Increased $InsP_3Rs$ in the junctional sarcoplasmic reticulum augment Ca^{2+} transients and arrhythmias associated with cardiac hypertrophy

Dagmar Harzheim[a], Mehregan Movassagh[b], Roger S.-Y. Foo[b], Oliver Ritter[c], Aslam Tashfeen[d], Stuart J. Conway[e], Martin D. Bootman[a,1], and H. Llewelyn Roderick[a,f,1,2]

[a]Department of Molecular Signaling, Babraham Institute, Cambridge, CB22 3AT, United Kingdom; [b]Addenbrooke's Centre for Clinical Investigation, Cambridge, CB2 0QQ, United Kingdom; [c]Department of Medicine I, University of Wuerzburg, 97080 Wuerzburg, Germany; [d]Department of Chemistry, University of St. Andrews, St. Andrews, KY16 9ST, United Kingdom; [e]Department of Chemistry, University of Oxford, Oxford, OX1 3TA, United Kingdom; and [f]Department of Pharmacology, University of Cambridge, Cambridge CB2 1PD, United Kingdom

Communicated by Michael J. Berridge, The Babraham Institute, Cambridge, United Kingdom, May 19, 2009 (received for review April 9, 2009)

Cardiac hypertrophy is a growth response of the heart to increased hemodynamic demand or damage. Accompanying this heart enlargement is a remodeling of Ca^{2+} signaling. Due to its fundamental role in controlling cardiomyocyte contraction during every heartbeat, modifications in Ca^{2+} fluxes significantly impact on cardiac output and facilitate the development of arrhythmias. Using cardiomyocytes from spontaneously hypertensive rats (SHRs), we demonstrate that an increase in Ca^{2+} release through inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) contributes to the larger excitation contraction coupling (ECC)-mediated Ca^{2+} transients characteristic of hypertrophic myocytes and underlies the more potent enhancement of ECC-mediated Ca^{2+} transients and contraction elicited by $InsP_3$ or endothelin-1 (ET-1). Responsible for this is an increase in $InsP_3R$ expression in the junctional sarcoplasmic reticulum. Due to their close proximity to ryanodine receptors (RyRs) in this region, enhanced Ca^{2+} release through $InsP_3Rs$ served to sensitize RyRs, thereby increasing diastolic Ca^{2+} levels, the incidence of extra-systolic Ca^{2+} transients, and the induction of ECC-mediated Ca^{2+} elevations. Unlike the increase in $InsP_3R$ expression and Ca^{2+} transient amplitude in the cytosol, $InsP_3R$ expression and ECC-mediated Ca^{2+} transients in the nucleus were not altered during hypertrophy. Elevated $InsP_3R2$ expression was also detected in hearts from human patients with heart failure after ischemic dilated cardiomyopathy, as well as in aortic-banded hypertrophic mouse hearts. Our data establish that increased $InsP_3R$ expression is a general mechanism that underlies remodeling of Ca^{2+} signaling during heart disease, and in particular, in triggering ventricular arrhythmia during hypertrophy.

calcium | ECC | IP3 | SHR | signalling

In response to increased hemodynamic requirements or damage the heart undergoes a hypertrophic growth response. Hypertrophy is induced by physiological stimuli, such as exercise or pregnancy and by pathological conditions such as hypertension and ischemic heart disease. Although hypertrophy can initially be an adaptive compensatory response, chronically it may become decompensated. As a result, cardiac function is decreased and the heart exhibits an increased propensity for arrhythmias that together ultimately lead to heart failure and death (1).

Ca^{2+} is a fundamental regulator of cardiac function causing myocyte contraction via excitation-contraction coupling (ECC) (2), and stimulating the gene transcription that underlies hypertrophy (3). Accompanying cardiac hypertrophy and failure is a remodeling of Ca^{2+} signaling (4). Whilst enhanced Ca^{2+} transients facilitate greater myocyte contraction during adaptive hypertrophy, Ca^{2+} fluxes are diminished during heart failure and thereby contribute to decreased cardiac output (5). Remodeling of the Ca^{2+} signaling proteome also underlies the increased arrhythmias associated with hypertrophy and heart failure (6).

In addition to the RyRs that mediate ECC-dependent Ca^{2+} fluxes, cardiomyocytes also express $InsP_3Rs$, albeit outnumbered by RyRs at approximately 50:1 (7). Mammals have 3 $InsP_3R$ isoforms (types 1–3) (8), with $InsP_3R2$ being the main isoform in cardiomyocytes (9, 10). Although Ca^{2+} flux via these $InsP_3Rs$ is relatively small in comparison to the large Ca^{2+} transients occurring during every heartbeat, recent data suggests that $InsP_3Rs$ have an important role in cardiac physiology. We, and others have shown, that Ca^{2+} release through $InsP_3Rs$ contributes to the inotropic, arrhythmogenic, and hypertrophic effect of $G\alpha_q$-coupled agonists such as the vasoactive peptide ET-1 (11–16). Whether altered $InsP_3R$ signaling also contributes to remodeling of Ca^{2+} homeostasis during cardiac hypertrophy is not yet determined. An increase in $InsP_3R$ expression has however been reported during heart failure in humans (17). Moreover, $InsP_3$-induced Ca^{2+} release (IICR) is increased in SR microsomes prepared from hypertrophic myocytes (18).

Here, we hypothesized that enhanced Ca^{2+} release via $InsP_3Rs$ contributes to remodeling of ECC-mediated Ca^{2+} transients, and to the increased arrhythmogenic Ca^{2+} signals observed in ventricular cardiomyocytes during compensated hypertrophy. To test these hypotheses, in a model that reflects the slow development of hypertrophy in humans, Ca^{2+} fluxes and contractility were investigated in hypertrophic ventricular myocytes isolated form SHRs (19). We found that the increase in amplitude of ECC-mediated Ca^{2+} transients and propensity for extra-systolic spontaneous Ca^{2+} signals, characteristic of hypertrophic myocytes, was caused by augmented $InsP_3R$ signaling. This profound effect of enhanced $InsP_3R$ activity in hypertrophic myocytes was due to an increase in $InsP_3R$ expression, specifically in the junctional SR membrane in close proximity to RyRs. At this location, Ca^{2+} release via $InsP_3Rs$ acted to sensitize RyRs, thereby enhancing Ca^{2+} release during ECC and inducing spontaneous elementary Ca^{2+}-release events and extra-systolic Ca^{2+} transients. $InsP_3R2$ expression was also increased in hypertrophic cardiomyocytes isolated from aortically banded mice and in human hearts displaying ischemic dilated cardiomyopathy. We propose that $InsP_3Rs$ play a fundamental role in the physiology of hypertrophic hearts contributing to remodeled cardiac function and triggering ventricular arrhythmia.

Results

SHR Cardiomyocytes Develop Hypertrophy. As previously described, at 6 months, cardiomyocytes from SHRs are hypertrophic (20). Cardiomyocyte width was increased in SHRs compared to WKY

Author contributions: D.H., M.D.B., and H.L.R. designed research; D.H. and H.L.R. performed research; M.M., R.S.-Y.F., O.R., A.T., S.J.C., and H.L.R. contributed new reagents/analytic tools; D.H. and H.L.R. analyzed data; and D.H., M.D.B., and H.L.R. wrote the paper.

The authors declare no conflict of interest.

[1]M.D.B. and H.L.R. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: llewelyn.roderick@bbsrc.ac.uk.

This article contains supporting information online at www.pnas.org/cgi/content/full/0905485106/DCSupplemental.

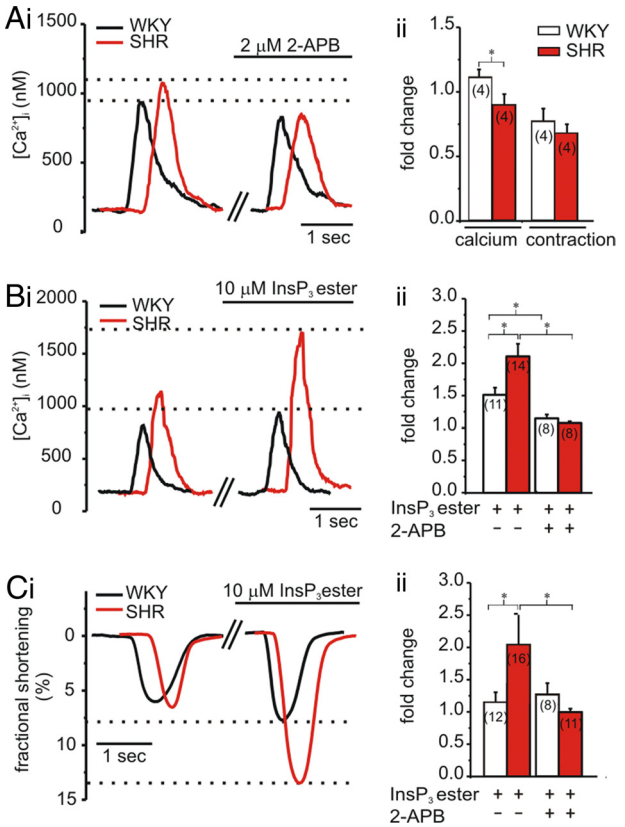

Fig. 1. Systolic Ca^{2+} transient amplitude and cellular contraction recorded from indo-1 AM-loaded ventricular myocytes isolated from 6-month-old WKY rats and SHRs. Data presented were obtained 20 min after application of 2-APB or $InsP_3$ ester. Representative traces are shown in *i*; values normalized to pre-application are shown in *ii*. For better comparison, single traces have been time-shifted. (*A*) Effect of 2 μM 2-APB on Ca^{2+} transient amplitude and fractional shortening. (*B*) Effect of 10 μM $InsP_3$ ester ± 2 μM 2-APB on Ca^{2+} transient amplitude. (*C*) As in *B* on fractional shortening. N numbers are indicated. *, $P < 0.05$; Student's *t* test.

controls (Table S1) resulting in a decrease in the cellular length/width ratio (WKY: 3.41 ± 0.11 vs. SHR: 2.71 ± 0.12; $P < 0.001$). Messenger RNA levels of the hypertrophic marker atrial natriuretic factor (ANF) (21) were also greater in SHR cardiomyocytes at the age of 6 months than in WKY controls (12.68 ± 4.04 vs. 0.69 ± 0.32, $P < 0.05$). In 12-week-old animals, cell size and ANF mRNA levels were not different between the 2 strains (Table S1).

Ca^{2+} Release via $InsP_3Rs$ Is Increased During Hypertrophy and Remodels ECC. Consistent with previous observations (20), the amplitude of electrically evoked systolic Ca^{2+} transients was greater in cardiomyocytes from 6-month-old SHRs than in WKY controls (SHR: 0.70 ± 0.07 mM, WKY: 0.50 ± 0.06 mM; $n = 48$ and 42, respectively, $P < 0.05$). Fractional shortening of myocytes under basal conditions was however not significantly different between the 2 strains (WKY: 10.35 ± 1.50%, SHR: 7.59 ± 0.98%).

To reveal whether increased Ca^{2+} release via $InsP_3Rs$ contributes to remodeling of Ca^{2+} signaling and myocyte function during hypertrophy, we measured global Ca^{2+} transients and cellular contraction under conditions where $InsP_3Rs$ were either inhibited or activated. As Ca^{2+} transients are greater under basal conditions in hypertrophic SHR than in WKY myocytes, the systolic Ca^{2+} amplitude was normalized to that before treatment. Inhibition of $InsP_3Rs$ with 2-APB (2 μM) (11) caused a greater reduction in ECC-mediated Ca^{2+} transient amplitude in SHR myocytes compared with WKY controls (Fig. 1*A*). Concurrently, 2-APB also decreased the magnitude of contraction in SHRs (Fig. 1*Aii*). These data suggested that Ca^{2+} release via $InsP_3Rs$ contributes to the greater basal ECC-associated Ca^{2+} transients observed in SHR myocytes.

Direct activation of $InsP_3Rs$ with $InsP_3$ ester (11) promoted a greater increase in Ca^{2+}transient amplitude and inotropy in SHR compared to WKY myocytes (Fig. 1 *B* and *C*), which was abrogated in both strains by 2-APB (Fig. 1 *Bii* and *Cii*). No difference in ECC-mediated Ca^{2+} transient amplitude or cellular contraction was observed between myocytes isolated from 12-week-old WKY rats or SHRs (Fig. S1*A*).

$InsP_3R2$ Expression Is Increased in Hypertrophic Cardiomyocytes. Next, we analyzed whether an increase in $InsP_3R$ expression underlies altered $InsP_3$ signaling during hypertrophy. At 6 months, $InsP_3R2$ mRNA and protein levels were higher in SHR than in WKY myocytes, whereas at 12 weeks, $InsP_3R2$ mRNA and protein levels were lower in SHRs compared to WKY controls (Fig. 2*A* and *B*). RyR2 protein levels were not different between the 2 strains at the age of 12 weeks or 6 months (Fig. 2*B*).

Immunofluorescent labeling revealed that in WKY myocytes, $InsP_3R2$ was predominantly expressed in the perinuclear regions with weaker staining along the SR membrane, where RyRs are localized (Fig. 2*C*). $InsP_3R2$ was also expressed in the perinuclear regions of SHR cardiomyocytes, but compared to WKY cells its expression was significantly greater along the RyR2-stained striations outside the nuclear region. Thus, the ratio of cytosolic/nuclear $InsP_3R2$ immunofluorescence was increased (Fig. 2*D*). No difference in RyR2 immunostaining between the 2 strains was observed (Fig. 2*C*). In both WKY and SHR myocytes, $InsP_3R2$ co-localized with RyR2s (intensity profile along indicated line, Fig. 2 *C* and *F*) and Pearson's coefficient (Fig. 2*E*), indicating that like RyR2s, $InsP_3R2$s are located at dyadic junctions alongside T-tubule membranes (Fig. 2*C*). The co-localization of these 2 channels was markedly increased in hypertrophic myocytes (Fig. 2 *E* and *F*). We concluded that, as a result of hypertrophic remodeling, the number of $InsP_3R2$s located in the junctional SR membrane is increased, thereby mediating their greater co-localization with RyR2s.

$InsP_3R2$ expression was also increased in hearts from mice after aortic banding (Fig. 2*G*) and in human patients with ischemic dilated cardiomyopathy (Fig. 2*H*). These data suggested that increased $InsP_3R2$ expression is a general hallmark of hypertrophy.

Increased $InsP_3R$ Expression in the Junctional SR Causes a Spatially Restricted Remodeling of ECC-mediated Ca^{2+} Transients during Hypertrophy. To establish how increased $InsP_3R$ expression in the junctional SR membrane impacted on ECC-mediated Ca^{2+} signals, we performed confocal Ca^{2+} imaging experiments. In addition to directly stimulating $InsP_3Rs$ with a membrane-permeant $InsP_3$ ester, we tested the effect of physiologically activating $InsP_3Rs$ with $InsP_3$ generated following ET-1 stimulation (11, 22). In SHR myocytes, stimulation with ET-1 and $InsP_3$ ester increased the amplitude of nuclear and cytosolic Ca^{2+} transients during electrical pacing (Fig. 3 *A–E*). Contrastingly, in WKY myocytes, only nuclear systolic Ca^{2+} transients were augmented (Fig. 3 *C* and *E*). The enhancement of systolic Ca^{2+} transients by ET-1 and $InsP_3$ ester was sensitive to 2-APB, further indicating that this effect was mediated by $InsP_3Rs$ (Fig. 3 *Bii–Eii*). The increase in nuclear Ca^{2+} transient amplitude in SHRs was comparable to that observed in WKY myocytes (Fig. 3 *C* and *E*). To accommodate for variation between cells in the absolute magnitude of Ca^{2+} changes, the ratio of nuclear to cytosolic Ca^{2+} transient amplitude was calculated. This ratio was increased in WKY myocytes following ET-1 or $InsP_3$ ester stimulation whereas no change was observed in SHR myocytes (Fig. S2*A*). The difference in ratio between the 2 strains is explained by restriction of the ET-1- and $InsP_3$ ester-stimulated increase in Ca^{2+} transient amplitude to the nuclear compartment in WKY myocytes, whereas, in SHR myocytes nuclear and cytosolic Ca^{2+} transient amplitude were both increased. These data indicate

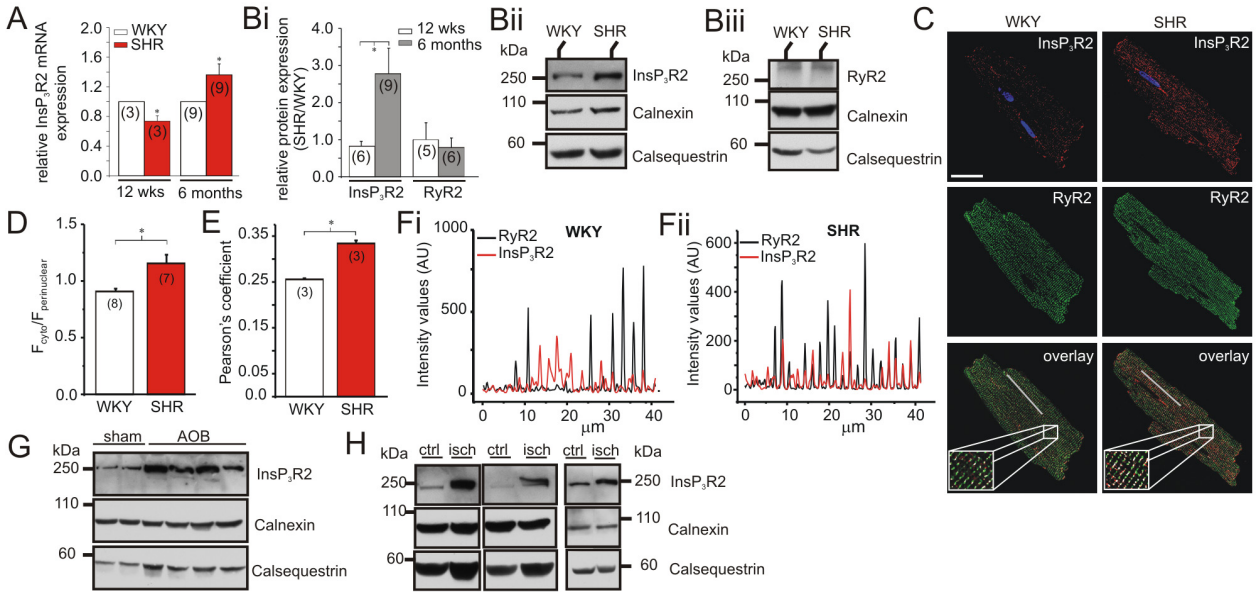

Fig. 2. Expression of $InsP_3R2$ during hypertrophy. (*A*) Relative $InsP_3R2$ mRNA levels. Values for SHRs have been normalized to age-matched WKY rats. (*Bi*) Relative $InsP_3R2$ and RyR2 protein levels. SHR/WKY ratios have been determined for 12-week- and 6-month-old rats. Representative immunoblots for $InsP_3R2$ (*ii*) and RyR2 (*iii*) are shown. Seventy-five micrograms membrane proteins from ventricular cardiomyocytes were loaded per lane. (*C*) Immunofluorescent staining for $InsP_3R2$ and RyR2. DAPI was used to stain the nuclei. (Scale bar, 30 μm.) Pixels positive for $InsP_3R2$ and RyR2 are shown in white. (*D*) Ratio of cytosolic/perinuclear fluorescence intensity in a 3-μm ring in these 2 regions. (*E*) Pearson's coefficient for co-localization of RyR2 and $InsP_3R2$. (*F*) Profiles of RyR2 and $InsP_3R2$ fluorescence intensity sampled along the longitudinal axis of a WKY (*i*) or SHR (*ii*) myocyte, as depicted by the white lines on the overlay images in *C*. (*G*) Representative immunoblots detecting $InsP_3R2$ in hearts from mice that have undergone aortic banding (AOB) and control mice. (*H*) As in *G* in human disease hearts from patients showing ischemic cardiomyopathy (isch) as well as control hearts (ctrl). Forty micrograms membrane proteins from left ventricles were loaded per lane. Calnexin and calsequestrin were used as loading controls. N numbers are indicated. *, $P < 0.05$; Student's *t* test.

that in non-hypertrophied myocytes, Ca^{2+} release via $InsP_3Rs$ impacts more profoundly on nuclear Ca^{2+} transients, whereas in hypertrophic myocytes, increased junctional $InsP_3R$ expression specifically augments the cytosolic Ca^{2+} transients. There was no difference in the ratio of nuclear to cytoplasmic Ca^{2+} transients between 12-week-old WKY and SHRs under basal conditions, or during ET-1 or $InsP_3$ ester stimulation (Fig. S1*B*).

Maximal Ca^{2+} release from nuclear and cytosolic Ca^{2+} stores induced by 10 mM caffeine (RyR agonist) was not significantly different between WKY and SHR myocytes (Fig. S2*B*), indicating that differences in Ca^{2+} store content do not underlie the changes in ECC-associated Ca^{2+} transients during ET-1 and $InsP_3$ ester stimulation.

Extra-Systolic $InsP_3$-dependent Ca^{2+}-release Events Are Increased in SHR Myocytes. In atrial cardiomyocytes, which express approximately 6 fold more $InsP_3Rs$ than ventricular myocytes, Ca^{2+} release via $InsP_3Rs$ underlies the induction of extra-systolic Ca^{2+} transients (11, 12). We therefore investigated whether the increased $InsP_3R$ expression and activity observed in SHR ventricular myocytes caused them to exhibit more extra-systolic Ca^{2+}-release events. Extra-systolic events were determined as rises in Ca^{2+} concentration that were temporally distinguished from signals induced by field stimulation and that also impacted on contraction (see arrows Fig. 4*A*). Stimulation with ET-1 or $InsP_3$ ester caused a 2-APB-sensitive increase in the number of extra-systolic Ca^{2+} transients in both SHR and WKY myocytes (Fig. 4*B* and Table S2). However, the number of cells that exhibited extra-systolic Ca^{2+} transients and the frequency of events per cell were greater in SHRs than WKYs (ET-1: WKY: 26% vs. SHR: 50%; $InsP_3$ ester: WKY: 29% vs. SHR: 57%, Table S2 and Fig. 4*B*). In both strains, extra-systolic Ca^{2+}-release events began to occur within a few minutes of $InsP_3$ ester or ET-1 stimulation and increased throughout the time-course of the experiment (Fig. 4*B*). The rate at which the frequency of the extra-systolic Ca^{2+} transients increased following $InsP_3$ ester or ET-1 stimulation was greater for SHR myocytes than WKY myocytes. After 1,000 s, the incidence of extra-systolic Ca^{2+} transients was significantly higher in SHRs than WKY cells (Fig. S3). No difference in the frequency of extra-systolic Ca^{2+} transients was observed between the 2 strains at 12 weeks (Fig. S1 *C* and *D*). These data indicate that activation of $InsP_3Rs$ was responsible for the initiation of the extra-systolic Ca^{2+} transients and provides an explanation for the increased frequency of extra-systolic Ca^{2+} transients during hypertrophy.

Enhanced Ca^{2+} Release via $InsP_3Rs$ Increases the Rate of Rise of Systolic Ca^{2+} Transients and Elevates Diastolic [Ca^{2+}] in SHRs. A hypertrophy-associated increase in $InsP_3R$-mediated Ca^{2+} flux via junctional $InsP_3Rs$ acting to induce Ca^{2+} release via neighboring RyRs could provide a mechanism to accentuate Ca^{2+} signaling during ECC. To test this hypothesis, the effect of ET-1 and $InsP_3$ ester on the rate of rise of pacing-evoked systolic Ca^{2+} transients was measured. During both ET-1 and $InsP_3$ ester stimulation, the rate of rise of the Ca^{2+} transient was faster in hypertrophic SHR than in WKY cells (Fig. 5*A*). The effects of ET-1 and $InsP_3$ ester were abrogated by adenoviral-mediated expression of a cherry fluorescent protein-tagged $InsP_3$ 5′-phosphatase, which disrupts $InsP_3$ signaling (5′P; Fig. 5*B*) (16). There was no difference in the rate of rise of the systolic Ca^{2+} transient in myocytes from 12-week-old WKY and SHRs (Fig. S1*E*).

As RyR opening is controlled by $[Ca^{2+}]_i$, we next tested whether Ca^{2+} release via $InsP_3Rs$ modulated the efficiency of Ca^{2+}-induced Ca^{2+} release (CICR) by changing diastolic [Ca^{2+}] levels. Under basal conditions, diastolic Ca^{2+} levels were not different between strains (WKY: 103.39 ± 8.86 nM vs. SHR: 95.74 ± 14.21 nM). Following stimulation with ET-1 or $InsP_3$ ester, diastolic [Ca^{2+}] was increased in SHR myocytes (ET-1: 96.1 ± 5.6 nM to 132.6 ± 15.2 nM, $InsP_3$ ester: 69.5 ± 19.9 nM to 184.5 ± 36.7 nM, Fig. 5*C*), whereas no change was seen in WKY cells (Fig. 5*C*). 2-APB or 5′P expression abrogated the increase in diastolic [Ca^{2+}] caused by

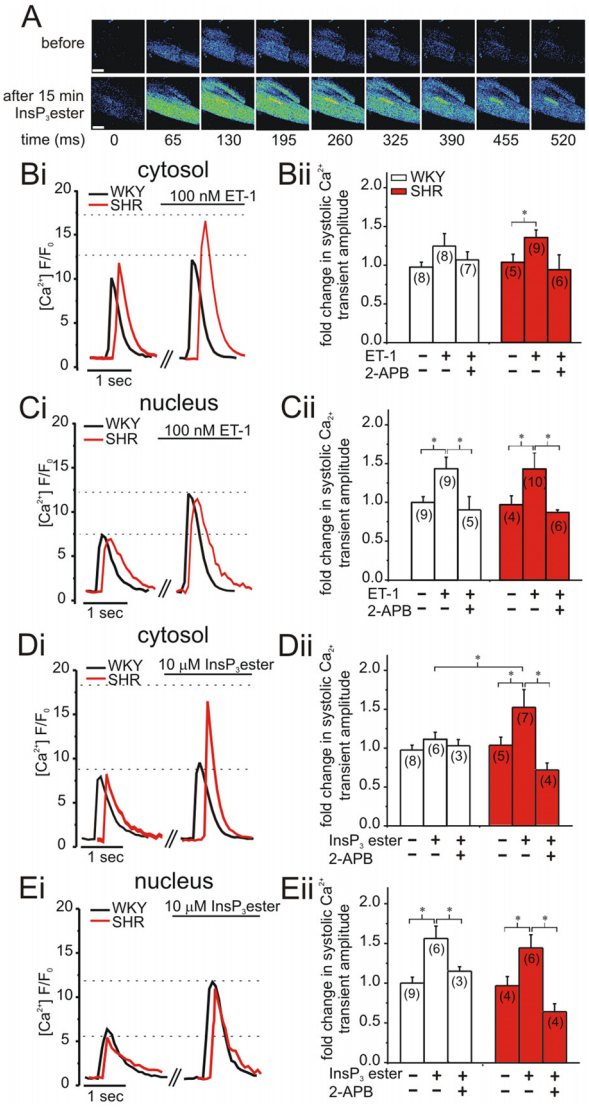

Fig. 3. Confocal analysis of systolic nuclear and cytosolic Ca^{2+} transient amplitude in fluo-4 AM-loaded ventricular myocytes. Data presented were determined 15 min after application of ET-1; $InsP_3$ ester or 2-APB. Representative traces are shown in *i*; values normalized to pre application are shown in (*ii*). For better comparison, single traces have been slightly time-shifted. (*A*) Representative SHR myocyte displaying Ca^{2+} transients at a series of time points before and after application of 10 μM $InsP_3$ester. (Scale bar, 10 μm.) (*B*) Effect of 100 nM ET-1 $\pm$ 2 μM 2-APB on cytosolic peak amplitude. (*C*) As in *B* for nuclear peak amplitude. (*D*) Effect of 10 μM $InsP_3$ ester $\pm$ 2 μM 2-APB on cytosolic peak amplitude. (*E*) as in *D* for nuclear peak amplitude. N numbers are indicated. $*$, $P < 0.05$; Student's *t* test.

$InsP_3$ ester or ET-1 in SHRs (Fig. 5 *C* and *D*) without effecting diastolic $[Ca^{2+}]$ under basal conditions. At 12 weeks of age, there was no difference in diastolic $[Ca^{2+}]$ during stimulation of SHR myocytes with ET-1 or $InsP_3$ ester (Fig. S1*F*).

Frequency of Elementary $InsP_3$-dependent Ca^{2+}-release Events Is Increased during Hypertrophy. To further resolve the consequences of increased $InsP_3R$ expression for Ca^{2+} signaling, elementary Ca^{2+}-release events were analyzed. Under normal paced conditions, Ca^{2+} events during the diastolic period were of greater amplitude in the hypertrophic SHR myocytes than in WKY cells (WKY: $\Delta F/F_0 = 0.26 \pm 0.01$ vs. SHR: 0.34 ± 0.04, Table S3 and Fig. 5*E*). Under conditions where RyRs were blocked with 1 mM tetracaine, $InsP_3$ ester application stimulated elementary Ca^{2+}-release events (Fig. 5*E*) that occurred at a greater frequency in

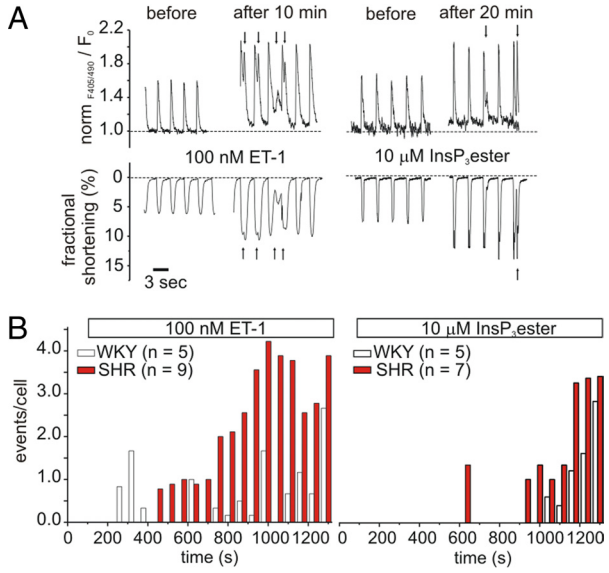

Fig. 4. Analysis of extra-systolic Ca^{2+} release events in indo-1 AM-loaded ventricular myocytes during hypertrophy. (*A*) Representative traces for global Ca^{2+} transients and cellular contraction recorded from SHR myocytes before and after stimulation with 100 nM ET-1 or 10 μM IP_3 ester. Arrows indicate extra-systolic events. (*B*) Extra-systolic Ca^{2+} release events per cell during stimulation with 100 nM ET-1 or 10 μM $InsP_3$ ester.

hypertrophic myocytes (WKY: 2.12 ± 0.46 vs. SHR: 6.84 ± 0.65, Table S3). These data suggest that $InsP_3R$-mediated Ca^{2+} signals contribute to the greater amplitude of diastolic Ca^{2+} events observed in SHR myocytes and may underlie the elevated diastolic $[Ca^{2+}]$ observed in SHR myocytes stimulated with $InsP_3$ or ET-1.

Our data suggest a model to explain the enhanced ECC-mediated Ca^{2+} signals and increased extra-systolic Ca^{2+}-release events observed during hypertrophy (Fig. 5*F*). Key to this model is a hypertrophy-associated increase in $InsP_3R$ expression in the dyadic region. Thus, more $InsP_3Rs$ are in close proximity to RyRs in the SR membrane (Fig. 2 *E* and *F*). Ca^{2+} released via these $InsP_3Rs$ sensitizes their adjacent RyRs, bringing them closer to threshold for activation. Under conditions of increased $[InsP_3]$, elementary $InsP_3$-dependent Ca^{2+}-release events are increased in frequency and diastolic $[Ca^{2+}]$ is elevated. Consequently, RyRs are triggered to generate extra-systolic Ca^{2+} signals and to accelerate the rate of rise of pacing-evoked Ca^{2+} transients (Fig. 5*F*).

Discussion

Here we demonstrate that enhanced Ca^{2+} signaling via $InsP_3Rs$ located in the dyadic cleft remodels Ca^{2+} signaling during hypertrophy.

In agreement with previous data, we found that the amplitude of ECC-mediated Ca^{2+} transients under basal conditions was significantly greater as a result of hypertrophy in SHR myocytes (in the absence of any other stimulation) (20). Significantly, we determined that this increased amplitude of basal ECC-mediated Ca^{2+} transients was due to augmented Ca^{2+} release via $InsP_3Rs$. These data demonstrated that $InsP_3Rs$ could contribute to ECC-mediated Ca^{2+} fluxes without additional neurohormonal input, thereby modifying myocyte Ca^{2+} signaling.

A greater role for $InsP_3Rs$ in regulating ECC-mediated Ca^{2+} transients during hypertrophy was revealed following their direct activation with cell-permeant $InsP_3$ ester. These data showed that increased activation of $InsP_3Rs$ could augment the amplitude of ECC-mediated Ca^{2+} transients mediated via RyRs even further. Consistent with previous reports (20, 23, 24), no increase in SR releasable Ca^{2+} was observed in hypertrophic SHR myocytes,

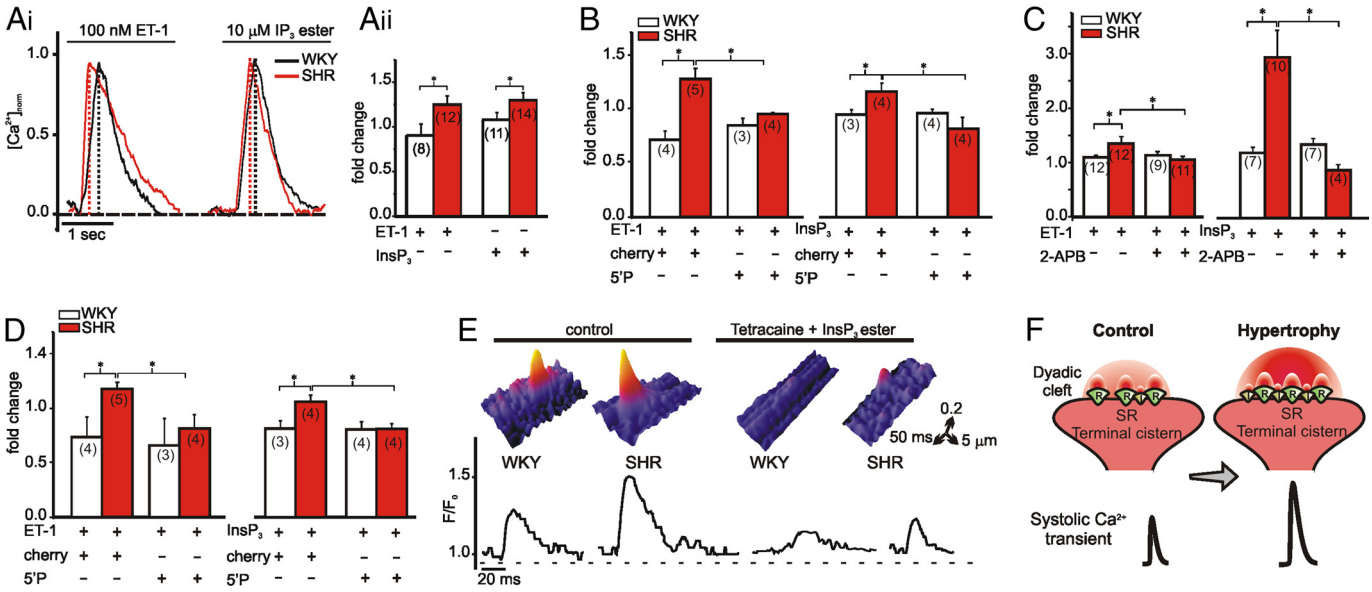

Fig. 5. Analysis of global Ca^{2+} transient kinetics, diastolic [Ca^{2+}], and elementary Ca^{2+} release during hypertrophy. (*A*) Rate of rise of Ca^{2+} transient (peak amplitude/time to peak) after 20 min stimulation with 100 nM ET-1 or 10 μM $InsP_3$ ester ($InsP_3$). Representative traces are shown in *i*; values normalized to pre application are shown in *ii*. Cells have been loaded with indo-1 a.m. and electrically paced at 0.3 Hz. (*B*) As in *Aii* for ventricular myocytes infected with control cherry virus or $InsP_3$ 5′-phosphatase (5′P) virus. (*C*) Changes in diastolic [Ca^{2+}] during application of 100 nM ET-1 ± 2 μM 2-APB or 10 μM $InsP_3$ ester ± 2 μM 2-APB, normalized to before application. (*D*) As in *C* for ventricular myocytes that have been infected with control cherry virus or 5′P virus. (*E*) Surface plot of representative elementary Ca^{2+} release events during normal pacing (control) and during pacing in the presence of 1 mM tetracaine + 10 μM $InsP_3$ ester. F/F_0 traces are shown below. (*F*) Schematic indicating how $InsP_3R$-mediated Ca^{2+} release augments systolic Ca^{2+} transients during hypertrophy. (*I*) $InsP_3R2$; R, RyR2. N numbers are indicated: ∗, $P < 0.05$; Student's *t* test.

thereby indicating that the enhancement of ECC-associated Ca^{2+} flux by $InsP_3Rs$ was not due to an increase in store loading. $InsP_3R2$ expression was elevated as a result of hypertrophy thereby providing a mechanism for increased Ca^{2+} release via $InsP_3Rs$. $InsP_3R$ expression in the heart has previously been reported to be modified following disease. In particular, $InsP_3R$ expression is increased in atrial myocytes of humans and dogs during atrial fibrillation (AF) (25, 26). Furthermore, elevated $InsP_3R$ levels and increased $InsP_3$ binding was reported in the left ventricle during human heart failure (17). Consistent with these reports and our observations in rats, we found that $InsP_3R2$ expression was significantly elevated in cardiac tissue from aortically-banded hypertrophic mice and from human hearts showing ischemic dilated cardiomyopathy. Due to its very low expression and insensitivity to hypertrophy in rat cardiac fibroblasts (Fig. S4), we considered that the changes in $InsP_3R2$ expression detected in human and mouse cardiac tissue was due solely to $InsP_3R2$ in cardiac myocytes. Our findings in rats, mice, and humans therefore suggested that increased $InsP_3R$ expression is a general feature of cardiac disease, raising the possibility that increased Ca^{2+} release via $InsP_3Rs$ contributes to pathological changes in Ca^{2+} signaling.

The enhanced $InsP_3R2$ expression had a striking spatial aspect in that $InsP_3R$ expression was specifically increased in the junctional SR. Detailed analysis showed that these junctional $InsP_3Rs$ co-localized with RyRs, which reside primarily in the dyadic cleft. This profound remodeling in $InsP_3Rs$ expression and distribution had significant functional consequences. In particular, the increased number of dyadic $InsP_3Rs$ augmented the amplitude of the cytosolic ECC-mediated Ca^{2+} transients and enhanced the positive inotropic effect of $InsP_3$ ester. Similarly, cytosolic ECC-mediated Ca^{2+} transient amplitude and contraction were enhanced when $InsP_3Rs$ were engaged by $InsP_3$ generated following application of ET-1. Given that ET-1 is a potent pro-hypertrophic agonist, and its levels are elevated during heart failure, these findings have significant implications for cardiac function during hypertrophy (16, 22, 27). The activation of $InsP_3Rs$ in SHR myocytes by ET-1 is in agreement with data from our laboratory and elsewhere showing that stimulation of the $InsP_3$ signaling cascade in cardiomyocytes with ET-1 modifies Ca^{2+} fluxes and contractility (11, 13, 28). The increase in nuclear Ca^{2+} transient amplitude during ECC by ET-1 and $InsP_3$ ester was not altered during hypertrophy reflecting the lack of a change in $InsP_3R$ expression in this region. Together, these data suggested that Ca^{2+} release via $InsP_3Rs$ in the dyadic region primed ECC-mediated Ca^{2+}-induced Ca^{2+} release via RyRs (see Fig. 5*F*). Specifically, Ca^{2+} release via $InsP_3Rs$ could elevate diastolic [Ca^{2+}] closer to the threshold for activation of RyRs. Thus, we established that increased Ca^{2+} release via $InsP_3Rs$ in hypertrophic myocytes can significantly contribute to remodel ECC-mediated Ca^{2+} signals.

At the most fundamental level, in the absence of RyR activity, SHR myocytes exhibited an increased frequency of elementary $InsP_3$-dependent Ca^{2+}-release events. Interestingly, the amplitudes of those events were no different between WKY and SHR myocytes. This is not surprising given that Ca^{2+} puffs are fundamental Ca^{2+} signals that are conserved between cells as diverse as *Xenopus* oocytes and HeLa human epithelial cells (29). At the molecular level, Ca^{2+} puffs arise via the stochastic recruitment of neighboring $InsP_3Rs$ (a cluster) until a threshold number required for puff generation is reached (30). Thus, it is plausible that greater $InsP_3R$ expression in SHR myocytes simply increases the probability of recruiting this puff-generating threshold number of receptors without altering the properties of puffs. As a result, only the frequency of elementary events is increased in SHRs. The greater abundance of these elementary events may explain the elevated diastolic [Ca^{2+}] observed in SHR myocytes stimulated with $InsP_3$ ester and ET-1. These data are consistent with the requirement for $InsP_3Rs$ for the ET-1-stimulated increase in diastolic [Ca^{2+}] observed in atrial myocytes (which have ≈6-fold greater $InsP_3R$ expression than ventricular myocytes) (10, 12). As elementary Ca^{2+}-release events (Ca^{2+} sparks and puffs) are the building blocks of higher order Ca^{2+} transients, it was not surprising that SHR myocytes also exhibited an increased frequency of extra-systolic Ca^{2+} transients. Similarly, stimulation of atrial myocytes with $InsP_3$ or $InsP_3$-generating ago-

nists such as ET-1, potently induced arrhythmogenic Ca^{2+}-release events that were dependent on $InsP_3R2$ expression (11–13, 28).

By bringing Ca^{2+} levels closer to the threshold for activation of RyRs, $InsP_3$-mediated sensitization of RyRs also served to increase the rate of rise of ECC-mediated Ca^{2+} transients. This may remediate the deterioration in Ca^{2+} signaling that occurs as hypertrophy progresses to failure. In particular, extra Ca^{2+} release via dyadic $InsP_3Rs$ may compensate for the decreased coupling efficiency between L-type Ca^{2+} channels and RyRs due to a deterioration in the T-tubular network and increased width of the dyadic cleft that occurs during disease (31).

The arrhythmogenic effect of $InsP_3R$ activity in the ventricles may have profound consequences. Coupled with increased systemic levels of $InsP_3$-generating agonists, such as ET-1 during hypertension and heart failure, it provides a possible mechanistic explanation why hypertrophic hearts are more likely to develop potentially lethal ventricular arrhythmias (32).

As $InsP_3R2$ is increased during cardiac hypertrophy, yet is dispensable for the normal physiological function of the healthy heart (12), it may represent an ideal target to which pharmacological modulators could be developed to intervene in both the induction of the hypertrophic gene program and the generation of arrhythmias.

Materials and Methods

Detailed methods for myocyte isolation, adenoviral infection, photometric, and confocal measurements of $[Ca^{2+}]_i$, immunoblotting, immunofluorescence, quantitative RT-PCR, and cell length measurements are provided elsewhere (14, 33) and in *SI Methods* and Fig. S5.

Animal Models. Male SHRs and normotensive Wistar-Kyoto (WKY) rats were obtained from Harlan and were housed under control conditions with ad libitum food and water. All experiments were performed in accordance with the guidelines from the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986. Constriction of the transverse thoracic aorta was performed on 3-month-old male mice as described in *SI Methods*. The sham procedure was identical but without aortic ligation.

Patients. Left ventricular tissue samples of human failing hearts were from individuals undergoing heart transplantation due to end-stage heart failure. All samples were obtained from male caucasians, aged 41–62. Samples from nonfailing donor hearts were provided by the U.K. Human Tissue Bank. After cardiectomy, left ventricular samples were frozen in liquid nitrogen and stored at −80 °C. Detailed information about the patients can be found in *SI Methods*. All experiments involving human tissue samples have been approved by the Cambridgeshire Research Ethics Committee.

Recordings of Myocyte Contraction and $[Ca^{2+}]_i$. All experiments, unless otherwise stated, were performed at 22 °C on myocytes electrically paced with field electrodes at 0.33 Hz. This condition is referred to as the basal condition. Detailed procedures can be found in *SI Methods*.

Statistics. Data are expressed as mean ± SEM. Statistical comparisons were carried out with Student's *t* test or 2-way ANOVA. Statistically significance was accepted at $P < 0.05$.

ACKNOWLEDGMENTS. We thank V. Sorrentino (University of Sienna) for the RyR2 Ab, the BI antibody facility for generating the rat anti-$InsP_3R2$ antibodies, Anne Segonds-Pichon for statistical analysis, and Reinhard Seifert (Caesar, Bonn) for his help with the analysis of the data. D.H. also thanks Wolfson College for a Junior Research Fellowship. The work was supported by the British Heart Foundation (PG/06/034/20637). H.L.R. is a Royal Society Research Fellow.

1. Vakili BA, Okin PM, Devereux RB (2001) Prognostic implications of left ventricular hypertrophy. *Am Heart J* 141:334–341.
2. Bers DM (2002) Cardiac excitation-contraction coupling. *Nature* 415:198–205.
3. Heineke J, Molkentin JD (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 7:589–600.
4. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signaling: Dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4:517–529.
5. Gomez AM, et al. (1997) Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. *Science* 276:800–806.
6. Kahan T, Bergfeldt L (2005) Left ventricular hypertrophy in hypertension: Its arrhythmogenic potential. *Heart* 91:250–256.
7. Moschella MC, Marks AR (1993) Inositol 1,4,5-trisphosphate receptor expression in cardiac myocytes. *J Cell Biol* 120:1137–1146.
8. Sudhof TC, et al. (1991) Structure of a novel InsP3 receptor. *EMBO J* 10:3199–3206.
9. Perez PJ, Ramos-Franco J, Fill M, Mignery GA (1997) Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. *J Biol Chem* 272:23961–23969.
10. Lipp P, et al. (2000) Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. *Curr Biol* 10:939–943.
11. Mackenzie L, et al. (2002) The role of inositol 1,4,5-trisphosphate receptors in Ca2+ signalling and the generation of arrhythmias in rat atrial myocytes. *J Physiol* 541:395–409.
12. Li X, Zima AV, Sheikh F, Blatter LA, Chen J (2005) Endothelin-1-induced arrhythmogenic Ca2+ signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ Res* 96:1274–1281.
13. Zima AV, Blatter LA (2004) Inositol-1,4,5-trisphosphate-dependent Ca2+ signalling in cat atrial excitation-contraction coupling and arrhythmias. *J Physiol* 555:607–615.
14. Proven A, et al. (2006) Inositol 1,4,5-trisphosphate supports the arrhythmogenic action of endothelin-1 on ventricular cardiac myocytes. *J Cell Sci* 119:3363–3375.
15. Kockskämper J, et al. (2008) Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. *J Mol Cell Cardiol* 45:128–147.
16. Higazi DR, et al. (2009) Endothelin-1-stimulated insP3-induced Ca2+ release is a nexus for hypertrophic signaling in cardiac myocytes. *Mol Cell* 33:472–482.
17. Go LO MM, Watras J, Handa KK, Fyfe BS, Marks AR (1995) Differential regulation of two types of intracellular calcium release channels during end-stage heart failure. *J Clin Invest* 95:888–894.
18. Kawaguchi HSH, et al. (1993) Increased calcium release from sarcoplasmic reticulum stimulated by inositol trisphosphate in spontaneously hypertensive rat heart cells. *Mol Cell Biochem* 119:51–57.
19. Bing OH, et al. (1995) The spontaneously hypertensive rat as a model of the transition from compensated left ventricular hypertrophy to failure. *J Mol Cell Cardiol* 27:383–396.
20. Shorofsky SR, et al. (1999) Cellular mechanisms of altered contractility in the hypertrophied heart: Big hearts, big sparks. *Circ Res* 84:424–434.
21. Masciotra S, Picard S, Deschepper CF (1999) Cosegregation analysis in genetic crosses suggests a protective role for atrial natriuretic factor against ventricular hypertrophy. *Circ Res* 84:1453–1458.
22. Russell FD, Molenaar P (2000) The human heart endothelin system: ET-1 synthesis, storage, release, and effect. *Trends Pharmacol Sci* 21:353–359.
23. Keller E, Bond M, Schomisch Moravec C (1997) Progression of left ventricular hypertrophy does not change the sarcoplasmic reticulum calcium store in the spontaneously hypertensive rat heart. *J Mol Cell Cardiol* 29:461–469.
24. McCall E, et al. (1998) Ca flux, contractility, and excitation-contraction coupling in hypertrophic rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 274:H1348–1360.
25. Zhao ZH, et al. (2007) Inositol-1,4,5-trisphosphate and ryanodine-dependent Ca2+ signaling in a chronic dog model of atrial fibrillation. *Cardiology* 107:269–276.
26. Yamada J, et al. (2001) Up-regulation of inositol 1,4,5 trisphosphate receptor expression in atrial tissue in patients with chronic atrial fibrillation. *J Am Coll Cardiol* 37:1111–1119.
27. Sugden PH, Clerk A (2005) Endothelin signalling in the cardiac myocyte and its pathophysiological relevance. *Curr Vasc Pharmacol* 3:343–351.
28. Kockskamper J, et al. (2008) Endothelin-1 enhances nuclear Ca2+ transients in atrial myocytes through Ins(1,4,5)P3-dependent Ca2+ release from perinuclear Ca2+ stores. *J Cell Sci* 121:186–195.
29. Tovey SC, et al. (2001) Calcium puffs are generic InsP3-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J Cell Sci* 114:3979–3989.
30. Smith IF, Parker I (2009) Imaging the quantal substructure of single IP3R channel activity during Ca2+ puffs in intact mammalian cells. *Proc Natl Acad Sci USA* 106:6404–6409.
31. Xu M, et al. (2007) Intermolecular failure of L-type Ca2+ channel and Ryanodine receptor signaling in hypertrophy. *PLoS Biol* 5:e21.
32. Versailles JT, Verscheure Y, Le Kim A, Pourrias B (1982) Comparison between the ventricular fibrillation thresholds of spontaneously hypertensive and normotensive rats–investigation of antidysrhythmic drugs. *J Cardiovasc Pharmacol* 4:430–435.
33. Bootman MD, Harzheim D, Smyrnias I, Conway SJ, Roderick HL (2007) Temporal changes in atrial EC-coupling during prolonged stimulation with endothelin-1. *Cell Calcium* 42:489–501.