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Human G109E-Inhibitor-1 Impairs Cardiac Function and Promotes Arrhythmias

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Abstract

A hallmark of human and experimental heart failure is deficient sarcoplasmic reticulum (SR) Ca-uptake reflecting impaired contractile function. This is at least partially attributed to dephosphorylation of phospholamban by increased protein phosphatase 1 (PP1) activity. Indeed inhibition of PP1 by transgenic overexpression or gene-transfer of constitutively active inhibitor-1 improved Ca-cycling, preserved function and decreased fibrosis in small and large animal models of heart failure, suggesting that inhibitor-1 may represent a potential therapeutic target. We recently identified a novel human polymorphism (G109E) in the inhibitor-1 gene with a frequency of 7% in either normal or heart failure patients. Transgenic mice, harboring cardiac-specific expression of G109E inhibitor-1, exhibited decreases in contractility, Ca-kinetics and SR Ca-load. These depressive effects were relieved by isoproterenol stimulation. Furthermore, stress conditions (2 Hz +/− Iso) induced increases in Ca-sparks, Ca-waves (60% of G109E versus 20% in wild types) and after-contractions (76% of G109E versus 23% of wild types) in mutant cardiomyocytes. Similar findings were obtained by acute expression of the G109E variant in adult cardiomyocytes in the absence or presence of endogenous inhibitor-1. The underlying mechanisms

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included reduced binding of mutant inhibitor-1 to PP1, increased PP1 activity, and dephosphorylation of phospholamban at Ser16 and Thr17. However, phosphorylation of the ryanodine receptor at Ser2808 was not altered while phosphorylation at Ser2814 was increased, consistent with increased activation of Ca/calmodulin-dependent protein kinase II (CaMKII), promoting aberrant SR Ca-release. Parallel in vivo studies revealed that mutant mice developed ventricular ectopy and complex ventricular arrhythmias (including bigeminy, trigeminy and ventricular tachycardia), when challenged with isoproterenol. Inhibition of CaMKII activity by KN-93 prevented the increased propensity to arrhythmias. These findings suggest that the human G109E inhibitor-1 variant impairs SR Ca-cycling and promotes arrhythmogenesis under stress conditions, which may present an additional insult in the compromised function of heart failure carriers.

Keywords

heart failure; protein phosphatase 1; inhibitor-1; calcium cycling; cardiac function

1. INTRODUCTION

Heart failure remains the leading cause of cardiovascular disease and mortality worldwide. A major and universal characteristic of human and experimental heart failure is aberrant SR calcium handling, reflecting both impaired Ca-transport and Ca-release [1]. The depressed SR Ca-transport is associated with decreased levels of the Ca-ATPase (SERCA2a) pump along with increased inhibition by phospholamban (PLN) [2]. PLN is a prominent regulator of Ca cycling and a primary mediator of the β-adrenergic effects, resulting in enhanced cardiac output [3]. In the dephosphorylated state, PLN inhibits SERCA2a and shifts its Ca activation toward lower apparent Ca affinity. However, upon cAMP-dependent protein kinase A (PKA) mediated phosphorylation, the inhibition on SERCA2a by PLN is relieved [3]. In failing hearts, PLN is highly dephosphorylated due to down-regulation of β-adrenergic receptor signaling and increased activity of protein phosphatase 1 (PP1) [4, 5]. Indeed, cardiac overexpression of PP1 at levels similar to those observed in human failing hearts resulted in depressed function, remodeling, heart failure and early death [5, 6]. Accordingly, reduction of PP1 activity by shRNA knockdown improved diastolic function and halted remodeling in the MLP (muscle LIM protein) knockout model of heart failure [7].

The activity of PP1 is modulated by an endogenous regulatory protein, called inhibitor-1. Inhibitor-1 is a ~28 kDa protein (171 amino acid) and phosphorylation at threonine-35 (Thr35) by PKA enhances its ability to bind and inhibit PP1 [4]. Inhibition of PP1 results in increased phosphorylation of PLN and activation of SERCA2a [3, 4]. In heart failure, the expression levels and phosphorylation of inhibitor-1 at Thr35 are decreased, contributing to increased PP1 activity [6, 8]. The functional role of inhibitor-1 in the heart has been elucidated through generation and characterization of various genetically altered mouse models. Inhibitor-1 ablation was associated with increased PP1 activity, decreased PLN phosphorylation levels at serine 16 (Ser16) and threonine 17 (Thr17) and depressed cardiac contractility [6]. Accordingly, chronic or inducible overexpression of a truncated (AA: 1–

65) and constitutively active (T35D) form of inhibitor-1 (active inhibitor-1 or I-1c) was associated with inhibition of PP1 activity, increased Ser16 and Thr17 phosphorylation of PLN and enhanced cardiac function [9, 10]. In addition, I-1c protected from ischemic injury or attenuated hypertrophic response and delayed heart failure progression [9, 10]. These beneficial effects of I-1c were challenged by a report, which showed that I-1c induced cardiomyopathy upon aging and promoted catecholamine associated ventricular tachycardia [11]. However, further gene-delivery studies in failing hearts, using adeno-associated virus AAV9, indicated that long-term expression of I-1c maintains PLN phosphorylation, enhances contractility and attenuates remodeling [12]. Importantly, the effects of I-1c in the heart appeared to be mainly mediated by alterations in phosphorylation of PLN [12]. These findings have been further supported by studies in a larger animal model of heart failure, where intracoronary injection of AAV9.I-1c prevented further deterioration of cardiac function and led to a decrease in scar size [13]. Thus, the active inhibitor-1 appears to represent a new molecular inotrope that may be beneficial in alleviating the detrimental effects of heart failure.

These studies point to the overall importance of SR calcium cycling in the fine-tuned regulation of myocardial contraction-relaxation dynamics and suggest that defects at any point in the Ca cycling pathways may result in cardiac dysfunction and development of heart failure [3]. Indeed, human genetic alterations in key SR calcium-handling proteins, such as PLN, HRC (histidine-rich Ca binding protein), CSQ (calsequestrin) and RyR2 (ryanodine receptor type-2) have been shown to associate with impaired cardiomyocyte contractility and aberrant SR Ca-cycling, resulting in either arrhythmogenic heart disease or accelerated progression of heart failure [14–16]. Thus, given the key role of inhibitor-1 in heart function, the current study was designed to examine whether there are naturally occurring variants in the human gene, which may alter inhibitor-1 activity, impacting cardiac Ca-handling and function. Along these lines, we identified a substitution in exon 5 of human inhibitor-1, which results in a glutamic acid substitution for glycine at position 109 (G109E). Cardiac specific expression of this variant resulted in increased PP1 activity, diminished phosphorylation of PLN but increased phosphorylation of RyR2 at Ser2814, promoting diastolic SR Ca leak and arrhythmogenic responses under stress conditions. Similar findings were observed by acute expression of this variant in cardiomyocytes. These findings suggest that alterations in inhibitor-1 activity may have a greater impact than the expected alterations in SR calcium transport and promote aberrant overall calcium handling, leading to increased propensity to arrhythmias.

2. MATERIALS AND METHODS

The Material and Methods are detailed in the online data supplement.

2.1. Genetic Screening of Study Subjects

In an initial screen for inhibitor-1 variants [17], we identified the G109E variant in Exon 5 of the human protein phosphatase 1, regulatory (inhibitor) subunit 1A gene (PPP1R1A). Subsequently, we screened human DNA samples from 378 heart failure patients with dilated cardiomyopathy (DCM) enrolled in the University Hospital and Cincinnati Heart Failure/ Transplant Program, and 209 normal controls. The study protocols were approved by the

Institutional Review Board of the University of Cincinnati. There were no human heart samples available for further cellular or biochemical studies.

2.2. Generation and Characterization of Transgenic Mice

We generated transgenic mice in the FVB/N background [18] with cardiac specific expression of the human G109E inhibitor-1. Ten to twelve week-old male mice were used for all studies. Handling and maintenance of animals was approved by the ethics committee of the University of Cincinnati. Binding properties of inhibitor-1 to PP1 were determined using recombinant proteins [19]. Cardiomyocyte experiments and Ca sparks were recorded as previously described [15]. Diastolic SR Ca-leak was measured using the tetracaine protocol [20]. After-contractions and *in vivo* isoproterenol-induced arrhythmias were recorded as we reported [15].

2.3 Biochemical Assays

Hearts were homogenized in buffer containing protease and phosphatase inhibitors. CaMKII activity was assayed, using the CycLex Kit (MBL International, Woburn, MA). PP1 activity was assessed, using RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit (Molecular Probes, Inc., Eugene, OR). The generation of the full length Maltose Binding Protein (MBP)-PP1 (aa 1–330) construct and expression of glutathione S-transferase (GST) and MBP fusion proteins as well as protein binding between PP1 and inhibitor-1 wild type (WT) or G109E variant were performed as we previously described [19].

3. RESULTS

3.1. Identification of Human G109E Inhibitor-1

We identified a genetic polymorphism in human inhibitor-1, which results in substitution of glycine by glutamic acid at position 109 (G109E). The incidence of G109E was ~7% in either normal or dilated cardiomyopathy patients, although this variant was primarily identified in the black population (Supplemental Table 1). All carriers were heterozygous with the exception of one homozygous individual and there were no apparent clinical characteristics associated with the G109E variant in dilated cardiomyopathy patients.

3.2. Functional Assessment of G109E Variant in the Heart

To examine the effects of human G109E-inhibitor-1 variant in cardiac function, we generated transgenic (TG) mice with cardiac-specific overexpression (2.5 fold) of mutant inhibitor-1 (Fig. 1A). Inhibitor-1 is a well-recognized regulator of protein phosphatase 1 activity resulting in attenuation of enzymatic activity especially in its PKA-phosphorylated form. However, the G109E variant resulted in significant increases in cardiac PP1 activity, compared to WT controls (Fig. 1B). To determine whether the increased PP1 activity was associated with alterations in the binding of G109E inhibitor-1 to this enzyme, we generated GST-inhibitor-1-WT and GST-inhibitor-1-G109E recombinant proteins (Fig. 1C) and evaluated their interaction with MBP-PP1, using blot overlay assays. Western blot analysis determined that, in comparison to WT, G109E exhibited significantly reduced PP1 binding (Fig. 1D; n=4, $P<0.05$). Thus, the G109E variant exhibits diminished binding to PP1, resulting in increased PP1 activity.

Further functional analysis of the TG hearts indicated that G109E depressed fractional shortening (28%) and rates of contraction (28%) and relaxation (23%), compared to WT cardiomyocytes (Figs. 2A, B, C, and D). However, β-adrenergic stimulation (isoproterenol; 100nM) resulted in enhancement of contractile parameters in both WT and G109E cells and the maximally stimulated parameters were similar between the two groups (Figs. 2A, B, C, and D). Consistent with the contractile parameters, analysis of Ca transients demonstrated that the Ca amplitude (Fura-2 ratio, 340/380 nm) was decreased by 20% (Figs. 3A and B) and the time constant for Ca-decay (Tau) was prolonged by 21% in G109E cells, compared to WTs (Fig. 3C). The depressed Ca-kinetics were associated with a small but significant increase in diastolic Ca in G109E cells (Fig. 3D). Isoproterenol stimulation increased the Ca kinetics and the maximally stimulated parameters were not different between WT and G109E cardiomyocytes (Figs. 3B, C and D).

3.3. Reduced SR Ca Content but Unchanged Sodium-Ca-Exchanger Activity

Since the Ca transient amplitude depends on SR Ca load, we next measured SR Ca content in ventricular myocytes (Fig. 3E). The amplitude of the caffeine-induced Ca release was decreased by 27%, which indicates lower SR Ca content in G109E cells under basal conditions (Fig. 3F). Isoproterenol stimulation normalized the SR Ca-load, which was similar between WT and mutant cardiomyocytes (Fig. 3F). There were no alterations in sodium-Ca-exchanger (NCX) function, assessed by the time to 50% decay of the caffeine induced Ca transient peak (T50), in G109E cardiomyocytes compared to WTs under basal or isoproterenol stimulation conditions (Fig. 3G). These results suggest that a decrease in SR Ca load accounts for the reduction in systolic $[Ca^{2+}]i$ transients and the associated lower contraction observed in G109E myocytes.

3.4. G109E-Inhibitor-1 Is Associated with Dephosphorylation of Phospholamban

The depressed contractile parameters and Ca-kinetics in TG hearts suggested alterations in the phosphorylation of key phosphoproteins by increased PP1 activity. Under basal conditions, phosphorylation of PLN at Ser16 and Thr17 was significantly decreased in mutant hearts, compared to WTs, assessed by quantitative immunoblotting (Figs. 4A and B). However, the phosphorylation of RyR2 was not altered at Ser2808, while it was increased at Ser2814 (Figs. 4A and B), suggesting sub-compartment alterations of PP1 activity in SR. This apparent specificity of inhibitor-1 for PP1/PLN, prompted us to carry out additional studies with recombinant proteins and immunoprecipitations. Pull down assays, using recombinant proteins in mouse cardiac homogenates (Supplemental Fig. 1A), showed that PP1 binds mainly to PLN and to a much lower extent to RyR2 (Supplemental Fig. 1B and 1C). These findings were supported by pull-down assays using GST and GST-G_M (PPP1R3A), the subunit that targets PP1 to PLN (Supplemental Fig. 1D), which demonstrated the interaction of G_M with PLN but lack of binding to RyR2 (Supplemental Fig. 1E). Furthermore, the potential preferential association between PLN and PP1 was examined by co-immunoprecipitation of PP1 with the SR phosphoregulatory proteins. In agreement with the pull down assays, the results indicated a preferential association between PP1 and PLN rather than RyR2 (Supplemental Fig. 1F). These findings are similar to those previously reported by Aoyama et al [21] and Miyazaki et al [7].

Interestingly, upon Iso stimulation, the phosphorylation levels of PLN at Ser16, and Thr17 as well as RyR2 at Ser2808 increased in G109E hearts, reaching similar levels to those in stimulated WT hearts. However, phosphorylation of RyR2 at Ser2814 remained higher in G109E hearts (Figs. 4A and B). The increased phosphorylation of Ser2814 in RyR2 prompted us to examine the activity of CaMKII. Since activation of CaMKII results in its auto-phosphorylation, we assessed the degree of Thr286 phosphorylation. Indeed, Thr286 was hyperphosphorylated in G109E hearts under basal conditions (Fig. 4C) and this reflected increased CaMKII activity (Fig 4D). Importantly, isoproterenol stimulation was associated with further increases in CaMKII activity of G109E hearts (Fig. 4D), consistent with elevated Ser2814 phosphorylation of RyR2 (Figs. 4A and B).

Furthermore, we determined the phosphorylation status of Ser23/24 in troponin I (TnI) and Ser282 in myosin binding protein C (MyBPC). There were no alterations by G109E (Supplemental Figs. 2 A and B), consistent with previous reports on localization of PP1 [19] and inhibitor-1 mainly in the cardiac SR-microsomal fraction. In addition, assessment of L-type Ca-channel (LTCC) activity in G109E mouse cardiomyocytes, using whole-cell voltage clamp, indicated that the Ca currents in response to a series of depolarizing steps from a holding potential of -50 mV (Supplemental Fig. 3A) and the average Ca current-voltage relationship (Supplemental Fig. 3B) were significantly increased in G109E, compared to WT ventricular cells under both basal and Iso stimulation conditions. These increases were not associated with any alterations in Cav1.2 protein levels (data not shown). Thus, increased LTCC current density and consequently the Ca influx through this channel may compensate for the increased SR Ca leak in an attempt to maintain SR Ca load in G109E cells.

3.5. Increased Ca Sparks, Waves, and Diastolic SR Ca leak in G109E Cardiomyocytes

Previous studies have shown that increased phosphorylation of Ser2814 in RyR2 enhances SR Ca leak [22], which may serve as a molecular trigger for arrhythmias. To determine the functional effect of increased RyR2 phosphorylation by G109E, we examined the frequency and properties of Ca sparks in cardiomyocytes with or without 100 nM isoproterenol (Fig. 5). Ca-spark frequency was significantly higher in G109E cardiomyocytes with respect to WT cardiomyocytes in either the absence or presence of isoproterenol (Fig. 5A). However, spark amplitude, full-width-at-half maximal amplitude (FWHM) and full-duration-at-half-maximal amplitude (FDHM) were not altered (Figs. 5B, C and D). Moreover, the SR Ca leak (Ca spark frequency × amplitude × duration × width) was increased in the G109E compared with the WT group (Fig. 5E). In addition, the inducibility of Ca waves was examined in G109E myocytes, in the absence and presence of isoproterenol (1 μM). In the absence of isoproterenol, no cardiomyocytes showed Ca waves (data not shown). However, under isoproterenol stimulation, Ca waves were developed in 60% of G109E cardiomyocytes, compared with 20% of WT cells (Figs. 5F and G). Accordingly, G109E cells exhibited increases in spontaneous after-contractions (76% of G109E cells within 5 seconds after pacing was stopped, compared with 23% of WT cells; Fig. 5H). Taken together, these findings suggest that human G109E inhibitor-1 enhances the propensity for spontaneous Ca release from the SR, resulting in increased susceptibility to arrhythmia.

Since the abnormal spontaneous Ca release events in G109E cells led to RyR2-mediated leak partly as Ca sparks, the total diastolic SR Ca leak was then measured, using the tetracaine protocol in the absence and presence of 100 nM isoproterenol (Fig. 6). The SR Ca leak was significantly higher in G109E cardiomyocytes compared with WT controls under basal conditions and this leak was further increased under isoproterenol stimulation (Figs. 6A, B, C, D, and E). Wild-type cells did not exhibit an increase in SR Ca-leak by isoproterenol stimulation, suggesting that their SR Ca load was below the required necessary threshold [23] under our experimental conditions. Furthermore, the ratio of SR Ca leak to SR Ca load was significantly greater in G109E compared with WT cardiomyocytes under both basal and Iso-stimulation conditions (Fig. 6F), suggesting that G109E enhances the spontaneous Ca release from the SR via RyR2. However, the increased SR Ca-leak was overcome under Iso and SR Ca-content was normalized in G109E cells (Fig. 3F). This may be attributed to: a) PKA activation; b) phosphorylation of PLN in G109E to similar levels as stimulated WTs (Figs. 4A and 4B); and c) activation of SERCA2a to eliminate increases in diastolic Ca levels, as evidenced in Fig. 3D.

3.6. G109E Mice Have Increased Incidence of Ventricular Ectopy upon Catecholamine Challenge

Since the human inhibitor-1 genetic variant (G109E) was associated with after-contractions in cardiomyocytes under stress conditions, we subjected the G109E mice to catecholaminergic stress to determine whether that would induce cardiac arrhythmias *in vivo.* Thus, the surface electrocardiogram (ECG) was monitored under basal and intraperitoneal injection of caffeine and isoproterenol (Figs. 7A, D, G). ECG tracings showed no arrhythmogenic events in either WT (data not shown) or G109E (Figs. 7B, E, H) mice under basal conditions. However, caffeine and isoproterenol combination caused arrhythmias (Fig. 7A), including frequent premature ventricular complexes (PVCs) in the form of bigeminy and trigeminy and bidirectional ventricular tachycardia (VT) in G109E mice under stress conditions (Fig. 7C).

Furthermore, to determine whether increased CaMKII activity in G109E mice contributed to the cardiac arrhythmias *in vivo*, TG and WT mice were subjected to KN-93, the selective CaMKII inhibitor, prior to induction of stress conditions. In parallel experiments, the inactive analogue KN-92 was used as control. Interestingly, while *in vivo* application of KN-92 had no effect on prevention of Iso-dependent arrhythmias (Figs. 7D and F), KN-93 completely prevented the arrhythmias in G109E mice (Figs. 7GI, and K). Thus, CaMKII inhibition in G109E hearts reduced cardiac arrhythmias *in vivo,* suggesting that increased CaMKII activity may contribute to the arrhythmias associated with the G109E mutation.

To further confirm the prominent role of CaMKII in promoting arrhythmias in G109E hearts, we measured the levels of reactive oxygen species (ROS) in cardiomyocytes since there is evidence that leakiness of RyR2 may be also attributed to its protein oxidation by elevated intracellular oxidative stress. We used a general oxidative stress florescent indicator, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and observed no significant differences between G109E and WTs in ROS-positive cardiomyocytes (36.11% ± 4.51% in G109E, versus 40.27% ± 3.76% in WT) under basal conditions, suggesting that

intracellular ROS levels may not contribute to the leakiness of RyR2 and arrhythmogenesis in G109E mice (supplemental Fig. 4). As a positive control, cardiomyocytes were exposed to H_2O_2 to maximally increase intracellular oxidative stress, which resulted in a complete shift to ROS-positive cells in both groups.

3.7. Acute Expression of G109E Inhibitor-1 in Cardiomyocytes Depresses Function and Elicits After-Contractions

Since the observed effects of G109E *in vivo* may be associated with potential compensatory or aberrant responses of chronic expression in the heart, we acutely expressed this variant in adult rat cardiomyocytes and determined its functional role. Infection with either Ad.WT-inhibitor-1 or Ad.G109E-inhibitor-1 mutant resulted in similar increases in inhibitor-1 expression levels, compared to Ad.GFP (GFP: green fluorescent protein). Expression of WT-inhibitor-1 did not alter contractile or Ca-kinetic parameters in agreement with previous findings [24]. However, expression of G109E elicited decreases in contractile parameters (Figs. 8A, B and C), Ca-kinetics (Figs. 8D, E) and SR Ca-load (Fig. 8F). Furthermore, G109E induced aftercontractions under identical stress conditions as in transgenic cardiomyocytes above (data not shown). Similar findings were obtained by adenoviral expression of G109E inhibitor-1 in cardiomyocytes from I-1 knockout mice or in the absence of endogenous inhibitor-1. Controls included infected KO cardiomyocytes with similar levels of WT inhibitor-1 as the mutant (Supplemental Figs. 5A, B, C and D). These findings further support the adverse effects of G109E on Ca-cycling especially under stress conditions.

4. DISCUSSION

This is the first study to identify a human variant in the inhibitor-1 gene, entailing substitution of glycine by glutamic acid at position 109 (G109E), which is associated with increased PP1 activity, impaired SR Ca-cycling and increased propensity to arrhythmias. The findings indicate that a primary defect in SR Ca-transport may elicit aberrant SR Ca-release even in the face of reduced SR Ca-load. The functional role of this mutant inhibitor-1 was elucidated *in vivo* by cardiac-specific overexpression and *ex vivo* by acute expression through adenoviral infection of adult cardiomyocytes. G109E inhibitor-1 elicited decreases in contractile and Ca-kinetic parameters as well as diminished SR Ca-load under basal conditions, while stress conditions promoted aberrant SR Ca-release. Furthermore, this substitution and the increased PP1 activity resulted in specific dephosphorylation of PLN at both Ser16 and Thr17, while there were no alterations in PKA phosphorylation levels of Ser2808 in RyR2, Ser23/24 in TnI and Ser282 in MyBPC. The depressed SR Ca re-sequestration by dephosphorylated PLN elicited elevation of diastolic Ca, activation of CaMKII and hyper-phosphorylation of RyR2 at Ser2814, leading to increased Ca-leak and after-contractions. In addition, *in vivo* studies revealed that mutant mice developed complex ventricular arrhythmias and ventricular ectopy under stress conditions. The apparent arrhythmia episodes elicited by G109E were prevented by inhibition of CaMKII activity through KN-93 treatment, while KN-92 had no effect.

Increases in PP1 activity, identified in failing hearts, have been suggested to promote dephosphorylation of important regulatory phosphoproteins, leading to overall decreased

function [4, 25], similar to mutant G109E hearts. The mechanisms underlying regulation of Ca cycling by G109E appear to involve its reduced binding to PP1 and diminished inhibition of enzymatic activity, impacting phosphorylation of PLN and SERCA2a function. This apparent specificity of G109E for PP1/PLN may be conferred by the regulatory subunits of the enzyme, that direct binding of its catalytic subunit (PP1c) to different cellular targets [26]. Specifically, the PP1 anchoring subunit is Gm (PPP1R3A) for PLN and spinophilin (PPP1R9B) for RyR2 in the heart [27–29]. The local amount of PP1 with PLN or RyR2 is likely to depend on the amount/expression levels of these targeting subunits, efficiency in their binding to PP1 as well as their efficiency in targeting PP1 to its substrate. We have observed a similar “preferred regulation” of PP1/PLN by Hsp20 [19]. Furthermore, human and experimental failing hearts, which exhibit 2.5-fold increases in PP1 activity [5], show dephosphorylation of only PLN and not RyR2 [29–34]. In addition, RyR2 is dephosphorylated by protein phosphatase 2a (PP2a), which associates to the channel through its regulatory subunit PR130 (PPP2R3A) [29]. Accordingly, studies in failing hearts have shown that PLN is hypo-phosphorylated whereas RyR2 is hyper-phosphorylated [35], suggesting sub-compartment specific alterations in protein phosphatase activity. Similar to these observations, the G109E hearts exhibited increased phosphorylation of RyR2 at Ser2814, despite increased PP1 activity and dephosphorylation of PLN. Collectively these findings suggest that, analogous to the human failing heart, G109E may alter PP1 activity in specific sub-cellular micro-domains.

The increased Ser2814-RyR2 phosphorylation in G109E hearts was associated with activation of CaMKII and reflected enhanced SR Ca leak. Importantly, the G109E cardiomyocytes exhibited after-contractions, when paced in the presence of β-adrenergic stimulation similar to the occurrence of cardiac arrhythmias *in vivo* under stress conditions. These findings in the G109E mouse are similar to previous studies, which have demonstrated that human genetic defects in other SR Ca cycling genes are linked to catecholaminergic arrhythmias [15–16]. Therefore, this variant in the inhibitor-1 gene may act in a similar manner and the underlying mechanisms involve impaired activity or dysregulation of the RyR2 channel [1, 36–37].

Interestingly, the enhanced RyR2 mediated SR Ca leak in G109E myocytes occurred in the face of a significant decrease in SR Ca content, indicating that stimulated release was not merely a consequence of increased SR Ca load. Recent studies have shown that alterations in the properties of RyR2 such as more frequent opening, causing Ca-leak during diastole, may result in Ca-waves [37]. However, at least in the steady state, Ca waves occur only in the combined presence of leaky RyRs and β-adrenergic stimulation. Thus, G109E-inhibitor-1 carriers or mice may have leaky RyRs throughout life but develop arrhythmias only during stress conditions.

Previous reports have linked either protein kinase A (Ser2808) or CaMKII (Ser2814) phosphorylation of RyR2 to increased SR Ca leak [23, 38–40] although most findings have associated only the CaMKII dependent phosphorylation with diastolic SR Ca leak in heart failure [32, 40–41,]. In our study, the alterations of RyR2 function and SR Ca release in G109E myocytes reflected only increased phosphorylation of RyR2 at the CaMKII site, Ser2814, without any alterations at the PKA site, Ser2808. These observations indicate that

the increases in diastolic Ca in G109E cardiomyocytes through increased inhibition of SERCA2a by dephosphorylated PLN may trigger activation of CaMKII and subsequent phosphorylation of RyR2, leading to increased SR Ca leak and arrhythmia. Furthermore, *in vivo* inhibition of CaMKII by KN-93 resulted in blockage of arrhythmias, elicited by β-adrenergic stimulation, confirming the role of CaMKII in the observed arrhythmias by G109E. Thus, altered interaction of G109E-inhibitor-1 with PP1 and dephosphorylation of PLN, resulting in amplified CaMKII phosphorylation of RyR2 and increased Ca leak, may constitute the underlying mechanisms for increased propensity of ventricular arrhythmias *in vivo*.

However, it should be pointed out that there are several potential limitations in extrapolating the current mouse findings to humans. In this study, the functional role of the human G109E variant was studied using an overexpression strategy in either isolated cardiomyocytes or *in vivo*, while a gene-targeted knock-in approach would have provided a more elegant setting to examine the G109E significance. Another limitation is that these findings in mouse hearts may not reflect similar alterations in large animal or human hearts as there are several species differences, including myosin heavy chain isoforms, Ca-cycling properties and heart rate.

Nevertheless, the current study has provided significant insights into the mechanisms associated with the naturally occurring G109E genetic variant in inhibitor-1 and showed that it does not only depress SR Ca cycling and contractility due to enhanced PLN/SERCA2a inhibition but also increases SR Ca leak during diastole due to aberrant RyR2 function. The underlying mechanisms include: a) decreased binding of G109E to PP1; b) reduced inhibition of PP1; c) dephosphorylation of PLN and increased inhibition of SERCA; d) decreased SR Ca-cycling and contractility; e) increased diastolic Ca-levels and activation of CaMKII; and f) increased Ser2814 phosphorylation of RyR2 (Supplemental Fig. 6). Hyperphosphorylation of RyR2 resulted in increased diastolic Ca-leak, triggering after-contractions under stress conditions and predisposed to increased ventricular arrhythmia. Thus, G109E carriers, who may be at risk for malignant ventricular arrhythmias and end-stage heart failure, may potentially benefit from inhibitor-1 based therapy, especially in light of recent promising findings in pre-clinical studies using I-1c gene delivery to restore function in failing hearts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Naturally occurring human G109E inhibitor-1 (I-1) impairs cardiac function.
- G109E I-1 exhibits diminished PP1inhibition, resulting in PLN dephosphorylation.
- Depressed SR Ca uptake increases diastolic Ca, CaMKII activity and pSer2814 in RyR2.
- Hyperphosphorylated RyR2 increases the frequency of Ca-sparks and Ca-waves.
- G109E I-1 leads to aftercontractions and arrhythmias under stress conditions.

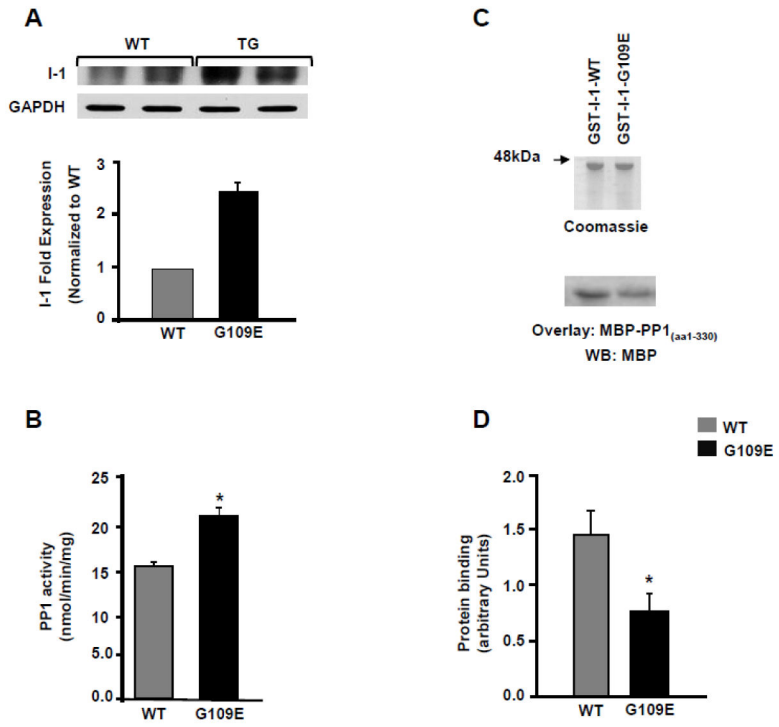

Figure 1. Assessment of inhibitor-1 expression levels and PP1 activity in G109E transgenic hearts and interaction of inhibitor-1 with protein phosphatase 1

A) Transgenic mice with cardiac specific overexpression of G109E were generated and the expression levels of inhibitor-1 (I-1) were assessed by quantitative immunoblotting. B) Assessment of protein phosphatase 1 (PP1) activity in WT and G109E transgenic mouse cardiac homogenates. C) Determination of I-1 and PP1 interaction. Upper panel: coomassie blue stained gels of purified I-1 recombinant proteins; lower panel: PP1 protein binding to WT-I-1and G109E-I-1; D) Quantification of I-1 binding to PP1. n=4; *P ≤ 0.05 vs WT-I-1.

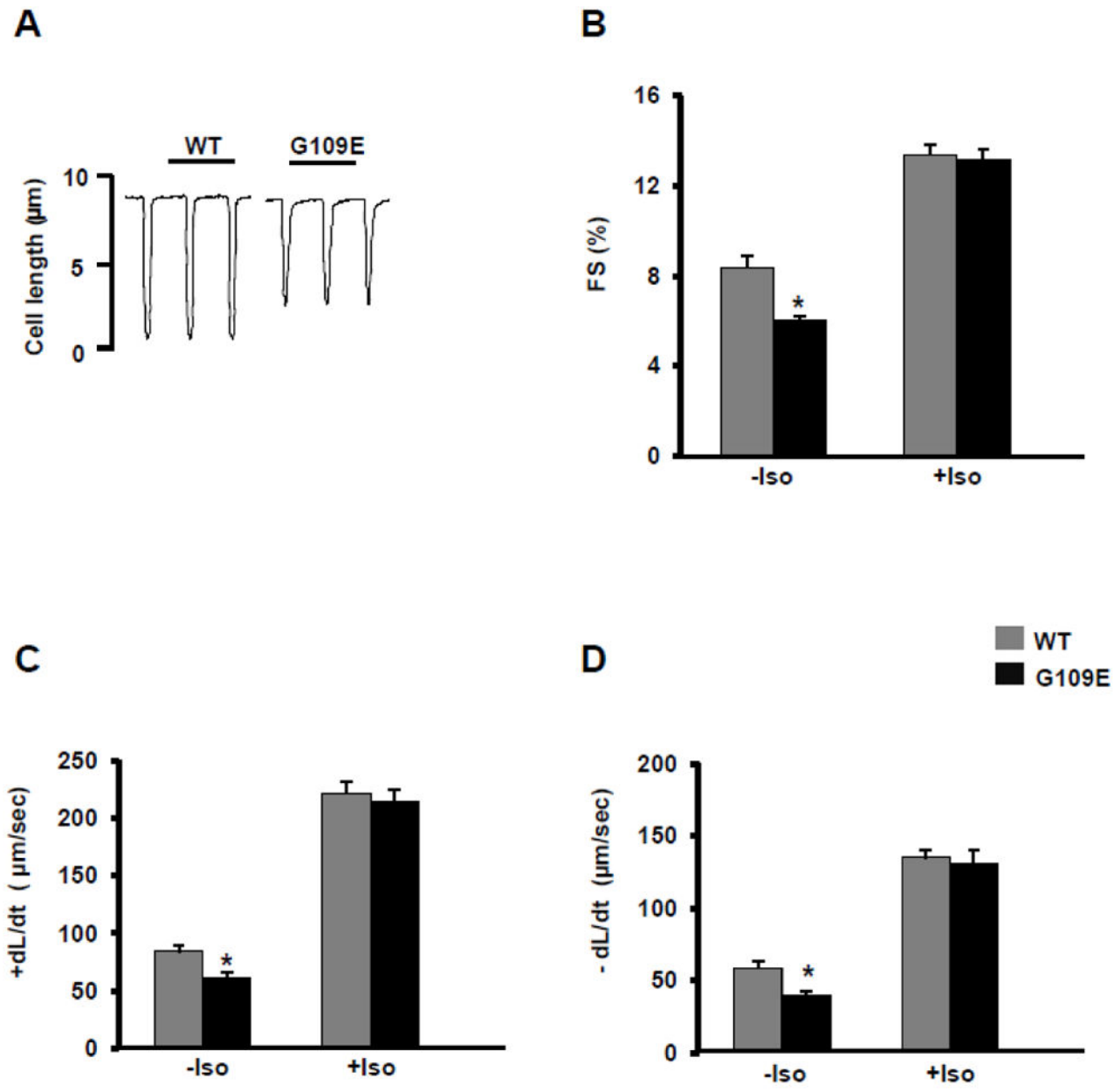

Figure 2. Mechanics of G109E cardiomyocytes and their responses to isoproterenol

A) Representative cell shortening tracings of WT and G109E cardiomyocytes under basal conditions. B) The fractional shortening (FS) in the absence and presence of 100 nM isoproterenol (Iso). C) Rates of contraction, +dL/dt, in the absence and presence of Iso. D) Rates of relaxation, −dL/dt, in the absence and presence of Iso. n = 55– 60 cells (−Iso) from 5 hearts; n = 31–42 cells (+Iso) from 5 hearts. Data are mean ± SEM based on the number of hearts. *P ≤ 0.05 vs WT hearts.

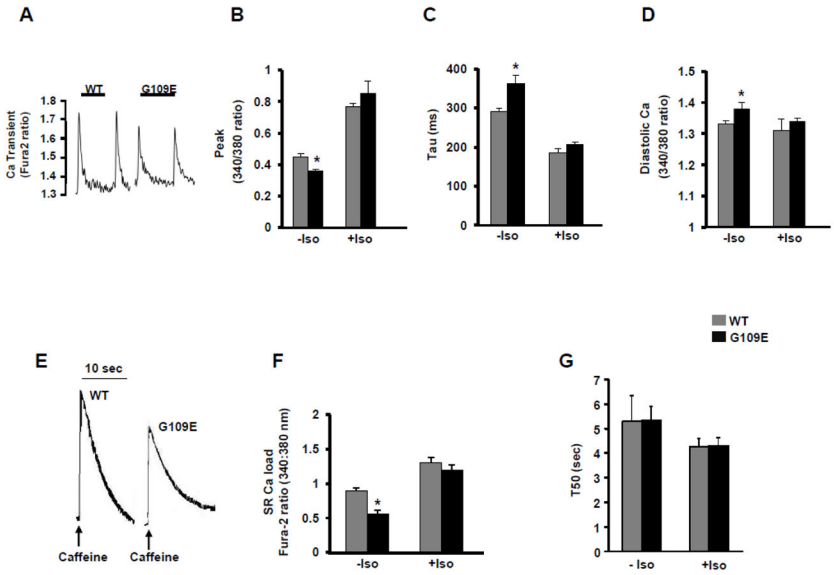

Figure 3. Ca kinetics, SR Ca content and NCX function in G109E cardiomyocytes

A) Representative tracings of Ca transients in WT and G109E cells under basal conditions. B) Ca transient amplitude, as indicated by the fura-2 ratio (340:380 nm) in the absence and presence of Iso. C) Tau of the transient decay in the absence and presence of Iso. D) Intracellular diastolic Ca levels. E) Representative tracings of caffeine-induced Ca transients. F) Amplitude of caffeine-induced Ca transients showing SR Ca load in myocytes from G109E hearts in the absence and presence of Iso. G) NCX activity assessed by T50 or the time to 50% decay of the caffeine induced Ca transient peak in the absence and presence of Iso. For Ca transients (−Iso): n = 40–45 and (+Iso) n=28–32 cells from 6 hearts. For SR Ca load (−Iso): n=14–16 cells and 12–14 cells (+Iso) from 3 hearts. Data are mean ± SEM, based on the number of hearts. *P ≤ 0.05 vs WT hearts.

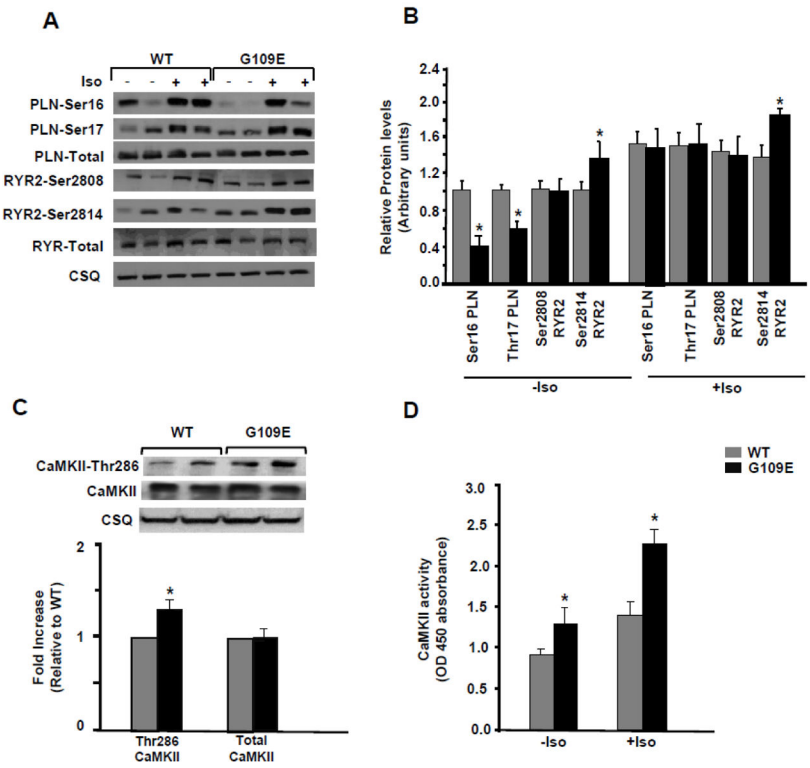

Figure 4. Assessment of phosphorylation levels of SR Ca regulatory phosphoproteins and CaMKII activity in G109E hearts

A) Protein expression and phosphorylation of phospholamban (PLN) and ryanodine receptor type-2 (RyR2) in G109E and WT cardiac homogenates under basal and Iso stimulation conditions. Calsequestrin (CSQ) was used as a loading control. B) Quantitation of relative protein and phosphoprotein levels. Samples for each protein were run on the same gel. n= 3. *P ≤ 0.05 vs WT hearts. C) Representative immunoblots of phosphorylated (Thr286) and total CaMKII as well as their relative quantification. D) CaMKII activity levels in absence and presence of Iso (n=6 hearts). Data are mean ± SEM. *P ≤ 0.05 vs. WT,

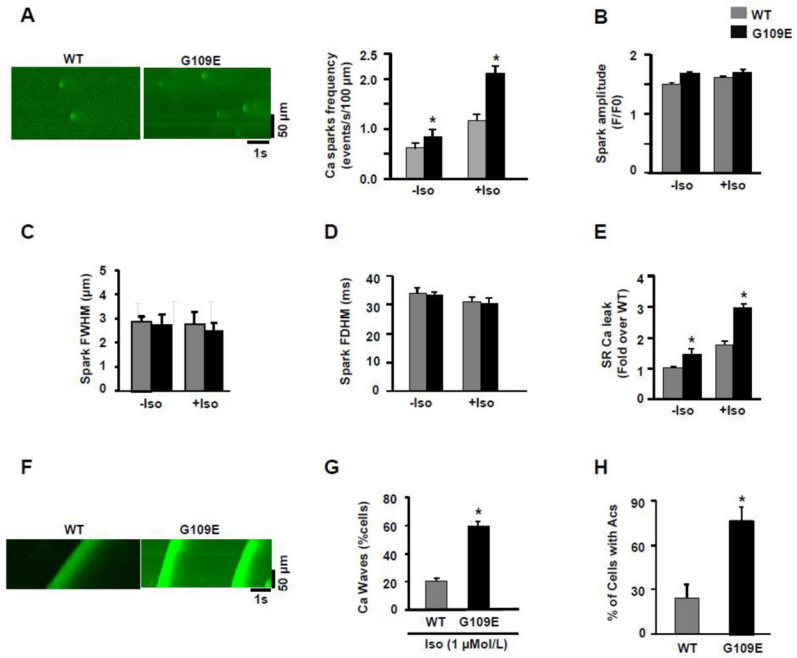

Figure 5. Ca spark characteristics, Ca waves and aftercontractions in G109E and WT cardiomyocytes

A) Mean data of Ca spark frequency in absence and presence of Iso under basal conditions. B) Mean data for Ca spark amplitude in absence and presence of Iso. C) Mean data of full width-at-half maximal amplitude (FWHM) in absence and presence of Iso. D) Mean data of full duration-at-half maximal amplitude (FDHM) in absence and presence of Iso. E) Mean data of SR Ca leak (calculated as Ca spark frequency × amplitude × FDHM × FWHM) in absence and presence of Iso. Data in A, B, C, D and E are presented as mean ± SEM of: n = 45 cells (−Iso) and n=70 cells (+Iso) from 6 G109E hearts; n=45 cells (−Iso) and n=43 cells (+Iso) from 5 WT hearts. *P ≤ 0.05 vs WT hearts, F) Representative line-scan images of Ca waves acquired in G109E and WT cardiomyocytes in the presence of 1 µM isoproterenol. G) Percentage of cells showing waves cells from 3 G109E hearts; n=15 cells from 3 WT hearts). *P ≤ 0.05 versus WT hearts. H) Cardiomyocytes were paced at 2 Hz at room temperature and after-contractions were recorded. n=17–27 cells from WT (n=3 hearts) and G109E (n=3 hearts). Data are mean ± SEM. *P ≤ 0.05 vs WT hearts.

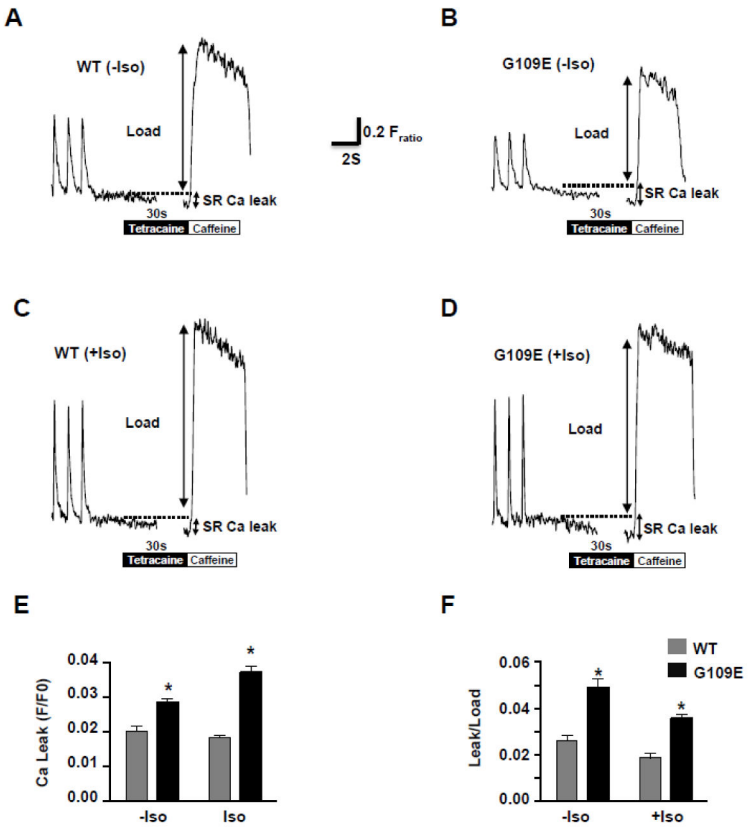

Figure 6. Diastolic SR Ca leak measurements in G109E and WT cardiomyocytes under basal and isoproterenol stimulation conditions

A, B, C and D) are representative traces of SR Ca leak in G109E and WT cardiomyocytes in the absence and presence of 100 nM Iso. Example traces were obtained from different myocytes. SR Ca leak was determined as the tetracaine sensitive drop in diastolic Fura-2 ratio. E) Quantification and comparison of average SR Ca leak in G109E and WT cardiomyocytes. F) Quantification of the SR Ca-leak/SR Ca-load relationship in G109E and WT myocytes. The height of the caffeine-induced Ca transient was used as a measure of total SR Ca content. Fractional SR Ca release was calculated by dividing the height of the last twitch transient by the height of the caffeine transient. In all experiments, 8–10 cells per experiment or heart and 3 hearts were used from G109E or WT controls. Data are mean ± SEM based on the number of hearts. *P ≤ 0.05 vs WT-I-1 hearts.

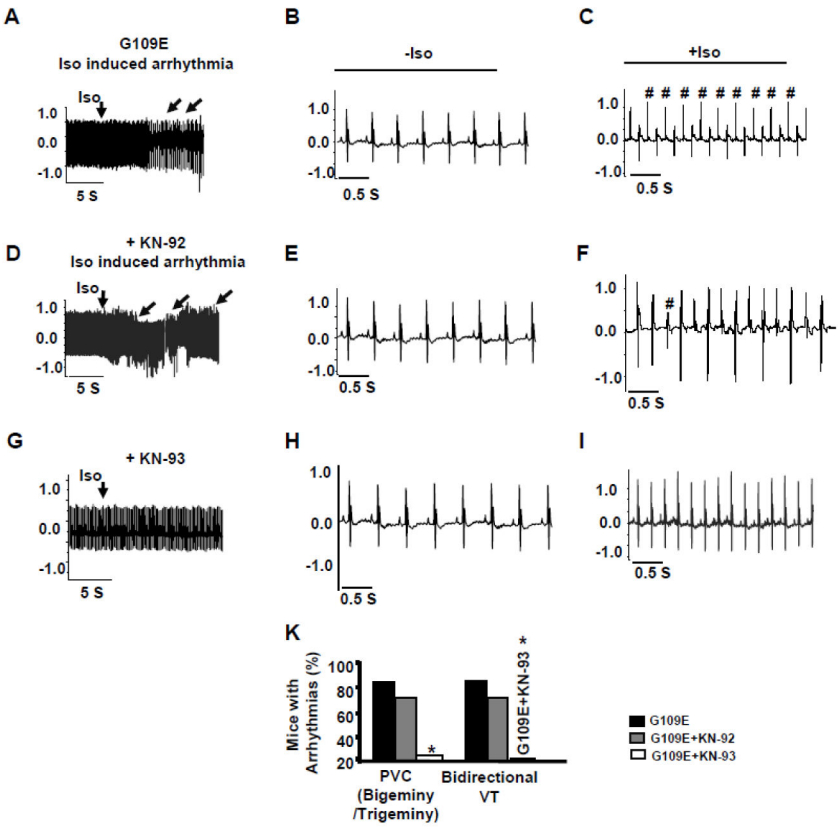

Figure 7. *In vivo* arrhythmia assessment in G109E mice after catecholamine challenge

G109E mice were anesthetized and monitored by surface ECG. A, D and G) Original ECG recordings in one representative G109E mouse under basal and Iso stimulation. The same mouse was used under all conditions and it was pretreated with KN-92 (D) or KN-93 (G) before Iso-challenge. Vertical arrows indicate the time of Iso injection and angled arrows indicate the regions of arrhythmias. Arrhythmias were inhibited by KN-93 (G) while KN-92 had no effects (D). B, E and H) expanded ECG tracings under basal condition. C, F, and I) Expanded ECG tracings showing Iso-induced arrhythmias in forms of premature ventricular complexes (PVCs, #), the initiation of bidirectional ventricular tachycardia (VT) proceeded by a PVC, and inhibition of arrhythmias by KN-93. K) G109E mice (n=6) and KN-92 treated mice (n=3) displayed complex forms of ventricular arrhythmias (bigeminy/trigeminy and VT) after caffeine and isoproterenol injection, which were inhibited by KN-93, the CaMKII inhibitor (n=4 mice). *P ≤ 0.05 G109E+KN-93 vs G109E.

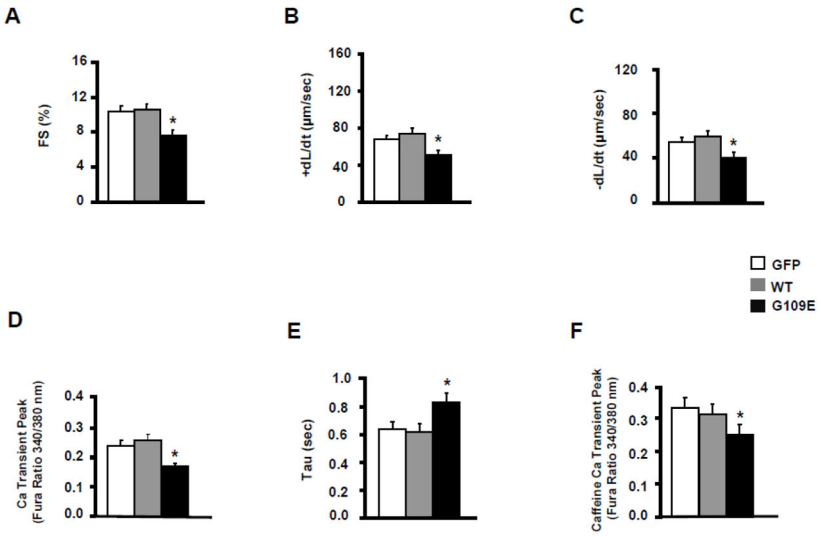

Figure 8. Contractility and Ca kinetics in adenovirally infected adult rat cardiomyocytes

A, B and C) Fractional shortening (%), rate of cell shortening (+dL/dt), and rate of cell re-lengthening (−dL/dt) in GFP, WT I-1, and G109E infected cardiomyocytes. D, E and F) Ca transient amplitude, decay (Tau) and Ca transient peak induced by 10 mM caffeine. n= 43–51 cells from 7 hearts. Data are mean ± SEM based on the number of hearts. *P ≤ 0.05 vs. GFP or WT hearts.