Increased InsP$_3$Rs in the junctional sarcoplasmic reticulum augment Ca$^{2+}$ transients and arrhythmias associated with cardiac hypertrophy

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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$_3$Rs) 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$_3$R expression in the junctional sarcoplasmic reticulum. Due to their close proximity to ryanodine receptors (RyRs) in this region, enhanced Ca$^{2+}$ release through InsP$_3$Rs 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$_3$R expression and Ca$^{2+}$ transient amplitude in the cytosol, InsP$_3$R expression and ECC-mediated Ca$^{2+}$ transients in the nucleus were not altered during hypertrophy. Elevated InsP$_3$R2 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$_3$R expression is a general mechanism that underlies remodeling of Ca$^{2+}$ signaling during heart disease, and in particular, in triggering ventricular arrhythmia during hypertrophy.

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 remodeling of Ca$^{2+}$ signaling (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$_3$Rs, albeit outnumbered by RyRs at approximately 50:1 (7). Mammals have 3 InsP$_3$R isoforms (types 1–3) (8), with InsP$_3$R2 being the main isoform in cardiomyocytes (9, 10). Although Ca$^{2+}$ flux via these InsP$_3$Rs is relatively small in comparison to the large Ca$^{2+}$ transients occurring during every heartbeat, recent data suggests that InsP$_3$Rs have an important role in cardiac physiology. We, and others have shown, that Ca$^{2+}$ release through InsP$_3$Rs contributes to the inotropic, arrhythmogenic, and hypertrophic effect of G$_q$-coupled agonists such as the vasoactive peptide ET-1 (11–16). Whether altered InsP$_3$R signaling also contributes to remodeling of Ca$^{2+}$ homeostasis during cardiac hypertrophy is not yet determined. An increase in InsP$_3$R expression has however been reported during heart failure in humans (17). Moreover, InsP$_3$-induced Ca$^{2+}$ release (ICR) is increased in SR microsomes prepared from hypertrophic myocytes (18).

Here, we hypothesized that enhanced Ca$^{2+}$ release via InsP$_3$Rs 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$_3$R signaling. This profound effect of enhanced InsP$_3$R activity in hypertrophic myocytes was due to an increase in InsP$_3$R expression, specifically in the junctional SR membrane in close proximity to RyRs. At this location, Ca$^{2+}$ release via InsP$_3$Rs 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$_3$R2 expression was also increased in hypertrophic cardiomyocytes isolated from aortically banded mice and in human hearts displaying ischemic dilated cardiomyopathy. We propose that InsP$_3$Rs 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

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Effect of 10 μM 2-APB on Ca\textsuperscript{2+} transient amplitude and fractional shortening. (A) Fractional shortening of myocytes under conditions where InsP\textsubscript{3}Rs were either inhibited or activated. 2-APB (2 μM) increased fractional shortening in both WKY and SHR myocytes (Fig. 1Ai). The difference in ratio between the 2 strains (WKY: 1.50; SHR: 0.98) was significant (P < 0.001).

To reveal whether increased Ca\textsuperscript{2+} release via InsP\textsubscript{3}Rs contributes to the greater basal ECC-associated Ca\textsuperscript{2+} transients observed in SHR myocytes.

Direct activation of InsP\textsubscript{3}Rs with InsP\textsubscript{3} ester (11) promoted a greater increase in Ca\textsuperscript{2+} transient amplitude and inotropy in SHR compared to WKY myocytes (Fig. 1B and C), which was abrogated in both strains by 2-APB (Fig. 1Bi and Ci). No difference in ECC-mediated Ca\textsuperscript{2+} transient amplitude or cellular contraction was observed between myocytes isolated from 12-week-old WKY rats or SHRs (Fig. S1A).

**InsP\textsubscript{3}R2 Expression Is Increased in Hypertrophic Cardiomyocytes.**

Next, we analyzed whether an increase in InsP\textsubscript{3}R expression underlies altered InsP\textsubscript{3} signaling during hypertrophy. At 6 months, InsP\textsubscript{3}R2 mRNA and protein levels were higher in SHR than in WKY myocytes, whereas at 12 weeks, InsP\textsubscript{3}R2 mRNA and protein levels were lower in SHRs compared to WKY controls (Fig. 2A and B). RyR2 protein levels were not different between the 2 strains at the age of 12 weeks or 6 months (Fig. 2B).

Immunofluorescent labeling revealed that in WKY myocytes, InsP\textsubscript{3}R2 was predominantly expressed in the perinuclear regions with weaker staining along the SR membrane, where RyRs are localized (Fig. 2C). InsP\textsubscript{3}R2 was also expressed in the perilacunar 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\textsubscript{3}R2 immunofluorescence was increased (Fig. 2D). No difference in RyR2 immunostaining between the 2 strains was observed (Fig. 2C). In both WKY and SHR myocytes, InsP\textsubscript{3}R2 co-localized with RyR2s (intensity profile along indicated line, Fig. 2C and F) and Pearson’s coefficient (Fig. 2E), indicating that like RyR2s, InsP\textsubscript{3}R2s are located at dyadic junctions alongside T-tubule membranes (Fig. 2C). The co-localization of these 2 channels was markedly increased in hypertrophic myocytes (Fig. 2E and F). We concluded that, as a result of hypertrophic remodeling, the number of InsP\textsubscript{3}R2s located in the junctional SR membrane is increased, thereby mediating their greater co-localization with RyR2s.

InsP\textsubscript{3}R2 expression was also increased in hearts from mice after aortic banding (Fig. 2G) and in human patients with ischemic dilated cardiomyopathy (Fig. 2H). These data suggested that increased InsP\textsubscript{3}R2 expression is a general hallmark of hypertrophy.

**Increased InsP\textsubscript{3}R Expression in the Junctional SR Causes a Spatially Restricted Remodeling of ECC-mediated Ca\textsuperscript{2+} Transients during Hypertrophy.**

To establish how increased InsP\textsubscript{3}R expression in the junctional SR membrane impacted on ECC-mediated Ca\textsuperscript{2+} signals, we performed confocal Ca\textsuperscript{2+} imaging experiments. In addition to directly stimulating InsP\textsubscript{3}Rs with a membrane-permeant InsP\textsubscript{3} ester, we tested the effect of physiologically activating InsP\textsubscript{3}Rs with InsP\textsubscript{3} generated following ET-1 stimulation (11, 22). In SHR myocytes, stimulation with ET-1 and InsP\textsubscript{3} ester increased the amplitude of nuclear and cytosolic Ca\textsuperscript{2+} transients during electrical pacing (Fig. 3A–E). Contrarily, in WKY myocytes, only nuclear Ca\textsuperscript{2+} transients were augmented (Fig. 3C and E). The enhancement of systolic Ca\textsuperscript{2+} transients by ET-1 and InsP\textsubscript{3} ester was sensitive to 2-APB, further indicating that this effect was mediated by InsP\textsubscript{3}Rs (Fig. 3Bi–Ei). The increase in nuclear Ca\textsuperscript{2+} transient amplitude in SHRs was comparable to that observed in WKY myocytes (Fig. 3C and E). To accommodate for variation between cells in the absolute magnitude of Ca\textsuperscript{2+} changes, the ratio of nuclear to cytosolic Ca\textsuperscript{2+} transient amplitude was calculated. This ratio was increased in WKY myocytes following ET-1 or InsP\textsubscript{3} ester stimulation whereas no change was observed in SHR myocytes (Fig. S2A). The difference in ratio between the 2 strains is explained by restriction of the ET-1- and InsP\textsubscript{3} ester-stimulated increase in Ca\textsuperscript{2+} transient amplitude to the nuclear compartment in WKY myocytes, whereas, in SHR myocytes nuclear and cytosolic Ca\textsuperscript{2+} transient amplitude were both increased. These data indicate...
that in non-hypertrophied myocytes, Ca^{2+} release via InsP_{3}R impacts more profoundly on nuclear Ca^{2+} transients, whereas in hypertrophic myocytes, increased functional InsP_{3}R 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 SHR hearts under basal conditions, or during ET-1 or InsP_{3} ester stimulation (Fig. S1B).

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. S2B), 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}R-dependent Ca^{2+}-release Events Are Increased in SHR Myocytes.** In atrial cardiomyocytes, which express approximately 6-fold more InsP_{3}R than ventricular myocytes, Ca^{2+} release via InsP_{3}R underlies the induction of extra-systolic Ca^{2+} transients (11, 12). We therefore investigated whether the increased InsP_{3}R 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 in Fig. 4A). 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. 4B 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 SHR than WKYs (ET-1: WKY: 26% vs. SHR: 50%; InsP_{3} ester: WKY: 29% vs. SHR: 57%, Table S2 and Fig. 4B). 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. 4B). 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 SHR 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. S1C and D). These data indicate that activation of InsP_{3}R 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_{3}R Increases the Rate of Rise of Systolic Ca^{2+} Transients and Elevates Diastolic [Ca^{2+}]_{i} in SHRs.** A hypertrophy-associated increase in InsP_{3}R-mediated Ca^{2+} flux via junctional InsP_{3}Rs 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. 5A). The effects of ET-1 and InsP_{3} ester were abrogated by adenosinergic-mediated expression of a cherry fluorescent protein-tagged InsP_{3} S’-phosphatase, which disrupts InsP_{3} signaling (5’P; Fig. 5B) (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. S1E).

As RyR opening is controlled by [Ca^{2+}]_{i}, we next tested whether Ca^{2+} release via InsP_{3}Rs 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. 5C), whereas no change was seen in WKY cells (Fig. 5C). 2-APB or 5’P expression abrogated the increase in diastolic [Ca^{2+}] caused by

**Fig. 2.** Expression of InsP_{3}R2 during hypertrophy. (A) Relative InsP_{3}R2 mRNA levels. Values for SHR hearts have been normalized to age-matched WKY rats. (B) Relative InsP_{3}R2 and RyR2 protein levels. SHR/WKY ratios have been determined for 12-week- and 6-month-old rats. Representative immunoblots for InsP_{3}R2 (ii) and RyR2 (iii) are shown. Seventy-five 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.

**Fig. 3.** Expression of InsP_{3}R2 during hypertrophy. (A) Relative InsP_{3}R2 mRNA levels. Values for SHR hearts have been normalized to age-matched WKY rats. (B) Relative InsP_{3}R2 and RyR2 protein levels. SHR/WKY ratios have been determined for 12-week- and 6-month-old rats. Representative immunoblots for InsP_{3}R2 (ii) and RyR2 (iii) are shown. Seventy-five 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.
Effects of ET-1 and InsP3 ester in SHR myocytes

**Fig. 4.** Analysis of extra-systolic Ca²⁺ release events in indo-1 AM-loaded ventricular myocytes during hypertrophy. (A) Representative traces for global Ca²⁺ transients and cellular contraction recorded from SHR myocytes before and after stimulation with 100 nM ET-1 or 10 μM InsP3 ester. Arrows indicate extra-systolic events. (B) Extra-systolic Ca²⁺ release events per cell during stimulation with 100 nM ET-1 or 10 μM InsP3 ester.

**Discussion**

Here we demonstrate that enhanced Ca²⁺ signaling via InsP₃Rs located in the dyadic cleft remodels Ca²⁺ signaling during hypertrophy.

In agreement with previous data, we found that the amplitude of ECC-mediated Ca²⁺ 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 Ca²⁺ transients was due to augmented Ca²⁺ release via InsP₃Rs. These data demonstrated that InsP₃Rs could contribute to ECC-mediated Ca²⁺ fluxes without additional neurohormonal input, thereby modifying myocyte Ca²⁺ signaling.

A greater role for InsP₃Rs in regulating ECC-mediated Ca²⁺ transients during hypertrophy was revealed following their direct activation with cell-permeant InsP₃ ester. These data showed that increased activation of InsP₃Rs could augment the amplitude of ECC-mediated Ca²⁺ transients mediated via RyRs even further. Consistent with previous reports (20, 23, 24), no increase in SR releasable Ca²⁺ was observed in hypertrophic SHR myocytes.

**Frequency of Elementary InsP₃-dependent Ca²⁺-release Events Is Increased during Hypertrophy.** To further resolve the consequences of increased InsP₃R expression for Ca²⁺ signaling, elementary Ca²⁺-release events were analyzed. Under normal paced conditions, Ca²⁺ events during the diastolic period were of greater amplitude in the hypertrophic SHR myocytes than in WKY cells (WKY: AF/F₀ = 0.26 ± 0.01 vs. SHR: 0.34 ± 0.04, Table S3 and Fig. 5E). Under conditions where RyRs were blocked with 1 mM tetracaine, InsP₃ ester application stimulated elementary Ca²⁺-release events (Fig. 5E) that occurred at a greater frequency in hypertrophic myocytes (WKY: 2.12 ± 0.46 vs. SHR: 6.84 ± 0.65, Table S3). These data suggest that InsP₃,R-mediated Ca²⁺ signals contribute to the greater amplitude of diastolic Ca²⁺ events observed in SHR myocytes and may underlie the elevated diastolic [Ca²⁺] observed in SHR myocytes stimulated with InsP₃ or ET-1.

Our data suggest a model to explain the enhanced ECC-mediated Ca²⁺ signals and increased extra-systolic Ca²⁺-release events observed during hypertrophy (Fig. 5F). Key to this model is a hypertrophy-associated increase in InsP₃R expression in the dyadic region. Thus, more InsP₃Rs are in close proximity to RyRs in the SR membrane (Fig. 2E and F). Ca²⁺ released via these InsP₃Rs sensitizes their adjacent RyRs, bringing them closer to threshold for activation. Under conditions of increased [InsP₃], elementary InsP₃-dependent Ca²⁺-release events are increased in frequency and diastolic [Ca²⁺] is elevated. Consequently, RyRs are triggered to generate extra-systolic Ca²⁺ signals and to accelerate the rate of rise of pacing-evoked Ca²⁺ transients (Fig. 5F).

InsP₃ ester or ET-1 in SHRs (Fig. 5C and D) without affecting diastolic [Ca²⁺] under basal conditions. At 12 weeks of age, there was no difference in diastolic [Ca²⁺] during stimulation of SHR myocytes with ET-1 or InsP₃ ester (Fig. S1F).

**Fig. 3.** Confocal analysis of systolic nuclear and cytosolic Ca²⁺ transient amplitude in fluo-4 AM-loaded ventricular myocytes. Data presented were determined 15 min after application of ET-1; InsP₃ 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 Ca²⁺ transients at a series of time points before and after application of 10 μM InsP₃ ester. (Scale bar, 10 μm.) (B) Effect of 100 nM ET-1 ± 2 μM 2-APB on cytosolic peak amplitude. (C) As in B for nuclear peak amplitude. (D) Effect of 10 μM InsP₃ ester ± 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.
are shown in the following disease. In particular, InsP3R expression is increased in the heart has previously been reported to be modified during normal pacing (control) and during pacing in the presence of 1 mM tetracaine. 

Fig. 5A. Analysis of global Ca\(^{2+}\) transient kinetics, diastolic [Ca\(^{2+}\)]\(_{\text{c}}\) and, 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 \(\mu\)M InsP3 ester (InsP3). Representative traces are shown in A; values normalized to pre application are shown in B. 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 InsP3 5’-phosphatase (5’P) virus. (C) Changes in diastolic [Ca\(^{2+}\)]\(_{\text{c}}\) during application of 100 nM ET-1 = \(\pm\) 2 \(\mu\)M 2-APB or 10 \(\mu\)M InsP3 ester = \(\pm\) 2 \(\mu\)M 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 \(\mu\)M InsP3 ester. F/F\_0 traces are shown below. (F) Schematic indicating how InsP3R-mediated Ca\(^{2+}\) release augments systolic Ca\(^{2+}\) transients during hypertrophy. (I) InsP3R2, R, RyR2. N numbers are indicated. * \(P < 0.05\); Student’s t test.

Fig. 5B. Depicts the effects of InsP3R expression on Ca\(^{2+}\) release in SHR myocytes. InsP3R expression was elevated as a result of hypertrophy thereby providing a mechanism for increased Ca\(^{2+}\) release via InsP3Rs. InsP3R expression in the heart has previously been reported to be modified following disease. In particular, InsP3R expression is increased in atrial myocytes of humans and dogs during atrial fibrillation (AF) (25, 26). Furthermore, elevated InsP3R levels and increased InsP3 binding was reported in the left ventricle during human heart failure (17). Consistent with these reports and our observations in rats, we found that InsP3R2 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 InsP3R2 expression detected in human and mouse cardiac tissue was due solely to InsP3R2 in cardiac myocytes. Our findings in rats, mice, and humans therefore suggested that increased InsP3R expression is a general feature of cardiac disease, raising the possibility that increased Ca\(^{2+}\) release via InsP3Rs contributes to pathological changes in Ca\(^{2+}\) signaling.

The enhanced InsP3R2 expression had a striking spatial aspect in that InsP3R expression was specifically increased in the junctional SR. Detailed analysis showed that these junctional InsP3Rs colocalized with RyRs, which reside primarily in the dyadic cleft. This profound remodeling in InsP3Rs expression and distribution had significant functional consequences. In particular, the increased number of dyadic InsP3Rs augmented the amplitude of the cytosolic ECC-mediated Ca\(^{2+}\) transients and enhanced the positive inotropic effect of InsP3 ester. Similarly, cytosolic ECC-mediated Ca\(^{2+}\) transient amplitude and contraction were enhanced when InsP3Rs were engaged by InsP3, 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 InsP3Rs in SHR myocytes by ET-1 is in agreement with data from our laboratory and elsewhere showing that stimulation of the InsP3 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 InsP3 ester was not altered during hypertrophy reflecting the lack of a change in InsP3R expression in this region. Together, these data suggested that increased Ca\(^{2+}\) release via InsP3Rs in the dyadic region primed ECC-mediated Ca\(^{2+}\)-induced Ca\(^{2+}\) release via RyRs (see Fig. 5F). Specifically, Ca\(^{2+}\) release via InsP3Rs could elevate diastolic [Ca\(^{2+}\)]\(_{\text{c}}\) closer to the threshold for activation of RyRs. Thus, we established that increased Ca\(^{2+}\) release via InsP3Rs 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 InsP3-dependent Ca\(^{2+}\) release events. Interestingly, the amplitudes of these 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 InsP3Rs (a cluster) until a threshold number required for puff generation is reached (30). Thus, it is plausible that greater InsP3R 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 SHR. The greater abundance of these elementary events may explain the elevated diastolic [Ca\(^{2+}\)]\(_{\text{c}}\) observed in SHR myocytes stimulated with InsP3 ester and ET-1. These data are consistent with the requirement for InsP3Rs for the ET-1-stimulated increase in diastolic [Ca\(^{2+}\)]\(_{\text{c}}\) observed in atrial myocytes (which have \(\sim\)6-fold greater InsP3R 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 InsP3 or InsP3-generating ago-
nists such as ET-1, potently induced arrhythmogenic Ca\(^{2+}\)-release events that were dependent on InsP\(_3\)R,2 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\(_3\)R 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\(_3\)R 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\(_3\)R,2 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. 55.

Animal Models. Male SHRs and normotensive Wistar-Kyoto (WKY) rats were obtained from Harlan and were housed under controlled 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 non-failing 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 significant was accepted at P < 0.05.

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