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Lower sarcoplasmic reticulum Ca^{2+} threshold for triggering afterdepolarizations in diabetic rat hearts

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Abstract

Background: Type-2 diabetes (T2D) increases the arrhythmias risk through incompletely elucidated mechanisms. Ventricular arrhythmias could be initiated by delayed afterdepolarizations (DADs) resulting from elevated spontaneous sarcoplasmic reticulum (SR) Ca^{2+} release (SR Ca^{2+} leak).

Objective: To test the role of DADs and SR Ca^{2+} leak in triggering arrhythmias in T2D hearts.

Methods: We compared rats with late-onset T2D that display pancreatic and cardiac phenotypes similar to those in humans with T2D (HIP rats) and their non-diabetic littermates (WT).

Results: HIP rats showed higher propensity for premature ventricular complexes and ventricular tachyarrhythmias while HIP myocytes displayed more frequent DADs and had lower SR Ca^{2+} content compared to the WT. However, the threshold SR Ca^{2+} at which depolarizing transient inward currents (I_{Ti} s) are generated was also significantly decreased in HIP myocytes and was below the actual SR Ca^{2+} load, which explains the increased DAD incidence despite reduced Ca^{2+} in SR. In agreement with these findings, Ca^{2+} spark frequency was augmented in myocytes from HIP vs. WT rats, which suggests activation of ryanodine receptors (RyR) in HIP hearts. Indeed, RyR phosphorylation (by CaMKII and PKA) and oxidation are enhanced in HIP hearts, while there is no RyR O-GlcNAcylation in either HIP or control hearts. CaMKII inhibition dissipated the difference in Ca^{2+} spark frequency between HIP and WT myocytes.

Conclusions: The threshold SR Ca^{2+} for generating depolarizing I_{Ti} s is lower in T2D due to RyR activation following hyperphosphorylation and oxidation, which favors the occurrence of DADs despite low SR Ca^{2+} loads.

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Conflict of Interest: None

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Keywords

type-2 diabetes; transient inward current; afterdepolarizations; ryanodine receptors; CaMKII

Introduction

Type-2 diabetes (T2D) is generally associated with structural, contractile and electrical abnormalities of the heart, even in the absence of coronary artery disease or hypertension.^{1–3} Diabetic cardiomyopathy is characterized by diastolic and systolic dysfunction,^{4–6} hypertrophy,^{1,3,7} fibrosis^{3,4} and arrhythmias, including ventricular tachyarrhythmias and fibrillation.^{8–11} However, the mechanisms driving electrical remodeling of the heart in T2D are poorly understood.

Ventricular tachyarrhythmias may occur following a premature excitation of the ventricle caused by simultaneous delayed afterdepolarizations (DADs) in a large number of neighboring myocytes.¹² At cellular level, DADs are produced by a transient inward current (I_{ti}) generated by an increase in cytosolic Ca^{2+} due to spontaneous SR Ca^{2+} release during diastole (SR Ca^{2+} leak). SR leak is critically regulated by the amount of Ca^{2+} in the SR. Several studies reported lower SR Ca^{2+} content in hearts from various T2D animal models,^{13–17} which resulted in reduced amplitude of cytosolic Ca^{2+} transients. In agreement with impaired SR Ca^{2+} load, Pereira et al. found lower Ca^{2+} spark frequency in T2D db/db mice compared to their non-diabetic controls.¹⁵ However, other studies reported that SR Ca^{2+} leak is actually increased in myocytes from db/db mice^{16,17} Thus, it is unclear whether/how SR Ca^{2+} leak is altered in T2D hearts. Moreover, the db/db mouse model is not particularly translational because these mice are extremely obese and their pancreatic phenotype is different from that observed in humans with T2D as they lack deposits of amylin amyloid in the islets (because rodent amylin is not amyloidogenic)¹⁸ and the presence of β -cell mass depletion is strongly dependent on the genetic background.¹⁹ In addition, these prior studies focused entirely on Ca^{2+} handling and/or ryanodine receptor (RyR) function, without connecting them to the electrical activity of the myocyte.

Here we used the HIP rat model¹⁸ of late-onset T2D to test the role of DADs and SR Ca^{2+} leak in triggering ventricular arrhythmias in T2D hearts. These rats present the major manifestations of T2D in humans including insulin resistance, hyperglycemia, amylin amyloid deposition and loss of β -cell mass.^{18,20} Moreover, we have previously demonstrated that HIP rats develop cardiac hypertrophy,^{13,21} heart dysfunction^{13,21} and ventricular arrhythmias.²²

Methods

Detailed methods are in the Online Supplement.

Experimental animals

All animal experiments conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at University of Kentucky. The T2D HIP rats used here are obese Sprague-Dawley rats that

express the human isoform of amylin, a pancreatic hormone co-secreted with insulin, specifically in the pancreatic β -cells.¹⁸ Secretion of amylin is increased in parallel with that of insulin in obesity and insulin resistance.²³ Human, but not rodent, amylin is amyloidogenic. Therefore, hypersecretion of amylin in humans leads to amylin deposition in the pancreas, which is an active participant in the gradual decline in β -cell mass that results in late onset T2D.²³ A similar mechanism occurs in HIP rats.^{18,20} Male HIP rats develop late-onset T2D (by 10–12 months of age)^{18,21} and show cardiac hypertrophy and dysfunction.^{13,21} Only males were used here because HIP females develop T2D at more advanced aged (~18 months). WT littermates served as non-diabetic controls. A total of N=32 HIP and N=32 WT rats were used in the study.

Standard surface ECGs were recorded on anaesthetized rats at baseline and for 20 min following injection of caffeine and dobutamine. Ventricular myocytes were isolated by Langendorff perfusion with collagenase.²¹

Experiments on isolated myocytes

DADs were recorded under current clamp for 10 s following pre-conditioning at 1Hz. Voltage-clamp was used to determine the threshold SR Ca^{2+} load required for generation of a depolarizing I_{ti} as in Pogwizd et al.²⁴ I_{ti} occurrence was monitored for 30 sec at -80 mV, followed by emptying the SR with caffeine. SR Ca^{2+} load was calculated as the sum of the integrals of I_{ti} and caffeine-induced current. The threshold SR Ca^{2+} load for I_{ti} occurrence was determined as the average of the two lowest loads that gave an I_{ti} and the two highest that did not.

SR Ca^{2+} leak was measured using the tetracaine method.²⁵ Ca^{2+} sparks were recorded in linescan mode in myocytes pre-conditioned by pacing at 0.5 Hz.

Tissue analyses

Western blot experiments were performed on heart homogenates. Protein bands were visualized with a SynGene Imaging machine and band intensity was quantified using ImageJ software. RyR oxidation was assessed by measuring the amount of free thiol residues contained by RyRs using the monobromobimane (mBB) method.²⁶ CaMKII activity was measured as a function of ^{32}P -ATP incorporation into the synthetic substrate syntide-2, as previously described.²²

Statistical analysis

Statistical differences between groups were determined using the student's t-test or Fisher's exact test, as appropriate. Data are presented as mean $\pm$ standard error. * $P<0.05$, ** $P<0.01$; *** $P<0.001$

Results

Increased incidence of arrhythmias and delayed afterdepolarizations in diabetic rats.

Similar to humans with T2D, diabetic HIP rats display abnormal electrical activity following a challenge with dobutamine and caffeine (Fig 1), including premature ventricular

complexes (PVC; Fig 1A) and both polymorphic and monomorphic ventricular tachyarrhythmias (VT; Fig 1B). The frequency of PVCs was significantly higher in HIP *vs.* WT rats (Fig. 1C). The incidence (Fig. 1D) and average total duration (Fig. 1E) of VTs was also increased in HIP rats.

PVCs are generally triggered by DADs occurring simultaneously in a large number of neighboring myocytes and they may initiate VTs. Thus, we compared the frequency of DADs occurring in myocytes from WT and HIP rats during a 10 s period following pre-conditioning at 1Hz (Fig 1F-G). HIP myocytes showed higher propensity for generating DADs compared to WT cells (Fig 1H), which may explain the increased arrhythmogenicity in HIP rats. Most DADs were in the form of classical small depolarizations (Fig 1G). However, two HIP myocytes displayed large, action potential-like DADs, characterized by a slow initial depolarization (Online Figure I).

Lower threshold SR Ca²⁺ load for induction of transient inward currents and larger SR Ca²⁺ leak in myocytes from diabetic rats.

DADs are generated by I_{ti} s triggered by Ca²⁺ released from the SR. SR Ca²⁺ leak increases with higher Ca²⁺ levels in the SR. However, prior studies found lower SR Ca²⁺ content in T2D hearts.^{13–17} We found that this is also the case in HIP rats ($89 \pm 3 \mu\text{mol/L}_{\text{cytosol}}$ *vs.* $110 \pm 6 \mu\text{mol/L}_{\text{cytosol}}$ in WT myocytes; Fig. 2A). The decreased SR Ca²⁺ load in HIP myocytes is likely the result of impaired SERCA activity, which is evidenced by the slower decline of twitch-induced Ca²⁺ transients (Online Figure II). To explain the higher DAD incidence despite reduced SR Ca²⁺ load in HIP myocytes, we measured the threshold SR Ca²⁺ at which depolarizing I_{ti} s are generated in myocytes from HIP *vs.* WT rats (Fig. 2B-E). In these experiments, the SR of voltage clamped-myocytes was pre-loaded with varying amounts of Ca²⁺ by depolarizing the myocyte to +50 mV for either 1 or 4 seconds (Fig 2B). Depolarization results in the reversal of the Na⁺/Ca²⁺ exchanger (NCX), which now transports Ca²⁺ into the cells. L-type Ca²⁺ channels will also open initially, and thus contribute to Ca²⁺ loading. Ca²⁺ is then taken from cytosol into the SR by SERCA. The occurrence of I_{ti} was measured on repolarization to –80 mV. After 30 s, caffeine was applied to release all Ca²⁺ remaining in the SR; this Ca²⁺ is then extruded from the cell by NCX, generating a large inward current (I_{NCX} in Fig. 2C-D). Assuming that I_{ti} is carried mainly by NCX,²⁷ SR Ca²⁺ load was calculated from the sum of I_{ti} and I_{NCX} integrals. Fig. 2C-D shows examples of such measurements in WT and HIP myocytes. These experiments indicate that the threshold SR Ca²⁺ content needed for generating Ca²⁺ waves that trigger I_{ti} s is greatly reduced in myocytes from HIP *vs.* WT rats (58 ± 7 *vs.* $118 \pm 22 \mu\text{mol/L}_{\text{cytosol}}$, $P=0.04$; Fig. 2E). The decreased threshold favors the occurrence of DADs at lower SR Ca²⁺ in HIP myocytes.

The data above suggest that SR Ca²⁺ leak is elevated in myocytes from HIP rats compared to the WT. We found that this is indeed the case (Fig. 3A). SR Ca²⁺ leak occurs as both Ca²⁺ sparks and smaller Ca²⁺ release events. Since occurrence of Ca²⁺ waves and consequent I_{ti} s is promoted by increased Ca²⁺ spark frequency, we recorded sparks in WT and HIP myocytes (Fig. 3B). Note the occurrence of a Ca²⁺ wave in the HIP cell (red arrow in Fig 3B). Ca²⁺ spark frequency was significantly higher in myocytes from diabetic HIP rats

compared to non-diabetic WT littermates (2.6 ± 0.2 vs. 1.7 ± 0.2 sparks/100 $\mu\text{m}\cdot\text{s}$; Fig. 3C). Ca^{2+} spark amplitude was lower in HIP rat myocytes (Online Figure IIIA), likely due to smaller SR Ca^{2+} content and thus reduced Ca^{2+} flux from the SR during a spark, while Ca^{2+} spark width and duration were similar in WT and HIP myocytes (Online Figure IIIB-C).

Increased RyR phosphorylation and oxidation in diabetic rat hearts

Higher SR Ca^{2+} leak at lower SR Ca^{2+} in HIP myocytes suggests that spontaneous RyR activity is enhanced compared to the WT. The total amount of RyRs is similar in hearts from HIP and WT rats (Online Figure IV). However, RyRs are activated by several post-translational modifications, including phosphorylation (by CAMKII at Ser2814 and PKA at Ser2808) and oxidation of thiol residues. We assessed the level of RyR phosphorylation using immunoblots and phospho-specific antibodies (Fig. 4A). While phosphorylation at both Ser2814 and Ser2808 sites was significantly enhanced in heart homogenates from HIP vs. WT rats (Fig. 4B-C), the effect was particularly strong at the CaMKII site (3fold increase vs. WT; Fig. 4B). Of note, phospholamban was also hyperphosphorylated by CaMKII in HIP hearts, whereas there were no significant differences in PKA-mediated phosphorylation between HIP and WT hearts (Online Figure V).

To assess RyR oxidation, we measured the relative amount of free thiols contained in RyRs using the mBB method (Fig. 4D-E). For each heart, the free thiol level was calculated as the ratio between the mBB fluorescence of RyRs under control conditions and the maximum mBB signal obtained following incubation with the reducing agent DTT (after correcting for the background fluorescence obtained following incubation with the oxidizing agent DTDP). The percentage of free RyR thiols was lower in myocytes from HIP compared to WT rats (Fig. 4E), which denotes increased RyR oxidation in T2D hearts.

Addition of O-linked β -N-acetylglucosamine (O-GlcNAc) to Ser/Thr residues is generally enhanced in T2D and plays a key role in diabetic cardiomyopathy.²⁸ Thus, we investigated whether RyRs are O-GlcNAcylated differently in WT and HIP hearts. For these measurements, heart homogenates containing Thiamet G (selective inhibitor of O-GlcNAcase, the enzyme that removes O-GlcNAc from proteins) were first immunoprecipitated with an anti-O-GlcNAc antibody, then separated by SDS-PAGE and probed with antibodies that recognize either O-GlcNAc or RyR (Fig. 4F). There was no O-GlcNAc protein band with the molecular weight corresponding to RyRs in hearts from either WT or HIP rats (Fig. 4F, left panel). Moreover, the RyR antibody produced no signal in O-GlcNAc immunoprecipitates (Fig. 4F, right panel). In contrast, the RyR band was clearly seen in the whole heart homogenate (Fig. 4F, right panel). These results suggest that there is little RyR O-GlcNAcylation in hearts from both WT and HIP rats. Thus, increased RyR phosphorylation and oxidation, but not O-GlcNAc modification, contribute to RyR hyperactivity in diabetic HIP rat hearts.

Enhanced CaMKII activity and its role in SR Ca^{2+} leak in diabetic hearts

Experiments in Fig. 4 suggest that CaMKII-dependent RyR hyperphosphorylation plays an important role in augmenting the SR Ca^{2+} leak in myocytes from diabetic rats. In agreement with this result, we found higher CaMKII activity in hearts from diabetic HIP rats compared

to WT (Fig. 5A). Ca^{2+} /calmodulin-independent CaMKII facilitates post-translational modifications of CaMKII, particularly autophosphorylation at the T287 site, oxidation at M281/M282 and O-GlcNAcylation at S280, that result in autonomous activation of the kinase.²⁹ Increased CaMKII autophosphorylation and O-GlcNAcylation were previously reported in HIP rat hearts.²² We found that CaMKII oxidation is also significantly elevated in hearts from diabetic HIP rats vs. WT (Fig. 5B). Thus, multiple mechanisms contribute to CaMKII activation in diabetic hearts.

In agreement with a major role of CaMKII-mediated RyR hyperphosphorylation in enhancing SR Ca^{2+} leak, CaMKII inhibition with either KN-93 or the more specific myristoylated-AIP greatly reduced Ca^{2+} spark frequency in HIP rats while having little effect in the WT (Fig. 5C).

Discussion

The mechanisms through which T2D increases the propensity for ventricular arrhythmias and sudden cardiac death are incompletely elucidated. Generally, diabetic hearts present structural alterations (fibrosis, etc) that predispose the heart to reentry arrhythmias.^{3,4} The contribution of triggered activity to the heightened arrhythmia risk is less clear. Using a rat model with late-onset T2D that largely recapitulates both the pancreatic^{18,20} and cardiac^{13,21,22} phenotypes present in humans with T2D we found that T2D significantly decreases both the amount of Ca^{2+} stored in the SR (Fig. 2A) and the threshold SR Ca^{2+} load at which depolarizing I_{Li} s occur (Fig. 2E). In myocytes from control rats the I_{Li} threshold is slightly above the SR Ca^{2+} content (Fig 6), which makes the occurrence of Ca^{2+} waves and DADs unlikely. In contrast, in myocytes from HIP rats the I_{Li} threshold is below the actual SR Ca^{2+} level (Fig 6), which favors the occurrence of DADs and premature beats. The lower I_{Li} threshold in HIP myocytes is due to RyR activation following hyperphosphorylation (mainly by CaMKII, but also PKA) and oxidation. Several studies have linked the T2D-induced impairment in cardiac contraction and relaxation to alterations in myocyte Ca^{2+} handling.^{3,13–17} Smaller and slower electrically-triggered Ca^{2+} transients and decreased SR Ca^{2+} content have been reported in some T2D animal models and are caused mainly by SERCA downregulation.^{3,13–17} SERCA expression and function are also reduced in diabetic HIP rats (Despa et al.²¹ and Online Figure II). Phospholamban hyperphosphorylation (Online Figure V) partially offsets the effect of reduced expression on SERCA activity. Moreover, we reported previously³⁰ that intracellular Na^+ concentration is elevated in HIP vs. WT hearts, which reduces Ca^{2+} extrusion through NCX allowing SERCA to compete better for cytosolic Ca^{2+} . Thus, phospholamban hyperphosphorylation and higher intracellular Na^+ limit the decrease in SR Ca^{2+} load in diabetic hearts, keeping it above the threshold for generating I_{Li} s.

Despite the lower SR Ca^{2+} load, SR Ca^{2+} leak is augmented in diabetic HIP rat hearts. This result is in agreement with some^{16,17} but not all¹⁵ prior studies in db/db mice, so far the only T2D model where SR Ca^{2+} leak was measured, as well as with a recent investigation in a model of pre-diabetes.³¹ Of note, the HIP rat is the only T2D animal model that reproduces the accumulation of amylin found in hearts from humans with T2D.^{13,21} We have previously found that amylin deposition directly perturbs myocyte Ca^{2+} cycling by increasing Ca^{2+} leak

across the sarcolemma,^{21,32} which accelerates the remodeling of Ca^{2+} handling proteins.²¹ Thus, the T2D-induced alterations in myocyte Ca^{2+} homeostasis in HIP rats are likely to resemble closer those occurring in humans with T2D.

Besides SR Ca^{2+} content, RyR open probability is enhanced by posttranslational modifications, including phosphorylation and oxidation. CaMKII-dependent RyR hyperphosphorylation has been previously reported in pre-diabetic³¹ and T2D hearts.¹⁷ We found that it also occurs in HIP rats, where CaMKII-mediated RyR phosphorylation is increased 3-fold compared to non-diabetic hearts (Fig. 4) and CaMKII inhibition normalizes Ca^{2+} sparks (Fig. 5). In addition, RyR phosphorylation by PKA and RyR oxidation are also elevated in HIP hearts (Fig. 4) and thus contribute to the higher SR Ca^{2+} leak. We expected that RyRs will also be O-GlcNAcylated in HIP hearts, similar to CaMKII.²² However, we could not detect RyR O-GlcNAcylation in either HIP or WT hearts.

Oxidative stress is a hallmark of diabetic cardiomyopathy.³ Our data indicate that oxidative stress has a dual role in enhancing SR Ca^{2+} leak in diabetic hearts. On one hand, it directly activates RyRs through oxidation of RyR thiol residues (Fig. 4). On the other hand, it oxidizes CaMKII and thus contributes to its activation and consequent RyR hyperphosphorylation (Fig. 5).

Our results show that RyR activation facilitates the occurrence of I_{ti} s at reduced levels of Ca^{2+} in the SR. Lower SR Ca^{2+} threshold for triggering an I_{ti} was previously shown to occur and increase the propensity for arrhythmias in a rabbit model of non-ischemic, arrhythmogenic heart failure.^{24,33} Thus, T2D and heart failure may share a similar cellular mechanism for ectopic events. Threshold SR Ca^{2+} for initiating spontaneous activity is also lowered by RyR mutations that cause catecholaminergic polymorphic ventricular tachyarrhythmias (CPVT).³⁴ Interestingly however, β -adrenergic stimulation increases the threshold for Ca^{2+} waves in myocytes expressing both wild-type RyRs and RyRs with a CPVT mutation.³⁵ This result implies that the combination of RyR CaMKII-mediated phosphorylation and oxidation is responsible for lowering the threshold SR Ca^{2+} load in T2D hearts and circumvents the opposing effect of PKA-mediated phosphorylation.

Clinical implications

Our findings suggest that increasing the threshold SR Ca^{2+} load for triggering I_{ti} s may be a useful therapeutic strategy for preventing DADs and arrhythmias in diabetic hearts. This could potentially be accomplished with dandrolene, a drug used for treating malignant hyperthermia caused by mutations in skeletal muscle RyR1. Indeed, dandrolene increased the wave initiation threshold in myocytes from failing rabbit hearts.³³

Conclusions

T2D decreases the threshold SR Ca^{2+} load at which a transient inward current is generated by enhancing the SR Ca^{2+} leak through CaMKII-mediated RyR hyperphosphorylation and RyR oxidation. The lower threshold facilitates the occurrence of DADs despite a reduction in SR Ca^{2+} content and thus constitutes an important mechanism for ventricular arrhythmias in T2D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Abnormal electrical activity in diabetic HIP rats.

(A-B) Premature ventricular complex (PVC) and ventricular tachyarrhythmia (VT) recorded in HIP rats during a stress test with caffeine and dobutamine. (C) Mean number of PVCs recorded within 20 min following injection of caffeine and dobutamine in WT and HIP rats. (D) Number of rats showing VT within 20 min following injection of caffeine and dobutamine. (E) Average VT duration. (F-G) Measurement of DADs in WT (F) and HIP (G) myocytes. Current-clamped myocytes were pre-conditioned by pacing at 1 Hz for 2 min. The occurrence of DADs was recorded for 10 s after pacing ended. Traces show the last two pacing-induced action potentials and the membrane potential during the following rest period. Red arrows point to DADs. (H) Frequency of DADs in myocytes from HIP and WT

rats. N=13/10 myocytes/rats for WT and 15/10 for HIP. Statistical significance in panels D and H was assessed with Fisher's exact test.

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Figure 2. Reduced threshold SR Ca^{2+} for I_{ti} occurrence in myocytes from HIP vs. WT rats. (A) Steady-state SR Ca^{2+} content in myocytes from HIP and WT rats assessed from the amplitude of caffeine-induced Ca^{2+} transient. (B) Voltage protocol used for measuring the I_{ti} threshold. Myocytes were conditioned by applying 100 ms depolarization pulses from -80 to $+50$ mV for 2 min (only last 3 are shown). Then, cells were depolarized to $+50$ mV for 1 or 4 seconds, to drive Ca^{2+} into the cell via reverse-mode NCX, thus increasing the SR Ca^{2+} content. I_{ti} was measured on repolarization to -80 mV (for 30 s). (C-D) Examples of current recordings following repolarization in a WT (C) and a HIP (D) myocyte. Caffeine was applied at the end to release all Ca^{2+} from the SR. The ensuing inward current is carried mainly by NCX (I_{NCX}). The sum of I_{ti} and I_{NCX} integrals indicates the SR Ca^{2+} load. (E) Threshold SR Ca^{2+} load for I_{ti} occurrence in HIP and WT myocytes.

Figure 3. Higher SR Ca^{2+} leak and Ca^{2+} spark frequency in HIP rat myocytes.

(A) Total RyR-mediated SR Ca^{2+} leak in WT and HIP myocytes. $N=4$ HIP and $N=6$ WT rats (3–8 myocytes/rat) (B) Representative line-scan recordings of spontaneous Ca^{2+} sparks in myocytes from HIP and WT rats preconditioned by steady-state pacing at 0.5 Hz (last stimulation-induced Ca^{2+} transient is indicated by black arrow). (C) Mean Ca^{2+} spark frequency in HIP and WT myocytes. $N=7$ HIP and $N=6$ WT rats (4–8 myocytes/rat). In (A) and (C), data were first averaged over cells from the same rat, then the mean SR leak/spark frequency per rat were averaged for each group.

Figure 4. Enhanced RyR phosphorylation and oxidation in diabetic HIP rat hearts.

(A) Immunoblots on heart homogenates with antibodies that recognize RyR phosphorylated at the CaMKII (S2814) and PKA (S2808) sites and total RyR. GAPDH is the loading control. (B-C) Ratio of phosphorylated-to-total RyR for the S2814 (B) and S2808 (C) phosphorylation sites in HIP and WT hearts. N=5 hearts/group and immunoblots were repeated 4 times. (D) Measurement of free thiols contained in RyRs. Myocytes from each heart were divided in three groups, lysed and incubated under control conditions (Ctl), with the reducing agent dithiothreitol (DTT) or with the oxidizing agent 2,2'-dithiodipyridine (DTDP). Lysates were then incubated with mBB and loaded on two SDS-PAGE gels for measurements of mBB fluorescence and, respectively, protein content (by Coomassie Blue staining). (E) Percentage of RyR free thiols calculated from experiments as in panel D. Measurements were performed in duplicate on myocyte lysates from 4 WT and 4 HIP rats. (F) Measurement of RyR O-GlcNAcylation. O-GlcNAcylated proteins immunoprecipitated, separated by SDS-PAGE and probed with antibodies that recognize either OGlcNAc (left panel) or RyR (right panel). Whole heart homogenate was used as positive control for RyR (Whole heart column). Similar experiments were performed on homogenates from 3 WT and 3 HIP hearts.

Figure 5. Increased CaMKII activity leads to higher Ca^{2+} spark frequency in HIP rat hearts. (A) CaMKII activity in hearts from WT and HIP rats. N=4 hearts/group. (B) Representative immunoblots for oxidized and total CaMKII in HIP and WT hearts and the relative amount of oxidized CaMKII. N=6 hearts/group and immunoblots were repeated 5 times. (C) Change in Ca^{2+} spark frequency produced by CaMKII inhibitors KN-93 and myristoylated-AIP (both 1 μM). Data were collected from 4–6 HIP and 4–6 WT rats (4–8 myocytes/rat). Spark frequency was first averaged over the cells from the same rat and the mean spark frequency per rat was averaged for each group.

Figure 6. Proposed mechanism for the occurrence of DADs in myocytes from diabetic hearts. In T2D, the threshold SR Ca^{2+} required for generating I_{tj} s and thus DADs decreases below the steady-state SR Ca^{2+} content.