

Acute atrial arrhythmogenesis in murine hearts following enhanced extracellular Ca^{2+} entry depends on intracellular Ca^{2+} stores

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Abstract

Aim: To investigate the effect of increases in extracellular Ca^{2+} entry produced by the L-type Ca^{2+} channel agonist FPL-64176 (FPL) upon *acute* atrial arrhythmogenesis in intact Langendorff-perfused mouse hearts and its dependence upon diastolic Ca^{2+} release from sarcoplasmic reticular Ca^{2+} stores.

Methods: Confocal microscope studies of Fluo-3 fluorescence in isolated atrial myocytes were performed in parallel with electrophysiological examination of Langendorff-perfused mouse hearts.

Results: Atrial myocytes stimulated at 1 Hz and exposed to FPL ($0.1 \mu\text{M}$) initially showed (<10 min) frequent, often multiple, diastolic peaks following the evoked Ca^{2+} transients whose amplitudes remained close to control values. With continued pacing (>10 min) this reverted to a regular pattern of evoked transients with increased amplitudes but in which diastolic peaks were absent. Higher FPL concentrations ($1.0 \mu\text{M}$) produced sustained and irregular patterns of cytosolic Ca^{2+} activity, independent of pacing. Nifedipine ($0.5 \mu\text{M}$), and caffeine (1.0 mM) and cyclopiazonic acid (CPA) ($0.15 \mu\text{M}$) pre-treatments respectively produced immediate and gradual reductions in the F/F_0 peaks. Such nifedipine and caffeine, or CPA pre-treatments, abolished, or reduced, the effects of 0.1 and $1.0 \mu\text{M}$ FPL on cytosolic Ca^{2+} signals. FPL ($1.0 \mu\text{M}$) increased the incidence of atrial tachycardia and fibrillation in intact Langendorff-perfused hearts without altering atrial effective refractory periods. These effects were inhibited by nifedipine and caffeine, and reduced by CPA.

Conclusion: Enhanced extracellular Ca^{2+} entry exerts *acute* atrial arrhythmogenic effects that is nevertheless dependent upon diastolic Ca^{2+} release. These findings complement reports that associate established, *chronic*, atrial arrhythmogenesis with *decreased* overall inward Ca^{2+} current.

Keywords atrial arrhythmogenesis, Ca^{2+} homeostasis, calcium entry, murine hearts, store calcium.

Atrial arrhythmias constitute the most common sustained disorders of cardiac rhythm encountered in clinical practice and result in substantial mortality and morbidity. For example, atrial fibrillation (AF) is associated with stroke, thromboembolism, heart failure and impaired quality of life; treatment strategies have largely proved inadequate (Thrall *et al.* 2006). It is attributed to irregular and rapid atrial electrical activity due to ectopic activity, single circuit or multiple wavelets of re-entry throughout the atria (Moe *et al.* 1964, Nattel 2002).

Persistent atrial arrhythmia appears to lead to changes in electrical properties of the cardiac tissue. This electrical remodelling may include alterations in cellular Ca^{2+} homeostasis including overload (Ryu *et al.* 2005, Yeh *et al.* 2008). Studies in both animal models and human atrial cardiomyocytes from patients in *established* AF report reduced current densities through L-type Ca^{2+} channels ($I_{\text{Ca,L}}$) (Yue *et al.* 1997, Bosch *et al.* 1999, Van Wagoner *et al.* 1999, Skasa *et al.* 2001, Yagi *et al.* 2002). The balance between inward currents through L-type Ca^{2+} channels (LTCCs) and outward K^+ currents are responsible for the plateau phase that extends the duration of action potentials in human cardiomyocytes. They are also responsible for Ca^{2+} entry that in turn regulates the contractile force (Fabiato & Fabiato 1975, Falk 1998). Their inhibition results in a shortening of both atrial action potential duration and effective refractory period (Morillo *et al.* 1995, Wijffels *et al.* 1995, Li & Nattel 1997). This in turn favours re-entry and persistence of the AF condition. Both human hearts with chronic AF (Christ *et al.* 2004) and a rabbit model of rapid atrial pacing (Bosch *et al.* 2003) showed parallel reductions in β -2 subunit expression and $I_{\text{Ca,L}}$ followed by changes in the α 1C subunit mRNA levels. This supports the concept that reduced α subunit expression underlies this reduction in voltage-dependent ($I_{\text{Ca,L}}$) despite increased single $I_{\text{Ca,L}}$ channel open probability as reported in hearts of patients with established AF (Klein *et al.* 2003). Conversely, it is well established that AF is associated with an abnormal sarcoplasmic reticular (SR) Ca^{2+} release reflecting increased open probabilities in the relevant RyR2 release channels resulting from their hyperphosphorylation (Nattel *et al.* 2007).

However, such earlier studies described results from models of *established* AF or *chronic* AF patients. There are relatively few reports on the features of *acute* atrial arrhythmogenesis that can include either atrial tachycardia (AT) or AF, and the possible involvement of LTCC activity. Reductions in $I_{\text{Ca,L}}$ form a characteristic and pathophysiologically important part of myocardial remodelling observed during long-lasting AF. However, recent studies indicate that they are not present in patients with non-persistent AF (Skasa *et al.* 2001).

Nevertheless, acute atrial arrhythmias (both fibrillation and flutter) occur with a 20–50% incidence following cardiothoracic surgery, particularly coronary artery bypass grafting. They most frequently occur 2 days after surgery and are rare after 7–15 postoperative days. However such episodes are usually short-lived. This incidence is significantly reduced by treatment with β -blockers and/or Ca^{2+} channel antagonists (Podesser *et al.* 1995, Yilmaz *et al.* 1996, Kim *et al.* 2002, Dobrilovic *et al.* 2005, Baker & White 2007, Iwamoto & Inoue 2007). Conversely, activation of β -adrenergic signalling leads to threefold to fourfold increases in calcium current. The latter results from protein kinase A phosphorylation of calcium channels (Hulme *et al.* 2006).

The present study accordingly goes on to investigate the possible roles of altered Ca^{2+} homeostasis on the *initiation* of *acute* atrial arrhythmias at both the cellular and the whole organ levels in intact murine hearts, through enhancing $I_{\text{Ca,L}}$. Murine hearts were used in view of their potential utility in future atrial studies of the effects of genetic modifications. Despite inevitable differences from human hearts, such genetically modified models have been successfully used to model mechanisms of ventricular arrhythmogenesis in LQTS3 (Thomas *et al.* 2008), Brugada syndrome (Stokoe *et al.* 2007) and catecholaminergic polymorphic ventricular tachycardia (Priori *et al.* 2001, Cerrone *et al.* 2005, Goddard *et al.* 2008). We provide experimental evidence that enhanced $I_{\text{Ca,L}}$ -mediated extracellular Ca^{2+} entry exerts acute atrial arrhythmogenic effects that nevertheless depend upon diastolic release of intracellularly stored SR Ca^{2+} . We compared the effects of FPL-64176 (methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylate; FPL) on regularly stimulated isolated murine atrial myocytes and atrial arrhythmogenesis in isolated Langendorff-perfused murine hearts. FPL is known to prolong opening of single LTCCs during depolarization and slow channel closing upon repolarization (Rampe & Lacerda 1991, Baxter *et al.* 1993, Lauven *et al.* 1999, Fan *et al.* 2000). FPL was used both alone and in combination with three agents with known effects upon cellular Ca^{2+} homeostasis. Of these, nifedipine acts as a competitive LTCC blocker (Triggle 2003) inhibiting inward Ca^{2+} current (Shen *et al.* 2000, Thomas *et al.* 2007). Caffeine is thought to increase the release of intracellularly stored Ca^{2+} , thereby ultimately depleting such stores, whether by sensitizing cardiac SR Ca^{2+} release-ryanodine receptors (RyR2s) to cytosolic Ca^{2+} or inhibiting phosphodiesterase activity, thereby increasing cellular cyclic adenosine monophosphate (cAMP) (Daly 2007). Finally, cyclopiazonic acid (CPA), is known to inhibit SR Ca^{2+} -ATPase activity (Seidler *et al.* 1989, Du *et al.* 1996) by blocking its Ca^{2+} access channel (Moncoq

et al. 2007, Palomeque *et al.* 2007), thereby altering levels of SR Ca.

Materials and methods

Inbred male and female 129/Sv wild-type mice (Harlan, Bicester, UK) kept in an animal house at room temperature were subjected to 12 h light–dark cycles, and fed sterile rodent chow with constant access to water. All procedures conformed to the U.K. Animals (Scientific Procedures) Act (1986). The following solutions were used in the course of the study: Solution A was prepared for electrophysiological experiments on Langendorff-perfused hearts. It consisted of normal bicarbonate-buffered Krebs–Henseleit (KH) solution containing (in mM): 119 NaCl, 25 NaHCO₃, 4.0 KCl, 1.2 KH₂PO₄, 1.0 MgCl₂, 1.8 CaCl₂, 10 glucose and 2.0 sodium pyruvate, and at pH 7.4 maintained by bubbling with 95% O₂–5% CO₂ (British Oxygen, Manchester, UK). Solution B was the basic stock solution from which other solutions (C–H) used in myocyte isolation were derived (in mM): 125 NaCl, 4.75 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 30 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), 10 glucose, 50 taurine and titrated to pH 7.4 with NaOH. It was filtered with a 0.2 µm filter (Millipore, Billerica, MA, USA) to remove microbes and small particles. Solution C: 750 µM Ca²⁺-containing perfusion solution was prepared by further adding 750 µM CaCl₂. Solution D: Ca²⁺-free nitrilotriacetic acid (NTA)-containing solution was prepared adding 5 mM NTA to solution B, and titrating the resulting solution to pH 6.95 with NaOH. Solution E: Enzyme solution was prepared by adding 1.5 mg mL⁻¹ collagenase (Worthington Type II), 2.0 mg mL⁻¹ hyaluronidase (Sigma, Gillingham Dorset, UK), and 100 µM CaCl₂ to solution B. Solution F: a further digestion buffer made by adding 1.0 mg mL⁻¹ collagenase and 1.0 mg mL⁻¹ BSA to solution B. Solution G: Enzyme washout solution was made by adding 1.0 mg mL⁻¹ BSA and 250 µM CaCl₂ to solution B. Solution H: Ca²⁺-containing solution was made by adding 1.2 mM CaCl₂ to solution B. After preparation, solutions D, E, F and G were always filtered using a 0.2 µm filter to remove microbes and small particles.

Measurement of cytosolic Ca²⁺ transients

Mice, aged 10–12 weeks at which viable atrial myocyte isolation could most readily be accomplished, were killed by cervical dislocation. A total of nine mice were used. Hearts were rapidly excised and cannulated in ice-cold KH solution (solution A). They were then mounted onto a Langendorff perfusion system and successively perfused with solution C (for 4 min),

solution D (4 min) and solution E (10–12 min) at 37 °C. The heart was then removed from the perfusion apparatus. The atrial appendages were then excised and chopped into several pieces in solution F. These were further incubated for another 5–10 min with gentle manual agitation using a 1 mL tip transfer pipette. All these latter steps were performed at 36–37 °C. Cells were then separated from the enzymic solution by centrifuging at 243 g for 3 min. The resulting isolated cells were then washed using solution G followed after 5 min, by centrifugation at 30 g for 2 min. The cells were then resuspended in solution H and after a 5 min interval, centrifuged again at 30 g for 2 min. The cells were then maintained at room temperature in solution H for the experiments that followed. The cells were then placed on a grade 1 circular laminin-coated coverslip (Menzel, Glasbearbeitungswerk, Germany) that formed the floor of a 1.5 mL perfusion chamber to which it was fixed with vacuum grease. They were then loaded with the acetoxymethyl (AM) ester of Fluo-3 (Molecular Probes, Leiden, The Netherlands) by incubation with 5 µM Fluo-3 AM in solution H (1.2 mM CaCl₂) for 10–20 min in the dark before washout of the Fluo-3 containing solution. They were then transferred onto the stage of a Zeiss LSM-510 laser scanning confocal system (Zeiss, Oberkochen, Germany) with a ×20 air objective on a Zeiss Axiovert 100 M inverted microscope. Fluo-3 fluorescence emission was excited with a 488 nm argon laser and measured at wavelengths between 505 and 550 nm. Images were then analysed using in-house custom-made software. Series of 500 frames (128 × 64 pixels per frame) were collected at a scanning frequency of 25 ms per frame to monitor fluorescence changes over time. Fluorescence (*F*) measurements were corrected for background signal in regions outside the cells. They were made within defined regions of interest (ROI; *F*) and were normalized to their baseline fluorescence (*F*₀) values. For each of the myocytes studied, peak *F*/*F*₀ values were calculated throughout each time series acquired. This typically contained around 12–13 peaks from which the mean peak *F*/*F*₀ could be obtained. A mean peak *F*/*F*₀ was then calculated for that series. Cells were paced at 1 Hz (5 V above excitation threshold of 30–60 V for 2 ms) with two field electrodes. All fluorescence studies were carried out at room temperature. Ca²⁺ transients were measured from ROIs covering entire cells.

Atrial electrophysiological experiments

Male and female mice (age 3–6 months), at which their aortic size permitted reproducible *in situ* cannulation, were randomly selected, heparinized with 50 IU heparin (IP) 10–15 min before being killed by cervical

dislocation (Schedule I: Animals Scientific Procedures Act 1986). A total of 72 mice were used. The heart was cannulated *in situ* using a straight-cut and smoothed 21-gauge needle previously filled with solution A, dissected and then fixed securely with a straight 60 g pressure micro-aneurysm clip (Harvard Apparatus, Edenbridge, UK). The cannulated heart was perfused with solution A at room temperature and then mounted onto a Langendorff system (Balasubramaniam *et al.* 2004) then perfused at a constant flow rate of 2–2.5 mL min⁻¹ (Watson-Marlow Bredel Peristaltic pumps, model 505S; Falmouth, Cornwall, UK) with solution A. The perfusate was first filtered through 200 μ m and 5 μ m membranes (Millipore UK, Watford, UK), and warmed to 37 °C by a water-jacketed heat exchange coil (Model C-58A; Techne, Cambridge, UK) before entering the coronary arterial network. Viable hearts regained a pink appearance and spontaneous rhythmic contractions upon warming. The hearts were perfused retrogradely for not less than 10 min in the absence of stimulation. Experiments were only performed in intact Langendorff preparations showing clear-cut 1 : 1 atrioventricular (AV) conduction during the intrinsic activity following cannulation.

The electrophysiological studies involved comparisons of records from simultaneous recordings made at two sites. Thus, in addition to the paired platinum-stimulating electrodes placed on the right atrium, two bipolar recording electrodes of 1 mm interpole spacing were placed on the left atrium and left ventricle respectively. Hearts were initially paced for not less than 5 min at 10 Hz to permit them to regain their physiological steady state. Three types of pacing protocols were then used. First, hearts were studied at their intrinsic rates in the absence of stimulation. Second, hearts were subject to regular pacing at 10 Hz using 2 ms square-wave stimuli set at two times the excitation threshold (Grass S48 stimulator; Grass-Telefactor, Slough, UK). Third, hearts were studied using a programmed electrical stimulation (PES) procedure adopted from clinical techniques previously used in ventricular studies (Saumarez & Grace 2000, Balasubramaniam *et al.* 2004) but recently introduced in clinical studies of atrial electrophysiology (Wellens 2003, Brembilla-Perrot *et al.* 2005). These began using standard baseline pacing stimuli at a frequency of 10 Hz for 20 s. Drive trains of eight paced beats (S1) were each followed by an extra stimulus (S2) every ninth beat, initially at an S1–S2 interval equal to the pacing interval. Each subsequent cycle reduced the S1–S2 interval by 1 ms until atrial refractoriness was reached. The resulting electrogram signals were amplified, band-pass filtered (30 Hz to 1 kHz; Gould 2400S; Gould-Nicolet Technologies, Ilford, Essex, UK) and digitized at a sampling frequency of 5 kHz (Spike2 software;

CED1401*plus*; Cambridge Electronic Design, Cambridge, UK). The present protocols thus differed from our previous studies on ventricular arrhythmogenesis (Balasubramaniam *et al.* 2004, Stokoe *et al.* 2007, Thomas *et al.* 2008) in pacing hearts from the atria rather than the ventricles. Furthermore, use of the previous 8 Hz pacing rate permitted atrial escape phenomena that precluded regular activation by the stimulus train. Accordingly we used a higher pacing rate of 10 Hz to ensure regular atrial stimulation under all the pharmacological conditions. In some hearts, this resulted in a gradual development of an AV block. Nevertheless, withdrawal of the regular pacing permitting resumption of normal intrinsic activity led to restoration of normal 1 : 1 AV conduction once pharmacological agents were withdrawn. These findings likely relate to AV node refractoriness at high pacing rates: we observed that the atria had shorter refractory periods (24 ± 7 ms, $n = 33$) than the AV node (61 ± 12 ms, $n = 5$).

Statistical analysis

Statistical analysis was carried out using a repeated-measures one-way ANOVA to compare data using SPSS software. Results from individual hearts acquired during pharmacological intervention were compared with their respective untreated controls using a one-way ANOVA for correlated samples (SPSS software). A probability of $P < 0.05$ was taken as statistically significant. Cross-tabulations with chi-squared or Fisher's exact test were used as appropriate for categorical variables. Data are expressed as means \pm standard errors of the mean (SEM). The numbers, n , denote either numbers of whole hearts or of peak F/F_0 values.

Agents

All drugs and other chemical agents were purchased from Sigma-Aldrich (Poole, UK) except where otherwise indicated. FPL-64176 was initially made up with dimethyl sulfoxide to make a stock concentration of 5.0 mM, and kept wrapped in foil to prevent light degradation, and was stored at -20 °C. Nifedipine was dissolved in 96% ethanol to make a 1.0 mM stock concentration, kept wrapped in foil to prevent light degradation and was kept refrigerated at 4 °C. Caffeine was dissolved directly in solution A or H as appropriate and kept at room temperature. CPA was prepared in 96% ethanol to make a 10 mM stock concentration, and stored at -20 °C. Final drug concentrations were achieved by dilution with solution A for electrophysiological experiments and solution H for experiments in single cells.

Results

FPL modifies Ca^{2+} homeostasis in atrial myocytes

The murine atrial myocytes that were obtained by our isolation procedure appeared as elongated cells with rounded but tapered ends with well-defined striations. They were viable for up to 6–8 h prior to fluorophore loading. We studied a total of 108 cells from nine hearts using confocal microscopy following loading with Fluo-3-AM. These were then subjected to regular pacing stimuli at a frequency of 1 Hz; higher pacing rates led to loss of capture in such isolated cells. The resulting fluorescence signals, F , were expressed normalized to their baseline values F_0 . Such values were obtained over ROIs that covered the entire cell area. In the absence of

added pharmacological agents, this resulted in regular successions of entrained Ca^{2+} transients with stable amplitudes of mean F/F_0 5.36 ± 0.89 ($n = 192$ peaks from 16 cells) that decayed to stable baselines with no evidence of diastolic events (Fig. 1Aa,B; Table 1). Such Ca^{2+} transients would be the result of cycles of depolarization-induced SR Ca^{2+} release into the cytosol and its subsequent return from cytosol to stores whose magnitudes and time courses would be sensitive to manoeuvres affecting either process. The isolated atrial myocytes thus provided stable preparations in which the effect of pharmacological agents on the overall magnitudes and transients of the resulting Fluo-3 Ca^{2+} signals could be investigated over prolonged periods.

The addition of FPL then resulted in significant disruptions of this regular pattern of Ca^{2+} transients.

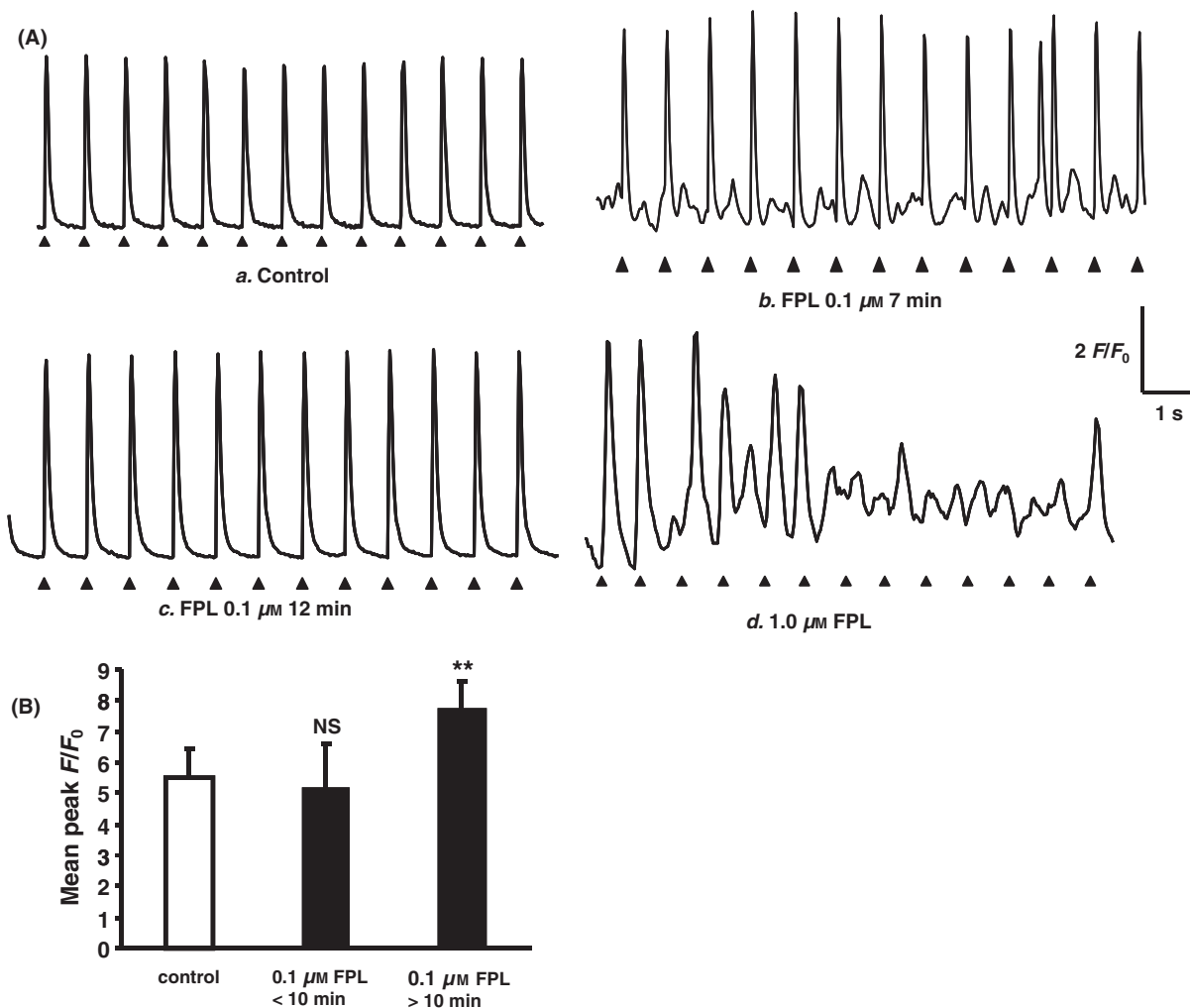


Figure 1 Effects of FPL on Ca^{2+} transients from regularly stimulated atrial myocytes. (A) Ca^{2+} transients obtained in a typical control myocyte (a) and cells 7 (b) and 12 min following addition of FPL ($0.1 \mu M$) (c), and immediately following addition of $1.0 \mu M$ FPL (d), showing diastolic transients (b) that eventually disappear (c), and sustained irregular patterns of cytosolic Ca^{2+} activity independent of the continued pacing (d). (B) Peak F/F_0 values (mean \pm SEM) obtained from control conditions (clear bar) and corresponding values obtained <10 min and >10 min following addition of $0.1 \mu M$ FPL (filled bars) (vs. control, NS: no significant difference, $**P < 0.01$).

Table 1 Occurrence of diastolic calcium release in atrial myocytes

Agents	Number of cells showing diastolic release/total no. of cells	Number of evoked peaks examined	Number of diastolic release events
Control	0/16	192	0
FPL (0.1 μM)	4/4**	50	55***
FPL (1 μM)	8/8**	Sustained irregular patterns of cytosolic Ca^{2+} activity	
Nifedipine	0/8	96	0
CPA pre-treated 20 min	0/16	192	0
Caffeine pre-treated 15 min	0/12	150	0
Nifedipine + 0.1 μM FPL	0/9	117	0
Nifedipine + 1 μM FPL	0/6	78	0
CPA + 0.1 μM FPL <10 min	3/3**	47	46***
CPA + 0.1 μM FPL >10 min	4/11*	143	46***, \$\$\$
CPA + 1.0 μM FPL	1/3	39	5***, \$\$\$
Caffeine + 0.1 μM FPL	0/6	78	0
Caffeine + 1 μM FPL	0/6	78	0

CPA, cyclopiazonic acid.

Test agents vs. control *** $P < 0.001$ on chi-squared testing; test agents vs. control, * $P < 0.05$, ** $P < 0.01$ on Fisher exact testing; test agents vs. FPL, \$\$\$ $P < 0.001$ on chi-squared testing against results obtained with 0.1 μM FPL.

The regular series of evoked deflections persisted with the introduction of low concentrations (0.1 μM) of FPL (Fig. 1Ab,B). The amplitudes of such evoked Ca^{2+} transients then initially remained close to control values, at 5.14 ± 1.46 ($P > 0.05$; $n = 50$ peaks from four cells). However, at early times (<10 min) following addition of FPL, there were additional smaller diastolic Ca^{2+} deflections that similarly extended over the entire cell area (Fig. 1Ab). These consisted of frequent, sometimes multiple diastolic peaks (55 diastolic peaks observed over 50 transients in four of four cells: see Table 1). However, with continued pacing beyond 10 min these subsidiary peaks disappeared, consistent with a reduction in available SR Ca upon which such events might depend. This left a sustained pattern of large evoked transients with a peak F/F_0 of 7.73 ± 0.86 ($P < 0.001$; $n = 50$ peaks from four cells) (Fig. 1Ac,B). Higher FPL concentrations (1.0 μM) initially resulted in an increased peak F/F_0 , but this was then often followed by a loss of the regular pattern of evoked activity. This was replaced by sustained irregular patterns of cytosolic Ca^{2+} activity that was independent of continued pacing (Fig. 1Ad, eight cells).

The effects of FPL are modified by agents known to modify Ca^{2+} homeostasis

The above findings demonstrated that FPL alters cellular Ca^{2+} homeostasis, consistent with previous reports.

FPL is thought to modify LTCCs through prolonging their opening during depolarization and their inactivation upon repolarization, enhancing the duration of the consequent tail currents (Rampe & Lacerda 1991). The FPL actions observed here accordingly could take place entirely through an increased entry of extracellular Ca^{2+} , the effect of such an entry on Ca^{2+} -induced Ca^{2+} release (CICR) from the SR Ca^{2+} store or both these effects. These possibilities were investigated using agents known to modify such Ca^{2+} entry, RyR2-mediated Ca^{2+} release or SR Ca^{2+} reuptake. The latter two manoeuvres would be expected to alter SR store Ca^{2+} through separate and therefore independent mechanisms.

First, nifedipine was used as a dihydropyridine-LTCC blocker to diminish extracellular Ca^{2+} entry. In the present experiments on atrial myocytes, nifedipine pre-treatment lasting 10 min promptly reduced peak F/F_0 , to 2.38 ± 0.19 ($n = 96$ peaks from eight cells) consistent with this known effect in reducing extracellular Ca^{2+} entry (Fig. 2Aa,b). It then abolished the diastolic Ca^{2+} transients produced by a subsequent addition of 0.1 μM FPL (Fig. 2Ac; Table 1). It also abolished the irregular activity produced by 1.0 μM FPL (Fig. 2Ad) leaving regular sequences of F/F_0 peaks with mean amplitudes of 3.08 ± 0.70 ($n = 117$ peaks) (Fig. 2B) and 3.78 ± 0.83 ($n = 78$ peaks) (Fig. 2B) respectively. These observations are consistent with a dependence of these altered patterns of Ca^{2+} homeostasis induced by

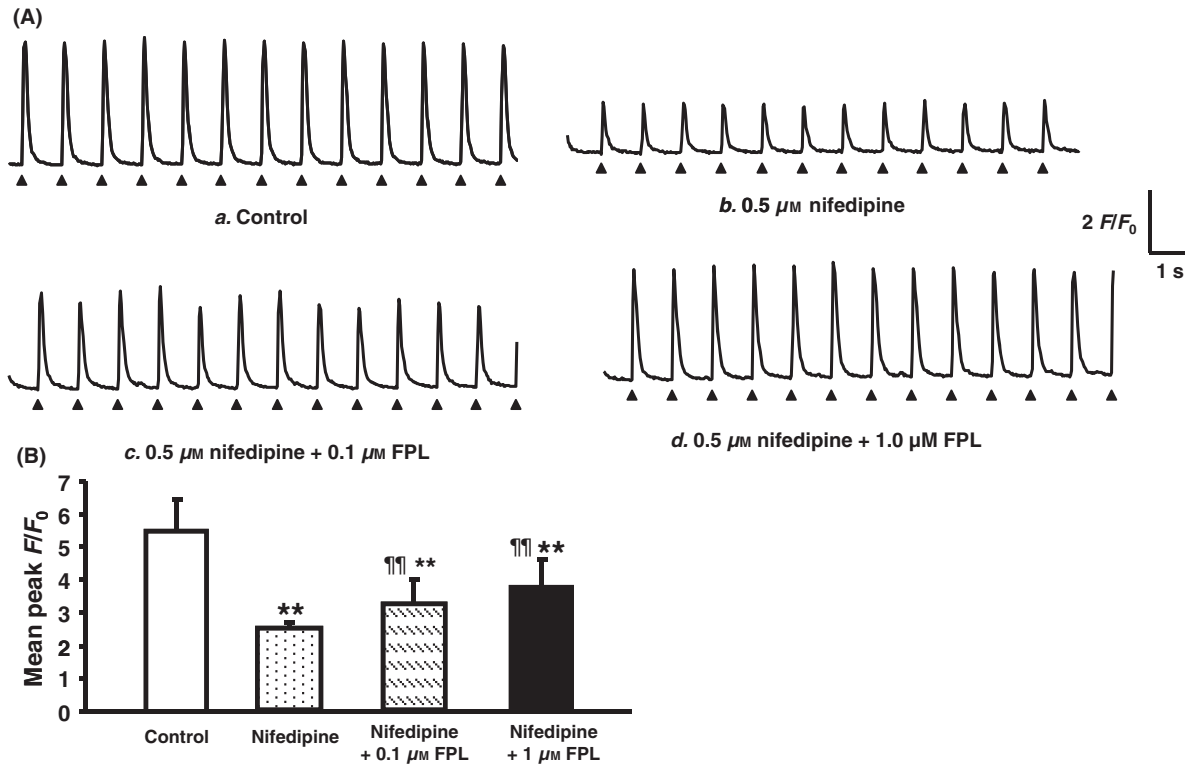


Figure 2 Nifedipine pre-treatment following addition of FPL on Ca²⁺ transients from regularly stimulated atrial myocytes. (A) Ca²⁺ transients obtained before (a) and immediately following introduction of 0.5 μM nifedipine (b), their partial recovery following the further addition of 0.1 and 1.0 μM FPL (c, d respectively): none of these records showed diastolic events. (B) Peak F/F₀ values (mean ± SEM) obtained under control conditions (clear bar) compared with (vs. control, **P < 0.01; vs. nifedipine ¶¶P < 0.01) corresponding values obtained following addition of nifedipine (0.5 μM) (second bar from left) and a further addition of FPL (0.1 and 1 μM) (third and fourth bars from left respectively).

FPL upon Ca²⁺ entry, whether directly through LTCC-mediated Ca²⁺ entry or indirectly through SR-mediated Ca²⁺ release. These latter possibilities were then resolved using the agents CPA and caffeine, as described below.

Second, caffeine was used as an agent to increase the release of intracellularly stored Ca²⁺ with a resulting gradual depletion of SR Ca²⁺. The present experiments in atrial cells demonstrated that a 15 min pre-treatment with caffeine (1.0 mM) similarly reduced the peak F/F₀ of the Ca²⁺ transients to 2.75 ± 0.21 (n = 150 peaks from 12 cells) consistent with such a hypothesis (Fig. 3Aa,b,B). Such transients showed prolonged kinetics, relative to those shown under control conditions consistent with our previously reported actions on the time course of CICR (Zhang *et al.* 2009). These were not observed with the remaining pharmacological interventions. This is illustrated in the full-width half-maximum values in Table 2. Following such pre-treatment, the addition of FPL (0.1 and 1.0 μM) resulted in a persistent, regular pattern of stimulation-induced responses, with reduced amplitudes of 3.04 ± 0.78 (n = 78 peaks from six cells) and 2.89 ± 0.28 (n = 78

peaks from six cells) (Fig. 3Ac,d,B) respectively but an absence of diastolic Ca²⁺ release. Caffeine pre-treatment thus rescued the cells from the effects of FPL alone.

Third, CPA was used as a further, independent means of depleting SR store Ca²⁺ through its inhibition of SR Ca²⁺-ATPase activity. In the present experiments, CPA (0.15 μM) resulted in a progressive reduction of the amplitude of the evoked transients to a peak F/F₀ of 3.64 ± 1.06 (n = 168 peaks). (Fig. 4Aa,b,B; Table 1). CPA *pre-treatment* also markedly reduced the incidence of cells that showed diastolic phenomena following further addition of 0.1 μM FPL. Thus, of 14 cells studied, only three cells which were examined at a period <10 min following addition of 0.1 μM FPL showed extensive diastolic Ca²⁺ release phenomena (following 46 of 47 examined peaks) (Fig. 4Ac,B; Table 1). The remaining 11 cells studied >10 min following addition of FPL showed a lower incidence of such transients. These followed 46 of 143 peaks and only occurred in 4 of the 11 cells studied (Fig. 4Ad). They similarly disappeared >10 min following addition of FPL, consistent with their dependence upon a limited Ca store.

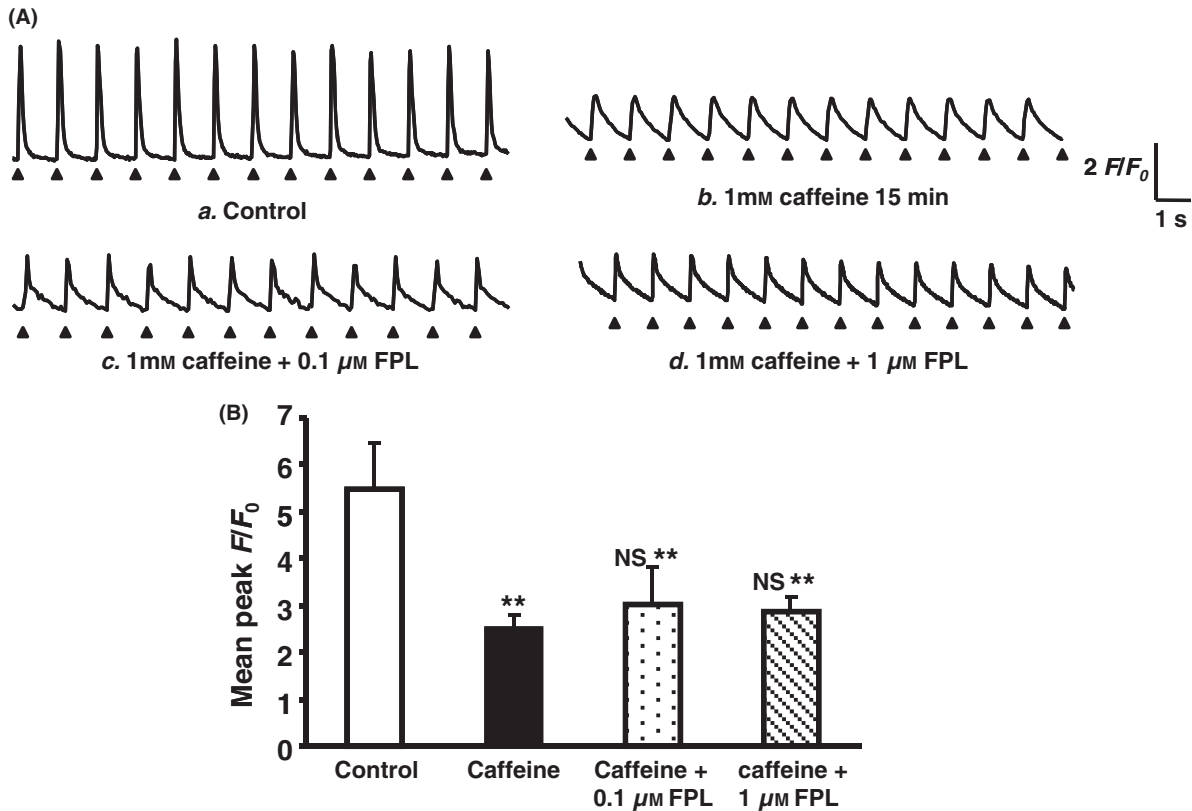


Figure 3 Caffeine pre-treatment following addition of FPL on Ca^{2+} transients from regularly stimulated atrial myocytes. (A) Ca^{2+} transients obtained before (a) and pre-treatment after 15 min following introduction of 1 mM caffeine (b), following introduction of 0.1 μM FPL (c, d): none of these records showed diastolic events. (B) Peak F/F_0 values (mean \pm SEM) obtained under control conditions (clear bar) compared with (vs. control $**P < 0.01$; vs. caffeine NS: no significant difference) corresponding values obtained following addition of caffeine (0.5 μM) (filled bar) and a further addition of FPL (0.1 and 1 μM) (third and fourth bars from left respectively).

Table 2 Full-width half-maximum (FWHM) values for Ca^{2+} transients obtained from regularly stimulated atrial myocytes under different conditions

Agents	No. of peaks	FWHM (ms, mean \pm SEM)
Control	12	118 \pm 7
0.1 μM FPL	12	117 \pm 13
Nifedipine	12	114 \pm 21
Caffeine 15 min	12	336 \pm 38*
CPA 20 min	12	125 \pm 11
Nifedipine + 0.1 μM FPL	12	123 \pm 21
Nifedipine + 1.0 μM FPL	12	121 \pm 17
Caffeine + 0.1 μM FPL	12	178 \pm 15*
Caffeine + 1.0 μM FPL	12	185 \pm 8*
CPA + 0.1 μM FPL	12	102 \pm 14
CPA + 1.0 μM FPL	12	117 \pm 8

CPA, cyclopiazonic acid.

* $P < 0.05$ when tested against control.

Pre-treatment with CPA also reduced the incidence of cells that showed an irregular pattern of Ca^{2+} activity in response to the addition of 1.0 μM FPL (Fig. 4Ae). Of the five such cells studied, two showed a persistent irregular pattern but three continued to show a regular pattern of activity in response to the regular pattern of stimulation. However, the latter consisted of a series of Ca^{2+} transients that declined to a peak F/F_0 of 1.82 ± 0.62 ($n = 39$ peaks; Fig. 4Af,B) with continued pacing beyond 10 min (Fig. 4Af,B; Table 1). Only one cell showed diastolic Ca^{2+} release in 5 of 13 peaks even 8–10 min following addition of FPL that nevertheless disappeared after continued pacing.

Cellular Ca^{2+} changes correlate directly with arrhythmogenic tendencies in intact perfused hearts

The above results directly correlated with findings from dual ventricular and atrial bipolar electrogram (BEG) recordings from Langendorff-perfused hearts

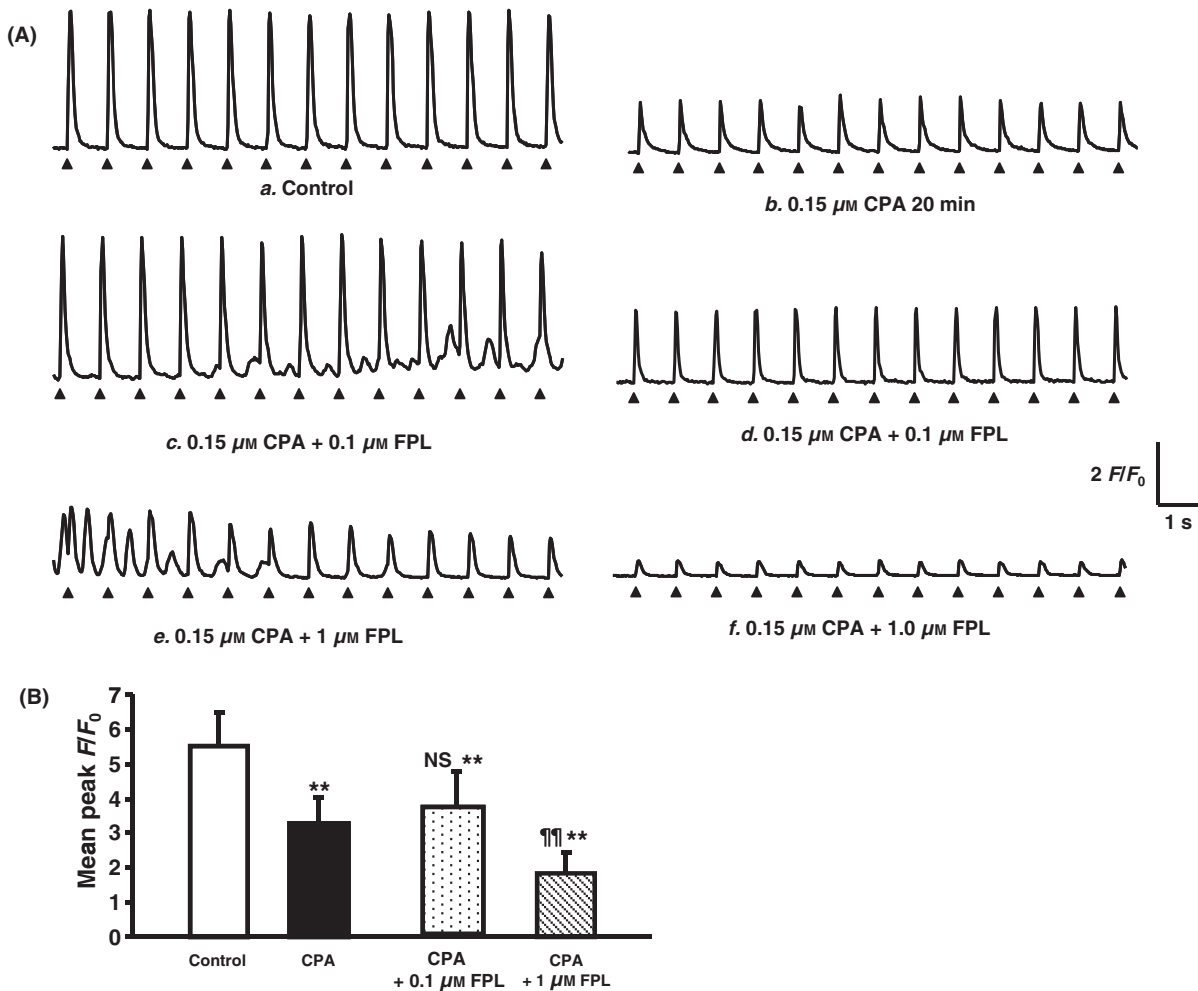


Figure 4 Cyclopiazonic acid (CPA) pre-treatment following addition of FPL on Ca^{2+} transients from regularly stimulated atrial myocytes. (A) Results obtained during control conditions (a) and 20 min following the addition of CPA ($0.15 \mu\text{M}$) (b) and further introduction of FPL at concentrations of $0.1 \mu\text{M}$ (c, d) and $1.0 \mu\text{M}$ (e, f) showing an initial appearance of diastolic signals (c, e) followed by their disappearance and a decline in the amplitude of the evoked Ca^{2+} transients (d, f). (B) Peak F/F_0 values (mean \pm SEM) obtained before (open bar) and following addition of CPA ($0.15 \mu\text{M}$) (filled bar) and a further addition of FPL (vs. control $**P < 0.01$; vs. CPA NS: no significant difference, $***P < 0.01$) (third and fourth bars from left).

implicating such alterations in Ca^{2+} homeostasis in atrial arrhythmogenic phenomena. The atrial recordings in Figs 5 and 6 show both atrial and ventricular deflections whereas atrial deflections were absent from ventricular BEG traces. Atrial pacing at 10 rather than 8 Hz avoided atrial escape phenomena and ensured regular activation by the stimulus train.

In the absence of test agents, none of the hearts showed arrhythmic activity, whether in the form of regular AT or irregular AF, during either intrinsic activity or regular pacing (Table 3). Three showed AT lasting 0.5, 1.0 and 5.0 s during PES. However, addition of FPL ($1.0 \mu\text{M}$) produced strong arrhythmic effects in all seven hearts studied. This was accompanied by evidence of third-degree AV block in all seven cases during intrinsic activity. We observed a total of 12

episodes of sinus pause, in two of seven hearts treated with FPL as also illustrated in Fig. 5A, with durations in the range of 0.4–1.6 s. These were not seen in untreated hearts. Thus, following addition of FPL, three hearts showed AT lasting 5.6, 5.7 and 14.0 s during intrinsic activity (Fig. 5A), two showed AT lasting 0.8 and 6.0 s (Fig. 5B) and two showed AF lasting 2.1 and 5.5 s respectively during regular pacing. During PES, five hearts showed AT lasting 3.8, 7.7, 0.4, 2.0 and 6.8 s and four showed AF lasting 3.4, 3.1, 0.6 and 18.7 s respectively (Fig. 5C,D). These arrhythmogenic phenomena took place in an absence of the altered refractoriness that has been associated with re-entrant phenomena occurring during atrial arrhythmogenesis in the experimental system used here. Tissue refractoriness has previously been closely associated with the

Table 3 Occurrence of arrhythmogenesis (AT or AF) under different protocols

Agents	Intrinsic (<i>n</i>)	Regular pacing (10 Hz) (<i>n</i>)	PES (<i>n</i>)
Control	0 (36)	0 (36)	3 (36)
FPL	3 (7)**	4 (7)**	7 (7)**
Nifedipine pre-treated	0 (12)*	0 (12)*	3 (12)##
CPA pre-treated	0 (11)*	0 (11)*	1 (11)***
20 min			
Caffeine pre-treated	0 (6)*	0 (6)*	0 (6)***
>15 min			
Nifedipine + FPL	0 (6)*	0 (6)*	5 (6)**
CPA + FPL	0 (6)*	0 (6)*	4 (6)**
Caffeine + FPL	0 (6)*	0 (6)*	1 (6)##

AT, atrial tachycardia; AF, atrial fibrillation; CPA, cyclopiazonic acid; PES, programmed electrical stimulation.

Test agents vs. control $P < 0.01^{**}$; test agents vs. FPL

$P < 0.05^{*}$; test agents vs. FPL $P < 0.01^{##}$ and $P < 0.001^{***}$ on Fisher exact testing. Each entry provides both the incidence of arrhythmogenesis and the number of hearts studied in brackets.

inducibility or otherwise of ventricular arrhythmogenesis in murine hearts (Maguire *et al.* 2003). We assessed atrial refractoriness in intact hearts using a decremental pacing protocol both before and following FPL activation. The atrial effective refractory period (AERP) was determined from the shortest S1–S2 interval that failed to elicit an atrial deflection (Thomas *et al.* 2007). The addition of FPL (1.0 μM) did not produce significant changes in AERP (control: 22.10 ± 7.80 ms, $n = 22$ hearts; FPL: 26.47 ± 6.50 ms, $n = 7$ hearts; $P > 0.05$).

Nifedipine, caffeine and CPA treatments reduced these arrhythmic effects of FPL. Nifedipine pre-treatment combined with a further addition of FPL in six hearts did not result in arrhythmogenesis during either intrinsic or regular pacing but five hearts showed AT (lasting 1.1, 1.1, 0.3, 2.6 and 29.2 s) during PES (Fig. 6A). Caffeine pre-treatment abolished FPL-mediated arrhythmic effects during intrinsic, regular pacing in six hearts. One heart showed AT during PES lasting 5.2 s (Fig. 6B). Finally, with pre-treatment with CPA combined with a further addition of FPL in five hearts, none of the hearts showed arrhythmogenesis during either intrinsic or regular pacing. During PES two hearts showed AT (lasting 0.6 and 5.4 s) and two showed sustained AF both lasting 5.0 s (Fig. 6C).

Discussion

The present study investigated the effect of enhancing LTCC opening on the *initiation* of acute atrial arrhythmias at both the cellular and the whole organ levels in

intact murine hearts. We provide an independent confirmation of the finding that enhanced extracellular Ca^{2+} entry through using the LTCC agonist FPL exerts acute atrial arrhythmogenic effects that nevertheless depend upon diastolic release of intracellularly stored SR Ca^{2+} . Thus treatment with FPL resulted in an appearance both of diastolic Ca^{2+} events at the cellular level, and atrial arrhythmogenesis despite an absence of changes in AERP at the level of intact perfused hearts. These phenomena – whether at the cellular or the whole organ level – were inhibited if the addition of FPL followed pre-treatment with the LTCC antagonist nifedipine, suggesting their dependence upon voltage-activated entry of extracellular Ca^{2+} . They were also abolished by pre-treatments with either caffeine or CPA. This provided two independent methods of reducing SR Ca^{2+} . The first would increase RyR2-mediated release of intracellularly stored Ca^{2+} . The second would inhibit Ca^{2+} -ATPase activity. The latter findings are consistent with a dependence of the effects of FPL upon a finite SR Ca^{2+} store. Together these findings thus attribute acute atrial arrhythmogenesis produced by FPL in an otherwise normal heart to an increased CICR that is dependent upon *both* Ca entry and SR Ca^{2+} .

There have been extensive studies on *chronic* or *established* AF (Yue *et al.* 1997, Bosch *et al.* 1999, Van Wagoner *et al.* 1999, Skasa *et al.* 2001, Yagi *et al.* 2002). Thus, persistent AF has been associated with a reduction in atrial action potential duration (Li & Nattel 1997). This has been attributed to reductions in current densities through L-type Ca^{2+} channels ($I_{\text{Ca,L}}$). Recent studies have correspondingly shown reduced expression in mRNA coding for the $\alpha 1\text{C}$ subunit of L-type channels in atria from patients with persistent AF (Brundel *et al.* 1999, Lai *et al.* 1999). Experimental studies that applied chronic atrial pacing at rates mimicking AF demonstrated depressed Ca^{2+} currents and numbers of dihydropyridine binding sites (Gaspo *et al.* 1999). There is also recent evidence for reduced L-type Ca^{2+} currents in the atrial myocardium of patients with persistent AF (Bosch *et al.* 1999, Van Wagoner *et al.* 1999). These changes in turn have been implicated in the electrical remodelling associated with AF (Yue *et al.* 1997, Bosch *et al.* 1999, Van Wagoner *et al.* 1999, Skasa *et al.* 2001, Yagi *et al.* 2002). These remodelling changes are not present in patients with non-persistent AF (Skasa *et al.* 2001). Conversely, AF is accompanied by abnormal SR Ca^{2+} release that likely reflects increased open probability in hyperphosphorylated RyR2-release channels (Hulme *et al.* 2006). In contrast, a recent report described transgenic (TG) mice with a cardiac over-expression of junctin and an accompanying 2.4 fold $I_{\text{Ca,L}}$ over-expression. This described the electrophysiological features of significantly lower heart rates in TG

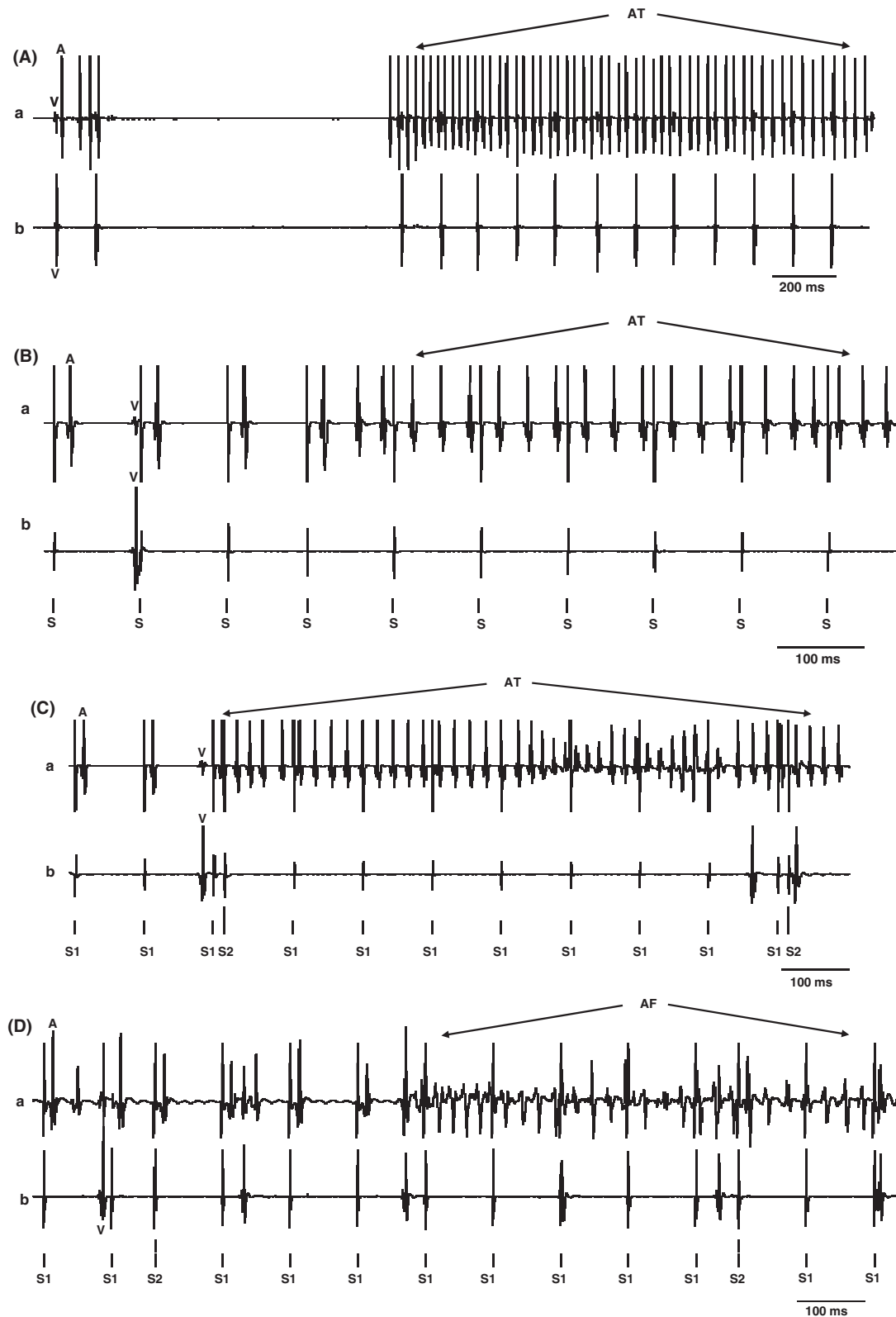


Figure 5 Examples of atrial arrhythmogenic effects of FPL. Traces of atrial (a) and ventricular (b) bipolar electrogram records obtained under the following conditions: (A) Spontaneous atrial tachycardia (AT) during intrinsic pacing. (B) AT during regular pacing. (C, D) AT (C) and atrial fibrillation (AF; D) respectively during programmed electrical stimulation (PES); timing of regular (S1) and extrasystolic stimulation (S2) indicated below each trace. In the individual traces, ventricular deflections are marked 'V' and atrial deflections are marked 'A'.

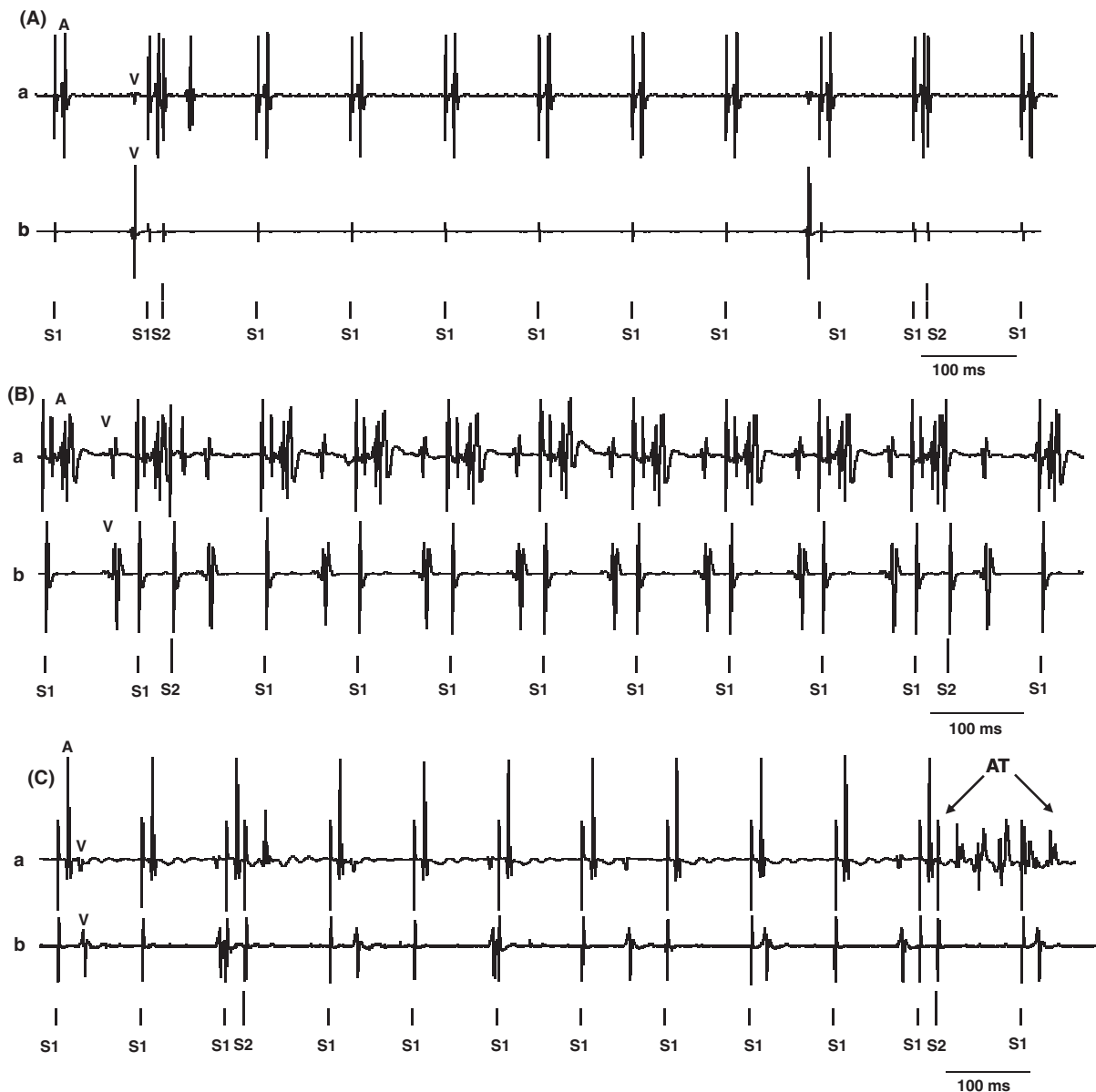


Figure 6 Nifedipine, caffeine and cyclopiazonic acid (CPA) pre-treatment abolish FPL-induced arrhythmogenic effects. Traces of atrial (a) and ventricular (b) bipolar electrogram records obtained during programmed electrical stimulation (PES) (timing of regular (S1) and extrasystolic stimulation (S2) indicated below each trace) under the following conditions: (A) No atrial tachycardia (AT) and atrial fibrillation (AF) after nifedipine ($0.5 \mu\text{M}$). (B) No atrial tachycardia (AT) and atrial fibrillation (AF) after 1 mM caffeine pre-treatment. (C) A brief AT after CPA ($0.15 \mu\text{M}$) pre-treatment. In the individual traces, ventricular deflections are marked 'V' and atrial deflections are marked 'A'.

compared with WT litters. There were also signs of AF on examination using limb-lead electrocardiography. The hearts also showed accompanying fibrotic and anatomical alterations. However, the precise causal relationships between these observations were not tested in detail (Hong *et al.* 2002).

However, there have been relatively few reports on factors bearing on the *acute initiation* of AF in otherwise structurally normal hearts despite its accepted clinical importance (see introduction for references).

Previous studies had examined the relationship between altered Ca^{2+} homeostasis at the cellular level (Venetucci *et al.* 2007), and acute *ventricular* arrhythmogenic tendency in intact hearts (Balasubramaniam *et al.* 2004, 2005, Goddard *et al.* 2008). At the cellular level, the specific LTCC modulator FPL applied at a concentration of $1.0 \mu\text{M}$ increases the amplitude of both Ca^{2+} currents and Ca^{2+} transients more than it increased the rate of rise of Ca^{2+} transients in rat ventricular myocytes. This would potentially increase the release

of intracellularly stored SR Ca^{2+} through enhancing CICR (Fan & Palade 2002). Correspondingly, FPL produced a Ca^{2+} -mediated ventricular arrhythmogenicity at the level of intact Langendorff-perfused murine hearts. However, these relationships could potentially differ for atrial arrhythmogenesis. Atrial myocytes show important differences from ventricular myocytes particularly in their tubular and SR membrane systems that might reflect functional differences in their Ca^{2+} homeostatic processes. They do not possess extensive T-tubular systems (Mackenzie *et al.* 2001, 2004) but instead have prominent transversely oriented SR, Z-tubular, elements. Instead, atrial cells show an abundant corbular SR containing non-junctional RyR2s (Jorgensen *et al.* 1993). Junctional RyR2- $I_{\text{Ca,L}}$ clusters are confined to the cell peripheries (Mackenzie *et al.* 2001). This may reflect atrial activation normally involving CICR in a pattern of centripetal propagation into the cell interior from superficial T-SR junctions (Mackenzie *et al.* 2001, 2004, Bootman *et al.* 2006).

The present experiments explored for both direct and indirect effects of an enhanced entry of extracellular Ca^{2+} on both Ca^{2+} homeostasis at both the cellular level and possible arrhythmogenic consequences at the level of whole hearts. They used the following pharmacological agents known to modify Ca^{2+} homeostasis: (1) FPL may modify LTCCs by prolonging channel opening during depolarization and channel closing upon repolarization enhancing the duration of the consequent tail currents (Rampe & Lacerda 1991) with possible actions upon SR Ca^{2+} release (Katoh *et al.* 2000, Copello *et al.* 2007). In rat ventricular myocytes, it increases both Ca^{2+} currents and Ca^{2+} transients more than it increases the rate of rise of Ca^{2+} transients, potentially increasing CICR. (2) Nifedipine is a known competitive dihydropyridine-LTCC blocker in ventricular cells with a K_{D} of 40 nM (Shen *et al.* 2000, Balasubramaniam *et al.* 2004, Thomas *et al.* 2007). (3) Caffeine increases the release of intracellularly stored Ca^{2+} either by sensitizing RyR2s to cytosolic Ca^{2+} or inhibiting phosphodiesterase activity, thereby increasing cellular cAMP (Daly 2007), consequently increasing their open probabilities (Traford *et al.* 2000). This would produce a gradual depletion of SR Ca^{2+} as reported previously for ventricular cells (Gaburjakova & Gaburjakova 2006, Venetucci *et al.* 2007). (4) CPA acts through inhibition of SERCA activity. At 100–200 nM concentrations it produces a ~50% reduction of such activity (Schwinger *et al.* 1997) whilst sparing Ca^{2+} sensitivity in the contractile myofilaments (Takahashi *et al.* 1995), Ca^{2+} currents (Bonnet *et al.* 1994), and Na^{+} - Ca^{2+} exchange activity (Goeger *et al.* 1988, Yard *et al.* 1994).

The experiments demonstrated that the addition of FPL disrupted the regular pattern of Ca^{2+} transients observed in regularly stimulated isolated Fluo-3-loaded

atrial myocytes studied under confocal microscopy. These findings correlated with an appearance of atrial arrhythmogenesis at the level of intact isolated hearts. Such effects took place in atria at lower FPL concentrations than were needed to produce the corresponding ventricular effects. These changes occurred despite an absence of alterations in AERP.

These pro-arrhythmic actions of FPL whether at the cellular or the whole-heart level were dependent not only upon entry of extracellular Ca^{2+} but also on availability of releasable SR store Ca^{2+} . Thus, nifedipine pre-treatment abolished the changes in Ca^{2+} homeostasis produced by a subsequent introduction of FPL at the level of single cells, on the one hand, and reduced FPL-induced arrhythmogenesis in whole hearts on the other. This directly correlates with the known clinical actions of Ca^{2+} channel antagonists in preventing acute post-operative AF (Podesser *et al.* 1995, Yilmaz *et al.* 1996, Kim *et al.* 2002, Dobrilovic *et al.* 2005, Baker & White 2007, Iwamoto & Inoue 2007). It is consistent with the potential arrhythmogenic effects of extracellular Ca^{2+} entry. Pre-treatments with CPA or caffeine would both be expected to deplete SR Ca^{2+} either through an inhibition of Ca^{2+} -ATPase activity or an increase in RyR2-mediated release of intracellularly stored Ca^{2+} . These manoeuvres similarly blocked FPL-mediated alterations in Ca^{2+} signalling at the level of isolated atrial myocytes as well as its arrhythmogenic effects in intact hearts.

These findings together are consistent with a model for the acute atrial arrhythmogenesis in an otherwise normal heart to a process of CICR, dependent upon both Ca entry and SR Ca^{2+} . These would act within a model for an acute atrial arrhythmogenesis in an otherwise normal heart that involves triggered activity. This thus only resulted in transient rather than sustained arrhythmogenic episodes in an absence of alterations in the refractory period. The arrhythmogenic phenomena parallel the diastolic Ca^{2+} events produced by FPL observed at the cellular level. Furthermore, they were rescued by pharmacological manoeuvres demonstrated to reduce either SR Ca^{2+} or extracellular Ca^{2+} entry at the cellular level. These manoeuvres correspondingly rescued the diastolic Ca^{2+} events at the cellular level brought about by FPL.

Conflict of interest

We report no conflict of interest.

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