

The role of inositol 1,4,5-trisphosphate receptors in Ca^{2+} signalling and the generation of arrhythmias in rat atrial myocytes

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Various cardio-active stimuli, including endothelin-1 (ET-1), exhibit potent arrhythmogenicity, but the underlying cellular mechanisms of their actions are largely unclear. We used isolated rat atrial myocytes and related changes in their subcellular Ca^{2+} signalling to the ability of various stimuli to induce diastolic, premature extra Ca^{2+} transients (ECTs). For this, we recorded global and spatially resolved Ca^{2+} signals in indo-1- and fluo-4-loaded atrial myocytes during electrical pacing. ET-1 exhibited a higher arrhythmogenicity (arrhythmogenic index; ratio of number of ECTs over fold-increase in Ca^{2+} response, 8.60; $n = 8$ cells) when compared with concentrations of cardiac glycosides (arrhythmogenic index, 4.10; $n = 8$ cells) or the β -adrenergic agonist isoproterenol (arrhythmogenic index, 0.11; $n = 6$ cells) that gave similar increases in the global Ca^{2+} responses. Seventy-five percent of the ET-1-induced arrhythmogenic Ca^{2+} transients were accompanied by premature action potentials, while for digoxin this proportion was 25%. The β -adrenergic agonist failed to elicit a significant number of ECTs. Direct activation of inositol 1,4,5-trisphosphate (InsP_3) receptors with a membrane-permeable InsP_3 ester (InsP_3 BM) mimicked the effect of ET-1 (arrhythmogenic index, 14.70; $n = 6$ cells). Inhibition of InsP_3 receptors using $2 \mu\text{M}$ 2-aminoethoxydiphenyl borate, which did not display any effects on Ca^{2+} signalling under control conditions, specifically suppressed the arrhythmogenic action of ET-1 and InsP_3 BM. Immunocytochemistry indicated a co-localisation of peripheral, junctional ryanodine receptors with InsP_3 Rs. Thus, the pronounced arrhythmogenic potency of ET-1 is due to the spatially specific recruitment of Ca^{2+} sparks by subsarcolemmal InsP_3 Rs. Summation of such sparks efficiently generates delayed afterdepolarisations that trigger premature action potentials. We conclude that the particular spatial profile of cellular Ca^{2+} signals is a major, previously unrecognised, determinant for arrhythmogenic potency and that the InsP_3 signalling cassette might therefore be a promising new target for understanding and managing atrial arrhythmia.

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In the heart, Ca^{2+} release from the sarcoplasmic reticulum (SR) is a key event during the process of excitation–contraction coupling and is believed to be triggered through the mechanism of Ca^{2+} -induced Ca^{2+} -release (CICR). Essentially, Ca^{2+} influx via voltage-operated Ca^{2+} channels is amplified by the liberation of Ca^{2+} from the lumen of the SR (Callewaert, 1992). The release is mediated by the concerted opening of specialised Ca^{2+} release channels, the ryanodine receptors (RyRs) on the membrane of the SR. Such rhythmic Ca^{2+} responses, i.e. the occurrence of a single Ca^{2+} signal following an action potential, represent the physiological behaviour of the cardiac cell. Under a variety of patho-physiological conditions, altered membrane currents can disturb the

heartbeat. The origin of such alterations can be either changes in the expression pattern of ion channels or direct modulation of the gating of channels (Keating & Sanguinetti, 2001). Modified gating of the potassium currents, such as the delayed rectifier, for example, may lead to delayed repolarisation and can result in the reactivation of the L-type Ca^{2+} current, referred to as early afterdepolarisations (EADs; Fozzard, 1992). Very rapid and dynamic modulation of membrane currents can occur on a beat-to-beat basis through the intracellular Ca^{2+} concentration. Thus, spontaneous Ca^{2+} release from the SR has been implicated to evoke depolarising membrane currents (such as I_{ti}) and delayed after-depolarisations (DADs) under conditions of cellular Ca^{2+} overload (Berlin *et al.* 1989; Fozzard, 1992).

Recent evidence points to the direct involvement of agents such as endothelins, angiotensins and α -adrenergic agonists in the generation of cardiac arrhythmia (for reviews see Golfman *et al.* 1991; Woodcock *et al.* 1998) but their detailed cellular and subcellular mode of action is largely unclear.

In contrast to ventricular arrhythmia, such as ventricular fibrillation, that are life-threatening conditions, atrial arrhythmias exhibit more modest effects on the circulation. Atrial arrhythmias are the most common forms of long lasting or continuous cardiac arrhythmia and the risk of stroke is increased 5- to 7-fold (Ezekowitz, 1999). Despite their widespread occurrence (e.g. almost 20% of the entire population at the age of 75 years display atrial arrhythmias; Ezekowitz, 1999) our understanding of their cellular mechanisms, particularly in relation to the hormonal agents mentioned above, is sparse.

One mechanism that has been suggested for the arrhythmogenic action of stimuli such as ET-1 involves coupling to the G_q -phospholipase C (PLC) signalling pathway, resulting in the mobilisation of the second messenger $InsP_3$ (Golfman *et al.* 1991). In many cell types, this messenger engages its subcellular binding site on the $InsP_3$ receptor ($InsP_3R$) and causes release of Ca^{2+} from the Ca^{2+} storing organelle (Berridge *et al.* 2000). In heart, the presence and importance of this intracellular Ca^{2+} -release channel is controversial (for example see Woodcock *et al.* 1998; Marks, 2000). In the present study, we investigated the effects of various inotropic agents in causing arrhythmogenic Ca^{2+} release events in rat atrial myocytes. Furthermore we correlated their arrhythmogenicity to the characteristic subcellular Ca^{2+} release patterns, with particular emphasis on the putative involvement of $InsP_3Rs$.

METHODS

Rat atrial myocytes were isolated from male Wistar rats (~250 g, 8 weeks in age) by established enzymatic isolation methods (Lipp *et al.* 2000). For this, the animal was killed by cervical dislocation after CO_2 anaesthesia, as approved by the Babraham Institute's ethical committee (Schedule 1 killing in accordance with Home Office regulations). The heart was removed quickly and placed into a Langendorff apparatus after which cell isolation was performed as described earlier (Lipp *et al.* 2000). Single cells were kept in an extracellular medium (EM) containing (mM): NaCl 135, KCl 5.4, $MgCl_2$ 2, $CaCl_2$ 1, glucose 10, Hepes 10, pH 7.35. The osmolarity of all solutions was verified with an osmometer (Roebbling Osmometer, Camlab, Cambridge, UK).

All experiments were performed at room temperature (20–22 °C). In our hands, at that temperature Ca^{2+} indicators (either indo-1 or fluo-4) are better retained inside the myocytes and are not transported out so quickly by unspecific anion transporters (see Thomas *et al.* 2000). This process will lead to a decreased indicator level and thus decreased signal-to-noise ratio. We have performed control experiments at 37 °C and found that apart from faster onsets of the effects (for all agonists, isoproterenol, digoxin, ET-1 and $InsP_3$ BM) there was no qualitative change in the results (data not shown). Due to the indicator problem and the fact that the

slower response of the cells to the application of the agonists allowed us to perform a more detailed analysis of the time course of those processes, we chose to do the study at room temperature.

The experiments involving global Ca^{2+} concentration measurements were carried out on a PhoCal system (Perkin Elmer Life Sciences, Cambridge, UK). For this, the cells were loaded with indo-1 (40 min in EM with 2 μM indo-1 AM, 20 min de-esterification). Indo-1 was excited at 360 nm and the fluorescence was simultaneously monitored at 405 nm (F_{405}) and 490 nm (F_{490}). The Ca^{2+} concentration is expressed as the background corrected ratio of F_{405}/F_{490} . The recording protocol comprised repetitive 30 s illumination and 60 s resting periods. All compounds were applied via a solenoid-driven perfusion system with an exchange time of ~1 s.

For confocal recording, the cells were loaded with fluo-4 (20 min in EM with 2 μM fluo-4 AM, 20 min de-esterification) and transferred onto the stage of a real-time confocal microscope (NORAN Oz, Middleton, USA). Confocal images (512 × 115 pixels) were acquired at a rate of 30 Hz (4 consecutive images at 120 Hz were averaged, z section < 1 μm). Analysis of the confocal movies was performed with a customised version of NIH Image or ImageJ (NIH, Bethesda, USA). The fluo-4 fluorescence (excitation 488 nm, emission > 500 nm) was converted into Ca^{2+} concentrations by methods described earlier (Lipp *et al.* 2000). Cells were electrically paced with two field electrodes (0.3 Hz, 40 V, 2 ms duration).

Patch-clamp experiments were performed in the whole-cell mode with pipettes (series resistance ~2 M Ω) filled with an intracellular solution containing (mM): KCl 135, NaCl 10, EGTA 0.1, MgATP 3, $MgCl_2$ 1, Hepes 10 at pH 7.3. The membrane potential was recorded with an EPC-9 patch-clamp amplifier system (HEKA, Germany) that also provided brief (~2 ms) current injections to evoke action potentials. The amplitude of the current injection varied between cells but was set 20% higher than the threshold current necessary for triggering an action potential under control conditions.

The immunocytochemistry was carried out as described elsewhere (Lipp *et al.* 2000). We used confocal microscopy (*UltraView*, Perkin Elmer Life Sciences, Cambridge, UK) to probe the subcellular distribution of either Texas Red- or fluorescein-conjugated secondary antibodies.

Averaged fluorescence from subcellular locations was further analysed with IGOR software (Wavemetrics, USA). Statistical significance was tested using Student's *t* test or the Kolmogorov-Smirnov test where indicated (Statistica 5.0, StatSoft, USA).

We identified ECTs as spontaneous Ca^{2+} increases whose amplitudes exceeded two times the standard deviation of the time-dependent variations in the Ca^{2+} concentration at rest. Detailed analysis of the ECTs was only performed when the Ca^{2+} transient peaked at least 300 ms after the peak of the electrically induced Ca^{2+} response.

RESULTS

Arrhythmogenicity and increases in the amplitude of electrically evoked Ca^{2+} transients are not directly correlated

Application of ET-1 to electrically paced rat atrial myocytes caused increases in the amplitude of the electrically evoked

Ca²⁺ responses that coincided with the occurrence of arrhythmogenic extra Ca²⁺ transients (ECTs; Fig. 1A and B). These ECTs are generally attributed to an increase in the cellular Ca²⁺ load. However, application of concentrations of isoproterenol and digoxin to cells that gave Ca²⁺ responses of similar amplitude as ET-1 (Fig. 1Ba) revealed that there was no direct correlation between the Ca²⁺ signal amplitude and likelihood of spontaneous Ca²⁺ release (i.e. number of diastolic spontaneous ECTs; Fig. 1Bb). In order to quantify the effect of each stimulus to evoke ECTs and also normalise for differences in the increase of the electrically evoked Ca²⁺ signals, we estimated the arrhythmogenic index (AI) for each condition as the ratio of the number of ECTs (per 30 s recording period) relative to the increase in the global Ca²⁺ signal (determined when the Ca²⁺ increase had reached a steady state). For this, we counted the number of ECTs during a 30 s recording period and divided that value by the fold increase in the normalised amplitude of the electrically evoked Ca²⁺ response (normalised to the control values before application of the agonist). Digoxin (AI, 4.1; *n* = 8 cells) was approximately half as effective as ET-1 (8.6; *n* = 8 cells), whilst isoproterenol gave very few ECTs (0.1; *n* = 6 cells) despite a rapid and robust enhancement of the Ca²⁺ responses that even superseded ET-1 or digoxin-induced increases (268 ± 23%; *n* = 6 cells for isoproterenol *vs.* ~170% for ET-1 (± 31) and digoxin (± 25); *n* = 8 cells for both). These data suggest that (i) increasing Ca²⁺ influx through phosphorylation of L-type Ca²⁺ channels (isoproterenol), (ii) loading of the SR with Ca²⁺ by phosphorylation of phospholamban (isoproterenol), (iii) increasing Ca²⁺ influx via Na⁺-Ca²⁺ exchange (digoxin) or (iv) sensitisation of ryanodine receptors (digoxin; McGarry & Williams, 1993) are not necessarily sufficient to cause spontaneous Ca²⁺ release. Clearly, the other agents used in this study did not mimic ET-1's arrhythmogenicity. It was particularly interesting to observe the lack of ECTs during the duration of isoproterenol application (here up to 10 min), a β-adrenergic agonist, despite the most robust increase in the Ca²⁺ transient amplitude, since β-adrenergic receptor activation is believed to be a major arrhythmogenic stimulus in ventricular tissue (Talukder *et al.* 2001). Indeed, in parallel experiments conducted on rat ventricular myocytes, we found that during similar observation periods the substantial increases of the amplitude of electrically evoked Ca²⁺ signals observed during application of the β-adrenergic agonist were accompanied by the occurrence of a large number of ECTs (data not shown). This suggests differences in the hormonal response in atrial and ventricular myocytes that we did not pursue further in this study.

These data suggested that there is a difference between the digoxin- and ET-1-induced ECTs, at least in their probability of occurrence for similar levels of Ca²⁺ transient increases. To further substantiate such a different characteristic of

the two types of ECTs, we performed a detailed analysis of the distribution of the time delays, with which the ECTs occurred after the electrically evoked Ca²⁺ signals during ET-1 and digoxin stimulation (Fig. 1C). The two histograms summarise the peak-to-peak times for ET-1 (red) and digoxin (blue). Although both distributions seemed to be skewed towards shorter intervals, this tendency was more apparent in the digoxin distribution. We thus analysed the statistical significance of a different spreading between these two groups. Since the two data sets were not normally distributed, we used a Kolmogorov-Smirnov test, which aside from mean values and variances also takes into account general shape differences between the two data sets. The analysis showed mean values of 1.15 ± 0.046 s for ET-1 (mean ± S.E.M.; *n* = 250) and 0.92 ± 0.043 s for digoxin (mean ± S.E.M.; *n* = 225) with *P* < 0.001 (see Fig. 1Cc). From these results we suggest that the ECTs generated during digoxin superfusion have the tendency towards shorter delay times than those during ET-1 stimulation, which seem to occur at longer time intervals after an evoked action potential. Such a difference might hint to diversity in the underlying mechanism of generation (see Discussion). This was further substantiated by the fact that ET-1 induced multiple ECTs following a single action potential-induced Ca²⁺ signal (54% of all ECTs) while digoxin predominantly caused single ECTs (only 14% of all ECTs were multiple ones; Fig. 1Cd).

From electrophysiological recordings performed in parallel, we further characterised the ET-1-induced ECTs and found that they caused either subthreshold delayed after-depolarisations (DADs, Fig. 1Dd), which would remain locally in the intact tissue or suprathreshold DAD-induced premature action potentials that could potentially propagate through neighbouring cells (75% of all ECTs; *n* = 83 from 4 cells; Fig. 1D). In contrast, digoxin-induced ECTs were mainly characterised as subthreshold DADs (70%; *n* = 79 from 4 cells; data not shown) and were thus less arrhythmogenic in terms of their potency to trigger premature action potentials.

The subcellular properties of ET-1- and digoxin-induced ECTs

To understand the different effects of the ET-1- and digoxin-evoked ECTs with respect to the generation of action potentials, we examined the subcellular properties of these ECTs using video-rate laser-scanning confocal microscopy. For ET-1-stimulated cells, all ECTs occurred as highly localised subsarcolemmal Ca²⁺ increases (Fig. 2A). Subthreshold DADs (Fig. 2A*bi*) appeared as a burst of Ca²⁺ sparks forming a non-regenerative 'ring' of Ca²⁺ around the cell periphery (Fig. 2A*c* and *e*), i.e. this ring did not cause CICR in deeper layers of the cytosol. In contrast to that, suprathreshold DADs (Fig. 2A*biii*) appeared initially as a ring of Ca²⁺ sparks subsequently triggering a Ca²⁺ signal in deeper layers of the cell indicative of an action

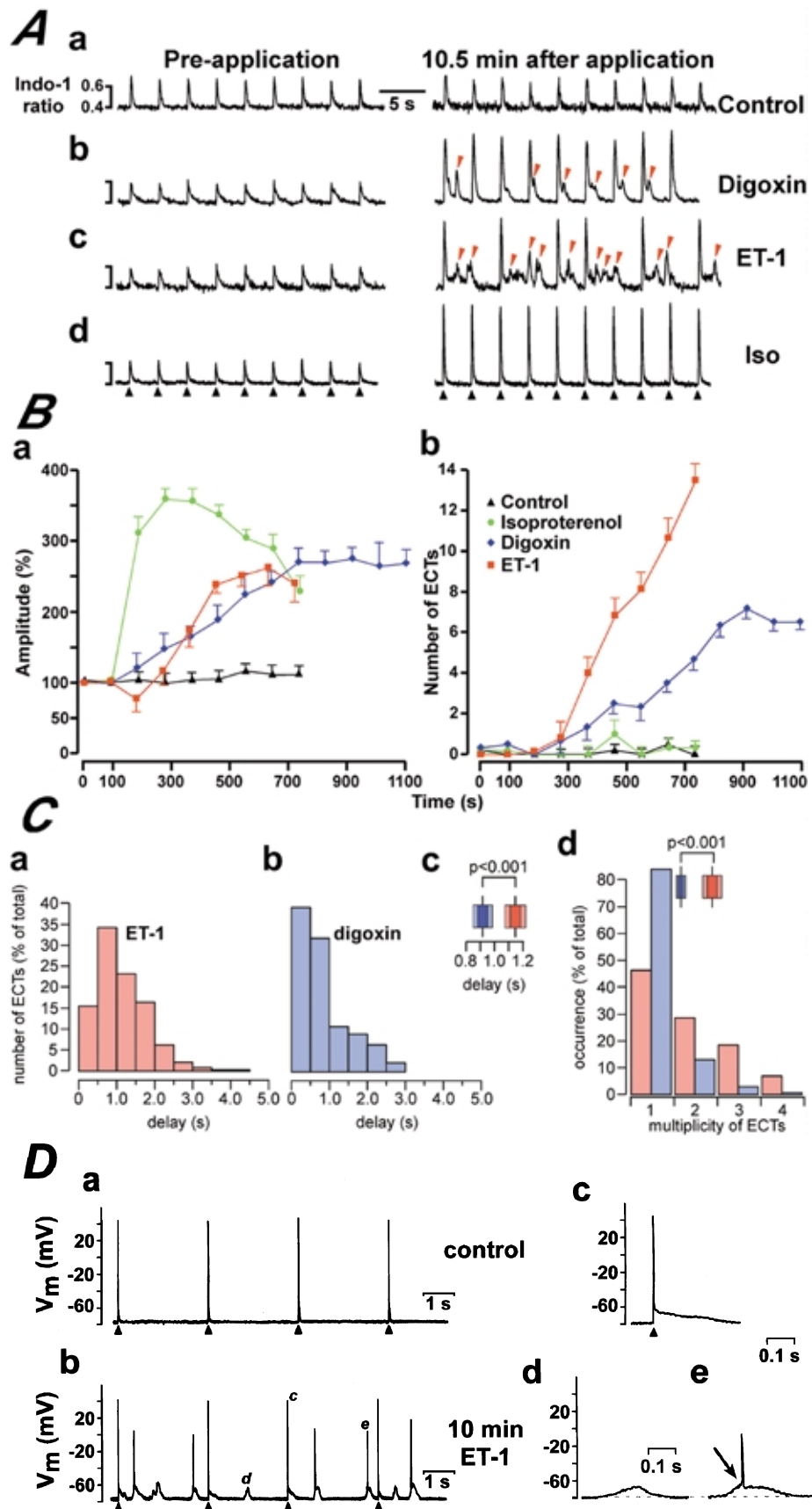


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potential (i.e. synchronised activation of many Ca²⁺ sparks; Fig. 2*Ad*; Mackenzie *et al.* 2001; similar observations were made elsewhere, Hüser *et al.* 1996; Kockskämper *et al.* 2001). Application of digoxin to myocytes did not evoke subsarcolemmal Ca²⁺ sparks, instead digoxin-evoked ECTs were almost exclusively Ca²⁺ waves (>90%; *n* = 36; data from 4 cells; Fig. 2*B*) of random subcellular origin (Berlin *et al.* 1989). All digoxin-evoked Ca²⁺ waves analysed displayed varying subcellular origins, with multiple wave origins in the same cell. Ca²⁺ waves either propagated through a part of the atrial myocyte before dying out or occupied a sufficient cytoplasmic volume to trigger a premature action potential (Fig. 2*Bbii*).

Experiments were conducted to further verify and confirm the identification of sub-threshold and supra-threshold DADs. We used simultaneous recording of the membrane voltage and Ca²⁺ concentration to characterise the basis of the subcellular pattern of the underlying Ca²⁺ release signal (Fig. 2*C*). Under control conditions (Fig. 2*Ca*) each external electrical stimulation is followed by a regular Ca²⁺ transient. The time series of confocal images in Fig. 2*Caiv* shows the typical 'Ca²⁺ ring' during ec-coupling described previously (Mackenzie *et al.* 2001). Seven minutes after application of ET-1 (100 nM) another train of stimuli was applied, of which Fig. 2*Cbi* and *ii* depict the last stimulation (black arrowhead) and the following train of spontaneous action potentials (marked by the red arrowheads). The detailed analysis of the selected spontaneous action potential (marked with the black circle) shown in Fig. 2*Cbiii* and *iv*

indicates that indeed the DAD (open arrowhead) is generated by an increased frequency of peripheral Ca²⁺ sparks (see also Fig. 2*Abi* and *ii*) while the transition from the subthreshold membrane depolarisation to the action potential (red arrowhead) is clearly marked by an abrupt and complete disappearance of Ca²⁺ sparks (Fig. 2*Cbiv*, see also 2*Abiii* and *iv*). From these data we suggest that the identification and distinction of sub-threshold and supra-threshold Ca²⁺ signals (and DADs) is indeed possible on the basis of the pattern of the underlying Ca²⁺ release signals. Sub-threshold as well as supra-threshold Ca²⁺ transients (and DADs) are generated by the gradual build-up of the frequency of peripheral Ca²⁺ sparks while the successful triggering of a premature action potential is indicated by the abrupt loss of visible Ca²⁺ sparks.

Involvement of InsP₃Rs in arrhythmogenicity and increases of the amplitude of electrically evoked Ca²⁺ signals

InsP₃Rs are expressed in the heart (Moschella & Marks, 1993; Gorza *et al.* 1993; Perez *et al.* 1997; Lipp *et al.* 2000) and their expression pattern changes during various cardiac pathologies that are associated with different forms of arrhythmia (Go *et al.* 1995; Marks, 2000). We therefore sought to examine the involvement of InsP₃Rs in ET-1-induced ECTs. In order to avoid the pleiotropic effects of phospholipase C (PLC) activation (Golfman *et al.* 1991), we evoked InsP₃R gating directly by superfusing cells with a membrane-permeant ester of InsP₃ (InsP₃ BM; Lipp *et al.* 2000). In contrast to ET-1, application of InsP₃ BM caused

Figure 1. ET-1 evoked increases in the amplitude of electrically evoked Ca²⁺ transients, ECTs and action potentials

A depicts representative traces showing the effects of application of the bath solution (control, *Aa*), digoxin (1 μM, *Ab*), ET-1 (0.1 μM, *Ac*) and isoproterenol (0.1 μM, *Ad*) on electrically paced atrial cells. The black arrowheads indicate the timing of electrical stimulation. ECTs were marked with red arrowheads for ET-1 and digoxin. *B*, summarises the responses of isolated indo-1-loaded electrically paced rat atrial myocytes to application of isoproterenol (green circles), digoxin (blue diamonds) and ET-1 (red squares). *Ba* depicts the change in relative amplitude of electrically evoked Ca²⁺ transients (data points were normalised against the Ca²⁺ transient amplitude prior to drug addition). *Bb* shows the development of ECTs. The data points represent averaged values of at least 6 cells for each condition. The drugs were applied after a 120 s control period. *C*, depicts the detailed analysis of peak-to-peak times for ET-1- and digoxin-induced ECTs. For this we measured the time delay between the peak of the electrically evoked Ca²⁺ signals and the peak of the ECT. The resulting histograms are shown in *Ca* for ET-1 and *Cb* for digoxin. The statistical analysis with a Kolmogorov-Smirnov test (Statistica 5.0, StatSoft, USA) showed a mean delay of 1.15 ± 0.046 s for ET-1 (mean ± s.e.m.; *n* = 250) and 0.92 ± 0.043 s for digoxin (mean ± s.e.m.; *n* = 225). The resulting *P* value (*P* < 0.001) demonstrated a significantly different distribution of the time delays. This is summarised in *Cc*, which also indicates the confidence intervals of 1 × s.e. (dark colouring) and 2 × s.e. (light colours) for ET-1 (red) and digoxin (blue). Panel *Cd* illustrates the distribution of the number of ECTs following a single action potential in the presence of ET-1 (red) and digoxin (blue). The statistical analysis with a Kolmogorov-Smirnov test indicated mean values of 1.82 ± 0.07 (mean ± s.e.m.; *n* = 250) for ET-1 and 1.07 ± 0.04 (mean ± s.e.m.; *n* = 225) for digoxin. The resulting *P* value (*P* < 0.001) suggested significantly different distributions. The inset illustrates the confidence intervals (see *Cc* for description). *D*, shows membrane potential recordings from patch-clamped atrial myocytes. The cells were stimulated as indicated by the black arrowheads. *Da* depicts membrane potential recordings under control conditions. 10 min after application of 0.1 μM ET-1, numerous diastolic membrane potential changes were recorded (panel *Db*). The events marked with corresponding characters in *Db* were redrawn at an expanded timescale in *Dc*, *d* and *e* to illustrate an evoked action potential (*Dc*), a premature action potential (*De*) and a subthreshold DAD (*Dd*). The transition from a subthreshold DAD to a premature action potential is indicated by the arrow in *De*.

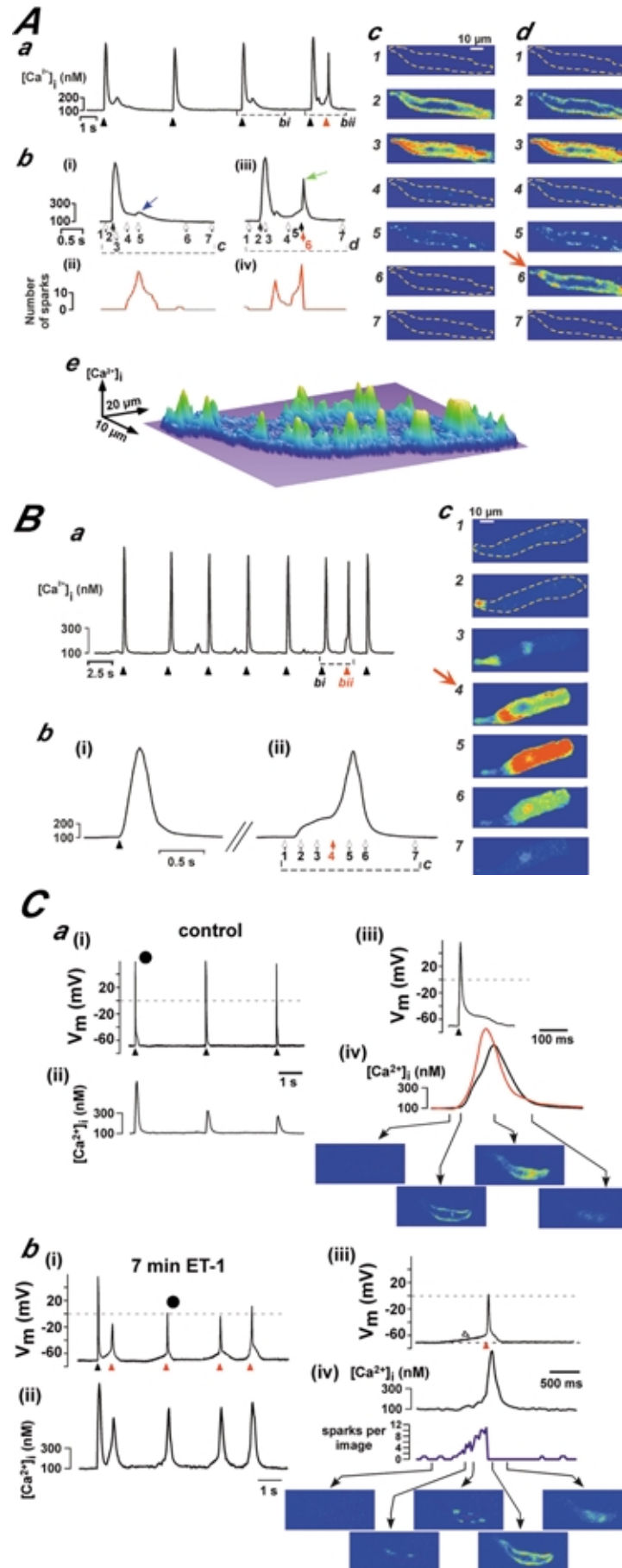


Figure 2. For legend see facing page

a much smaller increase in the amplitude of the electrically evoked Ca²⁺ transients ($58 \pm 24\%$; $n = 6$ cells; Fig. 3A). However, its ability to induce ECTs was comparable with 100 nM ET-1 (Fig. 3A and B). These findings support our earlier notion of the dissociation between increases in the global Ca²⁺ response and arrhythmogenicity. In the presence of ET-1 or InsP₃ BM cells often passed into a state of 'cellular fibrillation', indicated by the rapid drop in the Ca²⁺ transient amplitude of electrically evoked Ca²⁺ signals (Fig. 3A; filled black circles after 15 min). We apply this picturesque term of 'cellular fibrillation' if an atrial myocyte displayed Ca²⁺ transients at such a high frequency that: (i) the individual Ca²⁺ signals started to largely fuse and (ii) it was virtually impossible to identify individual Ca²⁺ signals caused by electrical stimulation. In addition, under these conditions no co-ordinated contraction of the myocyte could be observed (data not shown). Only the biologically active D-*myo*-inositol stereoisomer of InsP₃ BM was capable of evoking ECTs and 'cellular fibrillation', whereas the L-*myo*-InsP₃ BM isomer was inactive (data not shown), indicating that the action of the ester was highly specific.

Additional evidence for the role of InsP₃ in generating ECTs during ET-1 application was obtained using the membrane permeant InsP₃R antagonist 2-aminoethoxydiphenyl borate (2APB; Maruyama *et al.* 1997). This

compound rapidly and reversibly inhibited both the increase in the amplitude of electrically evoked Ca²⁺ signals and the ECTs occurring during InsP₃ BM application (Fig. 4A). The generation of ECTs was completely abrogated (Fig. 4Ab). Since several recent reports suggested effects of 2APB other than on the InsP₃ receptor (see for example Broad *et al.* 2001), we conducted a series of control experiments. We first tested whether 2 μM 2APB affects normal atrial ec-coupling and found that at this concentration 2APB did not cause any changes on normal atrial Ca²⁺ signals up to durations of more than 30 min ($n = 4$ cells from 3 hearts; data not shown). For this we applied 2APB onto atrial myocytes during constant electrical stimulation at 0.3 Hz and analysed the amplitude and the time course (upstroke and relaxation) of the Ca²⁺ transients (data not shown). Application of 2APB did not affect any of these parameters at lower concentrations (~2 μM). Higher concentrations of 2APB (> 30 μM) suppressed atrial ec-coupling (electrical pacing did not cause any increases in the intracellular Ca²⁺ concentrations and the cells stopped contracting) in a concentration and time-dependent manner (data not shown) indicating that at those high concentrations it started to display non-specific effects. From this we concluded that 2APB at the concentration of 2 μM used here appears to be an

Figure 2. Subcellular properties of ET-1- and digoxin-evoked ECTs

Aa shows the global Ca²⁺ concentration from a cell stimulated with 0.1 μM ET-1 for 8 min. The black arrowheads indicate the timings of electrical stimulations, and the red arrowhead depicts a Ca²⁺ signal from a premature action potential. The portions of traces bounded by dashed lines in *Aa* are shown on expanded timescales in *Abi* and *iii*. The numbered arrows beneath the traces indicate the time points at which the confocal images shown in *Ac* and *Ad* were taken. The expanded trace in *Abi* and the corresponding confocal images in *Ac* show an evoked action potential that was followed by a Ca²⁺ signal accompanied by a sub-threshold DAD (blue arrow) during which subsarcolemmal Ca²⁺ sparks were observed (see *Ac5*). Panels *Abii* and *Abiv* illustrate the time course of the occurrence of identifiable Ca²⁺ sparks. For this, every confocal image was analysed and the number of Ca²⁺ sparks plotted against time. The sudden disappearance of Ca²⁺ sparks in *Abiv* indicated the generation of an action potential (see panel C for further verification). The 3D-surface representation of *Ac5* shown in *Ae* emphasises the peripheral nature of Ca²⁺ release, indicated by the local elevations around the circumference of the cell. The evoked action potential shown in *Abiii* was followed by a ring of subsarcolemmal Ca²⁺ sparks that subsequently triggered a premature action potential (see corresponding confocal images in *Ad*, marked by a green arrow). *B* depicts a similar analysis as shown in *A* for a cell stimulated with 1 μM digoxin for 8 min. Panel *Ba* illustrates the global Ca²⁺ concentration response. The black arrowheads indicate the timings of electrical stimulation, and the red arrowhead denotes a premature action potential. The portion of the trace bounded by a dashed line in *Ba* is shown on an expanded timescale in *Bb*. The numbered arrows below the trace in *Bbii* indicate the times at which the confocal images shown in *Bc* were taken. These data are typical of the responses of 5 cells (from 4 hearts each) for both ET-1 and digoxin. *C* illustrates simultaneous recordings of the membrane potential (*Cai* and *iii* and *Cbi* and *iii*) and subcellular Ca²⁺ signals (*Caii* and *iv* and *Cbii* and *iv*). *Ca* depicts the recordings under control conditions, while *Cb* shows the same cell 7 min after application of 100 nM ET-1. The black arrowheads in *Cai* mark events of electrical field stimulation. The action potential marked ● was redrawn in *Caiii* and *iv* at an expanded time scale. Here, the red tracing was averaged from peripheral Ca²⁺ release sites, while the black tracing was obtained from a deeper layer inside the atrial myocyte. Selected self-ratioed confocal images are shown in the lower part of *Caiiv*. The data depicted in *Cb* was recorded at the end of a train of evoked action potentials (10 action potentials, 1 Hz). The black arrowhead marks the last stimulation of that train while the red arrowheads identify spontaneous action potentials. The premature action potential marked ● was redrawn in *Cbiii* and *iv* at an expanded time scale. Selected self-ratioed confocal images are shown in the lower part of *Cbiv*. The increase in the number of visible Ca²⁺ sparks during the DAD (marked by the open arrowhead) and the abrupt decline upon generation of the action potential (red arrowhead) are apparent. Similar results were obtained in 4 cells from 2 hearts.

appropriate tool for inhibiting InsP_3 action in cardiac myocytes without interfering with normal ec-coupling.

The antagonist 2APB caused a small but non-significant reduction in the amplitude of the ET-1 response (reduction to $75 \pm 38\%$; $P > 0.01$) and delayed its development (Fig. 4B; filled circles). The maximal ET-1-induced increase in pacing-evoked Ca^{2+} transient amplitude was $259 \pm 20\%$ in the absence, and $229 \pm 19\%$ in the presence of $2 \mu\text{M}$ 2APB. In contrast to its weak effect on ET-1-evoked increases in the Ca^{2+} transients, 2-APB reduced the occurrence of ECTs on average by 93% ($n = 6$ cells from 4 hearts; Fig. 4B; open circles). These data suggest a pivotal role for InsP_3 Rs in the arrhythmogenic component of the ET-1 response. The persistent amplitude increase of the electrically induced Ca^{2+} responses evoked by ET-1 during InsP_3 R inhibition most likely reflects the remaining activation of mechanisms downstream of PLC (Golfman *et al.* 1991) such as increased Ca^{2+} currents via PKC-dependent phosphorylation of L-type Ca^{2+} channels (He *et al.* 2000) which would allow for an increased cellular response.

Localisation of InsP_3 Rs and distribution of Ca^{2+} release likelihood

The immunolocalisation of InsP_3 Rs and RyRs in atrial myocytes indicated that the InsP_3 R type II (red staining in Fig. 5A) co-localised with part of the junctional RyR type II (green staining in Fig. 5A) population. A cartoon representation of these findings is given in Fig. 5Ac. These data support the idea of a signalling microdomain comprised of these two Ca^{2+} -release channels. We estimated the subcellular Ca^{2+} release likelihood during ET-1-, InsP_3 BM- and digoxin-induced ECTs (i.e. the subcellular locations where ECTs were most commonly observed) by averaging 4–6 consecutive confocal images around the peak of 8 subthreshold ECTs for every stimulus, and colour coded the resulting images so that warmer colours would indicate higher Ca^{2+} release likelihood and cooler colours would indicate lower release likelihood.

Our results indicated that spontaneous Ca^{2+} release was distributed in a characteristic fashion for each stimulus. In particular, the ECTs caused by both ET-1 and InsP_3 BM showed an increased likelihood of occurring in subplasma-

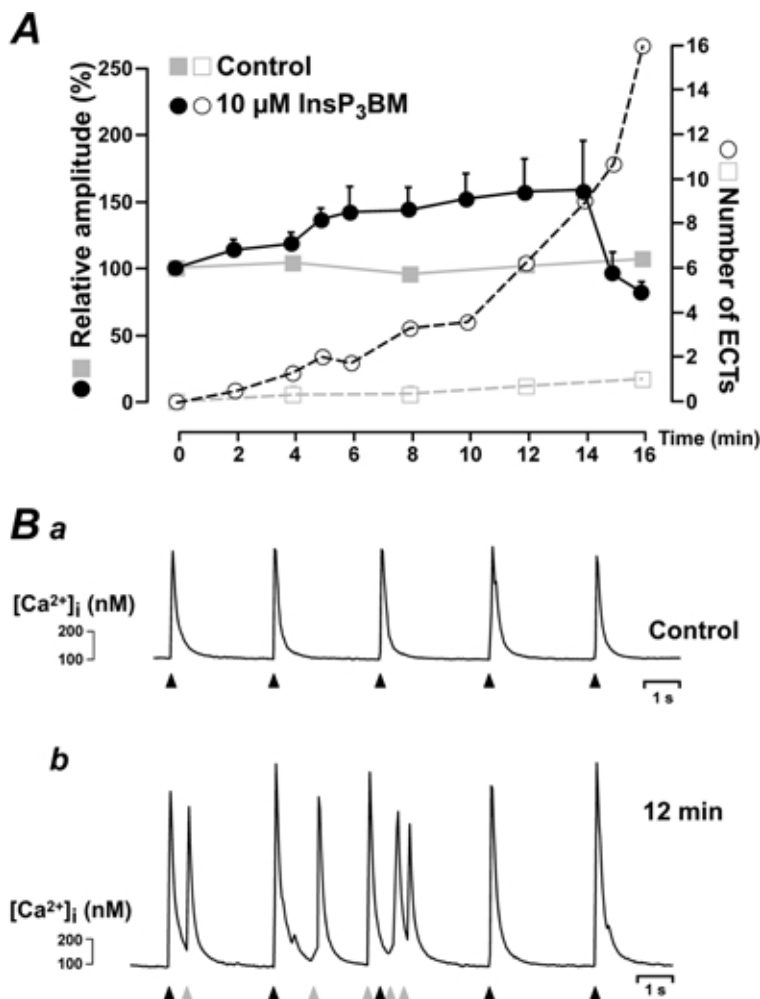


Figure 3. Direct InsP_3 R activation evoked increases in the amplitude of electrically induced Ca^{2+} transients and ECTs

A shows the time course of the relative amplitude of electrically evoked Ca^{2+} signals (filled symbols) and the occurrence of ECTs (open symbols) for control cells (grey symbols) and InsP_3 BM stimulated cells (black symbols). The data were averaged from 3 control cells and 6 InsP_3 BM-treated cells loaded with indo-1. *Ba* and *Bb* show the global Ca^{2+} signals (calculated from the fluo-4 fluorescence) in an atrial myocyte before, and 12 min after, InsP_3 BM addition, respectively. The black arrowheads indicate the timings of electrical stimulation. The diastolic ECTs were observed as subsarcolemmal Ca^{2+} rises and are marked with grey arrowheads. Similar results were observed in 6 cells from 3 hearts.

lemmal locations (Fig. 5Ba and b). This further substantiates the involvement of subsarcolemmal RyR/InsP₃R signalling microdomains in the action of ET-1 and InsP₃ BM. In contrast, digoxin displayed a largely homogeneous Ca²⁺ release likelihood (Fig. 5Bc), which was due to the Ca²⁺ waves exhibiting randomised subcellular origins (see also Berlin *et al.* 1989).

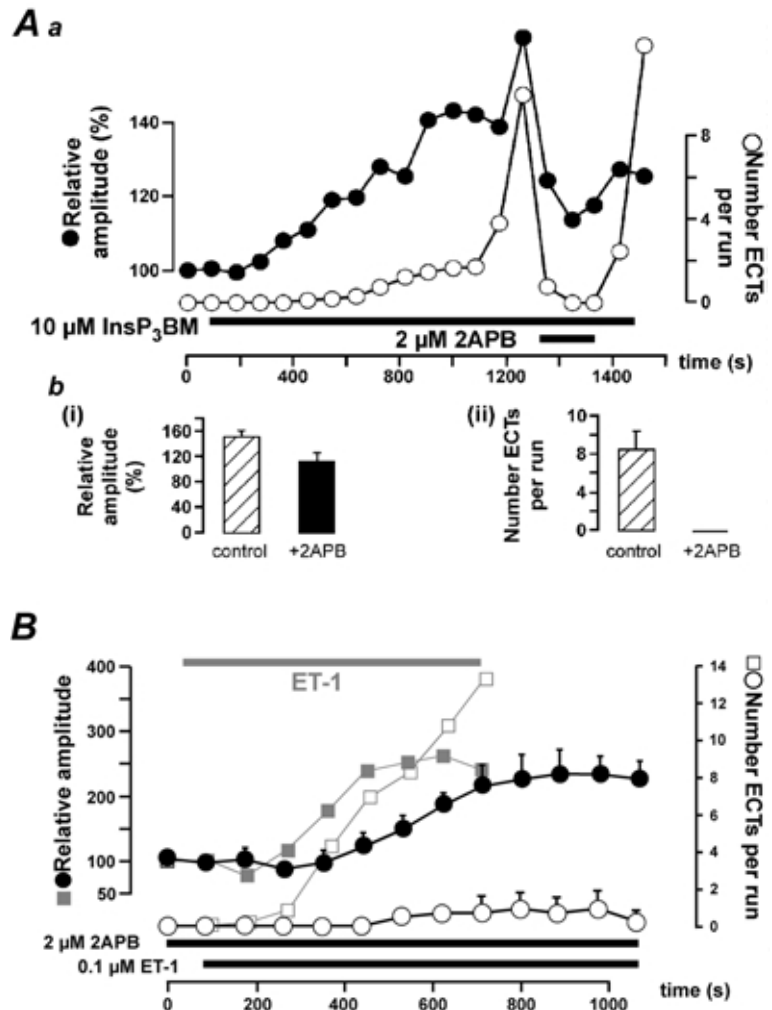
These spatially distinct distributions of the Ca²⁺ release likelihood are also reflected in the threshold Ca²⁺ concentration needed to trigger an action potential for the various stimuli (Fig. 5C). For this, we analysed 31 ECTs for each condition. We identified the transition from a sub-threshold Ca²⁺ signal (and DAD) to a transient indicative of a triggered action potential in two independent ways. (i) First, we averaged the global, whole cell fluorescence. In such traces, the transition between the spontaneous Ca²⁺ sparks and the action potential-induced Ca²⁺ sparks was clearly visible as the inflection point in the trace, where the Ca²⁺ concentration began to rise abruptly (see Fig. 2Abiii and Cb). (ii) Second, we analysed the occurrence of Ca²⁺ sparks. During the subthreshold DAD, Ca²⁺ sparks arise in a subsarcolemmal localisation in a random fashion, but

at the onset of an action potential they appear highly synchronised (see also Mackenzie *et al.* 2001). The global Ca²⁺ concentration at that time was taken as the threshold concentration. For Ca²⁺ waves that triggered an action potential, as seen most frequently during digoxin application, the threshold was identified as the occurrence of a partial ring of Ca²⁺ sparks, as seen in Figure 2Bc 3 and 4. The global Ca²⁺ concentration at that time was taken as the threshold concentration for an action potential. While ET-1 and InsP₃ BM displayed a consistently low threshold (~170 nM; hatched and open bar in Fig. 5C), the digoxin-induced ECTs had to generate a much more substantial global Ca²⁺ rise (threshold ~270 nM; black bar in Fig. 5C) before triggering an action potential.

These data suggest that the arrhythmogenicity of agents that act on cardiac myocytes cannot simply be estimated based on their ability to increase the cellular Ca²⁺ load or sensitisation of the Ca²⁺ release mechanism. Instead, the stimulus-specific spatial recruitment of Ca²⁺ release appears to be a central determinant for cellular arrhythmogenicity in atrial myocytes.

Figure 4. 2APB suppressed InsP₃ BM- and ET-1-evoked ECTs

Aa shows the reversible inhibitory effect of 2APB on InsP₃ BM-induced increases in the amplitude of electrically evoked Ca²⁺ responses (filled symbols) and ECTs (open symbols). The timing of InsP₃ BM and 2APB application is indicated by the black bars. Ab summarises the effect of 2APB on the positive inotropy (Abi) and ECTs (Abii); n = 9 cells from 3 hearts. The responses were analysed just before 2APB application (▨) and 3 min after 2APB administration (■). The effect of 2APB on ET-1 application is depicted in B. The greyed curves in the background show data taken from Fig. 1Ba and b to allow comparison of the effect of ET-1 in the absence of 2APB. The timing of ET-1 and 2APB application is indicated by the black bars.



DISCUSSION

Atrial arrhythmias are the most common form of long lasting cardiac arrhythmias and thus it is of immediate interest to understand their cellular basis and to investigate possible novel targets for managing these cardiac pathologies. Although the involvement of signalling pathways, such as those activated by ET-1, angiotensin-II

or α -adrenergic agonists, are implicated in the generation of cardiac arrhythmia, their cellular action is largely unknown. This seems surprising when taking into account recent evidence for their contribution to the death rate in chronic heart failure (Sakai *et al.* 1996). In the present study, we investigated possible cellular mechanisms of hormone action contributing to the generation of cardiac arrhythmia with particular emphasis on the atrium. We

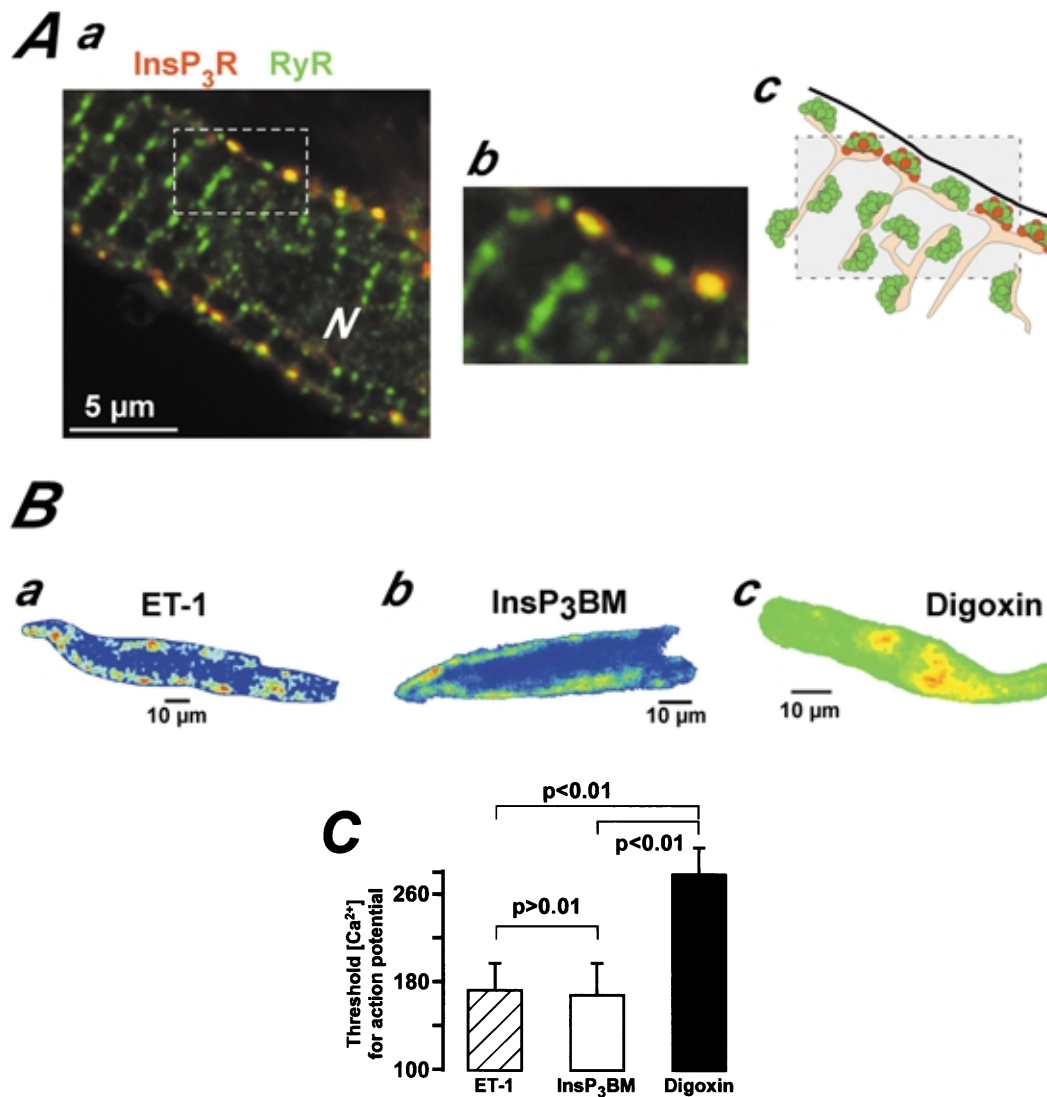


Figure 5. The localisation of InsP₃Rs and RyRs correlates with the distribution of Ca²⁺ release likelihood

Aa depicts a rat atrial myocyte probed for InsP₃Rs type II (red) and RyR type II (green). *N* denotes the cell nucleus. *Ab* shows the part of the cell marked in *Aa* with a dashed box at a higher magnification. A schematic cartoon of the arrangement of plasma membrane (thick black line), SR (orange filled) and Ca²⁺ release channel clusters (green-RyRs and red-InsP₃Rs) is depicted in *Ac*. *B*, distribution of SR Ca²⁺ release likelihood from ECTs during ET-1 (*Ba*), InsP₃ BM (*Bb*) and digoxin (*Bc*) application. For the images, confocal sections of 8 subthreshold ECTs were superimposed and averaged (each ECT was derived from 4–6 consecutive confocal images taken around the peak of the signal). The resultant image was colour coded with cool colours (blue, green) denoting low Ca²⁺ release likelihood and warm colours (red, yellow) indicating high Ca²⁺ release likelihood. Similar distributions were found in all cells analysed. For *Bc* the same cell was used as in Fig. 2*Bc*. *C* compares threshold Ca²⁺ concentrations for triggering premature action potentials during ET-1 (▨), InsP₃ BM (□) and digoxin (■) application.

used the rat atrial myocyte as an animal model cell system, since: (i) inhibition of ET-1 receptors had been shown to substantially increase the survival rate in a rat model of cardiac hypertrophy (Sakai *et al.* 1996) and (ii) ET-1 receptors promote phospholipid turnover in rats (Woodcock *et al.* 1995) which is associated with production of inositol phosphates. In addition, we have recently reported that activation of InsP₃ receptors in rat atrial myocytes can substantially increase the occurrence of spontaneous Ca²⁺ sparks, the principal building blocks of cardiac Ca²⁺ signalling (Lipp *et al.* 2000). Here, we present evidence, that amongst all agonists tested, ET-1 exhibited the highest rate of spontaneous diastolic Ca²⁺ transients despite similar degrees of increases in the amplitude of electrically evoked Ca²⁺ transients. In order to quantify this property, we calculated the 'arrhythmogenic index' (AI) as the ratio between the number of ECTs during a recording period (30 s) and the fold increase in the amplitude of the electrically evoked Ca²⁺ transients. The results of such calculations revealed the prominent status of ET-1 with an AI of 8.60 (digoxin 4.10; isoproterenol 0.11), which was only superseded by a direct activation of InsP₃Rs with its natural ligand derived from the membrane permeant ester, InsP₃ BM (AI, 14.70). We were surprised by the pronounced ability of InsP₃ BM to predominantly generate ECTs while the increases in the Ca²⁺ response amplitude were only modest. Nevertheless, these findings were in line with the experiments in which we blocked InsP₃Rs with 2APB. Here, we found that 2APB predominantly inhibited the occurrence of ET-1-induced ECTs while the amplitude of electrically evoked Ca²⁺ transients was not affected significantly (Fig. 4B). Both results taken together clearly point to the fact that the mechanisms, responsible for ECT generation and the increase in the amplitude of electrically evoked Ca²⁺ responses might be different. Following the application of ET-1, the majority of the ECTs are generated by the activation of InsP₃Rs in the periphery, while the Ca²⁺ response amplitude can be increased by PKC-dependent phosphorylation of L-type Ca²⁺ channels and the resulting increased Ca²⁺ influx during the action potential (He *et al.* 2000).

In addition, around 75 % of the ET-1-evoked ECTs were able to trigger spontaneous premature action potentials. The other agonists had a lower propensity to cause spontaneous activity and only 25 % of their ECTs were accompanied by premature action potentials while 75 % represented subthreshold DADs. Thus, despite the highest increase in the Ca²⁺ responses during application of the β -adrenergic agonist, it failed to generate substantial numbers of ECTs.

In addition, we found further supporting evidence for a mechanistical difference between the digoxin and ET-1-induced ECTs when analysing the time interval between

the preceding electrically induced Ca²⁺ signal and the peak of subsequent ECTs (Fig. 1C). ECTs seem to occur significantly earlier in the presence of digoxin than when cells were stimulated with ET-1. The reason for that is still unclear. We might hypothesise that in the presence of digoxin, spontaneous Ca²⁺ release is purely triggered by RyRs and thus the shorter time interval reflected a shorter functional refractoriness of the SR after the preceding Ca²⁺ release event. In contrast, the longer time interval in the presence of ET-1 might reflect a different activation mechanism involving InsP₃Rs. This notion is supported by the fact that in the presence of digoxin, we found predominantly single ECTs following the action potential-evoked Ca²⁺ transient while in the presence of ET-1, a much larger proportion of multiple ECTs occurred (see Fig. 1Cd). We interpret the multiplicity of ECTs upon ET-1 stimulation as being the result of an InsP₃ production that sensitises the RyRs to enter the spontaneous Ca²⁺ release cycle more frequently.

Subthreshold ECTs in individual myocytes by themselves contribute to the generation of microheterogeneities in the distribution of excitability and thus may add to an increase in the likelihood of re-entry and fibrillation (Nattel *et al.* 2000). Nevertheless these effects will remain rather local, probably even restricted to the single cell, because of the purely electrotonic propagation and the associated damping of the signal by the three dimensional structure of the cardiac tissue. In contrast to that, suprathreshold DADs trigger action potentials that can spread into larger proportions of the atrial tissue. Consequently, premature action potentials may increase the likelihood of local unidirectional conduction blocks and increase the dispersion of refractoriness; both processes that enhance the occurrence of re-entrant circuits and atrial fibrillation (Nattel & Yue, 2000). It has to be mentioned here, that a single cell producing a single premature action potential is very unlikely to trigger electrical activity in many neighbouring cells. Nevertheless, such changes of the electrical behaviour in response to ET-1 application will not be restricted to a single cell, rather the entire atrial tissue might be affected and thus, the likelihood of larger numbers of cells displaying electrical disturbances is increased.

We present evidence for the direct involvement of InsP₃Rs, which co-localise with junctional RyRs, in the ET-1 responses and conclude that InsP₃Rs and RyRs share the same signalling microdomain (Fig. 6A). Thus, despite similar increases in the global Ca²⁺ responses, the spatial properties of subcellular Ca²⁺ release determined the arrhythmogenicity of agonist application. Consequently, the arrhythmogenicity of particular agonists that act on the heart cannot simply be judged on their ability to modulate cardiac ec-coupling in general either by Ca²⁺

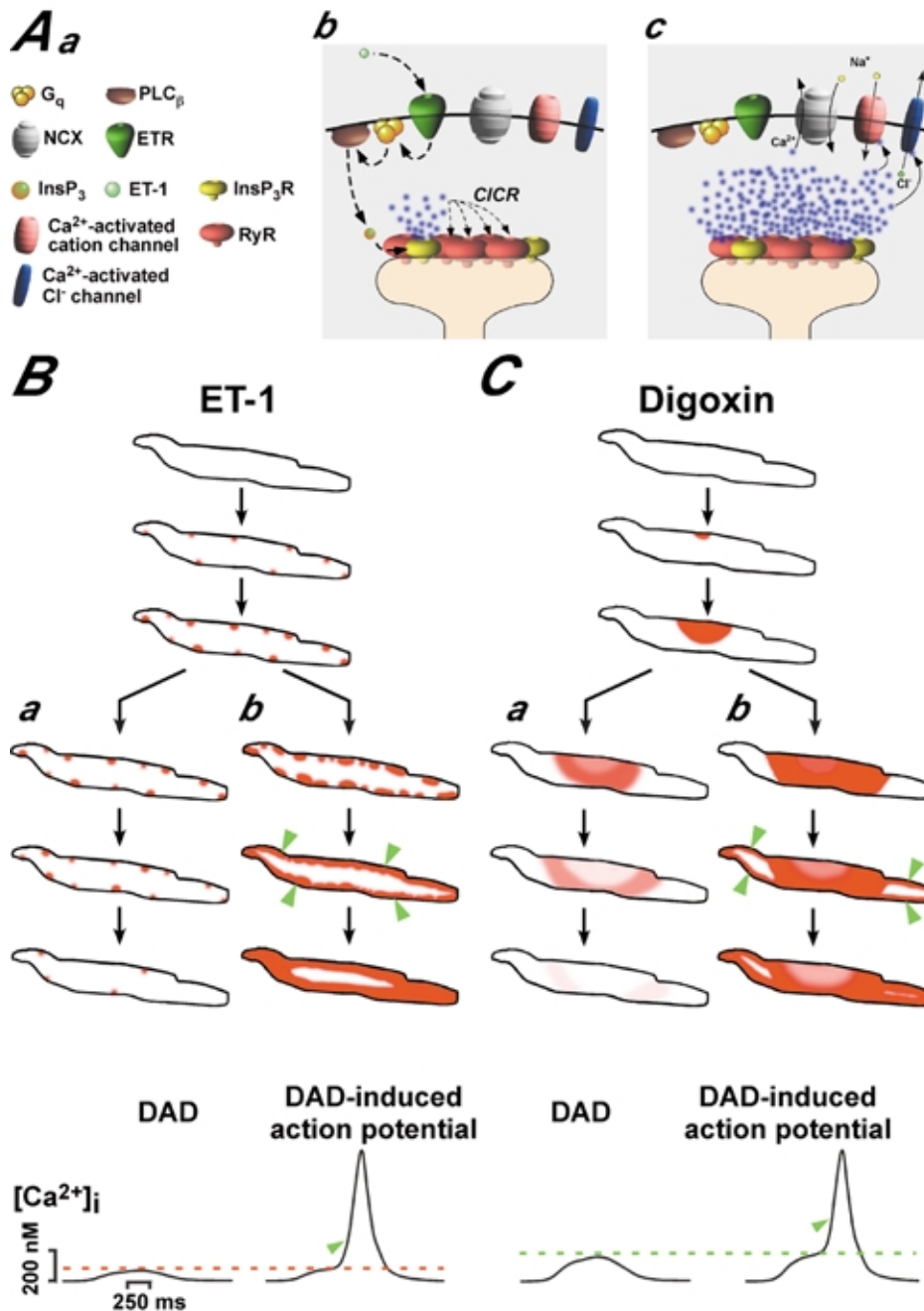


Figure 6. Activation of Ca^{2+} -dependent membrane currents and the relationship between subcellular Ca^{2+} release patterns, DADs and DAD-induced action potentials

A depicts a model of how InsP_3Rs impact on cardiac ec-coupling, Ca^{2+} signalling and membrane depolarisation. *Aa* introduces the symbols used in *Ab* and *Ac*. As depicted in Fig. 5*Ac*, InsP_3Rs and RyRs share the same peripheral signalling microdomain in the junction between plasma membrane and SR (*Ab*). Activation of ET-1 receptors mobilises InsP_3 (*Ab*) that stimulates InsP_3Rs to release Ca^{2+} , which then activates RyRs opening via CICR (*Ab*) and evokes depolarising membrane currents (*Ac*). *B* and *C* represent schematic diagrams for the subcellular pattern of Ca^{2+} release during subthreshold DADs (*Ba* for ET-1 and *Ca* for digoxin) and suprathreshold premature action potentials induced by ET-1 (*Bb*) and digoxin (*Cb*). The black lines illustrate the plasma membrane of rat atrial myocytes and the intensity of the red shading indicates the Ca^{2+} concentration. The ET-1 induces subsarcolemmal Ca^{2+} sparks that either evoke a DAD (*Ba*) or they are recruited to produce a larger Ca^{2+} elevation and the resulting depolarisation reaches threshold for triggering an action potential (*Bb*). Digoxin acts differently by setting up Ca^{2+} waves that are either confined in their propagation (*Ca*) and generate a subthreshold DAD (*Ca*) or they spread further throughout the cell (*Cb*) to trigger a DAD-induced action potential (*Cb*; threshold for digoxin indicated by the dashed green line). In both cases, the transition from a subthreshold to a suprathreshold Ca^{2+} signal is clearly indicated by the occurrence of the typical Ca^{2+} 'ring' (marked by the green arrowheads).

loading of the cells or sensitisation of the Ca²⁺ release process itself.

The ability of ET-1- and digoxin-induced diastolic ECTs to trigger premature action potentials results from the activation of Ca²⁺-sensitive membrane currents, such as Ca²⁺-dependent Cl⁻ or unspecific cation currents (Lederer & Tsien, 1976) and forwardmode Na⁺-Ca²⁺ exchange (Lipp & Pott, 1988; Fig. 6A). In principle, these currents only sense the subsarcolemmal Ca²⁺ concentration. Thus, Ca²⁺ signals confined to the sub-plasma membrane region, such as observed for ET-1 and InsP₃ BM (Fig. 5; see also cartoon in Fig. 6), effectively generate substantial depolarising currents for relatively small increases in the global Ca²⁺. In accordance with that, we observed a lower global Ca²⁺ threshold concentration for action potential generation in the presence of ET-1 and InsP₃ BM than for the digoxin application (Fig. 5C; see also cartoon in Fig. 6C). It is important to note here that although we did not monitor subcellular Ca²⁺ signals and changes of the membrane potential simultaneously for most of the cells (but see Fig. 2C), we are confident that the majority of action potential-induced ECTs represented DADs rather than EADs. Here, we define EADs as premature depolarisations that were clearly superimposed onto the shoulder of the rat atrial action potential. Although we were able to record EADs during our electrophysiological experiments, less than 2% of all after-depolarisations were identified as EADs (data not shown). In addition, we are able to distinguish EADs from DADs based on the particular recruitment of Ca²⁺ sparks. The highly synchronised activation of Ca²⁺ sparks (via the reactivating L-type Ca²⁺ channels) during EADs is contrasted by the rather slow and gradual build up of the spark frequency observed during DADs. DADs only cause synchronised Ca²⁺ spark recruitment when they successfully triggered a premature action potential (see Fig. 2). Thus DADs and EADs could be distinguished temporally on membrane potential recordings and spatially on confocal Ca²⁺ recordings.

Many agents including ET-1, angiotensin II and catecholamines have been implicated in the generation of atrial arrhythmia and fibrillation without explanations for their cellular effects (Du *et al.* 1995; Woodcock *et al.* 1998). Although these stimuli all activate the G_q-PLC signalling pathway and cause generation of InsP₃, the role of InsP₃Rs in cardiac myocytes is controversial, with suggestions that they play only a minor role during cardiac Ca²⁺ signalling or have a solely organelle specific function (Marks, 2000). In contrast, several laboratories have suggested a direct involvement of InsP₃ production in various heart pathologies. For example, the involvement of InsP₃Rs in inflammatory cardiac diseases accompanied by arrhythmia was suggested recently by Felzen and colleagues (Felzen *et al.* 1998). They used the PLC inhibitor U73122 and the InsP₃R antagonist heparin to block the InsP₃ limb of the

fas-mediated response to lymphocyte-myocyte interaction. In addition, Woodcock and colleagues demonstrated significant InsP₃ production following ischaemic periods that was associated with the occurrence of cardiac arrhythmia (Du *et al.* 1998). They also reported that various strategies that either blocked InsP₃ production directly or depleted the plasma membrane pool of the InsP₃-precursor phosphatidyl-4,5-bisphosphate suppressed the ischaemia-induced arrhythmias (Du *et al.* 1998). Furthermore, ischaemic insults substantially increase the production of InsP₃ during α -adrenergic application increasing the likelihood of InsP₃ signalling during such stresses (Woodcock *et al.* 1995). In the present study, we used isolated cardiac myocytes from healthy animals and possible periods of ischaemia during cell isolation were minimised and where unavoidable kept well below 2 min in total. Despite these precautions, we can, of course, not rule out absolutely that the cells suffered a period of ischaemia that somehow 'primed' them for a more substantial InsP₃ production during ET-1 application. However, the robust effects of InsP₃ BM on atrial myocytes indicates that these cells display a functional response to InsP₃ unrelated to any effects that ischaemia may have on hormone-induced InsP₃ production.

It was interesting to find substantial increases in the amplitudes of electrically evoked Ca²⁺ responses of rat atrial myocytes during digoxin application, since the rat heart is well known for its reduced sensitivity towards cardiac glycosides (Levi *et al.* 1994). The major reason for this relative insensitivity is most likely due to the reduced expression of the Na⁺-Ca²⁺ exchanger found in rat heart (for example see Sham *et al.* 1995), the Ca²⁺ entry pathway that is activated upon inhibition of the Na⁺-K⁺ pump. Indeed, in control experiments we found that digoxin concentrations that caused robust responses in atrial cells, failed to induce substantial Ca²⁺ transient increases and ECTs in ventricular cells (data not shown). At present, we do not fully understand these differences between atrial and ventricular cells and between species. However, our data hint to the possibility that similar stimuli can cause differential modulation of Ca²⁺ signalling. This is likely to be brought about by variations in ratios of homeostatic mechanisms such as Na⁺-Ca²⁺ exchange vs. Na⁺-K⁺ pump.

The concentrations of ET-1 used in the present study exceed reported apparent dissociation constant K_{Ds} for ET-1 binding to its receptors (low nanomolar values for ET-A and ET-B receptors; for a review see Golfman *et al.* 1991). Binding of ET-1 to its receptors has been described as 'quasi' irreversible (at least for the duration of even our longest experiments, see Golfman *et al.* 1991). Thus, for experiments using saturating ET-1 concentrations ([ET-1] >15–20 nM) the actual concentration will not alter steady-state occupancy of the receptors, instead it only affects the apparent association rate. The more rapid

onset of substantial ET-1 effects has recently been described for increased concentrations ($[ET-1] > 10$ nM; Talukder *et al.* 2001). Therefore, the high ET-1 concentrations used here, will ensure rapid occupancy of all the receptors and thus permits us to investigate effects of receptor binding on cellular Ca^{2+} signalling in the limited time for confocal microscopy. In addition, we are confident that even those high ET-1 concentrations do not cause substantial unspecific effects since 2-APB was able to specifically inhibit the arrhythmogenic actions of ET-1.

It is unlikely that cardiac myocytes would express $InsP_3$ Rs if they were solely involved in pathological responses. In keeping with this, our study clearly demonstrates a function for $InsP_3$ in cardiac physiology. The positive inotropy observed during activation of $InsP_3$ Rs could be considered as a beneficial result from PLC activation. Although we observed that the inotropy and ECTs developed concurrently, it may be possible by titrating the concentration of stimulus to provoke the positive response without the pathological Ca^{2+} release activity.

In summary, we have demonstrated that expression of functional $InsP_3$ Rs in the same subsarcolemmal signalling microdomain as RyRs underlies their ability and potency to modulate cardiac ec-coupling. Direct activation of the $InsP_3$ Rs with a membrane-permeable $InsP_3$ derivative, or their inhibition using 2APB, indicates that they can significantly contribute to the physiology (increases in the amplitude of electrically evoked Ca^{2+} signals) and pathophysiology (arrhythmogenicity) of agonist-stimulated atrial cardiomyocytes. The proximity between these two Ca^{2+} release channels, allows the $InsP_3$ signalling cassette to modulate ec-coupling in a way that one can almost speak of two parallel stimulation–contraction coupling mechanisms co-existing in cardiac muscle, the pharmac–contraction and the excitation–contraction coupling, with both signalling cassettes converging on the RyRs.

Apart from the physiological occurrence of $InsP_3$ Rs in normal atrial tissue, several reports pointed out a characteristic up-regulation of $InsP_3$ mRNA in hearts from hypertrophy and end-stage heart failure patients, while in parallel RyR mRNA was down-regulated (Marks, 2000). Furthermore, more than 50 % of those patients suffer initially from atrial arrhythmia and only later from ventricular arrhythmia, which in the light of our findings, might, at least in part, be explained by the changes of the $InsP_3$ R/RyR expression ratio. In addition to that, $InsP_3$ R expression also significantly increases during aging in humans (Go *et al.* 1995; Marks, 2000), a development that is again paralleled by an increase in the propensity to acquire particular forms of atrial arrhythmia, including fibrillation (Ezekowitz, 1999). Despite all this, it has to be pointed out here, that cardiac arrhythmia represents a multifactorial process. Many different mechanisms contribute to the occurrence of such arrhythmia, including the mechanisms

reported here but also an altered gene expression for a range of membrane currents (Keating & Sanguinetti, 2001). Nevertheless, our current study shows, that the $InsP_3$ signalling cassette might provide a novel target for the pharmacological management of atrial arrhythmias. However, further studies, especially on human preparations are necessary to refine our findings on the rat atrial myocytes and establish $InsP_3$ as a putative key arrhythmogenic agent.

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