Temporal changes in atrial EC-coupling during prolonged stimulation with endothelin-1

Martin D. Bootman a, Dagmar Harzheim a, Ioannis Smyrnias a, Stuart J. Conway b, H. Llewelyn Roderick a,c,*

a Laboratory of Molecular Signalling, The Babraham Institute, Babraham Hall, Babraham, Cambridge CB22 3AT, UK
b Department of Chemistry, School of Chemistry, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, UK
c Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

Received 1 May 2007; accepted 8 May 2007
Available online 14 June 2007

Abstract

Endothelin-1 (ET-1) is a potent Gq-coupled agonist with important physiological effects on the heart. In the present study, we characterised the effect of prolonged ET-1 stimulation on Ca2+ signalling within acutely isolated atrial myocytes. ET-1 induced a reproducible and complex sequence of effects, including negative inotropy, positive inotropy and pro-arrhythmic spontaneous Ca2+ transients (SCTs). The negative and positive inotropic effects correlated with the ability of Ca2+ to propagate from the subsarcolemmal sites where EC-coupling initiates into the centre of the atrial cells. We examined the spatial and temporal properties of the SCTs and observed them to range from elementary Ca2+ sparks, flurries of Ca2+ sparks, to Ca2+ waves and action potential-evoked global Ca2+ transients. The positive inotropic effect of ET-1 and its ability to trigger SCTs were mimicked by direct stimulation of InsP3Rs. An antagonist of InsP3Rs prevented the generation of SCTs and partially reduced the positive inotropy evoked by ET-1. Our data suggest that ET-1 engages multiple signal transduction pathways to provoke a plethora of different responses within an atrial myocyte. Some of the actions of ET-1 appear to be due to stimulation of InsP3Rs.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Calcium; Contraction; Atrial; Myocyte; Inositol; Endothelin

1. Introduction

Calcium (Ca2+) is the key regulator of heart contraction [1]. During each heart beat fluxes of Ca2+ occur across the cardiac myocyte plasma membrane, the sarcolemma, and from the internal Ca2+ store, the sarcoplasmic reticulum (SR). This excitation–contraction coupling (EC-coupling) process is initiated by an action potential that sweeps from the pacemaking region of the heart in the sino-atrial node. When the depolarising action potential reaches the excitable cells within the heart it causes depolarisation of the sarcolemma leading to activation of L-type voltage-operated Ca2+ channels (VOCs) and a consequent influx of Ca2+. The opening of VOCs provides a trigger Ca2+ signal to provoke more substantial Ca2+ release from closely apposed ryanodine receptor (RyR) clusters on the SR, by a process known as Ca2+-induced Ca2+ release (CICR) [2,3]. Activation of RyRs leads to the generation of ‘elementary’ Ca2+ signals known as ‘Ca2+ sparks’. These microscopic signals essentially reflect the simultaneous activation of a cluster of RyRs by CICR [4–6]. For ventricular cardiomyocytes, the spatial overlap and temporal summation of signals from multiple Ca2+ spark sites underlies the rapid homogenous Ca2+ transients that trigger co-ordinated ventricular myocyte contraction [5,7,8].

EC-coupling in atrial myocytes is substantially different from that in ventricular cells [9]. Atrial cells lack the T-tubule invaginations of the sarcolemma found in ventricular myocytes, and therefore express VOCs only on the sarcolemma surrounding the cells [10]. 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’) in the subsarcolemmal region are positioned to respond to the opening of the VOCs [11,12]. Ca²⁺ signals in atrial myocytes therefore originate around the periphery of the cells and are locally amplified by the junctional RyRs. Under control conditions, this peripheral Ca²⁺ signal does not propagate fully, or at all, into the centre of an atrial cell. This means that at the peak of the response, substantial Ca²⁺ gradients can be observed [12–15]. However, in addition to the junctional RyRs, atrial myocytes have a regular three-dimensional lattice of ‘non-junctional’ RyR clusters spaced ~2 μm apart, which pervade the entire cytoplasmic compartment [16]. It could be expected that the subsarcolemmal Ca²⁺ signal arising from the junctional RyRs would be sensed and amplified by the non-junctional RyRs via CICR. In this way, the trigger Ca²⁺ signal in the cell periphery could lead to centripetal propagation of a Ca²⁺ wave and complete engulfment of the cell. However, in the absence of positive inotropic stimulation, the non-junctional RyRs in the centre of atrial myocytes are largely unresponsive. This is due to cellular buffering mechanisms that inhibit inward propagation of the Ca²⁺ signal [17]. To stimulate contraction, the Ca²⁺ signal has to overcome the buffers and invade the cell centre where the bulk of the myofilaments exist [9].

The contraction of cardiac myocytes within the heart is regulated by various neurohormonal factors, which can increase or decrease blood pumping. Our work is focussed on understanding the mechanisms by which the potent vasoconstrictive peptide endothelin-1 (ET-1) alters cardiac Ca²⁺ signalling. One of the functions of ET-1 is to provide short-term inotropic support for a failing heart. However, in addition, ET-1 can lead to arrhythmogenesis and cardiac remodelling [18]. ET-1 concentration has been shown to be increased in cardiac tissues during pathological conditions such as congestive heart failure and myocardial infarction [19]. Furthermore, ET-1 can cause arrhythmias, independent of coronary vasoconstriction, in both normal and diseased hearts [20]. Understanding the mechanism of action of ET-1 is therefore clinically important.

The molecular processes underlying the inotropic and pro-arrhythmogenic effects of ET-1 are not entirely clear. Proposed mechanisms include stimulation of Ca²⁺ release [21,22], enhanced L-type Ca²⁺ current following activation of protein kinase C (PKC) [23] and sensitization of myofilaments following either PKC- or Rho kinase-mediated phosphorylation [24–26] or alkalinization resulting from increased Na⁺/H⁺ exchange [20]. ET-1 can be transported around the circulatory system following its secretion from vascular endothelial cells. It is also released from endothelial cells within the heart, and also by cardiac myocytes themselves. Whilst it is known that Gα₂–coupled hormones cause inotropic effects and can mediate changes in gene expression underlying conditions such as hypertrophy, it is not entirely clear what signal transduction pathways mediate the varied actions of such agonists. For endothelin, two receptor isotypes have been cloned (denoted ETα and ETβ). The predominant cardiac endothelin receptor isotype is ETα, although the ratio of the two receptor forms varies within the heart [19,20]. Both receptor types have been demonstrated to signal via the G protein Gq/11 to cause the activation of phospholipase C (PLC). Hydrolysis of phosphatidylinositol-4,5-bisphosphate by PLC on the inner leaflet of the plasma membrane yields two products; inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG) [27]. InsP₃ is water soluble, diffuses into cells and binds to specific receptors (InsP₃Rs) that channel Ca²⁺ from the lumen of the SR (or endoplasmic reticulum) to the cytosol [28–30]. DAG stays in the plasma membrane, where it can be metabolised further or activate numerous additional effectors such as protein kinase C (PKC) and Ras [31,32].

Numerous studies have demonstrated the expression of InsP₃R mRNA and protein in cardiac myocytes from various mammalian species [22,33–36]. Furthermore, InsP₃Rs have been purified from ventricular myocytes, incorporated into lipid bilayers and shown to be functional [37]. Agonist-stimulated increases in myocyte InsP₃ concentration have been monitored [38], and there are a growing number of studies showing that increased InsP₃ levels modulate Ca²⁺ release from internal stores within cardiac myocytes [39–44]. Despite these data, it is not widely accepted that InsP₃Rs can make a significant contribution to Ca²⁺ signalling in the heart. Within the adult mammalian heart, the ratio of InsP₃R:RyR expression is approximately 1:100 [45]. This would suggest that the flux of Ca²⁺ through InsP₃Rs could be swamped by the activity of RyRs [46]. However, the concerted opening of InsP₃Rs, or stimulation of regenerative CICR from neighbouring RyRs could cause InsP₃-mediated Ca²⁺ signals to have a profound effect on cardiac Ca²⁺ handling. In the present study, we characterised the effects of long-term incubation of atrial myocytes with ET-1 and the nature of the spontaneous Ca²⁺ transients (SCTs) that were evoked. In particular, we explored the potential role of InsP₃ as an intracellular messenger mediating the actions of ET-1.

2. Materials and methods

2.1. Materials

Collagenase type B was purchased from Worthington Biochemical Corporation. Endothelin-1 was obtained from Calbiochem. Salts for extracellular solutions were purchased form Sigma–Aldrich or BDH. Ca²⁺-sensitive fluorescent indicators were purchased from Molecular Probes. Rats were obtained from Harlan.

2.2. Myocyte isolation

Atrial myocytes were isolated as described previously [12]. In brief, Wistar rats weighing approximately 250 g
were anaesthetised in a CO₂ chamber and then killed by cervical dislocation. The heart was then dissected from the thorax and positioned on a modified Langendorff apparatus and perfused in a retrograde manner with HEPES buffer solution (NaCl 135 mM, KCl 5 mM, CaCl₂ 1 mM, HEPES 10 mM, glucose 10 mM and MgCl₂ 0.4 mM, pH 7.35) at 37 °C. This was followed by perfusion with a low Ca²⁺ buffer solution (NaCl 120 mM, KCl 5 mM, CaCl₂ 80 μM, HEPES 10 mM, taurine 20 mM, glucose 20 mM, MgSO₄ 5 mM, NTA 5.5 mM, and pyruvate 5 mM, pH 6.96) for 3.5 min. Following the low Ca²⁺ solution, an enzyme buffer solution (NaCl 120 mM, KCl 5 mM, CaCl₂ 35 μM, HEPES 10 mM, taurine 20 mM, glucose 20 mM, MgSO₄ 5 mM and pyruvate 5 mM, pH 7.4) was recirculated through the apparatus for 10 min. This solution also contained 118 lU ml⁻¹ type B collagenase (Worthington). Following dissociation of the myocytes, they were allowed to settle, and Ca²⁺ was reintroduced in an incremental manner to prevent intracellular Ca²⁺ overload and hypercontracture. The myocytes were kept at room temperature until ready for use. Only myocytes with no signs of deterioration and that had no spontaneous activity at rest were used for experiments. The myocytes were settled on poly-L-lysine-coated coverslips for 45 min before loading with a fluorescent Ca²⁺ indicator. All experiments were performed in accordance with the guidelines from the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986.

2.3. Photometry and confocal imaging

For photometry experiments, cells were loaded with indo-1 by incubation with the acetoxyethyl ester form of the fluorophore (3 μM for 30 min). The cells were then washed in fresh buffer and left for 30 min before use to allow complete de-esterification. For laser scanning confocal microscopy experiments, cells were loaded with fluo-3 using a similar procedure to that for indo-1, except that the myocytes were incubated with 2 μM fluo-3 acetoxyethyl ester for 20 min.

For the photometry recordings, coverslips bearing indo-1-loaded myocytes were placed on the stage of an inverted microscope (Nikon TE 200) and superfused with HEPES buffer solution containing 1 mM CaCl₂. Contraction (cell shortening) was measured optically with a video edge detector and recorded using Felix software (Photon Technology International). The cells were paced continuously but the contraction was sampled discontinuously (30 s recording period, 30 s pause). The amplitude of contraction was calculated as the difference between the maximum in cell length at the end of the diastolic phase and the minimum in cell length during each pacing cycle. The amplitude of contraction was averaged for each 30 s recording interval.

2.4. Contraction measurements

For contraction measurements, myocytes were paced in a pacing chamber (0.33 Hz, 80 V) on the stage of an inverted microscope (Olympus IX71) and superfused with HEPES buffer solutions containing 1 mM CaCl₂. Contraction (cell shortening) was measured optically with a video edge detector and recorded using Felix software (Photon Technology International). The cells were paced continuously but the contraction was sampled discontinuously (30 s recording period, 30 s pause). The amplitude of contraction was calculated as the difference between the maximum in cell length at the end of the diastolic phase and the minimum in cell length during each pacing cycle. The amplitude of contraction was averaged for each 30 s recording interval.

3. Results

3.1. Timecourse of the response to ET-1

Prolonged superfusion of atrial myocytes with ET-1 (100 nM) induced a complex sequence of changes in the amplitude of electrically evoked whole-cell Ca²⁺ transients. This is evident from Fig. 1A, which shows the peak sys-
Fig. 1. Effect of ET-1 on Ca\(^{2+}\) signalling in atrial myocytes. Panel A depicts the effect of continuous stimulation of atrial myocytes with 100 nM ET-1 on systolic Ca\(^{2+}\) signals. The data points show the normalised systolic Ca\(^{2+}\) transient amplitude monitored using photometry (data capture rate 2 ms). Data were obtained by averaging the peak systolic signal over 30 s periods, and sampling at 90 s intervals. The symbols indicate mean ± S.E.M. (n = 5). The photometry traces in panels B and C show representative indo-1 signals from single electrically paced atrial myocytes. For panel B, the myocyte was superfused with 100 nM ET-1 after the steady-state pacing condition had been established. The response depicted in panel C was obtained from an atrial myocyte that was superfused with control solution only. The traces in B and C are typical of at least 10 cells from 3 different cell preparations.

The typical response of a single atrial myocyte to continuous ET-1 stimulation is depicted in Fig. 1B. The pattern of negative inotropy, followed by positive inotropy was evident, as was the progressive increase in SCTs. In phase 4, the cell could be seen to miss a triggered systole. This was due to the incidence of spontaneous Ca\(^{2+}\) transients (SCTs) during the normally quiescent diastolic periods. The SCTs usually occurred concomitantly with the positive inotropic phases (2, 3 or 4), and progressively increased in frequency. High frequency SCTs can reduce systolic Ca\(^{2+}\) responses since they cause unloading of the SR Ca\(^{2+}\) pool [48]. Indeed, in phase 5 the SCTs were often so frequent that the electrical stimulation no longer evoked a response. Similar effects of ET-1 were observed in cells studied at room temperature or 37 °C, with the difference that the transition from negative to positive inotropic effect was achieved more rapidly at the higher temperature. The frequency of SCTs was slightly greater at 37 °C.
presence of multiple SCTs that made the myocyte electrically inert. A few minutes later in phase 5, the cell was displaying random arrhythmic Ca\textsuperscript{2+} signals that had little temporal correlation to the applied pacing regime. In the absence of ET-1, cells could be paced with no change in the characteristics of the evoked Ca\textsuperscript{2+} signals (Fig. 1C).

The pattern of systolic Ca\textsuperscript{2+} response depicted in Fig. 1A was mirrored by myocyte contraction. Whereas control cells maintained consistent twitch amplitudes for the duration of the electrical pacing, ET-1 caused both negative and positive inotropy (Fig. 2A). The ET-1-induced changes in contraction occurred over the same time course as the corresponding Ca\textsuperscript{2+} changes (Fig. 1). The amplitudes of contraction during the negative and positive inotropic phases were significantly different from that in control cells (Fig. 2B).

To further examine the relationship between changes in Ca\textsuperscript{2+} transient amplitude and magnitude of contraction, we investigated the spatial profile of Ca\textsuperscript{2+} signals during ET-1 stimulation using confocal microscopy. The three montages of images in Fig. 3Ai–iii depicts the same fluo-3-loaded atrial myocyte that was imaged before (control; left-hand montage) or after ET-1 superfusion (middle and right-hand montage). The cell images were obtained just as the cell was being electrically stimulated (stimulation is indicated by the horizontal arrow). In the control condition, it was evident that the Ca\textsuperscript{2+} signal arose initially in the subsarcolemmal region of the cell, and then spread centripetally inwards. At the peak of the response (Fig. 3Ai at 32 ms post-stimulation), the Ca\textsuperscript{2+} signal had propagated towards the centre of the myocyte, but had not completely engulfed the cell. During the negative inotropic phase, following application of ET-1 for 3 min, both the subsarcolemmal and central Ca\textsuperscript{2+} responses declined (Fig. 3Aii). In contrast, with prolonged application of ET-1 so that the cell could reach a positive inotropic state, the subsarcolemmal Ca\textsuperscript{2+} response was enhanced and the centripetal propagation of the Ca\textsuperscript{2+} signal spread completely throughout the cell. In addition to a larger Ca\textsuperscript{2+} signal during the positive inotropic phase, the cell was observed to have a substantially enhanced contraction (Fig. 3Aiii). The relative Ca\textsuperscript{2+} changes in the subsarcolemmal and central regions of the myocyte are depicted in Fig. 3B (note that for clarity only portions of the response that were devoid of SCTs are shown). It is evident that the subsarcolemmal region attained a higher peak Ca\textsuperscript{2+} response than the central region in both the control and negative inotropic phases. However, during the positive inotropic phase, the amplitudes of the Ca\textsuperscript{2+} signal in the subsarcolemmal and central regions were indistinguishable.

The centripetal propagation of the Ca\textsuperscript{2+} signal allowed it to reach the nucleus of the myocytes, which is generally located centrally in atrial cells. In the control situation, the fold change in fluo-3 emission was similar in the nucleus and surrounding cytoplasm (Fig. 3Bi). During the negative inotropic phase, when the centripetal propagation of the Ca\textsuperscript{2+} signal was weakest, the nucleus appeared to have a lesser fold change in fluo-3 fluorescence emission (Fig. 3Bii). However, the most striking effect of ET-1 on nuclear Ca\textsuperscript{2+} levels was observed during the positive inotropic phase (Fig. 3Biii), in which the fold change in fluo-3 emission was as great as that in the subsarcolemmal and cytoplasmic regions. In addition, there was an additional component to the nuclear Ca\textsuperscript{2+} response that distinguished it from the signals in the remainder of the cell. Whereas, the fluo-3 signal from the subsarcolemmal and central regions rapidly peaked and then decayed almost simultaneously, the nucleus displayed an extra surge of Ca\textsuperscript{2+} (see green trace in Fig. 3Biii). The fact that nuclear Ca\textsuperscript{2+} increased whilst the cytoplasmic and subsarcolemmal regions were decreasing suggests that the nucleus can produce autonomous signals. It is evident from the image montages in Fig. 3Aiii that the nucleus was initially isolated from the Ca\textsuperscript{2+} change in the surrounding cytoplasm until 32 ms after stimulation. At that point Ca\textsuperscript{2+} invaded the nucleus and triggered the secondary Ca\textsuperscript{2+} increase perhaps via a CICR mechanism. In addition, as has been demonstrated in other cell types [49], the nuclear Ca\textsuperscript{2+} rise recovered more slowly than in the rest of the myocyte.
3.2. The spontaneous Ca\textsuperscript{2+} transients (SCTs) stimulated by ET-1 were Ca\textsuperscript{2+} sparks, flurries of Ca\textsuperscript{2+} sparks, Ca\textsuperscript{2+} waves and action potential-evoked global Ca\textsuperscript{2+} transients

The incidence of SCTs correlated with the development of positive inotropy in the ET-1-stimulated atrial myocytes (Fig. 4A). Very few SCTs were recorded in control cells paced in the absence of ET-1. It was evident that ET-1 provoked the occurrence of three different types of SCT during diastole. Ca\textsuperscript{2+} sparks were obvious in confocal recordings as spatially restricted events (≤5 \textmu m in diameter) with a rapid appearance and decay (lifetime ≤150 ms). The incidence of Ca\textsuperscript{2+} sparks grew progressively with the duration of ET-1 stimulation. Both the number of Ca\textsuperscript{2+} spark sites and the rate of firing of those sites increased (Fig. 4B). Analysis of the positions of the spontaneous Ca\textsuperscript{2+} spark sites revealed that they predominantly occurred in the subsarcolemmal region of atrial myocytes. This is illustrated in Fig. 4C, which maps the positions of Ca\textsuperscript{2+} spark sites within an individual myocyte. The progressive activity of the Ca\textsuperscript{2+} spark sites in this cell is shown quantitatively in Fig. 4D. The amplitude of the Ca\textsuperscript{2+} sparks did not alter during ET-1 application (Fig. 4E).

Although Ca\textsuperscript{2+} sparks were often observed as temporally- and spatially discrete events during ET-1 stimulation, they also triggered more substantial Ca\textsuperscript{2+} signals, as exemplified...
Fig. 4. Concurrent development of positive inotropy and SCTs. Panel A illustrates the temporal coincidence of positive inotropy and SCTs in atrial myocytes. The symbols indicate mean ± S.E.M. (n = 5). The SCT frequency was calculated by counting the number of SCTs apparent in the photometry traces during the 30 s intervals when systolic Ca²⁺ was monitored. The black lines and symbols depict the responses of ET-1-stimulated cells. Whereas, the red lines and symbols relate to control cells superfused with extracellular buffer alone. Panel B illustrates the spatial and temporal properties of spontaneous diastolic Ca²⁺ sparks. The data in panels B–E were obtained from a single atrial myocyte, and are typical of similar observations made in four other cells. The data in panel E represent mean ± S.E.M. of the Ca²⁺ sparks observed in 30 s confocal recording periods.

in Fig. 5. Panels 5A and B illustrate whole-cell Ca²⁺ signals recorded from a single atrial myocyte that had been stimulated with ET-1 for 10 min. As described earlier, under this condition the cell was displaying positive inotropy and SCTs. The Ca²⁺ signals evoked by electrical pacing are indicated by the arrows, whereas the SCTs are marked with arrowheads. It is evident that the SCTs had different amplitudes. Fig. 5C–F depicts the temporal and spatial profiles of the two SCTs marked with red arrowheads. One of these events was essentially a flurry of Ca²⁺ sparks that appeared spontaneously and then rapidly declined. The globally averaged Ca²⁺ response of this SCT is shown on an expanded timescale in Fig. 5C (black trace) with a preceding pacing-evoked Ca²⁺ transient. Superimposed on the photometry trace is a record of the incidence of Ca²⁺ sparks during the SCT (orange trace in Fig. 5C). Although a single Ca²⁺ spark is not sufficient to significantly affect the globally averaged Ca²⁺ concentration within a myocyte, the flurry of Ca²⁺ sparks was able to cause a substantial deviation of diastolic Ca²⁺. The SCT declined because the Ca²⁺ spark activity did not persist. The spatial
Fig. 5. Transition from flurries of Ca$^{2+}$ sparks to action potentials in an ET-1-stimulated atrial myocyte. The traces in panels A and B depict whole-cell averaged Ca$^{2+}$ signals within a single atrial myocyte. The cell was electrically paced at the times shown by vertical arrows. Spontaneous diastolic Ca$^{2+}$ transients of different amplitudes are evident in the traces. The two SCTs indicated by red arrowheads are shown on an expanded timescale by the black traces in panels C and D. Superimposed on the whole-cell averaged Ca$^{2+}$ signals is the incidence of isolated Ca$^{2+}$ sparks (orange traces). The numbered arrows beneath the orange traces indicate the times at which the cell images in panels E and F were captured. The white scale bar in E represents 8 μm.

The pattern of the Ca$^{2+}$ increase during the SCT is depicted in Fig. 5E. The Ca$^{2+}$ sparks are particularly evident around the subsarcolemmal region of the cell.

The second, larger, SCT showed a markedly different type of response. The temporal profile of that SCT is shown in Fig. 5D (black trace). In that example, the SCT had the same amplitude as the prior pacing-evoked Ca$^{2+}$ transient, although it had a slower initial rising phase. The plot of Ca$^{2+}$ spark occurrence (orange trace in Fig. 5D) is overlaid on the Ca$^{2+}$ recording. It is evident that there was a progressive increase in Ca$^{2+}$ spark frequency, which was coincident with a gradual rise in diastolic Ca$^{2+}$. At the point marked with an asterisk (*), the number of obvious discrete Ca$^{2+}$ sparks declined to zero. The reason for the decline in apparent Ca$^{2+}$ sparks was the rapid transition to a global Ca$^{2+}$ signal. At that point, individual Ca$^{2+}$ sparks could no longer be identified. The sudden upstroke of the Ca$^{2+}$ transient was due to the triggering of an action potential by the preceding Ca$^{2+}$ sparks [21,50]. The spatial pattern of the Ca$^{2+}$ response for the larger SCT is illustrated in Fig. 5F. Initially, the larger SCT develops in the same manner as described previously for the smaller SCT (Fig. 5E), in that there is a flurry of subsarcolemmal Ca$^{2+}$ sparks. However, in the case of the larger SCT, the Cu$^{2+}$ spark activity does not simply decline, and instead triggers a large global Ca$^{2+}$ change (panel 6 in Fig. 5F).

Individual Ca$^{2+}$ sparks, flurries of Ca$^{2+}$ sparks and Ca$^{2+}$ spark-triggered action potentials were the most common forms of SCT observed within the ET-1-stimulated atrial myocytes. In addition, we also observed Ca$^{2+}$ waves that traversed the whole, or a portion, of a cell. Ca$^{2+}$ waves were particularly evident with prolonged ET-1 stimulation (n = 8). An example of a Ca$^{2+}$ wave is presented in Fig. 6A. As shown in the montage of images, the Ca$^{2+}$ wave initiated in the periphery of the cell, and spiralled around the nuclear boundary and engulfed the nucleus. As with the majority of Ca$^{2+}$ waves, this event appeared to be triggered by preceding subsarcolemmal Ca$^{2+}$ spark activity. The amplitudes of Cu$^{2+}$ waves were intermediate between flurries of Cu$^{2+}$ sparks and action potentials. This is evident in Fig. 6B, which shows the globally averaged Ca$^{2+}$ signal from the cell illustrated in Fig. 6A during the time when Ca$^{2+}$ waves were observed. The montage of images in Fig. 6A relate to the Ca$^{2+}$ wave identified by an asterisk (*) in Fig. 6B.

3.3. InsP$_3$ underlies the arrhythmic effect of ET-1 in atrial myocytes

We, and others, have demonstrated the expression of functional InsP$_3$Rs in adult atrial myocytes [22,33]. InsP$_3$Rs could provide a parallel Ca$^{2+}$ release pathway to act in concert with the RyRs that underlie EC-coupling. We therefore sought to determine whether they played a role in any of the responses induced by prolonged ET-1 stimulation. To directly activate InsP$_3$Rs a membrane-permeant InsP$_3$ ester was used. This compound has been employed by our lab and several others [21,51–57], and all its effects are consistent with a sole action on InsP$_3$Rs.

Similar to ET-1, continuous stimulation of atrial myocytes with InsP$_3$ ester provoked a progressive increase in the amplitude of systolic Ca$^{2+}$ transients and the occurrence of SCTs (Fig. 7A). However, the effect of InsP$_3$ ester was different from that of ET-1 in a couple of respects. Firstly, there was no negative inotropy observed in InsP$_3$ ester-treated cells. Also, the InsP$_3$ ester-evoked positive inotropy was not as profound
Fig. 6. Transition from flurries of Ca\(^{2+}\) sparks to Ca\(^{2+}\) waves in an ET-1-stimulated atrial myocyte. The montage of images in panel A illustrates the onset of a Ca\(^{2+}\) wave from preceding Ca\(^{2+}\) spark activity. The individual frames were captured sequentially at 8 ms intervals during the diastolic period. Panel B depicts the whole-cell averaged Ca\(^{2+}\) signals occurring in the cell shown in panel A at the time when the Ca\(^{2+}\) wave was recorded. The asterisk (*) in panel B indicates the actual Ca\(^{2+}\) wave that is represented in panel A.

and typically took longer to develop compared to the effect of ET-1. The photometry traces in Fig. 7B illustrate the effect of continuous InsP\(_3\) ester stimulation on a single atrial myocyte. The positive inotropic increase in systolic Ca\(^{2+}\) response is evident, as is the activation of SCTs. Furthermore, just like ET-1, the InsP\(_3\) ester had the capacity to drive the cells into a condition in which they could no longer be electrically paced.

These data suggest that InsP\(_3\) could mediate the positive inotropic and arrhythmic effects of ET-1. To examine this, we used the membrane-permeant InsP\(_3\)R antagonist 2-aminoethoxydiphenyl borate (2-APB) [53]. When applied on its own at a concentration of 2 \(\mu\)M, 2-APB had no effect on pacing-evoked Ca\(^{2+}\) transients (Fig. 7C). However, 2-APB had a marked consequence for the response of atrial myocytes to ET-1 (compare Figs. 1B and 7D). Both the negative and positive inotropic effects of ET-1 were observed in the presence of 2-APB, although the increase in systolic Ca\(^{2+}\) rise was significantly decreased by the InsP\(_3\)R antagonist. For example, at 12 min post-ET-1 addition the systolic Ca\(^{2+}\) rise was 917 ± 112. Whereas, in cells stimulated with ET-1 + 2-APB the systolic Ca\(^{2+}\) transient amplitude was 498 ± 80 nM (p < 0.05; n = 5). Another effect of 2-APB was to reduce the incidence of ET-1-evoked SCTs. All cells stimulated with ET-1 alone (n = 10) or ET-1 + 2-APB (n = 5) displayed SCTs, but they were substantially less common if 2-APB was present. Furthermore, no cells (n = 5) stimulated with ET-1 + 2-APB progressed to the condition where they were inert to the external pacing (Fig. 7D).

4. Discussion

ET-1 is known to exert several effects on cardiac function [20]. In the present study, we explored the consequence of prolonged stimulation of atrial myocytes with ET-1, and observed a complex pattern of response that included negative and positive inotropy, and the generation of pro-arrhythmic SCTs (Figs. 1 and 2). The positive inotropy and SCTs appeared concurrently (Fig. 4), and in some myocytes the SCTs became so frequent that they prevented the cells from being regularly paced (Fig. 1B). It was evident that the SCTs could take several different forms, ranging from isolated Ca\(^{2+}\) sparks to full-blown action potentials (Figs. 4–6). In addition, we determined that InsP\(_3\) was the major cause of SCTs in ET-1-stimulated cells (Fig. 7).

The inotropic effects of ET-1 correlated with alterations of the spatial profile of systolic Ca\(^{2+}\) signals. During the negative inotropic phase, both the subsarcolemmal and central Ca\(^{2+}\) responses were diminished. The signals underlying the negative inotropic action of ET-1 are not entirely clear. A suggested mechanism is a transient decrease in the current carried by VOCs during EC-coupling [58], possibly due to a reduction in intracellular cAMP or following activation of PKC [59]. The positive inotropic effect of ET-1 has been suggested to be mediated by several mechanisms, as described earlier. In this study, we demonstrated that InsP\(_3\) is responsible for a
Fig. 7. InsP$_3$ is a positive inotropic and pro-arrhythmic factor in atrial myocytes, and underlies the SCT-generating action of ET-1. Panel A depicts the effect of continuous stimulation of indo-1-loaded atrial myocytes with InsP$_3$ ester (10 μM) on systolic Ca$^{2+}$ signals and the frequency of SCTs. The data points show the normalised systolic Ca$^{2+}$ transient amplitude monitored using photometry (data capture rate 2 ms). Data were obtained by averaging the peak systolic signal over 30 s periods, and sampling at 90 s intervals. The SCT frequency was calculated by counting the number of SCTs apparent in the photometry traces during the 30 s intervals when systolic Ca$^{2+}$ was monitored. The symbols indicate mean ± S.E.M. (n = 5). The photometry traces in panels B–D show representative indo-1 signals from single electrically paced atrial myocytes, and are typical of at least 10 cells from 3 different cell preparations. The vertical arrows indicate the times at which the cells were electrically paced. For panel B, the myocyte was superfused with 10 μM InsP$_3$ ester after the steady-state pacing condition had been established. The response depicted in panel C was obtained from an atrial myocyte that was superfused with extracellular buffer supplemented with 2 μM 2-APB. Panel D shows the response a myocyte to superfusion with 100 nM ET-1 + 2 μM 2-APB.
portion of the positive inotropic effect of ET-1 (Fig. 7). Direct
stimulation of InsP3R with the InsP3 ester promoted positive
inotropy, whereas antagonism of InsP3Rs during ET-1 stimu-
lation reduced the increase in systolic Ca2+. The most likely
way in which InsP3 can act as a positive inotrope is to pro-
vide an extra source of Ca2+ during EC-coupling. Although
InsP3Rs require InsP3 for channel opening, the activation
of InsP3Rs is complex and their open probability is depen-
don the ambient Ca2+ concentration. Up to approximately
500 nM, Ca2+ works synergistically with InsP3 to activate
InsP3Rs [60–63]. InsP3Rs can therefore function as CICR
channels. It is plausible that they can sense and respond to
the trigger Ca2+ signal that enters through the VOCs at the
initiation of EC-coupling, and thereby provide a boost to the
systolic Ca2+ transient. A population of InsP3Rs has been
identified at the subsarcolemmal junctions where the VOCs
and RyR are closely opposed [21,33]. In this strategic loca-
tion, InsP3Rs are ideally placed to synergise with the other
Ca2+ channels and enhance EC-coupling.

The junctional location of InsP3Rs could also explain the
spatial origin of SCTs. The isolated Ca2+ sparks (Fig. 4),
flurries of Ca2+ sparks (both transient flurries and those that
triggered action potentials; Fig. 5) and Ca2+ waves (Fig. 6)
were all predominantly observed in the subsarcolemmal
region. Promiscuous firing of subsarcolemmal InsP3Rs, and
the subsequent recruitment of RyRs by CICR could explain
the prevalence of SCTs in the periphery of atrial myocytes.

An unexpected effect of ET-1 stimulation was to modify
nuclear Ca2+ concentration differentially from the surround-
ing cytoplasm (Fig. 3). The secondary surge of Ca2+ within
the nucleus depicted in Fig. 3Biiv was spatially and tem-
porally distinct from the decreasing Ca2+ levels within the
cytoplasm. The topic of autonomous nuclear Ca2+ signalling
has a long and controversial literature, with many discrepant
findings [64–66]. Whilst we have observed cytosolic Ca2+
signals that had a significant and longer-lasting effect on
nucleoplasmic Ca2+ [49], we had not previously found a nuclear Ca2+ signal that appeared to be uncoordinated with the
surrounding cytoplasmic Ca2+ concentration. Identifying
the source of the Ca2+ underlying the nuclear secondary
surge shown in Fig. 3 will require further work. However, it
is interesting to note that InsP3Rs have been observed in
the perinuclear region/nuclear envelope of cardiac myocytes
and could therefore be responsible [67].

In summary, ET-1 has a complex mixture of effects on
atrial myocyte EC-coupling. The signal transduction cas-
dades engaged by ET-1 are not fully established. However,
it appears that InsP3Rs are recruited by ET-1 and medi-
ate some of its actions. In particular, positive inotropy and
arrhythmogenesis. Consistent with our data, genetic ablation
of InsP3R expression in atrial myocytes prevents the positive
inotropic and SCT-generating actions of ET-1 [22]. We have
observed similar actions of InsP3 on ventricular myocytes
[50]. Although due to the lesser expression of InsP3Rs in ven-
tricular cells [33], the effects of InsP3 were not as profound as
with atrial myocytes. Given that InsP3Rs have a dual nature
of increasing physiological signalling (inotropy) and causing
pathological signals (SCTs), it is somewhat paradoxical that
InsP3Rs would be utilised by cardiac myocytes. The regular
activation of cardiomyocytes is critical to not disturb the car-
diac cycle. The data presented herein suggest that the modest
inotropic effect of InsP3Rs comes with a significant ability
to disrupt EC-coupling.

Acknowledgements

This work was supported by the British Heart Foundation
(Grant number PG/06/034/20637) and the BBSRC. HLR is
a Royal Society University Research Fellow.

References

198–205.
[3] H.L. Roderick, M.J. Berridge, M.D. Bootman, Calcium-induced cal-
events underlying excitation–contraction coupling in heart muscle, Sci-
W.J. Lederer, Local Ca(2+) signaling and EC coupling in heart: Ca(2+)+
spark and the regulation of the [Ca(2+)](i) transient, J. Mol. Cell.
signalling during excitation–contraction coupling in mammalian atrial
[10] F. Brette, C. Orchard, T-tubule function in mammalian cardiac myocytes,
G. Meissner, D.G. Ferguson, Immunolocalization of sarcoplasmic dithy-
dropridine receptor and sarcoplasmic reticular triadin and ryano
recruitment of calcium release sites underlies excitation–contraction
contractions in atrial and ventricular cells, Am. J. Physiol. 269 (1995)
H165–H1170.
[14] S.H. Woo, L. Cleemann, M. Morad, Ca2+ current-gated focal and local
Ca2+ release in rat atrial myocytes: evidence from rapid 2-D confocal
sarcoplasmic reticular calcium release in excitation–contraction cou-
Rabang, M.B. Cannell, C.W. Balke, L.T. Izu, Three-dimensional dis-
tribution of ryanoined receptor clusters in cardiac myocytes, Biophys.


[67] D.J. Bare, D.M. Bars, G.A. Mignery, InsP(3) receptors in ventricular myocytes are primarily in the nuclear envelope, associate with CaMKII delta, and are phosphorylation targets, Circulation 110 (2004) 159–160.