+ is the most versatile of all intracellular messengers, as its concentration can be widely modulated in spatial, amplitude and temporal domains (Berridge et al., 2000). The utility of Ca2+ as an intracellular messenger derives from the multitude of systems that work to regulate the fluxes of Ca2+ within cells. Ca2+ signals are generated by the entry of Ca2+ across the plasma membrane or release from intracellular Ca2+ storing organelles (mainly the endoplasmic or sarcoplasmic reticulum: ER/SR). Small fluxes of Ca2+ caused by the activation of individual/clusters of Ca2+ channels can give rise to local signals, or can be spatially and temporally summated to yield global cellular signals (Bootman et al., 2001).

Cardiac excitation-contraction coupling (EC-coupling) is initiated by depolarisation of the sarcolemma as an action potential sweeps over a cell (Bers, 2002). The change in membrane potential activates voltage-operated Ca2+ channels (VOCs). This leads to a trigger Ca2+ signal that evokes a more substantial Ca2+ release from closely apposed ryanodine receptor (RyR) clusters on the SR. The activation of a cluster of RyRs produces a localised Ca2+ transient known as a ‘Ca2+ spark’ (Cannell et al., 1995; Cheng et al., 1993). The distributions of VOCs and RyRs within ventricular and atrial myocytes are well known (Carl et al., 1995; Mackenzie et al., 2001). For ventricular myocytes, both channels are expressed with regular spacing throughout the cells. Consequently, action potential-evoked Ca2+ signals in ventricular myocytes physiologically take the form of homogeneous global increases, reflecting the spatial and temporal summation of many Ca2+ sparks (Cannell et al., 1995; Guatimosim et al., 2002).

Atrial cells, however, lack the well-developed T-tubule invaginations of the sarcolemma found in ventricular myocytes, and therefore express VOCs only on the plasma membrane surrounding the cells (Brette and Orchard, 2003). The distribution of RyRs in atrial cells is similar to that in ventricular myocytes, but with the important exception that only a small fraction of the RyRs (the ‘junctional RyRs’) are positioned to respond to the opening of the VOCs. Ca2+ signals in atrial myocytes therefore originate around the periphery of the cells and are locally amplified by the junctional RyRs.

In many species, including rat (Mackenzie et al., 2001; Woo et al., 2002), guinea pig (Berlin, 1995; Lipp et al., 1990), cat (Sheehan and Blatter, 2003) and human (Hatem et al., 1997), the sub-sarcolemmal Ca2+ signal does not propagate and...
regenerate fully, or at all, into the centre of atrial cells. This means that at the peak of the response, substantial Ca²⁺ gradients can be observed. This is surprising given that clusters of RyRs are expressed as a regular three-dimensional lattice with a spacing of ~2 µm. It would be expected that RyRs would convey the subsarcolemmal Ca²⁺ signal deep into the cell via Ca²⁺-induced Ca²⁺ release (CICR). However, the non-junctional RyRs in the centre of atrial myocytes can be largely non-responsive. The mechanisms that inhibit inward propagation of the Ca²⁺ signal are unknown. Furthermore, the function of the non-junctional RyRs, which form the majority of intracellular Ca²⁺ release channels, is unclear.

In this study, we examined how the interplay of cellular organelles and Ca²⁺ transport mechanisms, such as mitochondria, SERCA pumps, RyRs and inositol 1,4,5-trisphosphate receptors (InsP₃Rs), impact on the properties of physiological Ca²⁺ signals in adult rat atrial myocytes. We demonstrate that to stimulate significant contraction of atrial myocytes, Ca²⁺ signals need to reach the contractile machinery in the cell centre. Under control conditions, Ca²⁺ signals are limited to the periphery of atrial cells by mitochondria and SERCA pumps. This is similar to inhibition of Ca²⁺ wave propagation by mitochondria in pancreatic acinar cells (Tinel et al., 1999) and by the ‘superficial buffer barrier’ in smooth muscle (Sanders, 2001). Consequently, the twitch of cells under control conditions is modest. An increase in cellular contraction is achieved by either reducing the potency of mitochondrial/SERCA Ca²⁺ sequestration or boosting the triggers for CICR. The latter effect is utilised by physiological inotropic agents that modulate cardiac contractility. The non-junctional RyRs therefore constitute an inotropic reserve that is recruited under conditions where greater contractility is required.

Materials and Methods

Cell isolation and solutions

Rat atrial myocytes were isolated from male Wistar rats by an enzymatic isolation method described previously (Mackenzie et al., 2001). Single cells were maintained in an extracellular medium containing (in mM): NaCl 135, KCl 5.4, MgCl₂ 2, CaCl₂ 1, glucose 10, Hepes 10 pH 7.35.

Confocal Ca²⁺ recordings

Cells were loaded with fluo-4 (20 minute incubation at room temperature with 2 µM fluo-4-AM, followed by 20 minutes for de-esterification) or rhod-2 (20 minute incubation at room temperature with 10 µM rhod-2-AM followed by 30 minute de-esterification) and transferred onto the stage of a NORAN Oz, confocal microscope (Middleton, USA). Images (512×115 pixels; z-section <1 µm) were acquired at a rate of 30 Hz (averaging four consecutive images). Images (512×115 pixels; z-section <1 µm) were acquired at a rate of 30 Hz (averaging four consecutive images obtained at 120 Hz). Analysis was performed with NIH Image (NIH, Bethesda, USA) or ImageJ (http://rsb.info.nih.gov/ij). In some figures, the subcellular properties of Ca²⁺ signals are shown using ‘pseudolinear images’, obtained by averaging a 2-4 pixel-wide line. Unless indicated otherwise, a line running transverse to the longitudinal axis and spanning across a cell was sampled to generate the pseudolinear images. The sequential lines are arranged in a linear fashion. The fluo-4 fluorescence (ex 488 nm, em ≥500 nm) was calibrated as described previously (Thomas et al., 2000). Cells were electrically paced using a pair of field electrodes (1 Hz, 40 volts, 2 milliseconds duration; platinum electrodes were spaced 1 cm apart). All experiments were performed at room temperature (20-22°C), since cell viability and dye loading is prolonged at this temperature. We have previously reported similar heterogeneous Ca²⁺ responses in atrial cells at 37°C (Mackenzie et al., 2001). Statistical significance was calculated using Student’s t-test.

Prior to starting experiments, all cells were paced for a brief period to establish a steady-state Ca²⁺ response. During this period, there was a ‘positive staircase’ indicative of Ca²⁺ loading. Generally, it took between 10 and 20 depolarisations to reach equilibrium.

Cell length measurement

Changes in the length of atrial myocytes were monitored simultaneously with confocal Ca²⁺ imaging. Displacement of the edges of cells was tracked along their longitudinal axis. Cell length was only analysed for atrial myocytes that displayed a prominent linear contraction.

Results

Spatial properties of atrial myocyte EC-coupling

In all atrial myocytes examined (n=118), Ca²⁺ signals were initiated at the cell periphery (Fig. 1A), where VOCs and junctional RyRs are co-localised (see Fig. S1 in supplementary material). The peripheral Ca²⁺ signal occurred initially as discrete Ca²⁺ sparks that eventually fused to form a ring of elevated Ca²⁺ around the cells. In the majority of myocytes (87.3%; n=118), the subsarcolemmal Ca²⁺ signal did not fully propagate into the centre of the cells. At peak, the Ca²⁺ signal amplitude in the centre of cells averaged just 36.7±10.4% of that recorded from the periphery. Such gradients were evident during the entire course of a Ca²⁺ signal, even as it decayed (Fig. 1A).

The lack of full inward regeneration of the Ca²⁺ signal is illustrated more quantitatively in Fig. 1B, which depicts the response of six discrete subcellular regions transversely spanning across a typical atrial myocyte. The Ca²⁺ signal amplitude and rate of Ca²⁺ rise are significantly greater in the subsarcolemmal portions of the cell (regions ‘A’ and ‘F’ in Fig. 1B). As the Ca²⁺ signal penetrates deeper into the cell, it increases more slowly and to a lesser extent (regions ‘B’ to ‘E’ in Fig. 1B). This progressive diminution indicates that regeneration of the Ca²⁺ signal by CICR is failing as it penetrates into the cell.

Contraction of the myocytes would have led to an increase in the cellular fluo-4 concentration. However, it is unlikely that this affected the spatial pattern of Ca²⁺ signalling since the development of the subsarcolemmal Ca²⁺ signal was rapid and preceded the twitch. Furthermore, any change in fluo-4 concentration arising as a result of cell shortening is expected to occur across an entire cell, and would therefore have contributed to an increase in global cellular fluorescence. Such a change was likely to be very modest since the cells typically shortened by only a few percent of their length.

In cells pretreated with caffeine (10 mM) and ryanodine (1 µM) to block RyR activation, electrical stimulation produces a barely discernible Ca²⁺ rise (n=4, data not shown) (see also Michailova et al., 2002). This indicates that the Ca²⁺ influx signal through VOCs that triggers EC-coupling is a small component of the Ca²⁺ signals observed. Therefore, the Ca²⁺ signals depicted in Fig. 1, whether sub-sarcolemmal or centrally located, must largely arise from CICR.
The weak response of central regions within atrial cardiomyocytes was not due to the Ca\textsuperscript{2+} stores being empty or the RyRs being non-functional, since perfusion of electrically paced cells with a maximal (10 mM) caffeine concentration invariably caused homogenous cellular Ca\textsuperscript{2+} signals ($n=21$). The spatially inhomogeneous Ca\textsuperscript{2+} signals observed during EC-coupling must therefore arise from a heterogeneous triggering of CICR within individual cells. To probe the cause of the steep gradients of Ca\textsuperscript{2+} between the peripheral initiation sites and central non-junctional RyRs, we examined the effect of modulating the processes that affect Ca\textsuperscript{2+} release and homeostasis in atrial myocytes.

**Acute increases of Ca\textsuperscript{2+} influx alter spatiotemporal properties of EC-coupling without increasing SR Ca\textsuperscript{2+} loading**

The effect of increasing the Ca\textsuperscript{2+} influx that occurs following depolarisation was examined by acutely raising the extracellular Ca\textsuperscript{2+} concentration from 1 to 10 mM, whilst...
simultaneously electrically pacing the cells and recording both the intracellular Ca\(^{2+}\) concentration and changes in cell length. A typical response to this experimental protocol is depicted in Fig. 2. In order to quantify the spatial properties of the Ca\(^{2+}\) signals during EC-coupling, we calculated the ratio between the Ca\(^{2+}\) concentration recorded from a peripheral initiation site and from a central non-regenerative region. Thus, steeper Ca\(^{2+}\) gradients are indicated by higher ratio values. When paced in control conditions, cells displayed the spatially heterogeneous Ca\(^{2+}\) signal described above (Fig. 2). Upon application of 10 mM Ca\(^{2+}\) there was an immediate decrease of the Ca\(^{2+}\) gradient that entirely disappeared within 10 subsequent electrical stimulations (Fig. 2; the filled circles approach a ratio value of 1). Thus, the major effect of increasing extracellular Ca\(^{2+}\) was to provoke more substantial Ca\(^{2+}\) release from central regions that poorly responded under control conditions. In this example, the Ca\(^{2+}\) transient amplitude at the peripheral site increased by a modest 30% (black inset traces in Fig. 2, mean increase from six independent cells was 29.9±11.2%; \(P<0.05\)), whereas in the centre of the cell the Ca\(^{2+}\) signal was enhanced by ~500% (white inset traces in Fig. 2; mean increase 219.0±23.3%, \(P<0.01\), \(n=6\)).

Concomitant with greater homogeneity of the evoked Ca\(^{2+}\) signals, there was a substantial increase in the contraction of the cells (yellow inset traces in Fig. 2). Under control conditions the cell contracted by ~2 \(\mu\)m, and this increased sevenfold following perfusion of the cell with extracellular solution containing 10 mM Ca\(^{2+}\) for 10 seconds. Upon returning to 1 mM extracellular Ca\(^{2+}\), atrial cells typically regained 85.6±6.2% \((n=6)\) of the initial Ca\(^{2+}\) gradient and the twitch returned to control levels (Fig. 2). These data indicate that changing the spatial pattern of atrial myocyte Ca\(^{2+}\) signals, so that Ca\(^{2+}\) is elevated in the central regions where it can engage the contractile machinery, is a key determinant of twitch amplitude.

The positive inotropic effect of acutely elevating the extracellular Ca\(^{2+}\) concentration, as described above, could have been due to either greater Ca\(^{2+}\) influx and/or an increase in SR Ca\(^{2+}\) load. The latter possibility was investigated by examining the extent of the caffeine-releasable Ca\(^{2+}\) pool in cells paced under control conditions or following acute exposure to elevated extracellular Ca\(^{2+}\). The average amplitude of Ca\(^{2+}\) signals evoked by 10 mM caffeine following pacing in 10 mM Ca\(^{2+}\) was 102±2.3% \((n=8; P>0.05)\) of the control response, revealing that a brief exposure to 10 mM extracellular Ca\(^{2+}\) was insufficient to alter the SR Ca\(^{2+}\) load. Therefore, increased Ca\(^{2+}\) influx through VOCs causes enhanced CICR from the junctional SR, which then provides sufficient Ca\(^{2+}\) to increase the responsiveness of central release sites.

Sensitisation of RyRs changes the spatial pattern of Ca\(^{2+}\) signals during EC-coupling

As described above, the peripheral junctional RyRs respond strongly during EC-coupling under control conditions, but CICR progressively diminishes as the Ca\(^{2+}\) signal propagates inward. A plausible explanation for the lack of central RyR response could be that their sensitivity for CICR is insufficient to allow them to respond to the Ca\(^{2+}\) influx signal. We therefore examined the consequences of sensitising RyRs on the spatial pattern of Ca\(^{2+}\) signalling in atrial myocytes.

Caffeine promotes RyR activation by sensitising them to Ca\(^{2+}\) (Rousseau and Meissner, 1989). The first approach to sensitising RyRs during EC-coupling therefore utilised a concentration of caffeine (0.1 mM), which by itself was not sufficient to activate Ca\(^{2+}\) release (Fig. 3). Responses before caffeine application and after removal of caffeine are shown in Fig. 3Ba and Bc, respectively. The weaker response of the central non-junctional RyRs (grey traces) is clearly

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**Fig. 3.** Sub-millimolar caffeine induces recruitment of central RyRs and enhances contraction during atrial EC-coupling. (A) The experimental protocol used to reversibly sensitisate RyRs with 0.1 mM caffeine. (Ba-c) Traces showing the amplitude of electrically evoked Ca\(^{2+}\) responses at a peripheral site (black traces) and a central non-regenerative region (grey traces) before (a), during (b) and after washout (c) of 0.1 mM caffeine. The locations of the subcellular regions that were sampled are shown on the inset cell image. The dashed white line indicates the cell boundary, and the solid white line, the nucleus (N). (Ca-c) Traces depicting the twitch of the cell; a and c were obtained before caffeine application and after washout, respectively and b illustrates the enhancement of contraction observed during caffeine addition. A similar enhancement of contraction was observed in six out of six cells studied.
distinguishable from the more profound Ca\(^{2+}\) signals that occurred at a peripheral junctional location (black traces). However, when the cells were paced in the presence of caffeine (Fig. 3Bb), the evoked Ca\(^{2+}\) signals were almost homogenous at peak. The amplitude of the Ca\(^{2+}\) signal in the central region increased by 370±20.8% relative to control amplitude (n=6, grey trace in Fig. 3Bb shows a typical example). There was an increase in the peripheral region relative to control responses, of just 13.4±5.4% (n=6). The net result of this was a more homogeneous Ca\(^{2+}\) release response across the cell, with central regions achieving 83.9±15% of peripheral responses while 0.1 mM caffeine was applied.

The effect of caffeine was therefore to promote Ca\(^{2+}\) release from the central RyRs, which would otherwise not have responded during EC-coupling. Consistent with the notion that Ca\(^{2+}\) signals are required in the central regions of atrial myocytes to trigger contraction, cells perfused with 0.1 mM caffeine showed a marked, and reversible, increase in cell shortening of 214.9±26.9% of control length change (n=6; P<0.02, representative examples are shown in Fig. 3C).

Another approach used to increase the sensitivity of the non-junctional RyRs to CICR was to enhance the SR Ca\(^{2+}\) store load. Increasing the concentration of Ca\(^{2+}\) within the SR has been demonstrated to sensitise RyRs (Koizumi et al., 1999; Lukyanenko et al., 2001). To achieve this, we utilised the phenomenon of post-rest potentiation that occurs in rat cardiomyocytes. Essentially, when rat heart cells are not electrically paced they load with Ca\(^{2+}\) from the extracellular medium via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange. The Ca\(^{2+}\) that enters the cells is sequestered by SERCA Ca\(^{2+}\)ATPases leading to substantial increases of Ca\(^{2+}\) concentration within the SR lumen.

Cells were initially electrically stimulated under control conditions to examine the spatial pattern of the Ca\(^{2+}\) response. The cells were then left unstimulated for 1 minute in the presence of extracellular medium containing 10 mM Ca\(^{2+}\). Subsequently the extracellular Ca\(^{2+}\) was returned to 1 mM and the cells re-paced to assess the effect of increased SR Ca\(^{2+}\) load. The amplitude of Ca\(^{2+}\) signals evoked by 10 mM caffeine was increased by 187±17% (n=7; P<0.05) compared to control cells following the 1 minute incubation in high Ca\(^{2+}\), confirming that this protocol increased SR Ca\(^{2+}\) load.

Stimulation of the cells under control conditions gave the usual spatially heterogeneous Ca\(^{2+}\) response with a substantial signal at the junctional site (Fig. 4Bai), and a much lesser Ca\(^{2+}\) transient at the non-junctional site (Fig. 4Bbi). Enhancing the Ca\(^{2+}\) load of the SR increased the amplitude of the Ca\(^{2+}\) transients at both the junctional (Fig. 4Baii) and the non-junctional (Fig. 4Bbii) regions, and the response became more homogeneous. Therefore, elevating the sensitivity of the RyRs to CICR by increasing the cellular Ca\(^{2+}\) load promoted the recruitment of the otherwise poorly responsive non-junctional channels. Furthermore, increasing the SR Ca\(^{2+}\) load led to a substantial increase in the contraction of the cells during EC-coupling (Fig. 4C).

SERCA pumps and mitochondria prevent inward regenerative CICR in atrial myocytes

We sought to determine the mechanisms responsible for restricting the Ca\(^{2+}\) signal in the peripheral region. Initially, we
determined the contribution of SERCA pump activity using the specific inhibitor cyclopiazonic acid. Our aim was to achieve a partial inhibition of SERCA activity, whilst retaining sufficient Ca\(^{2+}\) sequestration for EC-coupling to occur. To this end, we used 5 µM CPA, which did not provoke a detectable change in the diastolic Ca\(^{2+}\) concentration or cause significant unloading of the SR Ca\(^{2+}\) content (n=6; data not shown). The effects of CPA application on a typical atrial cell are depicted in Fig. 5. Prior to CPA application, this cell showed a spatially heterogeneous Ca\(^{2+}\) signal. This is evident from the ratio of amplitudes of the Ca\(^{2+}\) response in a peripheral junctional region versus that in a central non-junctional location (green
circles in Fig. 5B), which indicates that the subsarcolemmal Ca²⁺ response was almost twofold higher. The lack of full regeneration of the central Ca²⁺ signal is also apparent from the traces and pseudo-linescan image shown in Fig. 5C. Upon application of CPA, the amplitude of Ca²⁺ signals increased substantially at both the junctional and non-junctional regions. For the cell shown in Fig. 5, the Ca²⁺ signal at the peripheral site rose by 260%, whilst that in the central region increased by almost 700%. Although Ca²⁺ signals were increased at both junctional and non-junctional locations, CPA effectively made the response almost homogenous. Before CPA, central regions displayed Ca²⁺ signals with a mean amplitude of 32.7±3.1% relative to the periphery. Following CPA, the central regenerative regions achieved a relative mean peak amplitude of 72.1±8.7% (n=5, P<0.05) compared to that observed in the peripheral region (Fig. 5Ca,b).

Another consequence of CPA was to change the modest contraction of the cells under control conditions (Fig. 5Ca–iii) into a much more profound twitch (Fig. 5Cb–iii, mean increase in contraction length was 184.6±45.8%; n=5, P<0.5). These results indicate that a modest inhibition of SERCA activity can alter the spatial pattern of Ca²⁺ signals in an atrial myocyte so that the central RyRs can respond to the trigger signal initiated at the periphery of the cells. SERCA pumps have a similar localisation to RyRs in atrial cells (see Fig. S1 in supplementary material) and are therefore strategically located to hinder diffusion of Ca²⁺ and thereby modulate Ca²⁺ signalling.

In the continued presence of CPA, the cells did not reach a steady-state Ca²⁺ signal or contraction amplitude (Fig. 5B). Instead, four out of five cells displayed an oscillatory behaviour where both the Ca²⁺ signal and contraction alternated between higher and lower magnitudes. This phenomenon is known as ‘alternans’ (Blatter et al., 2003). Alternans derive from changing proportions of Ca²⁺ release within cardiac cells (Diaz et al., 2004), and have profound clinical and pathophysiological importance during various heart diseases (Euler, 1999). During the period of alternans, the central RyRs exhibited much greater variation of their Ca²⁺ release. The amplitude of the Ca²⁺ response at the non-junctional RyRs varied on average by 47±9% (n=5), whereas the peripheral junctional response changed by 16±7% (Fig. 5C).

In many cell types mitochondria have a profound role in shaping Ca²⁺ transients (Duchen, 2000; Rizzuto et al., 2004). A significant proportion of the cell volume of atrial myocytes is occupied by energised mitochondria (see Fig. S1 in supplementary material), suggesting that these organelles could have a significant impact on cellular Ca²⁺ transients. Mitochondria sequester Ca²⁺ via a uniporter located on the inner mitochondrial membrane (IMM). Since the energy for Ca²⁺ uptake is provided by the electrochemical gradient (∆ϕmit) across the IMM, a specific method to determine mitochondrial participation in Ca²⁺ signalling is to depolarise them using inhibitors of electron and proton transport, such as antimycin and oligomycin.

Treatment of tetramethylrhodamine ethyl ester (TMRE)-loaded atrial myocytes with oligomycin (20 µM) and antimycin (20 µM) caused a progressive decline in TMRE fluorescence, indicating depolarisation of ∆ϕmit. The effect of the oligomycin-antimycin mixture was rapid in on-set, requiring only 2–3 minutes to decrease the TMRE signal to undetectable levels. The half-time for reduction of background-corrected TMRE fluorescence was 23.0±6.8 seconds (n=4; see Fig. S2 in supplementary material) Although the oligomycin-antimycin provides a rapid depolarisation of mitochondria that would prevent Ca²⁺ sequestration into these organelles, such agents will also inhibit ATP production, which by itself could impact on Ca²⁺ signalling.

To examine putative changes in ATP levels, we monitored mag-fura-2 fluorescence following oligomycin-antimycin treatment (Di Lisa et al., 1995; Westerblad and Allen, 1992) (see Fig. S1 in supplementary material). Since most of the cellular Mg²⁺ is bound to ATP, and Mg²⁺ ions display a more than tenfold higher affinity for binding to ATP than to ADP, reduction of cellular ATP can be acutely monitored as an increase in Mg²⁺ concentration.

In non-electrically paced cells, antimycin/oligomycin caused only a shallow increase in mag-fura-2 fluorescence, suggesting that there was very little decline in ATP levels. In
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contrast, when added to cells being continually paced, there was a rapid rise in the signal (see Fig. S2 in supplementary material). The greater effect of antimycin-oligomycin on mag-fura-2 fluorescence in electrically paced cells reflected the progressive utilisation of ATP during EC-coupling.

In light of the reduction of ATP levels by oligomycin-antimycin in paced cells, we adopted the experimental protocol illustrated in Fig. 6Aa. This method allowed us to depolarise the mitochondria, but not decrease the cellular ATP concentration. Cells were initially subjected to a period of electrical stimulation under control conditions. The stimulation was then stopped and antimycin/oligomycin was applied for 3 minutes. After this period, the pacing was re-started. Prior to addition of antimycin-oligomycin, cells displayed low-amplitude responses that showed the typical peripheral restriction (Fig. 6B). In contrast, after mitochondrial depolarisation the amplitude of the Ca$^{2+}$ response at central non-junctional regions increased to 93.1±1.9% ($n=4$) of the subsarcolemmal response (Fig. 6C). Similar to the effect of inhibiting SERCA pumps with CPA, depolarising mitochondria also induced alternans in the Ca$^{2+}$ signal (80% of cells; $n=5$).

Confocal imaging of rhod-2-loaded atrial myocytes (Fig. 6Da) showed that under control conditions the mitochondria around the periphery of cells sequestered Ca$^{2+}$ during electrical stimulation (Fig. 6Db). In contrast, mitochondria in the centre of the myocytes showed a modest Ca$^{2+}$ rise of just 11.1±3.9% of that seen in peripheral mitochondria ($n=4$; Fig. 6Db).

When atrial myocytes are not electrically paced, they overload with Ca$^{2+}$, as described above, and with prolonged loading spontaneous global Ca$^{2+}$ waves can be triggered. We observed that such Ca$^{2+}$ waves led to equivalent Ca$^{2+}$ uptake in both peripheral and central mitochondria (Fig. 6Dc). Therefore, all the mitochondria within atrial cells were capable of buffering Ca$^{2+}$, but during EC-coupling the centrally located mitochondria only modestly respond because their peripheral counterparts have sequestered the Ca$^{2+}$, thus preventing its inward propagation. Since treatment of cells with antimycin and oligomycin substantially reduced the changes in rhod-2 fluorescence during pacing (Fig. 6Dd), we conclude that there was only a minor contamination of the signal from cytosolically located dye.

Recruitment of non-junctional SR RyRs underlies hormone- and Ins$P_3$-mediated inotropy in atrial myocytes

The data presented above demonstrate that the spatial pattern of Ca$^{2+}$ signalling is a determinant of atrial myocyte contraction.

Fig. 6. Mitochondria buffer Ca$^{2+}$ signals in atrial myocytes. (Aa) The experimental protocol. Cells were initially paced under control conditions. The stimulation was then halted and the cells incubated for 3 minutes with 20 µM antimycin + 20 µM oligomycin. After this period, the pacing was continued. The arrowheads indicate the timing of the depolarising pulses. The cell image (Ab) shows the position of the line that was used to generate the pseudo-linescan plots. (B) The response of the cell to electrical pacing under control conditions. The inset plots showing subsarcolemmal and central Ca$^{2+}$ signals were obtained by sampling the linescan image along the regions shown by the red and black lines. (C) The reaction of the same cell following depolarisation of the mitochondrial membrane potential. The average cellular Ca$^{2+}$ signal is shown as single black traces in Ba and Ca. The data are representative of 5 cells (from 3 hearts). (D) Ca$^{2+}$ uptake into mitochondria. An image of a rhod-2-loaded atrial myocyte is depicted in Da. Ca$^{2+}$ uptake into subsarcolemmal and central mitochondria was assessed by sampling the fluorescence changes within the regions indicated by the filled circles. The changes in mitochondrial Ca$^{2+}$ are depicted by the correspondingly coloured traces for electrically evoked signals (Db) and a spontaneous Ca$^{2+}$ wave (Dc), which occurred in the same cell. (Dd) Electrical stimulation only modestly increased rhod-2 fluorescence following application of 20 µM antimycin + 20 µM oligomycin for 3 minutes. The rhod-2 loading depicted in Da is representative of >20 cells from several independent experiments. The responses to electrical stimulation and generation of spontaneous Ca$^{2+}$ waves are typical of six cells from three independent experiments.
Cardioactive hormones, such as adrenaline and endothelin-1 (ET-1), are known to evoke an increase in contraction magnitude (Endoh et al., 1998; Rockman et al., 2002). We therefore examined whether the positive inotropic response to these physiological stimuli was mediated by a change in the spatial properties of Ca\(^2+\) signalling during EC-coupling.

Application of the \(\beta\)-adrenergic agonist isoproterenol (0.1 \(\mu\)M) for 3 minutes resulted in a significant increase in both the cellular Ca\(^2+\) response and contraction, as shown for the representative cell depicted in Fig. 7. The average increase in contraction was 478±35\% (\(n=5\); \(P<0.05\)). Prior to isoproterenol application, electrically evoked Ca\(^2+\) signals were largely restricted to the peripheral junctional regions of the cell (Fig. 7B). After agonist stimulation, the Ca\(^2+\) signal patterns altered so that the central non-junctional regions displayed a response that was briefly even larger than that in the peripheral region (Fig. 7C).

Incubation of atrial myocytes with ET-1 (0.1 \(\mu\)M) had a similar effect to isoproterenol in that it also promoted the response of the central non-junctional RyRs and increased contractility of the cells (Fig. 8). The responses of a typical cell under control conditions and 5 minutes after ET-1 stimulation are depicted in Fig. 8B and C, respectively. The average change of contraction amplitude was 585±52\% (\(n=8\)). Both isoproterenol and ET-1 enhanced the Ca\(^2+\) response at the junctional sites within atrial cells, but this was less marked than the effect on the non-junctional regions. With ET-1 stimulation, for example, the junctional response increased by 38±15\% (\(n=8\)), whereas Ca\(^2+\) signals from the non-junctional regions grew by 449±75\% (\(n=8\)).

In addition to RyRs, cardiac myocytes also express InsP\(_3\) receptors (Lipp et al., 2000; Mohler et al., 2003; Zima and Blatter, 2004). In a previous study using single-cell photometry, we demonstrated that direct stimulation of these

**Fig. 7.** \(\beta\)-adrenergic stimulation induces positive inotropy by recruitment of central RyRs. (A) A cell image showing the locations of the subsarcolemmal site (red circle) and the central non-regenerative region (green circle) that were analysed. (B,C) The response of the cell before and following a 3-minute incubation with isoproterenol (0.1 \(\mu\)M). The red and green traces depict the change in Ca\(^2+\) concentration at the subsarcolemmal and central region, respectively. The blue traces depict the ratio of the Ca\(^2+\) response at the subsarcolemmal site relative to that inside the cell. The upward deflection represents a subsarcolemmal response with a lesser central Ca\(^2+\) signal. Note that in the isoproterenol stimulated cell (C), the ratio of subsarcolemmal/central Ca\(^2+\) response increases quickly, as in the control situation. However, the ratio more rapidly declines and undershoots, owing to the development of the central Ca\(^2+\) response. The contraction of the cell is shown by the black traces. The spatial properties of the Ca\(^2+\) signal are illustrated by the pseudo-linescan images in Bi and Ci. The dashed yellow lines and arrowheads indicate the points at which the cell was depolarised. The dashed black line on the cell image in A shows the portion of the cell that was sampled in generating the pseudo-linescan plots. The data shown are from a representative cell typical of eight other cells analysed (from three hearts).

**Fig. 8.** Endothelin-1 promotes atrial myocyte contraction by increasing the response of non-junctional RyRs. (A) A cell image showing the locations of the subsarcolemmal site (red circle) and the central non-regenerative region (black circle) that were analysed. (B,C) The response of a typical atrial myocyte under control conditions and following a 5-minute incubation with 0.1 \(\mu\)M ET-1. The red and black traces illustrate the Ca\(^2+\) response in the subsarcolemmal and central region, respectively. The dashed black line on the cell image shows the portion of the cell that was sampled in generating the pseudo-linescan plots. Similar responses were observed in eight other cells (from three hearts).
intracellular Ca\(^{2+}\) channels with a membrane permeant InsP\(_3\) ester (InsP\(_3\)BM) increases the amplitude of electrically evoked Ca\(^{2+}\) signals (Mackenzie et al., 2002). Confocal imaging revealed that InsP\(_3\)BM enhanced the Ca\(^{2+}\) response from non-junctional regions of atrial myocytes. The protocol for this experiment is depicted in Fig. 9A. Essentially, Ca\(^{2+}\) transients were recorded under control conditions and then 2, 4 and 6 minutes after incubating the cells in extracellular medium containing InsP\(_3\)BM (10 \(\mu\)M; Fig. 9B–d). The traces and cell images in Fig. 9B–d show the response of a typical cell and also the progressively enhanced signal from the central non-junctional region of the cell (red traces in Fig. 9B–d). Under control conditions, the amplitude of the Ca\(^{2+}\) signals in the central region was 35.3±14.7\% of that recorded in the periphery (\(n=5\)). After 6 minutes exposure to InsP\(_3\)BM, the central Ca\(^{2+}\) response increased to 108±20.2\% relative to the periphery (\(n=5\); Fig. 9). Concomitant with the greater Ca\(^{2+}\) signal from the non-junctional RyRs, the contractility of the cells was elevated by 252.9±32.9\% (\(n=5\); Fig. 9C).

**Discussion**

Modulation of the spatial profile of Ca\(^{2+}\) signals is critical for determining the physiological output of cells. Various cell types display local and global Ca\(^{2+}\) signals that can differentially activate cellular processes (Bootman et al., 2001; Marchant and Parker, 2000). We demonstrate that atrial cardiomyocytes alter the spatial properties of their Ca\(^{2+}\) signals to modulate contractility. The ventricles are the major effectors of blood pumping and refill in a largely passive manner because of the pressure of venous return. However, contraction of the atria contributes ~20\% to the volume of blood within ventricles before they contract. The contribution from atria becomes increasingly important with age or under stress.

A summary of the Ca\(^{2+}\) signalling toolkit components that generate and shape atrial Ca\(^{2+}\) signals is depicted in Fig. 10. EC-coupling is initiated at the periphery of cells where RyRs and VOCs are juxtaposed (Fig. 1; see also Fig. S1 in supplementary material). In the majority of cells, the Ca\(^{2+}\) response is sharply limited to the subsarcolemmal space. Since it does not occur in the vicinity of contractile machinery, the junctional Ca\(^{2+}\) signal triggers only a modest twitch (Figs 2 and 3). The lack of inward propagation of the subsarcolemmal Ca\(^{2+}\) response is surprising as atrial myocytes express abundant non-junctional RyRs that are functional and clearly have access to substantial Ca\(^{2+}\) stores. Major mechanisms that serve to limit the diffusion of Ca\(^{2+}\) are SERCA pumps and mitochondria (Figs 5 and 6). The reason why mitochondria and SERCAs affect the inward propagation of Ca\(^{2+}\) signals but not the initiation of CICR at the cell periphery is most probably because of the proximity of the VOCs and subsarcolemmal RyRs (~2 \(\mu\)m) in comparison to the distance between RyR clusters throughout the cell (~2 \(\mu\)m). The ability of mitochondria to sequester Ca\(^{2+}\) on a beat-by-beat basis in the heart has been controversial. Some studies have suggested that mitochondrial Ca\(^{2+}\) uptake is too slow to respond during each systole, and that they sense the average cytosolic Ca\(^{2+}\) level (Huser et al., 2000). However, our data are more consistent with observations that mitochondria sequester and release Ca\(^{2+}\) in synchrony with the rapid fluctuations of cytosolic Ca\(^{2+}\) (Robert et al., 2001).
Pharmacological manipulation of the sensitivity of RyRs toward CICR, or increasing the trigger Ca$^{2+}$ influx, allows the subsarcolemmal Ca$^{2+}$ signal to recruit central RyRs. This leads to global Ca$^{2+}$ signals that provoke a substantial increase in contraction (Figs 2-4). We, therefore, suggest that the non-junctional RyRs are poised on the verge of being able to respond during EC-coupling. Under control conditions, their sensitivity to CICR is insufficient for them to be activated by the limited amount of Ca$^{2+}$ that escapes sequestration by mitochondria and SERCA pumps. However, even a modest sensitisation, for example using low caffeine concentrations (Fig. 3), allows them to become responsive. The same change from peripheral to global Ca$^{2+}$ signalling underlies the positive inotropic effect of physiological stimulation with ET-1 or the β-adrenergic agonist isoproterenol (Figs 7 and 8). In addition to aiding ventricular filling, atrial myocytes secrete hormones such as atrial natriuretic peptide (ANP). The release of ANP is known to be dependent on cellular stretch and intracellular Ca$^{2+}$ signals, and in particular on Ca$^{2+}$ release from RyRs (Laine et al., 1994). A further effect of increasing contraction by globalising atrial Ca$^{2+}$ transients may be to induce the release of this hormone. Consistent with this, we have observed that ANP release is stimulated by simply increasing extracellular Ca$^{2+}$ over a range (0.1-10 mM) that promotes the globalisation of cellular Ca$^{2+}$ transients (data not shown).

The effect of stimulating β-adrenergic receptors is mediated by increased cellular cAMP levels, which will promote Ca$^{2+}$ influx, SR Ca$^{2+}$ loading and sensitivity of RyRs to CICR (Bers, 2002). As shown by the pharmacological manipulations of Ca$^{2+}$ signalling described above, any one of these factors would have been capable of modulating the spatial properties of the Ca$^{2+}$ signal. The signal transduction pathways underlying the response of atrial myocytes to ET-1 are less well established. It is known that ET-1 receptors couple to G$q$, activation of phospholipase C and subsequently increase Ins$^P_3$ concentration (Endoh et al., 1998). The physiological function of Ins$^P_3$Rs in the heart is controversial. In the present study, we demonstrate that a cell-permeant Ins$^P_3$ ester mimics the positive inotropic effect of ET-1 by stimulating Ca$^{2+}$ release from the non-junctional RyRs (Fig. 9). Since Ins$^P_3$Rs are co-activated by Ins$^P_3$ and Ca$^{2+}$, their opening will be tuned to that of VOCs, and would plausibly act to boost Ca$^{2+}$ signals so that they can overcome the mitochondria and SERCA pumps.

The inhibition of mitochondrial Ca$^{2+}$ uptake or SERCA activity resulted in the occurrence of Ca$^{2+}$ ‘alternans’. This phenomenon occurs in both ventricular and atrial myocytes and is characterised by beat-to-beat changes in the extent of Ca$^{2+}$ released by RyRs. The current model for alternans suggests that there are cyclical changes in SR luminal calcium, which leads to variation in the sensitivity of RyRs and amount of calcium released during each stimulation (Diaz et al., 2004). Cardiac alternans is an important determinant for arrhythmia, including atrial fibrillation, since it offers the substrate for spatial repolarisation gradients and re-entrant circuits (Euler, 1999). The mechanism by which inhibition of mitochondrial Ca$^{2+}$ uptake or SERCA activity led to alternans in the present study is unclear. None of the other treatments that caused a positive inotropic effect evoked significant alternans. It appears therefore that mitochondrial and SERCA functions are required to provide a steady-state balance to the SR Ca$^{2+}$ load.

A physiological role for the central RyRs within atrial myocytes has not been identified. We suggest that the non-junctional RyRs represent an inotropic reserve of channels that are recruited to enhance myocyte contractility. This contrasts with the development of positive inotropic effects in ventricular cells, where EC-coupling is enhanced by a greater Ca$^{2+}$ release from RyRs that are active under control conditions. However, the situation in atrial cells is similar to neonatal ventricular myocytes and Purkinje cells, which also lack T-tubules (Brette and Orchard, 2003). These cells have both junctional RyRs and an extensive distribution of non-junctional RyRs, and like atrial cells the Ca$^{2+}$ signals are restricted to subsarcolemmal regions following depolarisation (Cordeiro et al., 2001; Haddock et al., 1999). Purkinje cells are responsible for relaying action potentials, and are not considered to be significantly contractile. The function of the non-junctional RyRs within Purkinje cells is therefore unclear and not likely to be enhancing cellular twitch. One possibility is that the non-junctional RyRs are responsible for specifically conveying Ca$^{2+}$ signals into the nucleus to modulate transcription.

In summary, our data present an example of the importance of spatial localisation of Ca$^{2+}$ release in determining the physiological output of a cell. Atrial myocytes express a common arrangement of Ca$^{2+}$ signalling components, as idealised in Fig. 10, which contribute to shaping cellular Ca$^{2+}$ signals. Under basal conditions, Ca$^{2+}$ signals are restricted to subsarcolemmal regions, resulting in a modest contraction. This correlates with the minor contribution of the atria to pumping blood in individuals under normal, un-stressed conditions. The headroom for positive inotropic responses in response to physiological hormones is derived from the recruitment of otherwise silent RyRs in the cell centre. The central non-junctional RyRs are located closer to the myofibrils than their junctional counterparts, and activation of these channels provokes dramatic increases in cellular contraction.

![Diagram of Atrial Myocyte Ca$^{2+}$ Signalling Machinery](image-url)
References


