Inositol 1, 4, 5-Triphosphate Receptors and Human Left Ventricular Myocytes

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Abstract

Background—Little is known concerning the function of inositol 1,4,5-triphosphate receptors (IP3Rs) in the adult heart experimentally. Moreover, whether these Ca^{2+} release channels are present and play a critical role in human cardiomyocytes remains to be defined. IP3Rs may be activated following Gαq-protein-coupled receptors (GPCR) stimulation affecting Ca^{2+} cycling, enhancing myocyte performance and, potentially, favoring an increase in the incidence of arrhythmias.

Methods and Results—IP3R function was determined in human left ventricular (LV) myocytes and this analysis was integrated with assays in mouse myocytes to identify the mechanisms by which IP3Rs influence the electrical and mechanical properties of the myocardium. We report that IP3Rs are expressed and operative in human LV myocytes. Following GPCR activation, Ca^{2+} mobilized from the sarcoplasmic reticulum via IP3Rs contributes to the decrease in resting membrane potential, prolongation of the action-potential, and occurrence of early after-depolarizations. Ca^{2+} transient amplitude and cell shortening are enhanced, and extra-systolic and dysregulated Ca^{2+} elevations and contractions become apparent. These alterations in the electromechanical behavior of human cardiomyocytes are coupled with increased isometric twitch of the myocardium and arrhythmic events, suggesting that GPCR activation provide inotropic reserve, which is hampered by electrical instability and contractile abnormalities. Additionally, our findings support the notion that increases in Ca^{2+} load by IP3Rs promote Ca^{2+} extrusion by forward mode Na^{+}/Ca^{2+} exchange, an important mechanism of arrhythmic events.

Conclusions—Thus, the GPCR/IP3R axis modulates the electromechanical properties of the human myocardium and its propensity to develop arrhythmias.

Key words: myocyte, calcium transients, arrhythmia, Human Myocyte
Myocyte function relies on Ca\textsuperscript{2+} entry from the extracellular space and its release from the sites of storage in the sarcoplasmic reticulum (SR), the latter being mediated by activation of ryanodine receptors (RyRs).\textsuperscript{1} Following membrane depolarization, opening of the RyRs is triggered by Ca\textsuperscript{2+} influx through voltage-activated L-type channels, allowing translocation of Ca\textsuperscript{2+} to the cytoplasm where myofilament cross-bridge formation initiates cell shortening.\textsuperscript{1,2} Relaxation is promoted by a reduction in cytosolic Ca\textsuperscript{2+} through its re-uptake in the SR by the sarco-endoplasmic Ca\textsuperscript{2+} pump (SERCA), and Ca\textsuperscript{2+} extrusion via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX). Dysfunctional RyRs result in spontaneous Ca\textsuperscript{2+} release or enhanced Ca\textsuperscript{2+} leak, pathologies found in inherited and acquired arrhythmogenic diseases,\textsuperscript{3-7} strengthening the notion that defects in Ca\textsuperscript{2+} homeostasis impact on the electrical properties of the heart.

The release of Ca\textsuperscript{2+} from the endoplasmic reticulum via inositol 1,4,5-triphosphate receptors (IP3Rs) promotes spontaneous Ca\textsuperscript{2+} oscillations in human cardiac stem cells (hCSCs), inducing cell cycle reentry and modulating the fate of these cells, following their injection in the infarcted heart of immunosuppressed mice.\textsuperscript{8} Additionally, modulation of Ca\textsuperscript{2+} signals via IP3Rs controls apoptosis and lineage commitment of differentiating embryonic stem cells,\textsuperscript{9} a mechanism which may be operative in cardiomyogenesis. Although limited data in rodents and rabbits suggest that IP3Rs are expressed in left ventricular (LV) myocytes,\textsuperscript{10,11} whether the presence of IP3Rs in resident hCSCs is preserved in the derived human myocyte progeny,\textsuperscript{12,13} playing a role in Ca\textsuperscript{2+} homeostasis remains to be defined.

Impaired IP3R-Ca\textsuperscript{2+} signaling and alterations of the calcineurin-NFAT pathway lead to developmental defects and abrogate cardiac hypertrophy induced by isoproterenol or angiotensin II.\textsuperscript{14,15} Moreover, initial observations raise the possibility that enhanced IP3R activity may increase Ca\textsuperscript{2+} transient amplitude, the incidence of extra-systolic Ca\textsuperscript{2+} elevations and the
propensity for arrhythmias. Thus, the hypothesis may be raised that abnormalities in Ca\(^{2+}\) transients in the presence of G\(\alpha\)-protein-coupled receptors (GPCR) agonists may involve IP3R-mediated Ca\(^{2+}\) mobilization, cytosolic Ca\(^{2+}\) overload, perturbations in transmembrane potential, and/or sensitization of RyRs, leading to elementary release of Ca\(^{2+}\) and electrical instability.

Ca\(^{2+}\) mobilized by IP3Rs relies on intracellular levels of IP3, which is produced by phospholipase C (PLC). GPCR agonists promote PLC function that generates IP3 and diacylglycerol (DAG). IP3 stimulates IP3Rs and Ca\(^{2+}\) translocation from intracellular stores, whereas DAG activates transient receptor potential channels (TRPCs), and protein kinase C (PKC) isoforms, phosphorylating a wide spectrum of ion channels and contractile proteins. Additionally, GPCR ligands may initiate PLC-independent signaling mechanisms, modulating transmembrane ionic fluxes. Thus, whether IP3Rs contribute to the functional consequences of GPCR stimulation in cardiomyocytes remains to be elucidated.

Based on this premise, we have investigated the role of IP3Rs in human LV myocytes obtained from normal donor hearts not used from transplantation. This analysis in human cells was complemented with a number of assays in mouse myocytes to define the role of IP3Rs in Ca\(^{2+}\) mobilization, electrical activity, arrhythmic events, and myocyte contractile behavior following GPCR stimulation.

**Methods**

An expanded description of the methods is available in the online-only Data Supplement.

**Human Hearts and Myocyte Isolation**

Donor hearts not used for transplantation and explanted failing hearts were utilized in this study.
(Table 1 in the online-only Data Supplement). The protocol was approved by the Institutional Review Board (#2010P002475). Donor and failing human hearts were shipped in cold cardioplegia solution, and were processed immediately upon receipt.

Myocardial samples, obtained at autopsy from patients who died from causes other than cardiovascular diseases, were fixed in formalin and used as controls for the quantitative evaluation of myocyte apoptosis by the TdT assay. As previously performed in our laboratory, large LV myocardial samples were used; a branch of the left main coronary artery was cannulated and the distal arteries were ligated. Initially, the tissue was perfused with a solution containing (mmol/L): NaCl 126, KCl 4.4, MgCl2 5, HEPES 5, glucose 22, taurine 20, creatine 5, Na pyruvate 5, NaH2PO4 5, and 2,3-butanedione monoxime 10 (pH 7.4, adjusted with NaOH). A constant temperature of 37°C was maintained, and the buffer was gassed with 85% O2 and 15% N2. After ~10 minutes, 0.015 mmol/L CaCl2, 274 units/ml collagenase (type 2, Worthington Biochemical Corp), and 0.57 units/ml protease (type XIV, Sigma) were added to the perfusate for enzymatic dissociation of the tissue. At completion of digestion, the myocardium was cut in small pieces, subjected to repeated pipetting to obtain a single cell preparation and re-suspended in Ca2+ 0.015 mmol/L solution. Aliquots of cell suspensions were centrifuged for 5 minutes at 20g, and myocytes were fixed in 4% paraformaldehyde, or frozen for immunocytochemistry and biochemical assays. For electrophysiological and mechanical studies only rod-shaped myocytes exhibiting cross striations, and showing no spontaneous contractions or contractures were selected; cells were used within 12 hours following organ acquisition.

Mouse Hearts and Myocyte Isolation

C57Bl/6 mice were maintained in accordance with the Guide for Care and Use of Laboratory
Animals, and all animal experiments were approved by the local animal care committee (IACUC). For myocyte isolation, a protocol similar to that described above was employed. Antibodies for FACS analysis, and primers for PCR studies are listed in Table 2 and Table 3 in the online-only Data Supplement.

**Cell Shortening, Ca\(^{2+}\) Transients, and Patch-Clamp Studies**

Isolated LV myocytes were placed in a bath located on the stage of an Axiovert (Zeiss), IX51 and IX71 (Olympus) inverted microscopes for measurements of contractility, Ca\(^{2+}\) transients and patch-clamp studies. Experiments were performed at room temperature.

**Data Analysis**

The magnitude of sampling employed in each measurement is listed in Table 1 in the online-only Data Supplement. Data are presented as mean±SEM. Independent samples were compared using ANOVA analysis with Bonferroni correction or Fisher’s exact tests, as appropriate for continuous or categorical responses. The normality and homogeneity of variance of the data were checked first to meet the assumptions of ANOVA. Paired observations were evaluated with Wilcoxon signed-rank sum test or paired t-test as appropriate. For data with multiple measurements (myocytes) from the same donor (mouse/patient), linear mixed-effects models with patient/mouse as random effect, main effects for treatment group were applied to examine and compare response changes. The compound symmetry covariance matrix was specified to account for dependency among observations. Differences in mean changes between assessments were compared between groups and within each group by LSMEANS statement. Throughout, a \(P<0.05\) was considered statistical significance and all tests were two sided with a type I error of 0.05. The statistical analyses were performed using SAS version 9.3 (SAS Institute).
Results

Human Hearts

Human cardiomyocytes were obtained from the LV of 34 donor hearts; 14 males and 20 females with an average age of 44±4 years (Table 1 in the online-only Data Supplement). These hearts were declined for transplantation. In donor hearts, foci of replacement fibrosis and areas of diffuse interstitial fibrosis were not detected histologically. Similarly, the coronary vessels had minimal atherosclerotic lesions with essentially no reduction in luminal diameter. Inflammatory infiltrates were not identified. Myocyte apoptosis was comparable in three randomly selected donor hearts and age-matched control hearts obtained at autopsy from patients who died from causes other than cardiovascular diseases (Figure 1 in the online-only Data Supplement). Thus, myocyte survival in donor hearts was not affected by the preservation protocol. End-stage failure in explanted hearts was of ischemic (n=6) and non-ischemic (n=9) origin.

IP3Rs, Ca2+ Transients, and Human Myocyte Contractility

Molecular assays were conducted in isolated myocyte preparations with minimal levels of contamination from fibroblasts, endothelial cells and smooth muscle cells (Figure 2 in the online-only Data Supplement). Transcripts for the three IP3R subtypes were identified by qRT-PCR in human LV myocytes. The expression of IP3R type-2 was also confirmed by Western blotting. Similarly, the presence of IP3Rs in mouse LV myocytes was documented by both methodologies (Figure 1 and Figures 3 and 4 in the online-only Data Supplement).

The effect of IP3R activation on Ca2+ cycling and contractility of human LV myocytes was investigated by stimulating GPCRs with adenosine 5'-triphosphate (ATP) and endothelin-1 (ET-1), which both enhance the synthesis of IP3. ATP increased myocyte contractility, and this response was attenuated with the unspecific IP3R blocker 2-APB (Figure
The enhanced cell performance mediated by GPCR activation was coupled with an increase in Ca\(^{2+}\) transient amplitude. Inhibition of IP3Rs with the selective blocker xestospongin-C (XeC) did not modify baseline myocyte mechanics, but abrogated the changes mediated by GPCR stimulation (Figure 2B and 2C). The increase in contractility with these agonists led to episodes of after-contractions during relaxation in 27% of human cardiomyocytes; however, spontaneous and sustained Ca\(^{2+}\) elevations were observed rarely (Figure 2D-2F). The effects of GPCR activation on cardiomyocytes were equally present in myocardial trabeculae dissected from the LV wall. ATP and ET-1 enhanced isometric developed tension, which was abolished by IP3R antagonists (Figure 2G and 2H). Thus, IP3R inhibition in human cardiomyocytes and myocardium attenuates the positive inotropic action of GPCR stimulation.

**GPCRs and Electrical Instability**

To establish whether the electrical properties of human cardiomyocytes were influenced by activation of GPCRs, membrane potential and [Ca\(^{2+}\)], were measured simultaneously in current-clamped cells.\(^{25}\) In human cardiomyocytes, ATP and ET-1 decreased resting membrane potential (RMP), prolonged the action potential (AP), and increased early after-depolarizations (EADs), together with an increase in Ca\(^{2+}\) transient amplitude and extra-systolic Ca\(^{2+}\) elevations. Comparable responses were observed with the IP3R agonist, thimerosal (Figure 3A-3D).

Importantly, ATP and ET-1 had similar effects on the intact myocardium. Human samples were perfused in a Langendorff apparatus, and transmural pseudo-EKG and monophasic action potentials (MAPs) were recorded. GPCR agonists delayed the electrical recovery and prolonged MAPs (Figure 4A and 4B), mimicking the observations at the myocyte level. Moreover, tachycardia and premature ventricular contractions occurred (Figure 4C). To exclude the contribution of ischemia, potentially introduced during the preservation of the organ,
measurements were obtained in the presence of $K_{\text{ATP}}$ channel blockade, since these channels are activated by hypoxia. Inhibition of $K_{\text{ATP}}$ channels had no consequences on MAPs (Figure 5 in the online-only Data Supplement), indicating that the electrical manifestations dictated by GPCR stimulation were independent from myocardial ischemia. Thus, GPCR stimulation prolongs the AP and promotes the electrical instability of cardiomyocytes and the myocardium.

**GPCR Simulation in the Failing Human Heart**

Contractile reserve is severely reduced in heart failure (HF), and the GPCR/IP3R axis may represent an important signaling pathway modulating the inotropic state of the diseased myocardium. Thus, explanted failing hearts were studied (Table 1 in the online-only Data Supplement). By Western blotting, IP3R-2 expression was significantly increased in failing LV myocytes (Figure 5A). Importantly, stimulation of GPCRs with ATP in failing LV myocytes led to a reduction in RMP and prolongation of the AP (Figure 5B). Similarly, measurements of cell shortening in one failing LV cardiomyocyte revealed that this GPCR/IP3R activation increased contractility, elicited premature contraction and altered relaxation (Figure 5C).

The effects of GPCR stimulation on the myocardium were evaluated in an isometric system. In comparison with donor human hearts, the inotropic response of LV trabeculae to the $\beta$-adrenergic agonist isoproterenol was significantly attenuated in failing hearts (Figure 5D). In contrast, GPCR stimulation promoted an increase in developed tension in the diseased myocardium mimicking the findings obtained in healthy hearts (Figure 2H and Figure 5E).

Thus, the inotrope reserve is decreased in the failing heart muscle, while the GPCR/IP3R axis remains operative.

**IP3Rs and Mouse Cardiomyocytes**

To define the role of IP3Rs in myocyte contractility following GPCR activation, a loss of
function assay was introduced in LV mouse cardiomyocytes. Initial studies were performed to demonstrate that GPCRs agonists induce in mouse cardiomyocytes responses similar to those seen in human cells. ATP, ET-1, angiotensin II or phenylephrine stimulation led to an increase in 
Ca\(^{2+}\) transient amplitude and myocyte contractility. Diastolic Ca\(^{2+}\) was also elevated. Extra-
systolic Ca\(^{2+}\) and sustained Ca\(^{2+}\) increases were apparent, resulting in after-contractions and prolonged contractures, respectively (Figure 6 in the online-only Data Supplement). Increased IP3R affinity by thimerosal led to comparable results. In contrast, IP3R blockade (XeC) or inhibition of IP3 synthesis (U-73122) abolished the effects of GPCR stimulation (Figure 6 in the online-only Data Supplement). By employing two-photon microscopy working in line-scan mode, it was possible to demonstrate that extra-systolic Ca\(^{2+}\) and sustained increases in peak Ca\(^{2+}\) were distributed synchronously in the myocyte cytoplasm; this pattern of Ca\(^{2+}\) changes excludes that local releases of Ca\(^{2+}\) from the SR, or Ca\(^{2+}\) waves were involved in the process (Figure 7 in the online-only Data Supplement).

ATP and ET-1 may stimulate other effector pathways involving phospholipase-C (PLC), which activates PKC isoforms, resulting in the phosphorylation of ion channels and contractile proteins.\(^{19-21}\) To establish whether this mechanism was implicated in myocyte performance, studies in mouse LV myocytes were conducted in the presence of the PKC inhibitor chelerythrine.\(^{32}\) Chelerythrine failed to abrogate the inotropic response triggered by ATP and ET-1 (Figure 8 in the online-only Data Supplement), suggesting that PKC-independent pathways play a critical role in mediating the impact of GPCR stimulation in myocytes.

Based on this information, which strengthened the human results, the consequences of downregulation of IP3R type-2 on Ca\(^{2+}\) cycling in myocytes was tested using a small hairpin-RNA (sh-RNA) assay. An adeno-associated AAV9 vector carrying EGFP and sh-RNA targeting
IP3R type-2 (shRNA-IP3R2-EGFP) was injected intravenously in mice (Figure 9 in the online-only Data Supplement). Three weeks later, EGFP-positive and EGFP-negative LV myocytes were isolated (Figure 6A) and loaded with the Ca$^{2+}$ indicator Rhod-2. Ca$^{2+}$ transient was measured in field stimulated cells, in the absence and presence of ATP or ET-1. GPCRs activation failed to increase Ca$^{2+}$ transients in EGFP-positive mouse myocytes. Similarly, extra-systolic and sustained Ca$^{2+}$ elevations were not detected. Conversely, these responses were preserved in EGFP-negative cells (Figure 6B and 6C, and Figure 10 in the online-only Data Supplement).

As in human cells, stimulation of GPCR function with ATP or ET-1, or enhanced IP3R affinity by thimerosal decreased RMP, prolonged the AP, and increased the frequency of EADs in mouse myocytes. Ca$^{2+}$ transient amplitude increased, and extra-systolic Ca$^{2+}$ elevations were apparent (Figure 11 in the online-only Data Supplement). Similarly, spontaneous sustained depolarization to the plateau and long-lasting [Ca$^{2+}$]$_i$ increases were detected (Figure 12 in the online-only Data Supplement). Importantly, direct activation of IP3Rs by dialysis with IP3 in the myocyte cytoplasm had effects comparable to those seen with GPCR agonists or thimerosal (Figure 6D and 6E).

IP3R blockade, in the absence of GPCR agonists, did not alter the AP, Ca$^{2+}$ transients and myocyte contractility (Figure 13 in the online-only Data Supplement). Conversely, IP3R inhibition reversed the effects of ET-1 and ATP on cardiomyocytes (Figure 14 in the online-only Data Supplement). Electrical abnormalities similar to those observed in isolated myocytes were detected in the perfused mouse heart under conditions favoring IP3R function; the arrhythmic events triggered by regular pacing and programmed electrical stimulation$^{13}$ were markedly enhanced (Figure 15 in the online-only Data Supplement). Thus, IP3Rs mediate partly the
effects of GPCR stimulation on Ca\textsuperscript{2+} cycling and the electrical and mechanical properties of adult human and mouse cardiomyocytes.

**GPCR stimulation and Ca\textsuperscript{2+} Release from RyRs**

The increase in Ca\textsuperscript{2+} transient amplitude and extra-systolic Ca\textsuperscript{2+} elevations induced by GPCR agonists in myocytes may result from the prolongation of the AP and altered repolarization phase. Alternatively, Ca\textsuperscript{2+} mobilized by IP3Rs may sensitize RyRs, enhancing excitation–contraction-coupling gain and promoting spontaneous Ca\textsuperscript{2+} releases, which, in turn, may affect myocyte electrical properties.

To establish the role of the AP profile on the properties of Ca\textsuperscript{2+} transients following GPCR stimulation, AP-clamp studies were performed\textsuperscript{25} AP waveforms in Tyrode solution (Ctrl-APs) or with GPCR agonists (GPCR-APs) were employed as voltage-clamp commands, while monitoring intracellular Ca\textsuperscript{2+}. Despite GPCR stimulation, Ctrl-APs were characterized by physiological Ca\textsuperscript{2+} transients with no extra-systolic Ca\textsuperscript{2+} elevations (n=23/23) (Figure 7A and 7B, and Figure 16 in the online-only Data Supplement). Conversely, GPCR-APs showed increased Ca\textsuperscript{2+} transients in the presence (n=27/27) or absence (n=8/8) of GPCR agonists (Figure 7B and Figure 16 and 17 in the online-only Data Supplement). Thus, the alterations in Ca\textsuperscript{2+} transients following GPCR stimulation are secondary to changes in AP profile.

To test whether RyRs were sensitized by GPCR activation, Fluo-3 loaded myocytes were exposed to a family of depolarizing steps in a voltage-clamp mode\textsuperscript{25} to progressively activate L-type Ca\textsuperscript{2+} current (I\textsubscript{CaL}) and release of Ca\textsuperscript{2+} from RyRs. I\textsubscript{CaL}, Ca\textsuperscript{2+} transient amplitude, and the ability of Ca\textsuperscript{2+} influx to induce RyR-Ca\textsuperscript{2+} release (excitation contraction-coupling gain) did not change with activation of GPCRs (Figure 7C and 7D). These results tend to exclude that the release of Ca\textsuperscript{2+} following activation of IP3Rs enhances the sensitivity of RyRs.
To establish whether RyRs contribute to the changes in the electrical properties of myocytes after GPCR stimulation, these Ca\(^{2+}\) channels were blocked by ryanodine and the effects of ATP were studied. Under this condition, Ca\(^{2+}\) transients and myocyte shortening were inhibited (Figure 18 in the online-only Data Supplement); however, RMP decreased, the AP was prolonged, and arrhythmic events occurred (Figure 7E and 7F and Figure 19 in the online-only Data Supplement). These findings suggest that RyRs are not implicated in the electrical instability of cardiomyocytes mediate by GPCR activation.

**IP3Rs and Electrical Properties Following GPCR Stimulation**

Although GPCR stimulation prolongs the AP favoring an increase in Ca\(^{2+}\) transients, the mechanism(s) involved in the changes of the electrical activity of cardiomyocytes have not been defined as yet. Based on the fact that (a) IP3 dialysis or (b) enhanced IP3R affinity to IP3 recapitulate the effects of GPCR stimulation, and that (c) IP3R downregulation or (d) IP3R inhibition attenuate the effects of ATP and ET-1, the possibility was raised that mobilization of Ca\(^{2+}\) from the SR by IP3Rs contributes to the altered electrical properties of myocytes in the presence of GPCR agonists. Ca\(^{2+}\) translocation from the SR may enhance cytosolic Ca\(^{2+}\) load, which may promote Ca\(^{2+}\) extrusion via forward mode NCX; this process may contribute to the depolarization of the RMP, and the delayed repolarization phase of the AP.\(^{33,34}\)

To test this hypothesis, voltage-clamp studies were performed in myocytes loaded with Fluo-3. IP3R function led to elevations in diastolic Ca\(^{2+}\) that were followed by sustained and transient inward currents. Importantly, the increase in diastolic Ca\(^{2+}\) developed slowly over a period of several seconds, potentially reflecting IP3R-mediated Ca\(^{2+}\) release (Figure 8A). The changes in resting Ca\(^{2+}\) and inward currents were preserved, despite inhibition of RyRs (Figure 8B). Conversely, blockade of the NCX prevented inward currents in the presence of increased
cytosolic Ca\textsuperscript{2+} (Figure 8C).

To define the role of NCX following activation of IP3Rs, a nickel sensitive current, indicative of NCX activity, was measured.\textsuperscript{35} ATP, ET-1, and dialysis with IP3 induced large inward currents, consistent with enhanced Ca\textsuperscript{2+} extrusion via forward mode NCX (Figure 8D). Additionally, to strengthen the possibility that Ca\textsuperscript{2+} extrusion was enhanced following Ca\textsuperscript{2+} mobilization by IP3Rs, myocytes were voltage-clamped at -70 mV; Ca\textsuperscript{2+} transient was triggered by short depolarizing steps and caffeine puffs to monitor intracellular SR Ca\textsuperscript{2+} stores. In the presence of ATP or ET-1, this protocol resulted in a gradual decrease in Ca\textsuperscript{2+} transients (Figure 20 in the online-only Data Supplement), supporting the notion that Ca\textsuperscript{2+} leak occurs under conditions favoring IP3R function.

To test whether the mobilization of Ca\textsuperscript{2+} from the SR via IP3Rs was implicated in the prolongation of the AP, decreased RMP, and increased EADs, Ca\textsuperscript{2+} from the SR was depleted by inhibition of SERCA and exposure to caffeine.\textsuperscript{36,37} By this approach, ATP and ET-1 showed a markedly reduced effect on RMP, AP duration, and arrhythmia (Figure 21 in the online-only Data Supplement). Additionally, to establish the impact of increased Ca\textsuperscript{2+} load on the electrical instability of cardiomyocytes, these cells were studied under [Ca\textsuperscript{2+}], buffered conditions, to prevent changes in cytosolic Ca\textsuperscript{2+}.\textsuperscript{38} GPCR agonists had no effects on RMP and AP (Figure 8E).

Collectively, these observations indicate that Ca\textsuperscript{2+} mobilization from the SR via IP3Rs contributes to the alterations in the electrical properties of cardiomyocytes mediated by GPCR stimulation.

**Discussion**

The results of the current study provide a characterization of the electrical and mechanical
properties of human LV myocytes and emphasize the critical role that the GPCR/IP3R axis has in regulating Ca\(^{2+}\) homeostasis, contractile performance and the electrical stability of the human heart. IP3Rs are expressed and functional in human LV myocytes, and the Ca\(^{2+}\) mobilized from the SR by IP3Rs contributes to the decrease in RMP, prolongation of the AP, and to the occurrence of EADs and sustained depolarizations following GPCR stimulation. Ca\(^{2+}\) transient amplitude and cell shortening are enhanced, and extra-systolic and dysregulated Ca\(^{2+}\) elevations and contractions become apparent. These alterations in the electromechanical behavior of human cardiomyocytes are coupled with increased isometric twitch of the myocardium and arrhythmic events, indicating that the GPCR/IP3R effector pathway offers inotropic reserve, which is hampered by electrical instability and contractile abnormalities.

IP3Rs are present in hCSCs\(^8\) and are preserved in the formed myocyte progeny. However, the growth promoting effects of IP3Rs in hCSCs are lost in post-mitotic human cardiomyocytes.\(^{39}\) In both cases, IP3Rs modulate the release of Ca\(^{2+}\) from the SR, but its distal effect varies dramatically; the increase in cytosolic Ca\(^{2+}\) favors cell cycle reentry and asymmetric division of hCSCs,\(^8\) while, as shown here, the release of Ca\(^{2+}\) in adult human cardiomyocytes enhances cell contractile performance. Whether a link between these two distinct mechanisms actually exists is currently unknown, although IP3Rs have a critical independent biological function in the mother stem cell and the derived differentiated cardiomyocytes.

The contribution of IP3Rs to the electromechanical behavior of ventricular myocytes in small and large mammals is controversial.\(^{10,39}\) Our findings support the notion that in the human heart IP3Rs represent an important substrate able to increase myocyte contractility and the propensity for electrical instability coupled with GPCR agonists. Increases in the circulating levels of neurohumoral transmitters and cytokines physiologically and pathologically\(^{40,41}\) may
involve IP3R activation predisposing the heart to arrhythmic events. The increased incidence of arrhythmias and sudden death in patients with chronic heart failure\(^{42,43}\) may be related partly to upregulation of IP3Rs in the hypertrophied, dysfunctional cardiomyocytes.\(^{44,45}\)

The GPCR agonists ATP, ET-1, and angiotensin II stimulate IP3Rs in human cardiomyocytes, but other signaling pathways may be upregulated as well. These GPCR agonists activate PLC that generates not only IP3 but also DAG, which activates TRPCs and PKC isoforms.\(^{18,21}\) PKC phosphorylates ion channels and myofilament proteins, affecting the electrical and mechanical properties of cardiomyocytes.\(^{19-21}\) Additionally, ATP and ET-1 may enhance PLC-independent molecular events,\(^{21,22}\) which may trigger transmembrane ionic fluxes. Importantly, the effects of ATP and ET-1 on myocyte contractility were maintained in the presence of a PKC inhibitor. Similarly, the alterations in the electrical properties of cardiomyocytes were recapitulated by direct stimulation with IP3 or by enhancing IP3R affinity. Conversely, the effects of GPCR agonists were abrogated by: (a) downregulation of IP3Rs; (b) inhibition of IP3R function or reduced IP3 production; (c) depletion of \(\text{Ca}^{2+}\) from the SR; and (d) buffered intracellular \(\text{Ca}^{2+}\). Collectively, our data support the notion that the \(\text{Ca}^{2+}\) mobilized by IP3Rs is a critical determinant of the electrical abnormalities dictated by stimulation of GPCRs. However, we cannot exclude that DAG-sensitive and PLC-independent signaling pathways may participate in the electromechanical changes initiated by IP3R function. GPCR agonists modulate \(I_{\text{CaL}}\) and the delayed rectifier \(K^+\) currents, which can alter the AP profile.\(^{21,22}\) Moreover, changes in membrane potential may result from TRPCs and store-operated \(\text{Ca}^{2+}\) channels activated, respectively, by DAG and SR \(\text{Ca}^{2+}\) levels.\(^{18,46}\)

A significant issue concerned whether, following GPCR stimulation, \(\text{Ca}^{2+}\) mobilization from the SR via IP3Rs alters the electrical properties of myocytes directly, or indirectly by \(\text{Ca}^{2+}\)
release from RyR channels.\textsuperscript{10,11} The contribution of RyRs to the process was analyzed by combining Ca\textsuperscript{2+} imaging with electrophysiological measurements. Our findings indicate that IP3Rs contribute to the alteration of the AP, independently from an increase in sensitivity of the RyRs. Excitation contraction-coupling gain was preserved in the presence of GPCR agonists, documenting that RyR function was not enhanced by the changes in Ca\textsuperscript{2+}.

Despite inhibition of RyRs, cytosolic Ca\textsuperscript{2+}, NCX forward mode, and electrical abnormalities increased in LV myocytes following stimulation of the GPCR/IP3R axis. Collectively, the cascade of events initiated by GPCR ligands involves promotion of PLC enzymatic activity, IP3 production, opening of IP3R channels, and Ca\textsuperscript{2+} translocation from the SR to the cytoplasm. The latter favors the electrogenic extrusion of this cation via NCX, resulting in depolarization of the RMP and prolonged repolarization of the AP (Figure 22 in the online-only Data Supplement). These electrical changes positively impact on the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release since the delayed repolarization of the AP sustains Ca\textsuperscript{2+} influx, amplifying Ca\textsuperscript{2+} transients and cell shortening.\textsuperscript{25} By AP-clamp, remodeled APs, in the absence of GPCR ligands, elicit Ca\textsuperscript{2+} transients comparable to those associated with GPCR agonists. These findings indicate that the altered electrical activity has a critical role in the process, rather than suggesting a direct interaction between IP3R and RyR function. Moreover, the enhanced Ca\textsuperscript{2+} entry by the protracted AP may counteract the excess of Ca\textsuperscript{2+} extruded during diastole via NCX, restoring intracellular SR Ca\textsuperscript{2+} stores. This may account for the prolonged effects of IP3R in the presence of GPCR agonists. Conversely, with stable (time constrained) depolarizations in voltage-clamp, GPCR agonists are coupled with a progressive decrease in Ca\textsuperscript{2+} transient amplitude and SR Ca\textsuperscript{2+} load, emphasizing the importance that the changes in the AP profile have in mediating a sustained positive inotropic effect.
The extra-systolic elevations in Ca^{2+} and after-contractions detected in cardiomyocytes with GPCR agonists appear to be due to EADs, which may be the product of the alteration in the AP. In fact, changes in AP profile alone were sufficient to trigger alone extra-systolic Ca^{2+} elevations, pointing to the alterations in the electrical properties of cardiomyocytes as the primary mediator of Ca^{2+} cycling abnormalities. However, the ionic basis for the sustained membrane depolarization, protracted Ca^{2+} increases and myocyte contractures remains to be completely defined. IP3-independent effector pathways may participate in the process, although the cellular alterations were abrogated by attenuating IP3R function.

The spatiotemporal dynamics of Ca^{2+} mobilized by IP3Rs is dictated by the hierarchical recruitment of elementary Ca^{2+} release when [IP3] is increased; this condition originates Ca^{2+} blips, puffs, and global regenerative waves. In the absence of GPCR agonists, blockade of IP3R channels has little or no impact on the mechanical and electrical behavior of cardiomyocytes, indicating that extracellular activators of PLC are needed to increase [IP3], and trigger an IP3R response. As in other cell types, IP3R activation results in a time-dependent increase in cytosolic Ca^{2+} of cardiomyocytes that develops slowly over a number of seconds. With GPCR agonists, the temporal dynamic of IP3R-mediated Ca^{2+} release provides the basis for the sustained changes in the AP, cellular contractility, and incidence of electrical disorders. A similar sequence of events appears to be operative in failing human cardiomyocytes, in which IP3R transcripts and proteins are up-regulated, providing a potential etiology for arrhythmias and sudden death in this patient population.

Ca^{2+} release channels are differentially expressed during cardiac development and postnatally; in cardiomyocytes, the contribution of the ubiquitous IP3R to intracellular Ca^{2+} mobilization is progressively attenuated from the fetal to the neonatal and adult life, in favor of
the more specialized function of RyRs. Thus, the up-regulation of IP3Rs may be viewed as a component of a larger fetal reprogramming in the failing myocardium, together with the multiple pathological changes that characterize the progression of the disease state.

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**Conflict of Interest Disclosures:** None.

**References:**


Figure Legends:

Figure 1. IP3Rs in human and mouse cardiomyocytes. A, qRT-PCR show transcripts for the three IP3R subtypes in human and mouse LV myocytes. Representative curves and PCR products are shown together with the mRNA obtained from the human and mouse brain, that was used as positive control (red curves). The PCR products had the expected molecular weight. Human β2-microglobulin: housekeeping gene (see Figure 3 in the online-only Data Supplement).

MW: molecular weight. For sequences see Figure 4 in the online-only Data Supplement. B, Expression of IP3R protein by Western blotting in human and mouse LV myocytes with IP3R-2 and pan-IP3R antibodies, respectively. GAPDH: loading condition.

Figure 2. IP3Rs, Ca^{2+} cycling, and contractility in human cardiomyocytes. A, Sarcomere shortening in a field stimulated human cardiomyocyte, exposed first to ATP (10 μmol/L) and then to the IP3R blocker 2-APB (10 μmol/L). Higher time resolution traces are shown in the inset. B, Ca^{2+} transients (red-traces) and sarcomere shortening (black-traces) in a field stimulated
cardiomyocyte before (Tyrode) and after exposure to ET-1 (100 nmol/L). C, Quantitative data for Fluo-loaded cells are shown as mean±SEM. Tyr: Tyrode; XeC: xestospongin-C (2 μmol/L). *P<0.05 versus Tyr, †P<0.05 versus ET-1. D, After-contractions (arrowheads) in a LV myocyte exposed to ATP. E, Fraction of myocytes, 27%, with impaired relaxation (black portion of the bar). *P<0.05 versus Tyr. F, Ca²⁺ transients (red-traces) and sarcomere shortening (black-traces) in field stimulated cardiomyocyte exposed to ATP. An ectopic (arrows) and sustained Ca²⁺ elevation coupled with prolonged contraction is shown. G, Isometric tension in human LV trabeculae exposed to ATP (100 μmol/L) and then to the IP3R blocker 2-APB. H, Quantitative data are shown as mean±SEM. Base: baseline in the presence of Krebs-Henseleit buffer. *P<0.05 versus Base.

**Figure 3.** IP3Rs and electrical properties of human cardiomyocytes. A, APs (black-traces) and Ca²⁺ transients (red-traces) in a human myocyte exposed to ATP. In the lower panel, traces are shown at higher time resolution. B, AP (black-traces) and Ca²⁺ transients (red-traces) in a human myocyte before and after ET-1 exposure. C, EADs (black-traces) and Ca²⁺ transients (red-traces) of human myocytes with ATP, ET-1, and the IP3R-agonist, thimerosal (10 μmol/L). D, Quantitative data are shown as mean±SEM. *P<0.05 versus Tyr.

**Figure 4.** IP3Rs and electrical properties of human myocardium. A, ET-1 prolongs the electrical activation (pseudo-EKG, red-traces) and the AP (black-traces) of paced human myocardium. B, Quantitative data are shown as mean±SEM. Base: Krebs-Henseleit buffer. *P<0.05 versus Base. C, Premature-ventricular-contractions (PVCs; red-arrowheads) and triggered-activity (red-dashed-lines) in human myocardium with ET-1 and ATP (20 μmol/L).
Figure 5. IP3Rs and GPCR activation in the failing human myocardium. A, Expression of IP3R-2 protein by Western blotting in normal and failing human LV myocytes. GAPDH: loading condition. Quantitative data are shown as mean±SEM. *P<0.05 versus Normal Hearts. B, APs of a failing human myocyte before (black-trace, Tyr) and after (blue trace) exposure to ATP. Quantitative data are shown as mean±SEM. Tyr: Tyrode; *P<0.05 versus Tyr, using paired t-test. C, Cell shortening in a field stimulated human failing cardiomyocyte, before and following exposure to ATP. Arrow and arrowhead point to a premature contraction and altered relaxation, respectively. D and E, Quantitative data for isometrically twitching human LV trabeculae. Data are shown as mean±SEM. Iso: isoproterenol (100 nmol/L); Base: baseline in the presence of Krebs-Henseleit buffer. *P<0.05 versus Base, †P<0.05 versus Normal Heart in the presence of Iso.

Figure 6. Downregulation of IP3Rs, Ca²⁺ transients, and extra-systolic Ca²⁺ elevations. A, Left panel: EGFP-negative myocyte (arrowheads) and EGFP-positive myocyte (arrows); the EGFP-positive myocyte is shown again by native EGFP fluorescence in the right panel. These myocytes were collected from a mouse heart treated with AAV9 vector carrying EGFP and shRNA targeting IP3R-2. B, Ca²⁺ transients in EGFP-negative (black-traces) and EGFP-positive myocytes (green-traces) in the presence of ATP and ET-1. C, Fraction of myocytes with extra-systolic and sustained Ca²⁺ elevation (black portion of the bars). Quantitative data are shown as mean±SEM. *P<0.05 versus Tyr. †P<0.05 versus EGFP-negative myocytes. D, Electrical (black-traces) and Ca²⁺ transient (red-traces) properties of myocytes dialyzed with IP3 (50 μmol/L, phase-contrast micrograph). E, Quantitative data are shown as mean±SEM. *P<0.05 versus Base, using paired t-test.
Figure 7. The profile of the AP conditions the effects of GPCR activation on Ca\(^{2+}\) transients, and IP3R activation does not alter ryanodine receptor function. A and B, APs (V\(_{\text{membrane}}\): membrane potential, black-traces) and Ca\(^{2+}\) transients (Fluo-3, red-traces) in a mouse cardiomyocyte exposed to ET-1. APs with variable profiles were recorded in current-clamp mode (A) before (Ctrl-AP) and after activation of GPCRs (GPCR-AP) and used as voltage-clamp commands (V\(_{\text{command}}\), blue-traces) (B); Ca\(^{2+}\) transients (red-traces) and membrane currents (black-traces) are shown. C, I\(_{\text{Ca,L}}\) (black-traces) and Ca\(^{2+}\) transients (red-traces) elicited by depolarizing steps (blue-traces) by patch-clamp in the presence of ET-1 and ATP. D, Voltage relations for I\(_{\text{Ca,L}}\), Ca\(^{2+}\) transient amplitude and ECC gain in Tyr and after IP3R stimulation. E, Transmembrane potential (black traces) and cytosolic [Ca\(^{2+}\)]\(_{i}\) (red traces) in myocytes exposed to ryanodine before and after ATP stimulation. F, Effects of IP3R activation on the AP following blockade of RyRs (ryanodine, 10 \(\mu\)mol/L). Quantitative data are shown as mean±SEM. *P<0.05 versus ryanodine alone.

Figure 8. IP3Rs activate the NCX through changes in [Ca\(^{2+}\)]\(_{i}\). A, Transient and sustained inward currents (black-traces) and slowly developing diastolic [Ca\(^{2+}\)]\(_{i}\) elevations (red-traces) in mouse myocytes exposed to thimerosal. Membrane potential was held at -70 mV; short (5 ms) depolarizing steps to 0 mV were used to trigger Ca\(^{2+}\) transients. The Ca\(^{2+}\) fluorescent signal is reported at higher magnification in the lower traces. B, Transient and sustained inward currents (black-traces) and slowly developing [Ca\(^{2+}\)]\(_{i}\) elevations (red-traces) in mouse myocytes held at -70 mV following exposure to ATP and ryanodine. C, Slowly developing diastolic [Ca\(^{2+}\)]\(_{i}\) elevations (red-traces) in a mouse myocytes exposed to ATP and ryanodine in the presence of nickel (2.5 mmol/L), fail to induce transient and sustained inward currents (black-traces).
Membrane potential was held at -70 mV; short (5 ms) depolarizing steps to 0 mV were employed. D, Current voltage relations for the nickel (Ni)-sensitive current measured in voltage-clamp in the absence of IP3R stimulation and in the presence of ATP, ET-1, or IP3 dialysis (left). On the right, superimposed curves indicate that the Ni-sensitive current is enhanced following IP3R stimulation. To prevent RyR-Ca\(^{2+}\) release, ryanodine was employed. E, In the presence of buffered \([\text{Ca}^{2+}]_i\), ATP and ET-1 fail to alter the myocyte AP profile. Quantitative data are shown as mean±SEM.
Figure 1
Figure 2
Figure 2, cont’d
Figure 3
Figure 3, cont’d
Human Myocardium

Figure 4
Figure 5
Figure 6

Mouse Myocytes

(A) Micrographs showing control (Ctrl) and shRNA-IP3R2-EGFP transfected mouse myocytes. The arrow indicates a control myocyte, and the arrowhead indicates a transfected myocyte.

(B) Graphs showing the effects of ATP and ET-1 on Rhod-2 fluorescence in EGFP-negative and EGFP-positive myocytes. Ctrl indicates control, shRNA-IP3R2 indicates shRNA-IP3R2 transfected myocytes.

(C) Bar charts showing the transient amplitude of Ca²⁺ elevation and the percentage of cells with extra systolic Ca²⁺ elevation and sustained Ca²⁺ elevation after treatment with Tyr, ATP, and ET-1 in Ctrl and shRNA-IP3R2 transfected myocytes.
Figure 6, cont’d
Figure 7

Mouse Myocyte

A

Current-clamp

Tyrode

ET-1

V_{membrane}

Fluo

20 mV

200 ms

B

ET-1

Ctrl-AP

GPCR-AP

GPCR-AP

GPCR-AP

V_{command}

V_{clamp}

Fluo

0.3 F/F_0

100 pA

200 ms

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Figure 7, cont’d
Figure 7, cont’d
Mouse Myocytes

A

Thimerosal

Ionic currents

Fluo-3

Fluo-3

0.5 pA/F

0.1 pF/F

30 sec

B

Thimerosal

Ionic currents

ATP + ryanodine

ATP + ryanodine

0.5 pA/F

0.25 pA/F

30 sec

C

ATP + ryanodine + nickel

Ionic currents

Fluo-3

0.25 pA/F

0.1 pF/F

30 sec

Figure 8
Figure 8, cont’d
Inositol 1, 4, 5-Triphosphate Receptors and Human Left Ventricular Myocytes

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SUPPLEMENTAL MATERIAL

Methods

Mouse Hearts and Myocyte Isolation

Seventy female C57Bl/6 mice at 3 months of age were used in this study. Animals were maintained in accordance with the Guide for Care and Use of Laboratory Animals, and all animal experiments were approved by the local animal care committee (IACUC). Mice were anesthetized with pentobarbital (50 mg/kg body weight, i.p.), the heart was excised and placed on a stainless steel cannula for retrograde perfusion through the aorta. A protocol similar to that described for the isolation of human cells was utilized here as well. Isolated myocytes were suspended in Ca^{2+} 0.1 mmol/L solution.

FACS Analysis

Aliquots of human and mouse myocytes were fixed in 4% paraformaldehyde, washed in PBS and immunolabeled with antibodies specific for myocytes, fibroblasts, smooth muscle and endothelial cells, respectively (Table II). The cell preparation was filtered (40 μm), and samples were analyzed by flow-cytometry (FACSCantoTM II, BD Biosciences). Fluorophore-conjugated secondary antibodies were used as negative controls.

Cell Shortening and Ca^{2+} Transients

Isolated LV myocytes were placed in a bath on the stage of an Axiovert (Zeiss), IX51 and IX71 (Olympus) inverted microscopes for contractility, Ca^{2+} transients and patch-clamp measurements. Experiments were conducted at room temperature. Cells were bathed continuously with a Tyrode solution containing (mmol/L): NaCl 140, KCl 5.4, MgCl_2 1, HEPES 5, Glucose 5.5 and CaCl_2 1.0 (pH 7.4, adjusted with NaOH). Measurements were performed in field-stimulated cells by using IonOptix fluorescence and contractility systems (IonOptix) and by video edge detection (Crescent Electronics). Contractions were elicited by rectangular depolarizing pulses, 2 ms in duration, and twice-diastolic threshold in intensity, by platinum electrodes. Cell shortening was measured by edge-track detection system. Changes in mean sarcomere length were computed by determining the mean frequency of sarcomere spacing by fast Fourier transform, and then frequency data were converted to length. Ca^{2+}
transients were measured by epifluorescence after loading the myocytes with 10 µml/L Fluo-3 AM (Invitrogen). Excitation length was 480 nm with emission collected at 535 nm using a 40x oil objective. Fluo-3 signals were expressed as normalized fluorescence (F/F₀). Following treatment with IP3R modulators, some human myocytes were excluded from the analysis if prolonged contraction could not be discriminated from after-contraction during relaxation. To evaluate the impact of the Ca²⁺ sensitive dye on myocyte contraction, a comparison of the shortening properties between non-loaded and Fluo-loaded mouse myocytes was performed (Figure XXIII in the online only Data Supplement).

Modulators of IP3R function included Gα₉-protein coupled receptor agonists endothelin-1 (100 nmol/L, Sigma), ATP (10 µmol/L, Sigma), angiotensin II (50 nmol/L, Sigma), phenylephrine hydrochloride (10 µmol/L, Sigma).⁶⁻¹⁶ Additionally, thimerosal (10 µmol/L, Sigma) was utilized to enhance the affinity of IP3Rs to IP₃;¹³⁻¹⁶ IP3R function was inhibited by exposing myocytes to xestospongion-C (2 µmol/L, Sigma), 2-APB (2-aminoethyl diphenylborinate, 10 µmol/L, Sigma); phospholipase-C activity was attenuated with U-73122 (2 µmol/L, Sigma).⁹,¹²⁻¹⁴,¹⁶ PKC function was inhibited with chelerythrine (2 µmol/L, Sigma).¹⁷ RyR channels were blocked with ryanodine (10 µmol/L, MP Biomedicals).¹³,¹⁸

**Patch-Clamp Studies**

Data were acquired by means of the whole-cell patch-clamp technique in voltage- and current-clamp modes using Multiclamp 700A and 700B amplifiers (Molecular Devices). Electrical signals were digitized using 250 kHz 16-bit resolution A/D converters (Digidata 1322 and 1440A, Molecular Devices) and recorded using pCLAMP 9.0 and 10 software (Molecular Devices) with low-pass filtering at 2 kHz.¹⁻³,¹³,¹⁹

For measurements of the action potential (AP), current-clamp mode was employed. Cells were stimulated with current pulses 1.5 times threshold. Myocytes were bathed with Tyrode solution as described above. The composition of the pipette solution was (mmol/L): NaCl 10, KCl 113, MgCl₂ 0.5, K₃-ATP 5, glucose 5.5, HEPES 10 (pH 7.2 with KOH). The pipettes were pulled by means of a Narishige PB-7 glass microelectrode puller (Narishige, Tokyo, Japan) and when filled had a resistance of 1-2 MΩ.
In Fluo-3 loaded myocytes, the fluorescence signal intensity was collected using a photomultiplier, and a photon to voltage converter (IonOptix) connected to the patch-clamp A/D converter.\(^2\)

IP3 dialysis in myocytes was achieved by whole-cell configuration patch-clamp pipette, or by microinjection technique (FemtoJet, Eppendorf AG) in patch-clamped cells by dissolving 50 \(\mu\)mol/L D-myoinositol 1,4,5-triphosphate potassium salt (Sigma) in the pipette solution.\(^3,13,20\)

For \([\text{Ca}^{2+}]_i\), buffered conditions,\(^21\) the pipette solution contained 10 mmol/L EGTA and 5 mmol/L CaCl\(_2\), which results in \(~150\) nmol/L free \([\text{Ca}]_i\), calculated using Maxchelator program (http://www.stanford.edu/~cpatton/maxc.html). Membrane capacitance (\(C_m\)) was measured in voltage-clamp mode using a 5 mV voltage step and pCLAMP software algorithm.\(^1,3,13,19\) For action potential clamp (AP-clamp) assays, a set of AP waveforms were utilized as voltage-clamp commands in myocytes loaded with Fluo-3 AM.\(^2\) The 2 solutions were similar to those utilized in current-clamp experiments. In the experiments in which RyRs were blocked, myocytes were initially incubated with 10 \(\mu\)mol/L ryanodine and then superfused with the same solution. To deplete \([\text{Ca}^{2+}]_i\) from the SR, cells were incubated with 10 \(\mu\)mol/L thapsigargin and 10 mmol/L caffeine (Sigma), and subsequently superfused and dialyzed with 5 \(\mu\)mol/L thapsigargin.\(^22,24\) In these studies, myocytes were monitored for the absence of \([\text{Ca}^{2+}]_i\) transients and/or cell shortening. Treatment with IP3R agonist was performed after the acquisition of baseline data.

In excitation-contraction coupling (ECC) gain studies, \([\text{Ca}^{2+}]_i\) current and \([\text{Ca}^{2+}]_i\) transients were measured simultaneously in voltage-clamped myocytes.\(^2\) Cells were loaded with Fluo-3 AM and bathed with a modified Tyrode solution in which KCl was replaced with CsCl and 2 mmol/L 4-aminopyridine was added. The composition of the pipette solution was (mmol/L): NaCl 10, CsCl 113, MgCl\(_2\) 0.5, Tris-ATP 5, glucose 5.5, HEPES 10 and tetraethylammonium chloride 20 (pH 7.2 with CsOH). Cells were depolarized for 300 ms from holding potential (\(V_h\)) -40 mV to a family of test potentials ranging from -30 to +70 mV. Each voltage-clamp was preceded by a train of 4 conditioning pulses (100 ms, 1Hz) from -40 to 0 mV.\(^2\)
Contraction in patch-clamped myocytes was evaluated using ImageJ by reslice of the cell profile collected by videoclips and a CCD camera (Retiga EXi Fast 1394, QImaging).

The nickel sensitive current was determined as follows: myocytes were bathed with a modified Tyrode solution in which KCl was replaced with CsCl; 10 µmol/L ryanodine and 4 µmol/L nicardipine were then added.25 Pipette solution was identical to that used in ECC gain measurements. Following a 200 ms depolarization from -40 mV to +80 mV, a hyperpolarizing ramp 2 sec in duration was applied to reach -120 mV. The voltage-clamp protocol was utilized in the absence and presence of 2.5 mmol/L NiCl₂ in myocytes exposed to Tyrode, ATP, ET-1, or dialyzed by D-myo-IP3 salt. The nickel sensitive current was calculated as the difference between currents in the presence and absence of nickel.

To establish the time course of Ca²⁺ transients evoked by depolarizing steps and by caffeine, myocytes were bathed with Tyrode solution under voltage-clamp. Ca²⁺ transients were elicited by voltage steps 50 ms in duration from V_h – 70 mV to 0 mV, at 0.2 Hz. Every 2-4 min, a 20 mmoL caffeine puff 0.5 sec in duration was delivered using a microinjector (FemtoJet, Eppendorf AG). Cells were studied in the absence or presence of ATP or ET-1. Analysis of Ca²⁺ transients was not extended to portion of recordings in which large inward currents (~>1 nA), potentially reflecting store-operated Ca²⁺ entry, were observed.

**In Vivo AAV9-shRNA-IP3R2-EGFP Vector Delivery**

To compromise IP3R type-2 expression in myocytes, an AAV9 vector carrying transgenic small hairpin-RNA targeting IP3R type-2 and EGFP (AAV9shRNA-IP3R2-EGFP, Vector Biolabs) was administered in twelve mice by intraventricular injections of 80 µl of solution containing the vector at titer of 8.8x10¹¹ VG/ml resuspended in PBS. Administration was performed in anesthetized mice (1.5% isoflurane) using a 32G needle Hamilton syringe. Mice were sacrificed 3 weeks after.

Cardiomyocytes obtained from mice treated with AAV9-shRNA-IP3R2-EGFP vector were loaded with the Ca²⁺ indicator Rhod-2 (10 µmol/L; Invitrogen).³,¹³,¹⁶ Excitation length was 545 nm with emission collected at 605 nm using a 40x objective. Rhod-2 signals obtained from EGFP-positive and EGFP-negative myocytes were expressed as normalized fluorescence (F/F₀), where F₀ is the diastolic
fluorescent level subtracted by the background signal measured in the region adjacent to the cell. Mouse myocytes were loaded with Rhod-2, Fluo-4 (5 μmol/L; Invitrogen), or a combination of the two dyes to establish the difference in Ca\(^{2+}\) transient amplitude with these two Ca\(^{2+}\) indicators. Results are reported in Figure XXIV in the online only Data Supplement. Previous work documented that EGFP does not interfere with detection of Rhod-2, by emission spectra analysis and intracellular Ca\(^{2+}\) measurements.\(^{13}\)

Quantitative RT-PCR

Total RNA from isolated human and mouse LV myocytes, and mouse LV myocardium was extracted utilizing TRIZOL reagent (Invitrogen). cDNA was obtained from 1 μg total RNA in a 20 μl reaction containing Reverse Transcription buffer (Applied Biosystems), 4 mmol/L each of dNTP together with 50 U of MultiScribe reverse transcriptase (Applied Biosystems), 20 U of RNase inhibitor (Applied Biosystems) and 5 μmol/L of oligo-(dT)\(_{15}\) primer. This mixture was incubated at 37°C for 2 h. Subsequently, real-time RT-PCR was performed with primers designed using the Vector NTI Advance 11 (Invitrogen) software.\(^{2,3,11}\) Sequences of primers are reported in Table III. The 7300 Real Time PCR system (Applied Biosystems) was employed for quantitative RT-PCR. In each case, 1 μl of cDNA was combined with Power SYBR Green Master Mix (Applied Biosystems) in a 20 μl reaction. Cycling conditions were as follow: 95°C for 10 min followed by 40 cycles of amplification (95°C denaturation for 15 sec, 60°C annealing and extension for 1 min). The melting curve was then obtained. To avoid the influence of genomic contamination, forward and reverse primers for each gene were located in different exons. PCR products were run on 2% agarose/1x TBE gel to confirm the specificity of the reaction. Total RNA extracted from human and mouse brain (Clontech) was employed as controls.

Western Blotting

Whole cell protein extracts from cell pellets of isolated human or mouse myocytes were prepared using RIPA buffer (Sigma), supplemented with a cocktail of protease inhibitors (Roche) and phosphatase inhibitors (Sigma). Equivalent of 50 μg of proteins were separated on 4 to 20% SDS-PAGE, transferred onto PVDF membrane, blocked with 5% BSA and exposed to the following primary antibodies: rabbit polyclonal IP3R type-2 (Cell Signaling), rabbit polyclonal IP3R type I/II/III (Santa Cruz Biotechnology),
mouse GAPDH (Millipore) followed by horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology and Jackson ImmunoResearch Laboratories), and developed using Pierce SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Fisher Scientific). IP3R expression was normalized by GAPDH.

**Two-Photon Microscopy**

Myocytes were loaded with 10 µmol/L Fluo-3 AM (Invitrogen) and placed on the stage of a two-photon microscope (BX51WI Olympus microscope coupled with a Bio-Rad Radiance 2100MP system). Cells were bathed with a Tyrode solution, and field stimulated using platinum electrodes. Fluo-3 was excited at 900-960 nm wavelength with mode-locked Ti:sapphire femtosecond laser (Tsunami, Spectra-Physics), and the emission signal was collected at 535 nm. Images were acquired in line scan mode with a 2 ms sampling rate.\(^3\)\(^13\)

**Isometric Force in Human Trabeculae Carnea**

Thin LV trabeculae were dissected from the LV wall and mounted in a horizontal tissue bath (Steiert, Hugo Sachs Elektronik-Harvard Apparatus) connected to a force transducer (F10, Harvard Apparatus). Muscles were superfused with Krebs–Henseleit buffer (KHB; Sigma), containing in mmol/L: 118 NaCl, 4.7 KCl, 11 glucose, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 1.8 CaCl\(_2\) and 25 NaHCO\(_3\) gassed with 95% O\(_2\)/5% CO\(_2\) (pH 7.4) at 37°C. Tissue was stimulated by two platinum electrodes using field stimulation from an isolated stimulator output (frequency 0.5 Hz; impulse duration 2 ms; intensity 1.5-fold threshold). Each muscle was stretched to the length at which force of contraction was maximal. The developed tension was measured isometrically with the force transducer attached to a Bridge Amp (ADInstruments) and a 4 kHz A/D converter (Power Lab 4/30, ADInstruments). Contractile signal was recorded using LabChart 7 Pro software (ADInstruments). Muscle preparations were allowed to equilibrate for at least 60 min. The effect of ATP (100 µmol/L), endothelin-1 (100 nmol/L), and isoproterenol (100 nmol/L, Sigma) on developed tension, is expressed as fold change with respect to baseline values obtained in the absence of these agonists.

**Ex-vivo Electrical Properties of Human and Mouse Myocardium**
Human LV samples and mouse hearts were perfused, respectively, through a large branch of the left coronary artery and the aorta in a Langendorff apparatus at a constant pressure of 80 mm Hg with KHB solution. The temperature was maintained at 37°C by immersing the myocardial tissue in a water-heated glassware reservoir (Radnoti), containing preheated KHB. Human myocardium and mouse hearts were stimulated with a 2 ms square pulse at two-times its threshold level (4 channels stimulator, BMS 414, Crescent Electronics), using a mini-coaxial electrode (Harvard Apparatus). Myocardial electrical activity was monitored employing a two-lead mini ECG system (Harvard Apparatus) in which electrodes were placed on the endocardial and epicardial aspects of the human LV sample, or on the right atrium and apex of the mouse heart. Monophasic action potentials (MAPs) were recorded using a micro MAP-Tip electrode (Harvard Apparatus). ECG and MAP signals were amplified (Animal Bio Amp, ADInstruments), digitized using a 4 kHz A/D converter (Power Lab 8/30, ADInstruments) and recorded using LabChart 7 Pro software (ADInstruments) with low and high-pass filtering at 1 kHz and 0.3 Hz, respectively. A protocol of programmed electrical stimulation (PES) was used to assess the propensity of mouse hearts to develop ventricular arrhythmias. PES consisted of a train of pacing stimuli (S1) applied at 150 ms cycle length, with an extra stimulus (S2) inserted every eighth beat. The S1-S2 interval was progressively reduced until the S2 stimulus either failed to generate an action potential or induced arrhythmic events. The appearance of ventricular tachycardia (3 or more consecutive ectopic beats characterized by atria-ventricular dissociation) and/or ventricular fibrillation was established. ATP (20 µmol/L), ET-1 (100 nmol/L), and thimerosal (20 µmol/L) were used to promote IP3R function. In a subset of experiments, 10 µmol/L glibenclamide was employed to block I_KATP, a repolarizing current activated under ischemic conditions.

References


segregation to identify human cardiac stem cells that regenerate extensively the infarcted myocardium. Circ Res. 2012;111:894-906.


Supplemental Figures

Figure 1. Myocyte death. Myocyte apoptosis measured by TdT-labeling in control hearts obtained from 3 randomly selected donor hearts not used for transplantation and 3 age-matched control hearts collected at autopsy. Results are shown as mean±SEM.

Figure 2. Human and mouse cardiac cells. A, Myocytes, fibroblasts, smooth muscle cells and endothelial cells were identified by α-sarcomeric actinin (sc α-actinin, red dots), procollagen-1α (purple dots), α-smooth muscle actin (α-SMA, blue dots), and von Willebrand factor (vWF, human; green dots) or PECAM-1 (mouse, green dots), respectively. Black dots correspond to negative control. B, Fraction of cardiomyocytes (CMs), fibroblasts (Fbs), smooth muscle cells (SMCs), and endothelial cells (ECs) in human and mouse myocyte preparations. Results are shown as mean±SEM.

Figure 3. IP3Rs in human and mouse cardiomyocytes. mRNA level of each of the three IP3R subtypes with respect to the housekeeping gene, β2-microglobulin. Results are shown as mean±SEM.

Figure 4. Sequences of human and mouse transcripts. Nucleotide sequences were obtained in a sense and anti-sense direction to confirm the specificity of the amplified PCR products for both human (A) and mouse (B) genes.
Figure 5. Monophasic action potentials and I\textsubscript{\textsc{katp}} activation. Quantitative data for human LV tissue perfused in a Langendorff system indicate the minimal effects of the I\textsubscript{\textsc{katp}} blocker glibenclamide (10 $\mu$mol/L) on repolarization time. Data are shown as mean±SEM. Base: Krebs-Henseleit buffer.

Figure 6. IP3Rs, Ca\textsuperscript{2+} transients and contractility in mouse myocytes. A, Ca\textsuperscript{2+} transients (red-traces), sarcomere shortening (black-traces), extra-systolic Ca\textsuperscript{2+} elevations and after-contraction in a field stimulated myocyte in the presence of ET-1. B, Effects of ATP (10 $\mu$mol/L), angiotensin II (Ang II, 50 nmol/L), phenylephrine (Phe, 10 $\mu$mol/L), and thimerosal (Thi, 10 $\mu$mol/L) on extra-systolic Ca\textsuperscript{2+} elevations (red-traces) and after-contractions (black-traces) in myocytes. C, ET-1 and ATP induce spontaneous sustained Ca\textsuperscript{2+} elevations and contracture in myocytes. D, IP3R blockade with xestospongin-C (XeC, 2 $\mu$mol/L) prevents largely the effects of ET-1 on Ca\textsuperscript{2+} transients (red-traces) and myocyte contractility (black-traces). E, Quantitative data are shown as mean±SEM *$P<0.05$ versus Tyr. Tyr: Tyrode; Base: baseline prior to IP3R stimulation. F, Fraction of myocytes with extra-systolic and sustained Ca\textsuperscript{2+} elevations (black portion of the bars). *$P<0.05$ vs. Tyr, †$P<0.05$ versus ATP or ET-1.

Figure 7. Ca\textsuperscript{2+} changes in mouse myocytes. Normal Ca\textsuperscript{2+} transients (upper), extra-systolic (center), and sustained Ca\textsuperscript{2+} elevations (lower) are distributed uniformly throughout the length of stimulated myocytes. Line scan images were acquired by two-photon microscopy in myocytes loaded with Fluo-3 (green). Traces from the line-scan mode are shown below each image.

Figure 8. Inhibition of PKC does not abrogate the effects of GPCR activation on mouse myocytes. A, Quantitative data are shown as mean±SEM *$P<0.05$ versus Tyr or Base. Tyr: Tyrode; Base: baseline prior to IP3R stimulation; Che, chelerythrine (2 $\mu$mol/L). B, Fraction of myocytes with after-contractions and sustained contractures (black portion of the bars). *$P<0.05$ versus Tyr or Base.

Figure 9. Downregulation of IP3Rs. Transcripts for IP3R type-2 are downregulated in the LV myocardium of mice injected with an adeno-associated AAV9 vector carrying EGFP and small hairpin-RNA targeting IP3R type-2 (shRNA-IP3R2).

Figure 10. GPCR activation and Ca\textsuperscript{2+} transient in mouse myocytes. ATP and ET-1 induce sustained elevations of Ca\textsuperscript{2+} in EGFP-negative mouse myocytes with preserved expression of IP3R type 2. Cells
were obtained from the LV myocardium of mice injected with an adeno-associated AAV9 vector carrying EGFP and small hairpin-RNA targeting IP3R type-2 (shRNA-IP3R2); myocytes were loaded with the Ca\(^{2+}\) sensitive dye Rhod-2.

**Figure 11.** IP3R activation in mouse myocytes. **A,** Transmembrane potential (black-traces) and Ca\(^{2+}\) transients (red-traces) in current-clamped myocytes before and after exposure to ET-1 (upper traces), ATP (middle traces), and thimerosal (lower traces). IP3R stimulation results in prolongation of the AP and in EADs, which are coupled with increased Ca\(^{2+}\) transient and extra-systolic Ca\(^{2+}\) elevations. **B,** Quantitative data are shown as mean±SEM. *P*<0.05 versus Tyr. **C,** Fraction of myocytes with EADs (black portion of the bars). *P*<0.05 versus Tyr.

**Figure 12.** GPCR activation and myocyte function. **A,** Sustained membrane depolarization of the AP in human (upper black-trace) and mouse (lower black-trace) cardiomyocytes with ET-1 is associated with intracellular Ca\(^{2+}\) elevations (lower red trace). **B,** Fraction of mouse myocytes with sustained depolarizations (black portion of the bars). *P*<0.05 versus Tyr.

**Figure 13.** IP3R blockade in mouse myocytes. **A,** Electrical (black-traces) and Ca\(^{2+}\) transient (red-traces) properties of a mouse myocyte before and after blockade of IP3Rs with 2-APB (10 µmol/L). **B** and **C,** Quantitative data are shown as mean±SEM. *P*<0.05 versus Tyr.

**Figure 14.** Effects of activation and blockade of IP3Rs on mouse myocytes. **A,** Transmembrane potential (black-traces) and Ca\(^{2+}\) transients (red-traces) in a mouse myocyte exposed to ATP and then to the IP3R blocker 2-APB. Prolonged APs and sustained depolarizations coupled with the increase in Ca\(^{2+}\) transient amplitude are induced by ATP and are reversed by 2-APB. Circles indicate portions of the traces shown at higher time resolution in the inset. **B,** Transmembrane potential (black-traces) and Ca\(^{2+}\) transients (red-traces) in a mouse myocyte exposed to ET-1 and then to the IP3R blocker 2-APB. ET-1 prolonged the AP, and induced spontaneous depolarization and EADs coupled with increased Ca\(^{2+}\) transient amplitude; these effects were reversed by 2-APB. Circles indicate portions of the traces shown at higher time resolution in the inset.
Figure 15. IP3Rs alter the electrical activity of the mouse heart. A, IP3R activation and arrhythmia (red-dashed-lines) of the mouse heart with pacing (upper-traces), or programmed electrical stimulation (PES; lower-traces). B, Fraction of mouse hearts showing arrhythmic episodes (black portion of the bars). Ctrl: Krebs-Henseleit buffer. *P<0.05 versus Ctrl.

Figure 16. The profile of the AP conditions the effects of GPCR activation on Ca\(^{2+}\) transients. A and B, APs (\(V_{\text{membrane}}\): membrane potential, black-traces) and Ca\(^{2+}\) transients (Fluo-3, red-traces) in a mouse cardiomyocyte exposed to ATP. APs with variable profiles were recorded in current-clamp mode (A) before (Ctrl-AP) and after activation of GPCRs (GPCR-AP) and used as voltage-clamp commands (\(V_{\text{command}}\), blue-traces) (B); Ca\(^{2+}\) transients (red-traces) and membrane currents (black-traces) are shown.

Figure 17. The profile of the AP conditions the effects of GPCR activation on Ca\(^{2+}\) transients. APs (black-traces) and Ca\(^{2+}\) transients (red-traces) in a myocyte exposed to ET-1 (left); the recorded APs were employed as IP3R-APs command (blue-traces) in a myocyte (right) kept in Tyrode solution.

Figure 18. Effects of RyR blockade on Ca\(^{2+}\) cycling and contractility of mouse myocytes. A and B, Depolarizing steps in voltage-clamp mode (A and B, black traces) elicit Ca\(^{2+}\) transients (A, red traces) and cell shortening (B, reslice image) in a mouse myocyte in Tyrode solution. Long-lasting depolarizations to 0 mV (black traces, arrows) lead to Ca\(^{2+}\) elevations (A) and localized contractions in the form of waves (B). Higher time resolution traces and images are shown in the right panels. C and D, In the presence of ryanodine (10 \(\mu \text{mol/L}\)), depolarizing steps in voltage-clamp mode do not induce Ca\(^{2+}\) transients (C, red traces) and contractions (D, reslice image). Long-lasting depolarizations to 0 mV (arrows, black traces) generate small increases in intracellular Ca\(^{2+}\) (C) in the absence of waves of contraction (D, reslice image). Higher time resolution traces and images are shown in the right panels.

Figure 19. IP3R activation and arrhythmic events. Transmembrane potential (black-trace) and cytosolic Ca\(^{2+}\) (red-trace) in a current-clamped mouse myocyte exposed to thimerosal in the presence of ryanodine. Blockade of RyRs did not prevent spontaneous sustained depolarizations.

Figure 20. GPCR stimulation and SR Ca\(^{2+}\) release in voltage-clamped myocytes. A, Ca\(^{2+}\) transients elicited by depolarizing steps from -70 to 0 mV, and by caffeine (20 mmol/L) puffs (arrows) in mouse
myocytes in the absence (upper traces) and presence of ATP (lower traces). Δ indicates the time interval between traces displayed on the left and right panel. B, Changes in Ca$^{2+}$ transient amplitude over time for myocytes exposed to Tyrode (Tyr) or GPCR agonists (ATP or ET-1). Quantitative data are shown as mean±SEM. *P<0.05 versus Tyr, using paired t-test.

**Figure 21.** The effects of GPCR activation on mouse myocytes are abolished by depletion of Ca$^{2+}$ from the SR. A, Transmembrane potential in mouse myocytes exposed to ATP in control conditions (upper trace) or following depletion of SR Ca$^{2+}$ (lower trace). Thapsigargin (10 µmol/L), TG. B, Quantitative data are shown as mean±SEM. *P<0.05 versus Tyr. C, Transmembrane potential in mouse myocytes exposed to ET-1 in control conditions (upper trace) or following depletion of SR Ca$^{2+}$ (lower trace). Thapsigargin, TG. B, Quantitative data are shown as mean±SEM. *P<0.05 versus Tyr.

**Figure 22.** Excitation-contraction coupling and GPCR stimulation in myocytes. A, Schematic representation of the process of Ca$^{2+}$-induced Ca$^{2+}$ release under physiological conditions. During the AP (1), Ca$^{2+}$ enters the cell via L-type channels, triggering the opening of RyRs and release of Ca$^{2+}$ from the SR (2), which originates a cytosolic Ca$^{2+}$ transient (3). B, Signaling cascade initiated by GPCR activation. Ligand-GPCR interaction (1) promotes PLC enzymatic activity (2), leading to the formation of soluble IP3 (3). The latter triggers the opening of IP3Rs and Ca$^{2+}$ translocation from the SR to the cytoplasm (4). The increased cytoplasmic Ca$^{2+}$ load (5) induces the electrogenic extrusion of this cation (6); this phenomenon results in depolarization of the RMP, protracted repolarization of the AP, and the occurrence of EADs (7). The remodeled profile of the AP leads to a sustained Ca$^{2+}$ influx and potentiated Ca$^{2+}$ release via RyRs (8), enhancing Ca$^{2+}$ transients (9). Extra-systolic Ca$^{2+}$ elevations are coupled with EADs.

**Figure 23.** Sarcomere shortening in myocytes following loading with Fluo-4. Quantitative data are shown as mean±SEM. *P<0.05 versus Non-loaded Myocytes.

**Figure 24.** Ca$^{2+}$ indicators and Ca$^{2+}$ transients in mouse myocytes. A, Ca$^{2+}$ transient (black-traces) in a myocyte loaded with the Ca$^{2+}$ sensitive dyes Rhod-2 and Fluo-4; Ca$^{2+}$ transient was collected using the red fluorescent channel (emission wavelength 605 nm), and the green fluorescent channel (emission wavelength 535 nm). Background fluorescence was obtained in the region adjacent to the cell. Gray lines
indicate portions of the recording reported in panel B. **B**, Ca\(^{2+}\) transients obtained with the red and green fluorescent channels are normalized by their amplitude and partly superimposed. **C**, Quantitative data for cells loaded with Rhod-2 or Fluo-4 are shown as mean±SEM *P<0.05 versus Rhod-2.
Table 1. Magnitude of Sampling

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<td>ATP in the presence of</td>
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<td>LV mouse myocardium</td>
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<td>Control</td>
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<td>Ca(^{2+}) transients</td>
<td>Tyrode</td>
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Fig VIII

Fig IX

6C
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<tr>
<th>Mouse myocytes</th>
<th>Current-clamp, Ca(^{2+}) transients (paired analysis)</th>
<th>ATP</th>
<th>12</th>
<th>Fig XI-B,C and XII-B</th>
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<tr>
<td>Mouse myocytes</td>
<td>Current-clamp, Ca(^{2+}) transients (paired analysis)</td>
<td>D-my-o-Inositol 1,4,5-trisphosphate potassium salt (IP3) dyalisis</td>
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<td>Fig 6E</td>
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<td>2-APB</td>
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<td>Fig XIII-B</td>
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<td>Mouse myocytes</td>
<td>Cell shortening (paired analysis)</td>
<td>2-APB</td>
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<td>Fig XIII-C</td>
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<td>Perfused mouse heart</td>
<td>Monophasic action potential (MAP)</td>
<td>Control (Ctrl)</td>
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<td>Perfused mouse heart</td>
<td>PES protocol</td>
<td>Thimerosal (Thimer)</td>
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<td>Voltage-clamp (Ni-Tyr in the presence of ryanodine)</td>
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<td>7F</td>
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<td>Mouse myocytes</td>
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<td>10</td>
<td>8D</td>
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<td>Section</td>
<td>Description</td>
<td>Condition</td>
<td>Time</td>
<td>Figure</td>
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<td>Mouse myocytes</td>
<td>Voltage-clamp, Ca(^{2+}) transients (steps and caffeine puffs)</td>
<td>ATP</td>
<td>Tyrode, t=0</td>
<td>8</td>
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<td>ATP in the presence of ryanodine</td>
<td>Tyrode, t=7-10 min</td>
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<td>ATP/ET-1, t=0</td>
<td>ATP/ET-1, t=3-5 min</td>
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<td>ATP/ET-1, t=7-10 min</td>
<td>Mouse myocytes</td>
<td>Current-clamp (paired analysis)</td>
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<td>ATP in the presence of thapsigargin</td>
<td>ET-1</td>
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<td>ET-1 in the presence of thapsigargin</td>
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<tr>
<td>Mouse myocytes</td>
<td>Current-clamp, (buffered [Ca(^{2+})], paired analysis)</td>
<td>ATP</td>
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<td>ET-1</td>
<td>7</td>
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<td>Mouse myocytes</td>
<td>Sarcomere shortening</td>
<td>Non-loaded myocytes</td>
<td>27</td>
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<td>Fluo-loaded myocytes</td>
<td>13</td>
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Table 2. Antibodies for FACS Analysis

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<th>Antigen</th>
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<th>Manufacturer</th>
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<tr>
<td>sarcomeric α-actinin</td>
<td>mouse monoclonal</td>
<td>Sigma</td>
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<tr>
<td>α-smooth muscle actin</td>
<td>mouse monoclonal</td>
<td>Sigma</td>
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<tr>
<td>von Willebrand factor</td>
<td>sheep polyclonal</td>
<td>AbD Serotec</td>
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<td>PECAM-1</td>
<td>goat polyclonal</td>
<td>R&amp;D Systems</td>
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<td>procollagen 1α</td>
<td>goat polyclonal</td>
<td>Santa Cruz</td>
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Table 3. Primers for PCR Analysis

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<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>Human IP3R type-1</td>
<td>F: 5'- GAAGAGCACATCAAGGAAGAACAC -3'</td>
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<tr>
<td></td>
<td>R: 5'- TGCTGACCAATGACATGGCT -3'</td>
</tr>
<tr>
<td>Human IP3R type-2</td>
<td>F: 5'- TAGTCCTGGTGAAGTAAAGACCC -3'</td>
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<tr>
<td></td>
<td>R: 5'- CAGACTCATGGTCATTCCAACT -3'</td>
</tr>
<tr>
<td>Human IP3R type-3</td>
<td>F: 5'- TGTACTTCATTGTGCTGGTCCG -3'</td>
</tr>
<tr>
<td>Mouse IP3R type</td>
<td>F: 5' - primer sequence</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Mouse IP3R type-1</td>
<td>5' - GAGAGTTACGTGGCAGAGATGATC -3'</td>
</tr>
<tr>
<td>Mouse IP3R type-2</td>
<td>5' - TGTGGCATTACCTGTACTTCATCG -3'</td>
</tr>
<tr>
<td>Mouse IP3R type-3</td>
<td>5' - CTGGAGCATAACATGTGGAACTACC -3'</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

**Myocyte Apoptosis**

- Control Hearts (Autopsy)
- Donor Hearts

**Age**

- Control Hearts (Autopsy)
- Donor Hearts
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4

A human IP3R-1 sense

A human IP3R-1 antisense

A human IP3R-2 sense

A human IP3R-2 antisense

A human IP3R-3 sense

A human IP3R-3 antisense
Supplemental Figure 5

MAP Repolarization Times

- **KHB**
- **Glibenclamide**

<table>
<thead>
<tr>
<th></th>
<th>KHB</th>
<th>Glibenclamide</th>
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<td>50%</td>
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<tr>
<td>70%</td>
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<td>90%</td>
<td>[Bars]</td>
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</table>

mS
Supplemental Figure 6
Supplemental Figure 6

Mouse Myocytes

C  ET-1

Fluo-3
Sarcomere shortening

D  XeC  XeC+ET-1

Fluo-3
Sarcomere shortening
Supplemental Figure 6
Supplemental Figure 7
Supplemental Figure 8

A. Cell Shortening

B. Cells with Aftercontractions

Cells with Sustained Contracture
Supplemental Figure 9
Supplemental Figure 10
Supplemental Figure 11
**Supplemental Figure 12**

**A**

- **Human - ET-1**
- **Mouse - ET-1**
- **Fluo-3**

**B**

**Cells with Transient Depolarizations**

- **Tyr**
- **ATP**
- **Tyr - ET-1**
- **Tyr - Thimer**
Supplemental Figure 13

Panel A: Mouse Myocytes

Panel B: RMP and AP Repolarization Times

Panel C: Ca²⁺ Transient Amplitude and Cell Shortening
Supplemental Figure 14
Supplemental Figure 14
Supplemental Figure 15
Supplemental Figure 16
Supplemental Figure 17
Mouse Myocyte

Supplemental Figure 18
Supplemental Figure 19

Mouse Myocyte
Thimerosal + Ryanodine

Membrane potential

Fluo-3

0.2 F/F₀

4 sec

20 mV
Supplemental Figure 20

Mouse Myocytes

A

Tyrode

Δ = 7 min

4 sec

0.5 F/F₀

ATP

Δ = 8 min

4 sec

0.25 F/F₀

B

Ca²⁺ Transient Amplitude
(Depolarizing Step)

Ca²⁺ Transient Amplitude
(Caffeine Puff)

Fold Change

0.6 0.8 1.0 1.2

0 2 4 6 8 10 min

Fold Change

0.2 0.6 1.0 1.4

0 2 4 6 8 10 min

Tyr

ATP/ET-1

*
Supplemental Figure 21
Supplemental Figure 21

Mouse Myocytes

C

--- ET-1 ---

Membrane potential

20 sec 20 mV

--- TG ---

--- ET-1 ---

Membrane potential

20 sec 20 mV

D

RMP

mV

-70

-80

Tyre

ET-1

RMP

mV

-70

-80

Tyr

ET-1

Thapsigargin

AP Repolarization Times

% 50

100

0

50

100

0

10% 30% 50% 70% 90%

Tyre ET-1 Thapsigargin

Cells with EADs

% 50

100

0

100

0

10% 30% 50% 70% 90%

Tyre ET-1 Thapsigargin

Cells with Transient Depolarizations

---
Supplemental Figure 22
Supplemental Figure 23
Mouse Myocyte

Supplemental Figure 24

A

B

C

Ca^{2+} Transient Amplitude

Supplementary Figure 24