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 ETₐ and ETₐ) have been demonstrated to cause the activation of phospholipase C (PLC). Hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] by PLC on the inner leaflet of the plasma membrane yields two products; inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and diacylglycerol (DAG). Ins(1,4,5)P₃ is water soluble, diffuses into cells and binds to specific receptors (Ins(1,4,5)P₃Rs) that channel Ca²⁺ 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)P₃Rs 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)P₃ 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)P₃ levels modulate Ca²⁺ release from internal stores within cardiac myocytes (Felzner et al., 1998; Gilbert et al., 1991; Mackenzie et al., 2004a; Zima and Blatter, 2003).

The bulk of evidence for Ins(1,4,5)P₃ causing Ca²⁺ release in cardiac myocytes has derived from studies of atrial myocytes, which have a higher expression of Ins(1,4,5)P₃Rs (Li et al., 2005; Lipp et al., 2000). The role of Ins(1,4,5)P₃ in ventricular myocytes is less clear. Even if Ins(1,4,5)P₃Rs are expressed and functional, it has been noted that their potential contribution to Ca²⁺ signalling inside ventricular myocytes could be modest. Within the adult mammalian heart, the ratio of Ins(1,4,5)P₃Rs to RyR expression is approximately 1:100 (Go et al., 1995). This would suggest that the flux of Ca²⁺ through Ins(1,4,5)P₃Rs could be swamped by the activity of RyRs. However, concerted opening of Ins(1,4,5)P₃Rs, or stimulation of regenerative CICR from neighbouring RyRs could cause Ins(1,4,5)P₃-mediated Ca²⁺ signals to have a profound effect on cardiac Ca²⁺ handling.

Here, we characterised the effect of ET-1 on Ca²⁺ signals within acutely-isolated ventricular myocytes. We observed two profound effects: a positive inotropic amplification of depolarisation-induced Ca²⁺ transients and an increase in the generation of spontaneous diastolic Ca²⁺ 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)P₃R antagonism, those evoked by digoxin and isoproterenol were not. These data indicate that Ins(1,4,5)P₃ 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 Ca²⁺ 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 Ca²⁺ transients during the 25-minute recording. The stability of evoked responses is also evident from the average amplitudes of the pacing-induced Ca²⁺ transients, as depicted in Fig. 1B (28 myocytes from 22 independent experiments). An overlay of an electrically-evoked Ca²⁺ 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 Ca²⁺ signals, as well as their amplitudes, were the same at both time points, suggesting that there was no change in Ca²⁺ release or sequestration dynamics over this time.

Although the responses of control ventricular cells did not alter during recordings, the Ca²⁺ indicator, indo-1, which was used to record cytoplasmic Ca²⁺ 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,
Table 1. Temporal characteristics of pacing-evoked Ca2+ transients in control cells and in cells superfused with agonist

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time to reach amplitude peak</th>
<th>Time of decay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_0 ) (milliseconds)</td>
<td>( t_{1440} ) (milliseconds)</td>
</tr>
<tr>
<td>Control</td>
<td>54±2.1</td>
<td>68±2.9</td>
</tr>
<tr>
<td>ET-1 (100 nM)</td>
<td>54±2.7</td>
<td>64±1.5</td>
</tr>
<tr>
<td>Isoproterenol (100 nM)</td>
<td>61±2.8</td>
<td>74±2.7</td>
</tr>
<tr>
<td>Digoxin (10 μM)</td>
<td>67±3.5</td>
<td>76±3.1</td>
</tr>
</tbody>
</table>

The table shows the average (mean ± s.e.m.) increase and recovery times for field-stimulation-evoked Ca2+ transients in cells superfused with the reagents shown. The data were sampled either before agonist addition (\( t_0 \)) or after 24 minutes of pacing with the appropriate reagent (\( t_{1440} \)). The time to reach peak amplitude was calculated as the interval between depolarisation of the cell and the maximum increase of the Ca2+ transient. The time of decay was taken as the period between the peak Ca2+ rise and the time taken to for cytosolic Ca2+ to return to the pre-depolarisation diastolic level. The data were calculated from ten field-stimulation-evoked Ca2+ transients from 6 myocytes for each condition (i.e. \( n=60 \) for each data point).

is evident in the montage of images obtained 5 minutes after superfusion with isoproterenol (Fig. 3Ci).

Digoxin, another well-established positive inotrope on ventricular myocytes (Smith, 1978), also caused an increase in the amplitude of pacing-induced Ca2+ 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 Ca2+ events of varying amplitude and time of increase. They were temporally distinct from the pacing-evoked Ca2+ 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 Ca2+ transients indicated two sub-populations of events (Fig. 4B). One group of events occurred within 1 second of the previous field-stimulation-evoked Ca2+ 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 Ca2+ transients and regenerative Ca2+ waves, but non-propagating subcellular events, such as Ca2+ 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 Ca2+ sparks in atrial myocytes (Mackenzie et al., 2002). Confocal microscopy indicated that spontaneous Ca2+ sparks occurred in addition to the events that were recorded using photometry. In some cases, Ca2+ sparks were solely transient, spatially-restricted events, whereas other Ca2+ sparks acted as a trigger for the initiation of regenerative Ca2+ waves. The frequency of spontaneous Ca2+ sparks and number of active Ca2+-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 Ca2+ 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 Ca2+ signal (Fig. 5B). In fact, the majority of SCTs originated on the falling phase of field-stimulation-evoked Ca2+ signals (Fig. 2C). With cells superfused with ET-1 for several minutes, the SCTs were often observed as repetitive Ca2+ 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 Ca2+ sparks in cells superfused with ET-1 (Fig. 5A). In non-paced ET-1-stimulated myocytes and in control cells, the frequencies of Ca2+ 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 μM digoxin caused a similar positive inotrope effect as 100 nM ET-1, it did not increase the incidence of SCTs compared with control cells (Fig. 6A). By contrast, 10 μM digoxin lead to a significant increase in SCT frequency (Fig. 6B). Furthermore, all cells (\( n=8 \)) eventually displayed SCTs after being perfused with 10 μM digoxin for >500 seconds (Fig. 6C). The timing of digoxin-stimulated SCTs relative to a previous electrically-evoked Ca2+ 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 cells stimulated with 10 μM digoxin. 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 Ca2+ 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 Ca2+ transient (Fig. 7B). Unlike any of the other agents used in this study, isoproterenol evoked a substantial increase in the activity of non-propagating Ca2+ 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
Ins(1,4,5)P3-evoked inotropy and arrhythmias

isoproterenol are depicted in Fig. 8. Please note that the data show the time between a previous field-stimulation-evoked 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 Ca^{2+} waves or action-potential-evoked Ca^{2+} transients. The trace depicted in Fig. 9Ai shows part of a recording from a ventricular myocyte that had been superfused with ET-1 for 24 minutes. All field-stimulation-evoked 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 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 Ca^{2+} transient and subsequent SCT. Both 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 Ca^{2+} transients. This is the only mechanism by which Ca^{2+} events (detectable by photometry) could occur with a rate of increase similar to field-stimulation-evoked Ca^{2+} signals. By contrast, the SCTs with a slower rate of increase represented Ca^{2+} waves. The same designation of SCTs as Ca^{2+} waves or action-potential-evoked Ca^{2+} transients was evident from confocal imaging of cells. The montage of images shown in Fig. 2Diii depicts an ET-1-stimulated SCT as a rapidly rising, spatially homogenous Ca^{2+} signal with an intensity similar to the evoked 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 Ca^{2+} wave. Averaging the confocal fluorescence signal across the whole cell (i.e. mimicking the situation with photometry) showed that the Ca^{2+} wave was rising more slowly and had a lesser amplitude than the field-stimulation-evoked Ca^{2+} transient (data not shown).

The SCTs recorded in photometry experiments were classified into Ca^{2+} waves or action-potential-evoked 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 Ca^{2+} waves and action potential-evoked Ca^{2+} transients were dependent on the stimulus used. With ET-1, the majority of SCTs manifested as action-potential-evoked Ca^{2+} transients, whereas digoxin and isoproterenol largely caused 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 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 μM prevented the positive inotropic effect of ET-1 (Fig. 10Bi) and also reduced the incidence of SCTs back to control levels.

Fig. 3. Isoproterenol stimulates positive inotropy and SCTs in ventricular myocytes. (Ai-iv) Traces depict the effect of continuous superfusion of ventricular myocytes with isoproterenol (100 nM). The isoproterenol superfusion was initiated just after the trace in i was recorded. Arrows indicate field stimulation. (B) Normalised response (mean ± s.e.m.; n=12 cells) of ventricular myocytes to electrical pacing over a 25-minute period. *P<0.05; calculated using Student’s t-test). (C) Montages of confocal images obtained from a single ventricular myocyte loaded with Fluo-4 either (i) before, (ii) 5 minutes after or 12.5 minutes (iii) after the start of superfusion with isoproterenol. The cell was field stimulated at the times indicated by the white arrows. N.B. In panel Cii, the initial image of the cell (i.e. at 0 milliseconds) shows elevated Ca^{2+} at the extreme ends of the cell. This reflects the final stage of a Ca^{2+} wave that had propagated horizontally throughout the myocyte. Bar, spatial scale of 15 μm.
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\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) signal. (C,D) Number of spontaneous Ca\(^{2+}\) 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 Ca²⁺ 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 Ca²⁺ signal. (C,D) Number of spontaneous Ca²⁺ sparks recorded using confocal imaging of electrically (C) paced or (D) unpaced cells.
(compare Fig. 4A with Fig. 10Bii). When applied on its own to electrically paced cells, U73122 caused a nonsignificant 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 nonspecific 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 use 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).

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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 use 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 nonspecific 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).
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\textsuperscript{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\textsubscript{3} 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\textsuperscript{2+} signal (Fig. 9A). By contrast, isoproterenol and digoxin promoted events that were more likely to occur when Ca\textsuperscript{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\textsuperscript{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\textsubscript{3}R antagonist 2-APB (Fig. 11), and the similarity of the spontaneous events triggered by application of Ins(1,4,5)P\textsubscript{3} ester (Figs 11 and 12), support the notion that Ins(1,4,5)P\textsubscript{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\textsubscript{3}Rs (Duru et al., 2001).

Within atrial myocytes, which have a higher expression of Ins(1,4,5)P\textsubscript{3}Rs than ventricular cells (Lipp et al., 2000), elevation of Ins(1,4,5)P\textsubscript{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\textsubscript{3}Rs using the Ins(1,4,5)P\textsubscript{3} ester caused an increase in the amplitude of field-stimulation-evoked Ca\textsuperscript{2+} transients (Fig. 11D and supplementary material Fig. S4). The positive inotropic effect of the Ins(1,4,5)P\textsubscript{3} ester, albeit significant, was not as substantial as that observed with ET-1. This may be due to ET-1 causing a more profound increase in the cellular Ins(1,4,5)P\textsubscript{3} concentration, perhaps in a critical subcellular location such as T-tubule membranes (Robu et al., 2003), which we cannot precisely match using passive diffusion of the Ins(1,4,5)P\textsubscript{3}.

Fig. 11. ET-1 and Ins\textsubscript{1,4,5}P\textsubscript{3} ester both stimulate SCTs that are inhibited by 2-APB. (A) Response of a single ventricular myocyte to continuous superfusion with ET-1 (100 nM). The cell was stimulated with ET-1 following the acquisition of the top (0 seconds) trace. Positive inotropy and SCT frequency developed progressively during the application of ET-1. (B) Similar experiment to that illustrated in A, except that the cell was superfused simultaneously with ET-1 (100 nM) and 2-APB (2 \mu M). (C) Average SCT frequencies for cells superfused with either ET-1 alone (black bars) or ET-1 + 2-APB (grey bars). (D,E) Similar experiments to those illustrated in A and B, except that the cells were stimulated with a membrane-permeant Ins\textsubscript{1,4,5}P\textsubscript{3} ester instead of ET-1. (F) Average SCT frequencies for cells superfused with either Ins\textsubscript{1,4,5}P\textsubscript{3} ester alone (black bars) or Ins\textsubscript{1,4,5}P\textsubscript{3} ester + 2-APB (grey bars). *P<0.05 (significantly different from control); calculated using Student’s t-test.)

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It is therefore plausible that \( \text{Ins}(1,4,5)P_3 \)-evoked inotropy and arrhythmias are caused by SR Ca\(^{2+} \) overload. The consequent sensitisation of RyRs by the enhanced Ca\(^{2+} \) 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 Ca\(^{2+} \) 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 Ca\(^{2+} \) waves (Fig. 9). All spontaneous Ca\(^{2+} \)-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\(^{+}\)-Ca\(^{2+} \) exchange and Ca\(^{2+} \)-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 Ca\(^{2+} \) signal (Capogrossi et al., 1987; Mackenzie et al., 2002). The Ca\(^{2+} \) 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 Ca\(^{2+} \) waves have a limited spatial extent at any moment. For ET-1 to promote SCTs most commonly as action potentials, the trigger Ca\(^{2+} \) signal arising from \( \text{Ins}(1,4,5)P_3 \)Rs must be widespread and must occur in close proximity to the sarclemma. The exact location of \( \text{Ins}(1,4,5)P_3 \)Rs 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 \( \text{Ins}(1,4,5)P_3 \)Rs in ventricular cells, and to determine whether different populations of these channels have specific functions.

In conclusion, our data indicate that \( \text{Ins}(1,4,5)P_3 \)Rs are expressed and functional in adult ventricular myocytes. Activation of \( \text{Ins}(1,4,5)P_3 \)Rs within electrically paced myocytes leads to the generation of spontaneous Ca\(^{2+} \)-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 \( \text{Ins}(1,4,5)P_3 \)Rs 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 \( \text{Ins}(1,4,5)P_3 \). It is therefore plausible that \( \text{Ins}(1,4,5)P_3 \)Rs are a putative target for therapeutic intervention in some forms of arrhythmia.

### Materials and Methods

#### Materials

Collagenase 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. Ca\(^{2+} \)-sensitive fluorescent indicators were purchased from Molecular Probes (Cambridge Bioscience, UK). The PLC inhibitor U73122 was from Calbiochem, UK and the...
membrane-permeant Ins(1,4,5)P3, R antagonist 2-aminophosphorylethenyl 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 CO2 chamber and then killed by cervical dislocation. The heart was then dissected from the thorax and placed on the stage of an inverted microscope (Nikon Diaphot) and superfused with (Callewaert et al., 1991).

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 30 minutes to allow complete de-esterification. For laser-scanning confocal microscopy (LSCM) experiments, cells were loaded with Fluo-4 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, cover slips bearing indo-1-loaded myocytes were placed on the stage of an inverted microscope (Nikon Diaphot) and superfused with HEPES buffer containing 1 mM CaCl2. The cells were stimulated for 2 milliseconds at 40 V with a pair of platinum field electrodes placed at equal distance from the cell (distance between electrodes was 1 cm). The pacing frequency was 0.33 Hz. Indo-1 was excited at a wavelength of 405 nm; the fluorescence emission at 405 nm and 490 nm was sampled using two independent photomultiplier detectors every 10 milliseconds. 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 emission was determined from fields of view without cells, and this was automatically subtracted from the subsequent experimental records by the software. The cells were paced continually throughout an experiment. However, to reduce bleaching of the indicator, indo-1 fluorescence was sampled discontinuously (30-second illumination and recording period followed by 90 seconds without illumination). The fluorescence ratio was converted into Ca2+ concentration using a calibration procedure described previously (Cayre et al., 1992).

For the confocal recordings, cover slips bearing Fluo-4-loaded myocytes were placed on the stage of an inverted microscope (Nikon TE 300) and superfused with HEPES buffer containing 1 mM CaCl2. The pacing apparatus and conditions were the same as those described above for photometry. The LSCM images were captured using a NARAN Ox (Middleton, USA) and data were acquired using Naran's Intervision software running on a Silicon Graphics computer. 2D confocal images (512x115 pixels; confocal slit aperture 25 µm) were acquired at 120 Hz. Laser excitation of Fluo-4 was applied in 10-second bursts at pre-set times over a 12.5-minute period.

Supersession of cells was accomplished using a gravity-driven flow of solution over the top of the cells. Solution exchange around a cell took less than 1 second. All experiments were performed at room temperature (20-22°C), because cell viability and dye loading is prolonged at this temperature. Before the experiments, all cells were paced for a brief period to establish a steady-state Ca2+ response. During this period, there was a ‘positive staircase’ indication of Ca2+ loading. Generally, it took between 10 and 20 depolarisations to reach equilibrium.

**Designation of SCTs as action potentials or Ca2+ waves**

The SCTs recorded in this study were manifest as three different types of event – Ca2+ sparks, Ca2+ waves and action-potential-evoked Ca2+ transients. Ca2+ sparks were observed in all experimental recordings, because their amplitude was too low to make a significant change to the whole-cell fluorescence that was monitored in photometry experiments. However, within confocal recordings Ca2+ sparks were very obvious as rapid (time to reach amplitude peak <100 milliseconds) spontaneously recurring events (diameter <4 µm) evoked by the test or control stimulus.

The differentiation of the Ca2+ waves or action-potential-evoked Ca2+ transients was obvious from photometry and confocal recordings. SCTs that were manifest as action-potential-evoked Ca2+ transients or Ca2+ waves are consistent with findings in a previous study (Mackenzie et al., 2003), where we used simultaneous imaging and electrophysiology to verify that the spatial pattern of the SCTs correlated with the magnitude of delayed-after-depolarisations (DADs). Only SCTs that had the same spatial and temporal properties because field stimulation-evoked Ca2+ transients were found to correlate with substantial membrane depolarisation (Mackenzie et al., 2003). By contrast, Ca2+ waves produced sub-threshold DADs and the cells did not fully depolarise.

Occasionally, Ca2+ recordings showed complex signals that resulted from Ca2+ waves causing action potentials. In these cases, the slowly rising Ca2+ signal due to a Ca2+ wave was observed to abruptly accelerate when the action potential was triggered. Such ‘mixed’ events were not included in the analysis described here.

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**References**


