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Inositol 1,4,5-trisphosphate supports the arrhythmogenic action of endothelin-1 on ventricular cardiac myocytes

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Summary

Although ventricular cardiomyocytes express inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] receptors, it is unclear how these Ca2+ channels contribute to the effects of Gq-coupled agonists. Endothelin-1 augmented the amplitude of pacing-evoked Ca2+ signals (positive inotropy), and caused an increasing frequency of spontaneous diastolic Ca2+-release transients. Both effects of endothelin-1 were blocked by an antagonist of phospholipase C, suggesting that Ins(1,4,5)P3 and/or diacylglycerol production was necessary. The endothelin-1-mediated spontaneous Ca2+ transients were abolished by application of 2-aminoethoxydiphenyl borate (2-APB), an antagonist of Ins(1,4,5)P3 receptors. Incubation of electrically-paced ventricular myocytes with a membrane-permeant Ins(1,4,5)P3 ester provoked the occurrence of spontaneous diastolic Ca2+ transients with the same characteristics and sensitivity to 2-APB as the events stimulated by endothelin-1. In addition to evoking spontaneous Ca2+ transients, stimulation of ventricular myocytes with the Ins(1,4,5)P3 ester caused a positive inotropic effect. The effects of endothelin-1 were compared with two other stimuli, isoproterenol and digoxin, which are known to induce inotropy and spontaneous Ca2+ transients by overloading intracellular Ca2+ stores. The events evoked by isoproterenol and digoxin were dissimilar from those triggered by endothelin-1 in several ways. We propose that Ins(1,4,5)P3 receptors support the development of both inotropy and spontaneous pro-arrhythmic Ca2+ signals in ventricular myocytes stimulated with a Gq-coupled agonist.

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Key words: Inositol 1,4,5-trisphosphate, Arrhythmia, Calcium, Ryanodine, Inotropy, Endothelin

Introduction

Calcium (Ca2+) release from the sarcoplasmic reticulum (SR) is the key event linking membrane depolarisation and mechanical activity during excitation-contraction coupling (EC-coupling) in the heart. Depolarisation of the sarcolemma leads to activation of L-type voltage-operated Ca2+ channels (VOCs). The consequent influx of Ca2+ provokes more substantial Ca2+ release from closely apposed ryanodine receptor (RyR) clusters on the SR by a process known as Ca2+-induced Ca2+ release (CICR) (Callewaert, 1992; Roderick et al., 2003). The essential elementary events underlying EC-coupling are ‘Ca2+ sparks’, which result from activation of a cluster of RyRs (Cheng et al., 1993; Rios, 2005). The spatial overlap and temporal summation of signals from multiple Ca2+ -spark sites leads to the rapid homogenous Ca2+ transients that trigger coordinated ventricular myocyte contraction (Bootman et al., 2001; Guatimosim et al., 2002).

The force of contraction of the heart is regulated by the interplay of multiple neurohormonal factors. Endothelin-1 (ET-1) is one such agonist, and has a significant positive inotropic effect in myocytes from different mammalian species. It has also been demonstrated that ET-1 can cause arrhythmias (Russell and Molenaar, 2000). The processes underlying the inotropic and pro-arrhythmic effects of ET-1 are not entirely clear. Proposed mechanisms include stimulation of Ca2+ release (Li et al., 2005; Mackenzie et al., 2002), enhanced L-type Ca2+ current following activation of protein kinase C (He et al., 2000) and sensitization of myofilaments following either PKC-mediated phosphorylation (Pi et al., 2003) or alkalisation resulting from increased Na+-H+ exchange (Russell and Molenaar, 2000).

Although ET-1 can provide short-term inotropic support for failing hearts, this effect comes with the potential burden of arrhythmogenesis and remodelling (Zolk et al., 2004). ET-1 concentration is increased in cardiac tissues during pathological conditions such as congestive heart failure and myocardial infarction (Duru et al., 2001; Russell and Molenaar, 2000). Understanding the mechanism of action of ET-1 is therefore clinically important.

ET-1 is secreted by endocardial endothelial cells, as well as being released by cardiac myocytes themselves. Both ET-1
receptor isotypes (denoted ET<sub>A</sub> and ET<sub>B</sub>) have been demonstrated to cause the activation of phospholipase C (PLC). Hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P<sub>2</sub>] by PLC on the inner leaflet of the plasma membrane yields two products; inositol 1,4,5-trisphosphate [Ins(1,4,5)P<sub>3</sub>] and diacylglycerol (DAG). Ins(1,4,5)P<sub>3</sub> is water soluble, diffuses into cells and binds to specific receptors (Ins(1,4,5)P<sub>3</sub>Rs) that channel Ca<sup>2+</sup> from the lumen of the SR (or endoplasmic reticulum) to the cytosol (Roderick and Bootman, 2003). 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 (Springett et al., 2004; Walker et al., 2003).

Several laboratories have demonstrated the expression of Ins(1,4,5)P<sub>3</sub>R mRNA and functional protein in cardiac myocytes from various mammalian species (Lencesova et al., 2002; Li et al., 2005; Lipp et al., 2000; Perez et al., 1997; Yamada et al., 2001). Also, agonist-stimulated increases in myocyte Ins(1,4,5)P<sub>3</sub> concentration have been monitored by many groups in response to diverse agonists, including ET-1 (Remus et al., 2006). In addition, there are a growing number of studies showing that increased Ins(1,4,5)P<sub>3</sub> levels modulate Ca<sup>2+</sup> release from internal stores within cardiac myocytes (Felzen et al., 1998; Gilbert et al., 1991; Mackenzie et al., 2004a; Zima and Blatter, 2003).

The bulk of evidence for Ins(1,4,5)P<sub>3</sub> causing Ca<sup>2+</sup> release in cardiac myocytes has derived from studies of atrial myocytes, which have a higher expression of Ins(1,4,5)P<sub>3</sub>Rs (Li et al., 2005; Lipp et al., 2000). The role of Ins(1,4,5)P<sub>3</sub>Rs in ventricular myocytes is less clear. Even if Ins(1,4,5)P<sub>3</sub>Rs are expressed and functional, it has been noted that their potential contribution to Ca<sup>2+</sup> signalling inside ventricular myocytes could be modest. Within the adult mammalian heart, the ratio of Ins(1,4,5)P<sub>3</sub>R to RyR expression is approximately 1:100 (Go et al., 1995). This would suggest that the flux of Ca<sup>2+</sup> through Ins(1,4,5)P<sub>3</sub>Rs could be swamped by the activity of RyRs. However, concerted opening of Ins(1,4,5)P<sub>3</sub>Rs, or stimulation of regenerative CICR from neighbouring RyRs could cause Ins(1,4,5)P<sub>3</sub>-mediated Ca<sup>2+</sup> signals to have a profound effect on cardiac Ca<sup>2+</sup> handling.

Here, we characterised the effect of ET-1 on Ca<sup>2+</sup> signals within acutely-isolated ventricular myocytes. We observed two profound effects: a positive inotropic amplification of
depolarisation-induced Ca<sup>2+</sup> transients and an increase in the generation of spontaneous diastolic Ca<sup>2+</sup> transients (SCTs). The characteristics of the ET-1-induced SCTs were significantly different from those evoked by digoxin and isoproterenol in terms of their temporal occurrence and spatial extent. Furthermore, whereas the SCTs triggered by ET-1 were sensitive to Ins(1,4,5)P<sub>3</sub>R antagonism, those evoked by digoxin and isoproterenol were not. These data indicate that Ins(1,4,5)P<sub>3</sub> can lead to the generation of pro-arrhythmic SCTs and inotropy in ventricular myocytes.

Results
Characterisation of the positive inotropic response to ET-1
The aims of the present study were to investigate mechanisms underlying the action of cardiotonic agents on ventricular myocyte Ca<sup>2+</sup> signalling. In these experiments, cells were superfused with various agonists while being electrically paced using field electrodes. The photometry trace shown in Fig. 1A illustrates the typical response observed from a control myocyte superfused with extracellular medium alone. There was no obvious change in the pattern of field-stimulation-evoked Ca<sup>2+</sup> transients during the 25-minute recording. The stability of evoked responses is also evident from the average amplitudes of the pacing-induced Ca<sup>2+</sup> transients, as depicted in Fig. 1B (28 myocytes from 22 independent experiments). An overlay of an electrically-evoked Ca<sup>2+</sup> transient recorded in the first 0.5 minutes of observation with another that occurred within the same cell 25 minutes later is illustrated in Fig. 1C. The rate of increase and decay of the Ca<sup>2+</sup> signals, as well as their amplitudes, were the same at both time points, suggesting that there was no change in Ca<sup>2+</sup> release or sequestration dynamics over this time.

Although the responses of control ventricular cells did not alter during recordings, the Ca<sup>2+</sup> indicator, indo-1, which was used to record cytoplasmic Ca<sup>2+</sup> in the photometry experiments, was bleached to an appreciable degree. The bleaching of indo-1 was responsible for the progressive increase in noise during the diastolic portions of the trace in Fig. 1A. However, despite the decline in absolute fluorescence emission recorded at 405 nm and 490 nm during the experiment, the background-corrected 405:490 nm ratio was unaffected (Fig. 1B).

Superfusion of ventricular myocytes with ET-1 (100 nM)
caused a progressive increase in the amplitude of field-stimulation-evoked Ca\(^{2+}\) signals (Fig. 2A,B). This positive inotropic response became significant when the cells were superfused with ET-1 for ~8 minutes (Fig. 2B). 64% of cells (n=14) displayed a positive inotropic response to ET-1. The other cells showed no significant change in their Ca\(^{2+}\) transients. ET-1 also appeared to modestly accelerate the recovery of field-stimulation-evoked Ca\(^{2+}\) transients (see Table 1). In addition to its positive inotropic effect, ET-1 significantly increased the incidence of SCTs. The SCTs were evident as rapid extra Ca\(^{2+}\) transients that occurred shortly after field-stimulation-evoked signals (Figs 2Aiii and iv).

To further characterise ET-1-induced inotropy, Ca\(^{2+}\) signals in ventricular myocytes were observed using confocal microscopy. The images in Fig. 2C depict the spatial properties of pacing-evoked Ca\(^{2+}\) transients and an SCT. The Ca\(^{2+}\) transients evoked by field stimulation were always recorded as global events with a homogenous elevation of intracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\). The SCT shown in Fig. 2Ciii (at 840 milliseconds) was also evident as a homogenous Ca\(^{2+}\) signal. The SCT appears as a sudden global increase of fluorescence in the confocal images in Fig. 2Ciii (the fluorescence intensity declined after the pacing-evoked signal, but then increased homogenously again at 840 milliseconds). The majority of ET-1-evoked SCTs were synchronous global Ca\(^{2+}\) elevations.

Characterisation of the positive inotropic response to \(\beta\)-adrenergic receptor stimulation

To provide a comparison with the effects of ET-1, the actions of two additional positive inotropic agents – isoproterenol and digoxin – were analysed. Stimulation of \(\beta\)-adrenergic receptors is a well-known physiological mechanism for inducing positive inotropy in the heart (Bers, 2002). The example photometry-traces presented in Fig. 3A and the averaged population data in Fig. 3B illustrate the positive inotropic response evoked by isoproterenol (100 nM). 83% of cells (n=11) displayed a positive inotropic response to isoproterenol. A significant inotropic effect occurred within 100 seconds, and was more rapid with isoproterenol than with any other agonist used in this study. The positive inotropy mediated by isoproterenol began to decline steadily after reaching a peak at 400 seconds (Fig. 3A). Isoproterenol also stimulated the appearance of SCTs, which became progressively more frequent (see below). The high frequency of SCTs is likely to be the cause of the decline of the positive inotropy because they would have caused unloading of the SR. This phenomenon has been noticed by others (Capogrossi et al., 1988). Isoproterenol also accelerated the decay of Ca\(^{2+}\) transients (see Table 1). This is in keeping with the reported action of PKA on phospholamban, which leads to an increase in SERCA2a function, thereby enhancing the sequestration of cytosolic Ca\(^{2+}\) (Bers, 2002).

The montage of images shown in Fig. 3C illustrate the homogenous increase and decrease in fluorescence observed during electrical pacing of an isoproterenol-stimulated cell. SCTs are also evident in the confocal images following isoproterenol stimulation for 5 minutes (Fig. 3Cii) and 12.5 minutes (Fig. 3Ciii). In the majority of cases, isoproterenol-induced SCTs occurred during the, usually quiescent, diastolic periods, after recovery of the field-stimulation-evoked Ca\(^{2+}\) signals.

Three separate SCTs were evident in the sequence of images captured 12.5 minutes after superfusion with isoproterenol (Fig. 3Ciii). The homogenous increases in fluorescence observed at 630 and 1060 milliseconds indicate that these SCTs were caused by spontaneous action potentials (see Discussion). However, the images captured at 1690 and 1900 milliseconds reveal the initiation and propagation of a Ca\(^{2+}\) wave. Another SCT, which was also manifest as a Ca\(^{2+}\) wave,
is evident in the montage of images obtained 5 minutes after superfusion with isoproterenol (Fig. 3Cii). Digoxin, another well-established positive inotrope on ventricular myocytes (Smith, 1978), also caused an increase in the amplitude of pacing-induced Ca\textsuperscript{2+} transients (see supplementary material Fig. S1) and triggered SCTs (see below).

Characterisation of SCTs

In the photometry traces shown in Figs 2 and 3, SCTs are evident as randomly-occurring Ca\textsuperscript{2+} events of varying amplitude and time of increase. They were temporally distinct from the pacing-evoked Ca\textsuperscript{2+} signals. Few SCTs were observed in cells superfused with control medium alone. Only 27\% of control cells (n=28) showed SCTs that were evident in photometry recordings, with each cell generating only a couple of infrequent events. In the cells that did display SCTs, the events generally occurred after the myocytes had been continually paced for >20 minutes (Fig. 4A).

With control cells, the time of occurrence of the SCTs relative to the field stimulation-induced Ca\textsuperscript{2+} transients indicated two sub-populations of events (Fig. 4B). One group of events occurred within 1 second of the previous field-stimulation-evoked Ca\textsuperscript{2+} signal. The other sub-population of SCTs was evident within 1-3 seconds after field stimulation.

The photometry recordings shown above were obtained by averaging indo-1 fluorescence across intact single myocytes. This system is appropriate for recording global Ca\textsuperscript{2+} transients and regenerative Ca\textsuperscript{2+} waves, but non-propagating subcellular events, such as Ca\textsuperscript{2+} sparks, would not be evident. Therefore, to determine whether there were additional SCTs manifest as microscopic events, real-time confocal microscopy was employed. We have previously used this technique to monitor Ca\textsuperscript{2+} sparks in atrial myocytes (Mackenzie et al., 2002). Confocal microscopy indicated that spontaneous Ca\textsuperscript{2+} sparks occurred in addition to the events that were recorded using photometry. In some cases, Ca\textsuperscript{2+} sparks were solely transient, spatially-restricted events, whereas other Ca\textsuperscript{2+} sparks acted as a trigger for the initiation of regenerative Ca\textsuperscript{2+} waves. The frequency of spontaneous Ca\textsuperscript{2+} sparks and number of active Ca\textsuperscript{2+}-spark sites was low in myocytes superfused with control buffer alone, and it did not significantly change over a 15-minute recording period (Fig. 4C). With non-paced myocytes, there was also a very low frequency of SCTs observed (using photometry; data not shown) and Ca\textsuperscript{2+} sparks (visualised with confocal microscopy; Fig. 4D).

ET-1 evoked a substantial increase in SCT occurrence (Fig. 5A). The proportion of cells showing SCTs (70%; n=14) was significantly higher than in control myocytes. In addition, the frequency of SCTs in individual cells was progressively enhanced by superfusion with ET-1 (Fig. 5A). Unlike the SCTs observed in control cells, the events triggered by ET-1 superfusion were found to initiate within 1 second of the previous field-stimulation-evoked Ca\textsuperscript{2+} signal (Fig. 5B). In fact, the majority of SCTs originated on the falling phase of field-stimulation-evoked Ca\textsuperscript{2+} signals (Fig. 2C). With cells superfused with ET-1 for several minutes, the SCTs were often observed as repetitive Ca\textsuperscript{2+} rises (see below). The first event occurred within 1 second after field stimulation and was succeeded by a variable number of SCTs that could persist for the entire diastolic period. Confocal imaging of paced cells indicated that there was no significant increase in the occurrence of non-propagating Ca\textsuperscript{2+} sparks in cells superfused with ET-1 (Fig. 5C). In non-paced ET-1-stimulated myocytes and in control cells, the frequencies of Ca\textsuperscript{2+} sparks (Fig. 5D; observed with confocal imaging) and SCTs (photometry data not shown) were similar. The potent pro-arrhythmic effects of ET-1 were therefore only evident when the cells were electrically paced.

Although 1 \muM digoxin caused a similar positive inotropic effect as 100 nM ET-1, it did not increase the incidence of SCTs compared with control cells (Fig. 6A). By contrast, 10 \muM digoxin lead to a significant increase in SCT frequency (Fig. 6B). Furthermore, all cells (n=8) eventually displayed SCTs after being perfused with 10 \muM digoxin for >500 seconds (Fig. 6C). The timing of digoxin-stimulated SCTs relative to a previous electrically-evoked Ca\textsuperscript{2+} transient is depicted in Fig. 6D. The two clear populations of events that were evident in control cells (Fig. 4B) were not so obvious in digoxin-stimulated events. Rather, the SCTs showed a more wide-spread distribution of latencies, with a majority occurring more than 1 second after the previous field-stimulation-evoked Ca\textsuperscript{2+} transient.

Isoproterenol (100 nM) led to a rapid and significant increase in the generation of SCTs (Fig. 7A) in all cells (n=12). Similar to digoxin, the isoproterenol-evoked SCTs occurred with a wide distribution of latencies following the previous electrically-evoked Ca\textsuperscript{2+} transient (Fig. 7B). Unlike any of the other agents used in this study, isoproterenol evoked a substantial increase in the activity of non-propagating Ca\textsuperscript{2+} sparks in both paced (Fig. 7C) and unpaced (Fig. 7D) conditions.

The average latencies for the occurrence of SCTs under control conditions and following ET-1, digoxin and
isoproterenol are depicted in Fig. 8. Please note that the data show the time between a previous field-stimulation-evoked \( \text{Ca}^{2+} \) signal and the onset of the first SCT. Where SCTs occurred as repetitive events in a diastolic period, only the latency to the first event was measured. The plot of latencies shows that ET-1, digoxin and isoproterenol triggered SCTs with significantly different characteristics. The SCTs triggered by ET-1 occurred earlier than those seen in control cells, whereas the SCTs during superfusion with digoxin or isoproterenol began substantially later.

In addition to their timing, another significant difference between SCTs triggered by ET-1, digoxin or isoproterenol was whether they were manifest as \( \text{Ca}^{2+} \) waves or action-potential-evoked \( \text{Ca}^{2+} \) transients. The trace depicted in Fig. 9Aii shows part of a recording from a ventricular myocyte that had been superfused with ET-1 for 24 minutes. All field-stimulation-evoked \( \text{Ca}^{2+} \) transients were succeeded by SCTs. In the majority of cases, the SCTs had a similar amplitude and rate of increase compared with the electrically-evoked \( \text{Ca}^{2+} \) transients. This is clearly illustrated in Fig. 9Aii, where a section of the trace from Fig. 9Ai is shown on an expanded time scale. The dashed grey lines indicate the rising phases of an evoked \( \text{Ca}^{2+} \) transient and subsequent SCT. Both \( \text{Ca}^{2+} \) signals show a similar slope. By contrast, the SCTs observed in digoxin- or isoproterenol-stimulated cells generally had a lesser amplitude and a slower rate of increase. This is evident in Fig. 9Bi and ii, which depict responses from a cell superfused with isoproterenol for 24 minutes.

The rapidly-rising SCTs were due to spontaneous action-potential-evoked \( \text{Ca}^{2+} \) transients. This is the only mechanism by which \( \text{Ca}^{2+} \) events (detectable by photometry) could occur with a rate of increase similar to field-stimulation-evoked \( \text{Ca}^{2+} \) signals. By contrast, the SCTs with a slower rate of increase represented \( \text{Ca}^{2+} \) waves. The same designation of SCTs as \( \text{Ca}^{2+} \) waves or action-potential-evoked \( \text{Ca}^{2+} \) transients was evident from confocal imaging of cells. The montage of images shown in Fig. 2Dii depicts an ET-1-stimulated SCT as a rapidly rising, spatially homogenous \( \text{Ca}^{2+} \) signal with an intensity similar to the evoked \( \text{Ca}^{2+} \) transient. The simultaneous global development of the SCT indicates that it was due to a spontaneous action potential. By contrast, the isoproterenol-stimulated SCT depicted in Fig. 3Cii was evident as a \( \text{Ca}^{2+} \) wave. Averaging the confocal fluorescence signal across the whole cell (i.e. mimicking the situation with photometry) showed that the \( \text{Ca}^{2+} \) wave was rising more slowly and had a lesser amplitude than the field-stimulation-evoked \( \text{Ca}^{2+} \) transient (data not shown).

The SCTs recorded in photometry experiments were classified into \( \text{Ca}^{2+} \) waves or action-potential-evoked \( \text{Ca}^{2+} \) transients on the basis of their time of increase and amplitude (see Materials and Methods). It was evident that ET-1, digoxin and isoproterenol all stimulated the appearance of both types of event (Fig. 9C). However, the proportions of \( \text{Ca}^{2+} \) waves and action potential-evoked \( \text{Ca}^{2+} \) transients were dependent on the stimulus used. With ET-1, the majority of SCTs manifested as action-potential-evoked \( \text{Ca}^{2+} \) transients, whereas digoxin and isoproterenol largely caused \( \text{Ca}^{2+} \) waves (Fig. 9D). Therefore, the SCTs triggered by ET-1 were different from those evoked by digoxin or isoproterenol, based on analysis of their latencies (Fig. 8) or action potential versus \( \text{Ca}^{2+} \) wave characteristics (Fig. 9D).

**Ins(1,4,5)P3 supports the generation of SCTs by ET-1 and stimulates positive inotropy**

The typical positive inotropic effect of ET-1, and the concomitant generation of SCTs, is illustrated in Fig. 10Ai and Aii. Superfusion with the PLC inhibitor U73122 at 10 \( \mu \text{M} \) prevented the positive inotropic effect of ET-1 (Fig. 10Bi) and also reduced the incidence of SCTs back to control levels.
Fig. 4. Characteristics of SCTs observed during control pacing. (A) Average frequency (mean ± s.e.m.; n=28 cells) of SCTs during control pacing of isolated ventricular myocytes. To calculate the SCT frequency, the number of events occurring within defined stimulation periods of 30-seconds was counted. (B) Time of occurrence of the SCTs relative to the previous field-stimulation-evoked Ca\textsuperscript{2+} signal. Since the cells were electrically paced at 0.33 Hz, the maximal time of occurrence was 3 seconds. (C,D) Number of spontaneous Ca\textsuperscript{2+} sparks recorded using confocal imaging of electrically (C) paced or (D) unpaced cells.

Fig. 5. Characteristics of SCTs observed during ET-1 superfusion. (A) Average frequency (mean ± s.e.m.; n=14 cells) of SCTs during continuous superfusion of isolated ventricular myocytes with ET-1 (100 nM). (B) Time of occurrence of the SCTs relative to the previous field-stimulation-evoked Ca\textsuperscript{2+} signal. (C,D) Number of spontaneous Ca\textsuperscript{2+} sparks recorded using confocal imaging of electrically (C) paced or (D) unpaced cells.
Fig. 6. Characteristics of SCTs observed during superfusion with digoxin. (A,B) Average frequency of SCTs (mean ± s.e.m.; n=8 cells) during continuous superfusion of isolated ventricular myocytes with either (A) 1 μM or (B) 10 μM digoxin. (C) Proportion of cells that displayed SCTs in response to 1 or 10 μM digoxin. (D) Time of occurrence of the SCTs relative to the previous field-stimulation-evoked Ca\(^{2+}\) signal.

Fig. 7. Characteristics of SCTs observed during superfusion with isoproterenol. (A) Average frequency of SCTs (mean ± s.e.m.; n=12 cells) of SCTs during continuous superfusion of isolated ventricular myocytes with isoproterenol (100 nM). (B) Time of occurrence of the SCTs relative to the previous field-stimulation-evoked Ca\(^{2+}\) signal. (C,D) Number of spontaneous Ca\(^{2+}\) sparks recorded using confocal imaging of electrically (C) paced or (D) unpaced cells.
When applied on its own to electrically paced cells, U73122 caused no significant reduction of the field-stimulation-evoked Ca\(^{2+}\) signals and a slight decrease in the number of SCTs (Fig. 10Ci and Cii).

To examine the role of Ins(1,4,5)\(P_3\) in ET-1 responses, the membrane-permeant Ins(1,4,5)\(P_3\) antagonist 2-APB was used. The traces in Fig. 11A illustrate the typical development of inotropy and SCTs during prolonged incubation with ET-1. Following 24 minutes of superfusion with ET-1 (100 nM), the peak into-1 signal (measured as a ratio of 405 nm to 490 nm emission) had increased by 34%. In addition, the progressive increase in the incidence of SCTs is apparent, with trains of multiple SCTs being manifest in the later stages of the recording. Concurrent superfusion of ventricular myocytes with ET-1 (100 nM) and 2-APB (2 \(\mu\)M) significantly reduced the incidence of SCTs (Fig. 11B,C).

Although it is well-known that 2-APB can affect a number of cellular processes (Bootman et al., 2002; Peppiatt et al., 2003) at the concentration used in this study and previously (Mackenzie et al., 2002), it does not appear to have non-specific actions. In control experiments, cells that had been superfused continuously with 2 \(\mu\)M 2-APB displayed field-stimulation-evoked Ca\(^{2+}\) signals with unaltered peak amplitudes or kinetics (supplementary material Fig. S2). Furthermore, the SCTs triggered by isoproterenol or digoxin were not significantly inhibited by 2-APB (supplementary material Fig. S3), suggesting that the events triggered by these agonists have a different underlying mechanism and that 2-APB does not generally block spontaneous Ca\(^{2+}\) release. 2-APB did not affect the rate or extent of inotropic response in isoproterenol- or digoxin-stimulated cells (data not shown).

The most direct method for stimulating Ins(1,4,5)\(P_3\)Rs in situ while avoiding the pleiotropic actions of hormones is to

**Fig. 8.** Bar graph, showing the average latency for the initiation of SCTs during superfusion with control medium or the agonists shown. * indicates data significantly different from control (determined using Kruskal-Wallis one-way ANOVA with Bonferroni correction). The data represent mean ± s.e.m. (n=60 SCTs for each condition).

(compare Fig. 4A with Fig. 10Bii). When applied on its own to electrically paced cells, U73122 caused a significant reduction of the field-stimulation-evoked Ca\(^{2+}\) signals and a slight decrease in the number of SCTs (Fig. 10Ci and Cii).

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The most direct method for stimulating Ins(1,4,5)\(P_3\)Rs in situ while avoiding the pleiotropic actions of hormones is to

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**Fig. 9.** Differences in the characteristics of SCTs stimulated by ET-1, digoxin and isoproterenol. (A, B) Parts of photometry recordings from cells stimulated with either (Ai) 100 nM ET-1 or (Bi) 100 nM isoproterenol. (Aii, Bii) Traces on an expanded time scale. Arrows indicate instances when the cells were field-stimulated. The dashed grey lines illustrate the rate of increase of the field-stimulation-evoked Ca\(^{2+}\) signals and the subsequent SCTs. It is evident that for the ET-1-stimulated cell (Aii) the rate of increase of the SCT was comparable to the field-stimulation-evoked event (i.e. it was due to an action potential). By contrast, in the isoproterenol-stimulated cell, the SCT developed at a much slower rate (i.e. it was due to a Ca\(^{2+}\) wave). (C) Peak rate of Ca\(^{2+}\) signal increase during SCTs recorded from six cells incubated with ET-1, and six cells treated with isoproterenol. □ indicates the typical rate of increase of field-stimulation-evoked Ca\(^{2+}\) transients (\(\Delta E_{\text{pm}}\)). ■ SCTs that arose with a similar rate to those triggered by field stimulation. ○ SCTs that were consistent with the development of Ca\(^{2+}\) waves. Italicised numbers indicate the number of SCTs measured for each data point, they also illustrate the relative proportions of events observed in each of the individual cells. (D) Proportion of SCTs that occurred as an action potential (i.e. that had a rate of increase >2000 nM s\(^{-1}\)) for ET-1, digoxin or isoproterenol.
use the membrane-permeant Ins(1,4,5)P$_3$ ester first introduced by Tsien and colleagues (Li et al., 1997). We (Chen et al., 2004; Kasri et al., 2004; Mackenzie et al., 2002; Peppiatt et al., 2003) and several other groups (Johenning et al., 2002; Tovey et al., 2003; Vermassen et al., 2003) have used the Ins(1,4,5)P$_3$ ester to specifically activate Ins(1,4,5)P$_3$R-mediated Ca$^{2+}$ release. All reported effects of Ins(1,4,5)P$_3$ ester are consistent with a sole action of the compound on Ins(1,4,5)P$_3$Rs.

Incubation of ventricular myocytes with Ins(1,4,5)P$_3$ ester (10 μM) led to a progressive increase in SCTs, although at a lower frequency than observed with 100 nM ET-1 (Fig. 11D). When 2-APB was applied simultaneously with Ins(1,4,5)P$_3$ ester, the SCTs were significantly reduced (Fig. 11E,F). Application of Ins(1,4,5)P$_3$ ester also stimulated an increase in the amplitude of pacing-evoked Ca$^{2+}$ transients. This positive inotropic effect was abolished by 2-APB (supplementary material Fig. S4). These data show that direct activation of Ins(1,4,5)P$_3$Rs with the Ins(1,4,5)P$_3$ ester mimics the effects of ET-1 on ventricular myocytes.

As described above, ET-1 increased the proportion of cells that displayed SCTs. A similar effect was observed with Ins(1,4,5)P$_3$ ester (Fig. 12A). For myocytes treated with ET-1 or Ins(1,4,5)P$_3$ ester, 2-APB reduced the percentage of cells showing SCTs back to control levels (Fig. 12A). The SCTs observed during Ins(1,4,5)P$_3$ ester superfusion were similar in their characteristics to those evoked by ET-1. In particular, they tended to occur during the recovery-phase of a field-stimulation-evoked Ca$^{2+}$ transient. This is evident from the traces depicted in Fig. 11D. The average latency for SCT occurrence during Ins(1,4,5)P$_3$ ester superfusion was comparable with that for ET-1 (Fig. 12B). Concurrent application of 2-APB (2 μM) with ET-1 or Ins(1,4,5)P$_3$ ester increased the latency for SCT generation to control levels (Fig. 12B). These data are consistent with the notion that Ins(1,4,5)P$_3$Rs are largely responsible for the SCTs that occur during the recovery of field-stimulation-evoked Ca$^{2+}$ transients. Inhibition of Ins(1,4,5)P$_3$Rs by 2-APB prevents these early SCTs, leaving only the events that would ordinarily be present during control stimulation.

**Discussion**

Here, we investigated the contribution of Ins(1,4,5)P$_3$ to the well-characterised physiological and pathological actions of ET-1. It is established that ET-1 acts as a positive inotropic agent in the heart (Russell and Molenaar, 2000). However, in addition to stimulating the contraction of the heart, ET-1 can evoke arrhythmias (Duru et al., 2001). ET-1 binds to receptors on the surface of cardiac myocytes and stimulates the activity of PLC to produce the intracellular messengers DAG and Ins(1,4,5)P$_3$. Activation of PKC by DAG has been widely implicated in the positive inotropic effect of ET-1 (He et al., 2000; Pi et al., 2003), but the role played by Ins(1,4,5)P$_3$ has not been thoroughly examined in ventricular myocytes.

To determine the unique characteristics of SCTs evoked by ET-1, we used two additional stimuli – isoproterenol and digoxin – that also have established positive inotropic and arrhythmic actions (Baartscheer et al., 2003; Smith, 1978; Tsien et al., 1978). The mechanism underlying isoproterenol- and digoxin-induced SCTs is believed to principally involve enhanced SR Ca$^{2+}$ loading (Capogrossi et al., 1988). It has been demonstrated that an increase in the Ca$^{2+}$ content of the SR promotes the occurrence of spontaneous Ca$^{2+}$ waves (Kurebayashi et al., 2004; Lukyanenko et al., 1999), and that this hemorrhaging of Ca$^{2+}$ limits the inotropic effect of neurohormonal agents (Capogrossi et al., 1988). Consistent with these concepts, we found that the SCTs in response to
both isoproterenol and digoxin were most commonly observed to be Ca\(^{2+}\) waves (Fig. 3C and Fig. 8). Furthermore, the inotropy stimulated by isoproterenol gradually declined upon reaching the peak amplitude (Fig. 3). The decrease in the inotropic effect was temporally correlated with the increase in SCT frequency (Fig. 7).

It is evident from a number of criteria that the SCTs evoked by ET-1 were distinct from those triggered by isoproterenol or digoxin. Whereas 2-APB dramatically reduced the occurrence of SCTs during ET-1 stimulation (Fig. 11), the Ins(1,4,5)\(P_3\)R antagonist did not have a significant effect on the spontaneous events observed with isoproterenol or digoxin (supplementary material Fig. S3). In addition, the SCTs triggered by ET-1 occurred largely on the decreasing phase of a previous field-stimulation-evoked Ca\(^{2+}\) signal (Fig. 9A). By contrast, isoproterenol and digoxin promoted events that were more likely to occur when Ca\(^{2+}\) had recovered to diastolic levels (Figs 6 and 7). Furthermore, the majority of spontaneous events recorded during application of ET-1 had rates of increase and amplitudes consistent with them being action potentials (Fig. 9), rather than Ca\(^{2+}\) waves. The clear differences between SCTs induced by ET-1, isoproterenol and digoxin, point to distinct mechanisms for their generation. The sensitivity of ET-1-evoked SCTs to the PLC antagonist U73122 (Fig. 10) and the Ins(1,4,5)\(P_3\)R antagonist 2-APB (Fig. 11), and the similarity of the spontaneous events triggered by application of Ins(1,4,5)\(P_3\) ester (Figs 11 and 12), support the notion that Ins(1,4,5)\(P_3\)Rs are responsible. It has been noticed previously that the focal origin of ET-1-mediated arrhythmias within the heart favours a mechanism of triggered activity, such as that observed by stimulating Ins(1,4,5)\(P_3\)Rs (Duru et al., 2001).

Within atrial myocytes, which have a higher expression of Ins(1,4,5)\(P_3\)Rs than ventricular cells (Lipp et al., 2000), elevation of Ins(1,4,5)\(P_3\) gives rise to a substantial positive inotropic effect (Mackenzie et al., 2004a; Zima and Blatter, 2004). In the present study using ventricular myocytes, we similarly observed that direct stimulation of Ins(1,4,5)\(P_3\)Rs (Figs 11 and 12), support the notion that Ins(1,4,5)\(P_3\)Rs are responsible. It has been noticed previously that the focal origin of ET-1-mediated arrhythmias within the heart favours a mechanism of triggered activity, such as that observed by stimulating Ins(1,4,5)\(P_3\)Rs (Duru et al., 2001).

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It is therefore plausible that Ins(1,4,5)P₃Rs are inhibited by the Ca²⁺ signals that occur during L-type VOC opening but, as the cytosolic Ca²⁺ concentration slowly recovers, it reaches a threshold where Ins(1,4,5)P₃Rs become active, thereby triggering an SCT. In addition, the opening of some Ins(1,4,5)P₃Rs during Ca²⁺ flux through L-type VOC could explain the positive inotropic effects of Ins(1,4,5)P₃ (Mackenzie et al., 2002b). Owing to their dual requirement for Ins(1,4,5)P₃ and Ca²⁺, Ins(1,4,5)P₃Rs would be ‘tuned’ to the electrical activity of the cell through the activity of L-type VOCS. By contrast, with digoxin and isoproterenol the SCTs are caused by SR Ca²⁺ overload. The consequent sensitisation of RyRs by the enhanced Ca²⁺ load would make them prone to spontaneous opening without the need for a triggering signal. The RyRs could therefore open randomly after a field-stimulation-evoked Ca²⁺ signal.

It is unclear why ET-1 produced a higher proportion of SCTs that manifested themselves as action potentials, whereas isoproterenol and digoxin largely triggered Ca²⁺ waves (Fig. 9). All spontaneous Ca²⁺ release events that reach the plasma membrane cause cellular depolarisation. This is due to the activation of depolarising currents, for example arising from forward-mode Na⁺-Ca²⁺ exchange and Ca²⁺-activated Cl⁻ efflux (Boyden and ter Keurs, 2001). The magnitude of cellular depolarisation depends on the proportion of the membrane that interacts with the spontaneous Ca²⁺ signal (Capogrossi et al., 1987; Mackenzie et al., 2002). The Ca²⁺ waves that were largely evident in cells stimulated with isoproterenol or digoxin would be expected to cause a modest depolarisation that is sub-threshold for triggering an action potential, because such Ca²⁺ waves have a limited spatial extent at any moment. For ET-1 to promote SCTs most commonly as action potentials, the trigger Ca²⁺ signal arising from Ins(1,4,5)P₃Rs must be widespread and must occur in close proximity to the sarcolemma. The exact location of Ins(1,4,5)P₃Rs in adult ventricular myocytes is presently not clear. It has been suggested that they are present at the nuclear envelope (Bare et al., 2005) and diadic junctions (Mohler et al., 2003). However, further studies are required to firmly establish the locations of Ins(1,4,5)P₃Rs in ventricular cells, and to determine whether different populations of these channels have specific functions.

In conclusion, our data indicate that Ins(1,4,5)P₃Rs are expressed and functional in adult ventricular myocytes. Activation of Ins(1,4,5)P₃Rs within electrically paced myocytes leads to the generation of spontaneous Ca²⁺ release transients and enhanced EC-coupling. It is important to notice that during various cardiac pathologies, such as heart failure or mitral valve disease, the expression of Ins(1,4,5)P₃Rs is increased (Go et al., 1995; Yamada et al., 2001). In principle, this would make the heart even more susceptible to the pro-arrhythmogenic action of Ins(1,4,5)P₃. It is therefore plausible that Ins(1,4,5)P₃Rs are a putative target for therapeutic intervention in some forms of arrhythmia.

**Materials and Methods**

**Materials**

Collagenease type II was purchased from Worthington Biochemical Corporation (Lorne Laboratories Ltd, UK). Endothelin-1 (ET-1) was obtained from Calbiochem. Salts for extracellular solutions were purchased from Sigma-Aldrich or BDH. Ca²⁺-sensitive fluorescent indicators were purchased from Molecular Probes (Cambridge Bioscience, UK). The PLC inhibitor U75122 was from Calbiochem, UK and the
membrane-permeant Ins(1,4,5)P_3 antagonist 2-aminoethoxydiphenyl borate (2-APB) was from Sigma, UK. Rats were obtained from Harlan (Bicester, UK).

Myocyte isolation
Wistar rats weighing approximately 250 g were anaesthetised in a CO_2 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 (NaCl 135 mM, KCl 5 mM, CaCl_2 1 mM HEPES 10 mM, glucose 20 mM and MgCl_2 0.4 mM, pH 7.35) at 37°C. This was followed by superfusion with a low-Ca2+ buffer (NaCl 120 mM, KCl 5 mM, CaCl_2 80 μM HEPES 10 mM, tauroine 20 mM, glucose 20 mM, MgSO_4 5 mM, NTA 5.5 mM, and pyruvate 5 mM, pH 6.96) for 3.5 minutes. Following the low-Ca2+ buffer, an enzyme superfusion with a low-Ca^2+ buffer (NaCl 120 mM, KCl 5 mM, CaCl_2 35 μM, HEPES 10 mM, tauroine 20 mM, glucose 20 mM, MgSO_4 5 mM and pyruvate 5 mM, pH 7.4) was recirculated through the apparatus for 10 minutes. This solution also contained 118 international units mL^{-1} type II collagenase (Worthington, UK). Following dissociation of the myocytes they were allowed to settle, and Ca^2+ was reintroduced in an incremental manner to prevent intracellular Ca^2+ overload and hypercontracture. The myocytes were kept at room temperature until ready for use. Only rod-shaped myocytes without any signs of deterioration and without spontaneous activity at rest were used for experiments. The myocytes were settled on poly-L-lysine-coated coverslips for 45 minutes before loading them with a fluorescent Ca^2+ indicator. All experiments were performed in accordance with the guidelines of the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986.

Photometry and confocal imaging, and field stimulation
For photometry experiments, cells were loaded with indo-1 by incubation with indo-1/AM (3 μM for 30 minutes). The cells were then washed in HEPES buffer and left for 45 minutes to allow complete de-esterification. For laser-scanning confocal microscopy (LSCM) experiments, cells were loaded with Fluo-4/AM by using a similar procedure to that for indo-1, except that the myocytes were incubated with 2 μM Fluo-4/AM for 20 minutes.

For the photometry recordings, coverslips bearing indo-1-loaded myocytes were placed on the stage of an inverted microscope (Nikon Diaphot). These were imaged with complete de-esterified indo-1/AM-loaded myocytes using a confocal microscope (LSCM) system, and images were processed using software to image individual Ca^2+ concentrations in myocytes. For laser excitation, the myocytes were loaded with a fluorescent Ca^2+ indicator, and excited at various wavelengths, each with a power density of 10 to 20 mW/μm^2. The myocytes were then loaded with Fluo-4/AM and observed under the same conditions as described for the LSCM system, with the exception that the power density was reduced to 1 mW/μm^2 to prevent Ca^2+ overload and hypercontracture.

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For the photometry recordings, coverslips bearing indo-1-loaded myocytes were placed on the stage of an inverted microscope (Nikon Diaphot) and superfused with HEPES buffer containing 1 mM CaCl_2. The pacing apparatus and conditions were the same as those described above for photometry. The LSCM images were captured using a Nikon Eclipse TE300 microscope equipped with a Nikon DS-5M digital camera and captured using NIS-Elements software running on a Silicon Graphics computer. 2D confocal images were acquired using a Zeiss LSM510 confocal microscope, and 3D confocal images were acquired using a Zeiss LSM710 confocal microscope. The photomultiplier signals were digitised using an analogue to digital converter controlled by PhoCal (PerkinElmer, UK). The background fluorescence at 405 nm and 490 nm was used to normalise the photomultiplier signals. The photomultiplier signals were digitised using an analogue to digital converter controlled by PhoCal (PerkinElmer, UK). The background fluorescence at 405 nm and 490 nm was used to normalise the photomultiplier signals. The photomultiplier signals were digitised using an analogue to digital converter controlled by PhoCal (PerkinElmer, UK). The background fluorescence at 405 nm and 490 nm was used to normalise the photomultiplier signals.

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