CaMKII Serine 280 O-GlcNAcylation Links Diabetic Hyperglycemia to Proarrhythmia

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RATIONALE: Diabetic hyperglycemia is associated with cardiac dysfunction and increased arrhythmia risk, and CaMKII (calcium/calmodulin-dependent protein kinase II) function has been implicated. CaMKII activity is promoted by both oxidation and O-linked β-N-acetylglucosamine (O-GlcNAc) of known CaMKII sites.

OBJECTIVE: To investigate which posttranslational modifications occur in human diabetic hearts and how they alter electrophysiological and Ca²⁺ handling properties in hyperglycemia.

METHODS AND RESULTS: We assessed echocardiography, electrophysiology, Ca²⁺-handling, and protein expression in site-specific CaMKII mutant mice (O-GlcNAc-resistant S280A and oxidation-resistant MM281/2VV knock-ins, and global and cardiac-specific knockouts), in myocytes subjected to acute hyperglycemia and Ang II (angiotensin II) and mice after streptozotocin injections (to induce diabetes). Human patients with diabetes exhibit elevated CaMKII O-GlcNAcylation but not oxidation. In mice, acute hyperglycemia increased spontaneous diastolic Ca²⁺ sparks and waves and arrhythmogenic action potential changes (prolongation, alternans, and delayed afterdepolarizations), all of which required CaMKII-S280O-GlcNAcylation. Ang II effects were dependent on NOX2 (NADPH oxidase 2)-mediated CaMKII MM281/2 oxidation. Diabetes led to much greater Ca²⁺ leak, RyR2 S2814 phosphorylation, electrophysiological remodeling, and increased susceptibility to in vivo arrhythmias, requiring CaMKII activation, predominantly via S280 O-GlcNAcylation and less via MM281/2 oxidation. These effects were present in myocytes at normal glucose but were exacerbated with the in vivo high circulating glucose. PLB (phospholamban) O-GlcNAcylation was increased and coincided with reduced PLB S16 phosphorylation in diabetes. Dantrolene, which reverses CaMKII-dependent proarrhythmic RyR-mediated Ca²⁺ leak, also prevented hyperglycemia-induced APD prolongation and delayed afterdepolarizations.

CONCLUSIONS: We found that CaMKII-S280 O-GlcNAcylation is required for increased arrhythmia susceptibility in diabetic hyperglycemia, which can be worsened by an additional Ang II-NOX2-CaMKII MM281/2 oxidation pathway. CaMKII-dependent RyR2 S2814 phosphorylation markedly increases proarrhythmic Ca²⁺ leak and PLB O-GlcNAcylation may limit sarcoplasmic reticulum Ca²⁺ reuptake, leading to impaired excitation-contraction coupling and arrhythmogenesis in diabetic hyperglycemia.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: acetylglucosamine ■ action potential ■ electrophysiology ■ hyperglycemia ■ phosphorylation

Meet the First Author, see p 5

Cardiovascular complications including cardiomyopathy and arrhythmias are common sequelae of diabetes and the leading causes of mortality representing a major burden on human health.¹,² As the incidences of diabetes, prediabetes, and metabolic syndrome steadily increase worldwide while effective therapies for diabetic cardiomyopathy remain lacking, there is a pressing need for a better understanding of the exact molecular
mechanisms by which diabetes promotes cardiac disease, to uncover novel therapeutic targets in precision medicine. Diabetic hyperglycemia and oxidative stress are major contributors to detrimental cardiac remodeling in which impaired intracellular Ca\textsuperscript{2+} handling and enhanced activity of CaMKII (calcium/calmodulin-dependent protein kinase II) have been implicated. CaMKII delicately senses and translates the periodic intracellular Ca\textsuperscript{2+} concentration rises in cardiomyocytes during excitation-contraction coupling to kinase activity. Besides the Ca\textsuperscript{2+}/calmodulin-dependent activation of CaMKII that may lead to autophosphorylation (pT287), additional post-translational modifications (PTMs) by oxidation and O-GlcNAcylation can also be enhanced in diabetes. Oxidation of MM281/282 pair leads to the formation of oxidized Met281 and Met282, which can be further modified by the addition of O-GlcNAc. This dual modification can lead to increased CaMKII activity and enhanced proarrhythmic effects in diabetic hearts. Clinically, diabetes is associated with increased risk of arrhythmias and heart failure. Excessive O-GlcNAcylation and reactive oxygen species production and upregulated CaMKII have been implicated in the pathophysiology of diabetic cardiomyopathy. However, the molecular details of this signaling were unclear. Here, we identified CaMKII S280 O-GlcNAcylation and subsequent SR Ca\textsuperscript{2+} leak as the predominant regulators of increased arrhythmia risk in diabetic hearts. This finding provides mechanistic insights into disease pathophysiology and may lead to better-targeted therapies in diabetes.
Here, we identified CaMKII S280 O-GlcNAcylation as the predominant regulator of increased arrhythmia risk in diabetic hearts. CaMKII MM281/2 oxidation was independent of hyperglycemia, was mediated by an Ang II–NOX2 (NADPH oxidase 2) pathway and played a secondary, yet synergistic role to promote autonomous kinase activity. The upregulated CaMKII-induced sarcoplasmic reticulum (SR) Ca²⁺ leak drove the arrhythmogenic electrophysiological remodeling in diabetic hyperglycemia.

**METHODS**

**Data Availability**

The authors declare that all data and methods supporting the findings of this study are available in the Data Supplement or from the corresponding authors on reasonable request. Please see expanded methods and the Major Resources Table in the Data Supplement.

**Human Heart Samples**

Human right atrial appendage and left ventricular tissue samples were collected from patients undergoing on-pump coronary artery bypass graft surgery. Our informed consent practice, which required both verbal and written consent by each patient, conformed to the principles outlined in the Declaration of Helsinki. All human tissue work was performed as outlined in an Ethics Agreement approved by the Human and Disability Ethics Committee of New Zealand (LRS/12/01/001). For each of the 12 patients in the diabetic and nondiabetic cohorts, right atrial appendage samples were removed under normothermic conditions before cross clamping for cardiopulmonary bypass. For a subset of 6 patients in each cohort, a small wedge (1.5 x 1.5 x 10 mm) was surgically excised from the epicardium of the left ventricular anterior wall. Immediately after removal, tissue samples were placed in a sealed vial containing modified, low Ca²⁺ (0.5 mmol/L) Krebs-Henseleit buffer containing (in mmol/L): NaCl 118.5, KCl 4.5, NaH₂PO₄ 0.3, MgCl₂ 1, NaHCO₃ 25, and glucose 11. Within 5 to 10 minutes after removal, all tissues were flash-frozen and stored at −80°C.

**Mouse Models and Animal Procedures**

Several types of adult (10–12-week-old) C57BL/6J mice were used, including wild-type (WT, Jackson Laboratory, Stock No. 000664), NOX2 knockout (Jackson Laboratory, Stock No. 002365), CaMKIIβ cardiac-specific and global knockouts,21,22 CaMKIIβ-S280A-KI (that ablates the key O-GlcNAcylation site) created for this study23 but also used in 2 parallel studies24,25 and mutated Met281Val and Met282Val (MMVVK) that (ablates key oxidation sites).7 Diabetes was induced in 6- to 8-week-old male mice by intraperitoneal injections with low-dose streptozotocin (50 mg/kg body weight in 40 mmol/L sodium citrate, pH=4.0) for 5 consecutive days and littermates received sodium citrate as vehicle control. Blood glucose levels were measured in fresh blood samples collected from the middle tail vein using OneTouch UltraMini blood glucose monitoring system and test strips (LifeScan; measurement range: 20–600 mg/dL). Only mice exhibiting >300 mg/dL blood glucose levels following streptozotocin injections (>70% success rate) were included in the study.

Enzymatic isolation of left ventricular cardiomyocytes was performed as previously described.25 All animal handling and laboratory procedures were in accordance with the approved protocols (No. 19721 and No. 21064) of the Institutional Animal Care and Use Committee at University of California, Davis conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (eighth edition, 2011).
**Calcium Imaging**

Intracellular Ca\(^{2+}\) transients and diastolic Ca\(^{2+}\) events (sparks and waves) were measured using confocal microscopy in freshly isolated ventricular cardiomyocytes loaded with Fluo-4 AM. Paired experiments were performed at room temperature in Tyrode’s solution containing normal glucose (100 mg/dL) then high glucose (540 mg/dL).

**Cellular Electrophysiology**

Recordings were performed in isolated ventricular cardiomyocytes using whole-cell patch-clamp with physiological solutions at 37 °C (for ionic composition see Data Supplement). Action potentials (APs) were evoked with short suprathreshold depolarizing pulses at 1 to 10 Hz pacing frequencies in current-clamp experiments. Arrhythmogenic diastolic activities were assessed during a 3-minute period following 1-minute tachypacing at 10 Hz.

**In Vivo ECG and Pharmacological Stress Test**

In vivo experiments were performed in anesthetized (isoflurane, 1%–2%) diabetic (streptozotocin-treated) and control (vehicle-treated) mice. For pharmacological stress test, mice were injected with isoproterenol (IP 2 mg/kg) and caffeine (IP, 120 mg/kg). Premature ventricular complexes were counted during the next 60-minute period.

**Murine Echocardiography**

Transthoracic echocardiography was performed in anesthetized (isoflurane, 1.5%) animals before and after 4-week streptozotocin (or vehicle) treatment. Left ventricular B-mode, M-mode, and Doppler images were acquired using a Vevo 2100 echocardiography system (FUJIFILM VisualSonics) equipped with a 40 MHz transducer. Body temperature was carefully monitored, and anesthesia was adjusted to achieve between 400 to 500 beats/min heart rate in each animal.

**Fluorescence Recovery After Photobleaching**

Fluorescence recovery after photobleaching experiments were performed in intact rabbit ventricular cardiomyocytes expressing GFP (green fluorescent protein)-tagged WT or S280A CaMKIIδ, using an Olympus Fluoview-1000 confocal microscope as previously described. Isolated myocytes were plated on laminin-coated coverslips, cultured, and used for experiments within 18 to 22 hours of adenosiral infection. Myocytes were electrically field stimulated at 0.5 Hz for 5 minutes at room temperature before initiating fluorescence recovery after photobleaching by bleaching a circular region with a diameter of 7.3 μm to 30% to 40% of the initial fluorescence value. Images were subsequently collected every 2.71 seconds to measure fluorescence recovery.

**Statistical Analysis**

Data are presented as mean±SEM. Normality of the data was assessed by Shapiro-Wilk test. Statistical significance of differences for normally distributed data was determined using 2-tailed Student t test (paired on unpaired) or ANOVA, when applicable. If the data were not normally distributed, we used nonparametric tests. Categorical outcomes were evaluated using Fisher exact test. In cellular experiments, we performed hierarchical statistical analyses (nested tests) to account for intersubject variability and nonindependent sampling (as multiple cells may come from one animal). GraphPad Prism 9 was used for data analysis. P<0.05 was considered statistically significant.

**RESULTS**

**CaMKII O-GlcNAcylation Is Enhanced in the Diabetic Human Heart**

Figure 1A illustrates 5 known PTM sites in the highly conserved regulatory region of CaMKII, immediately adjacent to the CaM (calmodulin) binding site. These include oxidation (MM281/282) and O-GlcNAcylation (S280), autophosphorylation (pT287), and S-nitrosylation (C290), each of which promotes autonomous kinase activity after Ca\(^{2+}\)/CaM dissociation (memory).

To directly test whether CaMKIIδ S280 O-GlcNAcylation activates CaMKII autonomous activity, Figure 1B shows measurements of CaMKII-dependent incorporation of 32P from 32P-ATP into an artificial substrate, syntide-2 (as previously described and in the Data Supplement). Lysates of HEK293 cells expressing WT, S280A, and T287A CaMKIIδ were used. Maximal kinase activation was achieved by treating the CaMKII samples for 10 minutes with Ca\(^{2+}\) (200 μmol/L) and CaM (10 μmol/L; white bars), showing similar activation of all three CaMKIIδ forms by Ca\(^{2+}\)-CaM. That activation is acutely reversed when Ca\(^{2+}\) is chelated with EGTA (10 mmol/L; gray bars). Robust autonomous kinase activity in WT CaMKIIδ was achieved by inclusion of either ATP (100 μmol/L) or a combination of UDP-GlcNAc (100 μmol/L) and recombinant human OGT (0.5 μg) during the Ca\(^{2+}\)-CaM exposure and Ca\(^{2+}\)-quench with EGTA (pink and red bars, respectively). In CaMKII-S280A, only ATP-induced autophosphorylation induced autonomic activity, whereas in T287A only O-GlcNAcylation-induced autonomous CaMKII activation. Notably, the direct substrate for O-GlcNAcylation by itself (UDP-GlcNAc) was insufficient to activate autonomy in any case (teal bars) without inclusion of the endogenous enzyme (OGT) that mediates O-GlcNAcylation (red bars). These data demonstrate that S280 and T287 are independently required for O-GlcNAcylation-dependent and autophosphorylation-dependent CaMKIIδ autonomy, respectively.

In human atrial and ventricular samples from patients with type 2 diabetes, we measured increased O-GlcNAcylation, but not oxidation, of CaMKII versus nondiabetic human samples (Figure 1C and 1D). Patients in the diabetic cohort exhibited higher fasting blood glucose and hemoglobin A1c values were obese and slightly hypertensive but did not exhibit chronic kidney disease, atrial fibrillation, or myocardial infarction, compared with
age- and sex-matched nondiabetic patients (Table I in the Data Supplement). Importantly, the cardiac ejection fraction was preserved in both cohorts (ejection fraction >50%), although diabetic hearts exhibited diastolic dysfunction (60% increase in E/e'). Thus, cardiac CaMKII O-GlcNAcylation is elevated and might contribute to cardiac dysfunction in human diabetes.

To study the functional consequences of CaMKII O-GlcNAcylation in the heart, we used a knock-in mouse model in which a previously identified serine residue was mutated to alanine (S280A-KI) in CaMKIIδ conferring resistance to O-GlcNAcylation (Figure IA in the Data Supplement). CaMKII-S280A-KI mice exhibited unaltered baseline CaMKIIδ expression, and its functional readouts including CaMKII autophosphorylation (pT287) and phospholamban T17 phosphorylation (PLB pT17, a characteristic CaMKII target) at 12-week of age (Figure IB and IC in the Data Supplement). Likewise, the S280A-KI mice exhibited unaltered baseline cardiac contractile function on echocardiography, overall morphological parameters, and blood glucose levels (Table II in the Data Supplement). Thus, the new O-GlcNAc-resistant mutant mice exhibit a normal baseline phenotype.

**High-Glucose Induced Diastolic SR Ca²⁺ Leak and CaMKII Mobility Require CaMKII S280 O-GlcNAcylation**

CaMKIIδ phosphorylation of the cardiac RyR2 (ryanodine receptor 2) is a major contributor to pathological intracellular Ca²⁺ mishandling. We tested whether diabetic hyperglycemia might alter intracellular Ca²⁺ transients and SR Ca²⁺ release events (Ca²⁺ sparks and waves) in intact ventricular cardiomyocytes. We exposed freshly isolated cardiomyocytes to levels of high-glucose (540 mg/dL; 30 mmol/L) corresponding to what is observed in severe diabetes versus osmotically matched (glucose substituted with equimolar mannitol) low-glucose (100 mg/dL; 5.5 mmol/L) conditions. Acute high-glucose treatment (6 minutes) did
not alter the pacing-induced Ca\textsuperscript{2+} transient amplitude or kinetics, but slightly reduced SR Ca\textsuperscript{2+} content in WT, cardiac-specific CaMKII\textdelta-knockout (cKO), or S280A-KI mice (Figure 2A and 2B). However, high-glucose-induced a major increase in Ca\textsuperscript{2+} spark frequency, whether normalized to SR Ca\textsuperscript{2+} load or not (Figure 2C and 2D), but this effect was completely prevented in both the CaMKII\textdelta cKO and S280A-KI mice. Likewise, there were more arrhythmogenic Ca\textsuperscript{2+} waves induced by high-glucose in WT than cKO or S280A-KI mice (Figure II in the Data Supplement). We conclude that the glucose-induced increase in diastolic SR Ca\textsuperscript{2+} leak requires O-GlcNAcylation of CaMKII\textdelta at S280, which may contribute to arrhythmogenesis.

Figure 2. Glucose-induced diastolic sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak is CaMKII\textdelta (Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II)-S280 O-GlcNAcylation dependent. 
A, Experimental protocol and representative intracellular Ca\textsuperscript{2+} signals (quantified as changes in Fluo-4 fluorescence). 
B, High-glucose treatment (540 mg/dL, 6 min) did not change the amplitude and decay of intracellular Ca\textsuperscript{2+} transient (CaT) but reduced SR Ca\textsuperscript{2+} load similarly in CaMKII\textdelta wild type (WT), cardiac-specific knockout (cKO), and O-GlcNAc-resistant CaMKII\textdelta-S280A knock-in (n[CaT]=total number of cells/animals, WT/normal glucose=24/8, WT/high-glucose=13/8, cKO/normal glucose=15/7, cKO/high-glucose=10/7, S280A/normal glucose=17/6, S280A/high-glucose=11/6; n[SRL]= total number of cells/animals, WT=20/11, cKO=13/9, S280A=18/9). Nested t test. 
C, Representative diastolic Ca\textsuperscript{2+} sparks were increased by high-glucose treatment in WT which was prevented in cKO and S280A. 
D, Increased Ca\textsuperscript{2+} spark frequency normalized to SR load indicates sensitized, leaky ryanodine receptors (n=total number of cells/animals, WT=20/11, cKO=13/9, S280A=18/9). Nested t test. 
E, Enhanced fluorescence recovery after photobleaching (FRAP) by high-glucose treatment in cardiomyocytes expressing GFP (green fluorescent protein)-tagged WT-CaMKII\textdelta (n=16 cells from 6 animals in normal glucose and n=13 cells from 6 animals in high-glucose) but not in GFP-CaMKII\textdelta-S280A (n=16 cells from 6 animals in both normal and high-glucose) indicates increased activation-dependent mobility of CaMKII\textdelta. Nested t test.
It was recently shown that CaMKIIδ activation promotes its subcellular mobility, which may facilitate phosphorylation of downstream targets outside the dyadic cleft to regulate contractile function, electrophysiology, and gene transcription.27 Figure 2E shows fluorescence recovery after photobleaching experiments in cardiomyocytes expressing GFP-tagged WT or S280A CaMKIIδ. High-glucose treatment increased the fluorescence recovery after photobleaching kinetics for WT but not for CaMKIIδ-S280A (Figure 2E), indicating that this increased CaMKIIδ mobility is also dependent upon S280 O-GlcNAcylation.

High-Glucose Induces Arrhythmogenic APs by CaMKIIδ S280 O-GlcNAcylation

Enhanced CaMKIIδ activity is associated with cardiac arrhythmias. We tested whether acute diabetic hyperglycemia alters ventricular APD and arrhythmogenic activities. High-glucose treatment prolonged APD at lower pacing rates (1–3 Hz) and induced significant APD alternans in subsequent beats at high pacing rates (9–10 Hz; Figure 3A). Both of these arrhythmogenic AP changes were prevented in CaMKIIδ-cKO and S280A but not in the oxidation-resistant CaMKIIδ-MMVV (Figure 3B through 3D). Similar arrhythmogenic AP responses were observed in males and females (Figure III in the Data Supplement).

Acutely blocking O-GlcNAcylation using the specific OGT inhibitor OSMI-1 (50 μmol/L) or chelating intracellular Ca2+ using EGTA in the pipette (10 mmol/L) both prevented the arrhythmogenic AP alterations by high-glucose (Figure 3E and 3F). Conversely, blocking O-GlcNAcase using the specific OGA inhibitor Thiamet-G (Thm-G, 100 nmol/L) induced these same APD changes even at normal glucose concentration and further enhanced the hyperglycemia effects. These results indicate that CaMKII O-GlcNAcylation but not oxidation mediate the acute hyperglycemic arrhythmogenic effects on APD.

Ang II promotes both CaMKII activation and reactive oxygen species (ROS) production and could contribute to the diabetes disease phenotype. When we added Ang II (100 nmol/L) on top of high glucose it exacerbated the high-glucose effects on APD in WT myocytes (Figure 3E and 3F) but not in MMVV and NOX2-KO mice (NOX2−/−). Notably, both the high-glucose and Ang II effects on APD were completely absent in CaMKIIδ-cKO myocytes. Taken together, these data indicate that NOX2-mediated CaMKIIδ oxidation at MM281/2 as a separate and additive effect of Ang II to that caused by high glucose alone.

Diastolic arrhythmogenic activities such as delayed afterdepolarizations (DADs) and spontaneous APs (sAPs) were also enhanced by acute hyperglycemia and Ang II (Figure 4A through 4D and Figure IV in the Data Supplement). Indeed, the changes closely paralleled the glucose and Ang II effects on APD with respect to the 5 different mouse lines studied. That is, high glucose induction of DADs and sAPs required CaMKIIδ-S280 and O-GlcNAcylation, but not NOX2 or MM281/2 on CaMKIIδ, whereas Ang II required NOX2 and CaMKIIδ-MM281/2. CaMKIIδ was essential to both high glucose and Ang II effects, and those were only additive in the WT mice. As for APD effects above, these suggest 2 distinct signaling pathways that activate CaMKIIδ at neighboring regulatory sites. Dan-trolene, which shifts back to normal the pathological and leaky RyR2 conformation that is induced by CaMKII phosphorylation,29 prevented hyperglycemia-induced arrhythmogenic AP changes and diastolic DADs (Figure V in the Data Supplement).

Diabetes Increases Arrhythmia Susceptibility by CaMKII Activation

Next, we tested the contribution of these CaMKIIδ PTM sites to the disease phenotype in chronic diabetes. On repeated low-dose streptozotocin injections, mice developed severe hyperglycemia and were subject to morphometric and functional evaluation following 4 weeks of diabetes (Figure 5A). This time point was chosen based on literature data showing that 4 weeks following streptozotocin injections mice already exhibit cardiac remodeling without chronic kidney disease.30 This allowed us to focus primarily on the hyperglycemia effects on the heart rather than the secondary renal complications. Similar levels of hyperglycemia were seen in WT, S280A, and MMVV diabetic animals (Figure 5B), and similar weight losses with unaltered heart weight to body weight ratios were observed in all 3 mouse lines (Figure 5C). The ejection fraction and ventricular wall thickness in echocardiography were unchanged following streptozotocin in all experimental groups, indicating preserved systolic function at this early diabetic stage (Figure 5B and 5D). However, streptozotocin induced some changes in diastolic function (enlarged left atria, 21% decrease in E/A, 26% increase in E/e′) in WT, which were attenuated in CaMKIIδ-S280A and slightly also in MMVV (Figure 5E). Streptozotocin-treated WT mice also exhibited higher incidence of premature ventricular complexes on in vivo ECG recordings following caffeine+isoproterenol stress test, whereas premature ventricular complex incidence was decreased in streptozotocin-treated CaMKIIδ-KO (Figure 5F), indicating a CaMKIIδ-dependent marked increase in arrhythmia susceptibility in diabetes. In line with this, RyR2 S2814 phosphorylation in diabetic WT hearts was found to be 1.93x higher than in nondiabetic WT hearts, while phosphorylation of RyR2 S2814 was absent in CaMKIIδ-KO (Figure 5G). Moreover, there was a 26% increase in PLB O-GlcNAcylation in streptozotocin-treated WT and 6% increase in streptozotocin-treated CaMKIIδ-KO (Figure 5G), which was reported to...
occur at Ser16.15 There was a tendency for increased PLB expression (by 26%) in diabetic hearts, whereas PLB phosphorylation on S16 site was decreased by 22% and phosphorylation on T17 site was unchanged in diabetes (Figure VI in the Data Supplement).

**Diabetes Promotes Diastolic SR Ca\(^{2+}\) Leak via CaMKII S280 O-GlcNAcylation**

We assessed myocyte Ca\(^{2+}\) handling in ventricular cardiomyocytes freshly isolated from diabetic hearts. Streptozotocin induced a slight reduction in intracellular Ca\(^{2+}\) transient amplitude and rate of [Ca\(^{2+}\)] decay in all 3 genotypes (Figure 6A and 6B). SR Ca\(^{2+}\) load was preserved in normal glucose and slightly reduced in high-glucose conditions (Figure 6B), independent of CaMKII\(\delta\) PTMs. However, diabetes increased Ca\(^{2+}\) sparks and waves in WT in normal glucose and even further in high-glucose (as occur in vivo in streptozotocin-treated diabetic animals, Figure 6C and 6D and Figure IIC in the Data Supplement). Focusing on the Ca\(^{2+}\) spark frequency normalized to SR Ca\(^{2+}\) load (Figure 6D, right) streptozotocin induced an elevation of baseline Ca\(^{2+}\) sparks in WT and CaMKII\(\delta\)-MMVV-KI but not in S280A myocytes. Moreover, restoring glucose concentration to the levels seen in vivo in the streptozotocin mice (540 mg/dL) further increased Ca\(^{2+}\) sparks in all but the S280A myocytes. This has 2 important

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**Figure 3.** Glucose-induced arrhythmogenic action potentials are CaMKII\(\delta\) (Ca\(^{2+}\)/calmodulin-dependent protein kinase II)-S280 O-GlcNAcylation dependent.

A, Action potential duration (APD) prolongation and alternans (S, short; L, long) were increased by acute high-glucose treatment in wild-type (WT) myocytes (n=16 cells from 9 animals). B, Arrhythmogenic APD responses were prevented by CaMKII\(\delta\)-cardiac-specific knockout (cKO; n=21 cells from 8 animals). C and D, CaMKII\(\delta\)-S280A knock-in (n=15 ells from 8 animals) but not mutated Met281Val and Met282Val (MMVV; n=17 cells from 9 animals) was resistant to glucose-induced acute APD changes. Nested t test. E, Glucose-induced APD changes were prevented by CaMKII\(\delta\)-cKO, S280A, intracellular Ca\(^{2+}\) buffering (EGTA) or the O-GlcNAc transferase inhibitor OSMI-1 (αR-[[1,2-dihydro-2-oxo-6-quinolinyl]sulfonyl]amino]-N-(2-furanylmethyl)-2-methoxy-N-(2-thienylmethyl)-benzeneacetamide) but enhanced by the O-GlcNAcase inhibitor Thiamet-G (Thm-G) in WT. Ang II (angiotensin II) further enhanced arrhythmogenic APD changes, which was prevented by CaMKII\(\delta\)-cKO, MMVV, or in NOX2\(^{-/-}\) (NADPH oxidase 2; n=total number of cells/animals is reported in the figure). Nested 1-way ANOVA, followed by Dunnett multiple comparisons test was used to compare 3 groups. Nested t test was used to compare 2 groups.
implications: (1) in the in vivo situation the WT diabetic mice have nearly 4x the rate of arrhythmogenic SR Ca^{2+} leak as in normal WT mice and (2) that the reversal of high glucose during cell isolation and incubation at normal 100 mg/dL glucose only partially reverses the chronic activation (ie, there is both an acutely reversible and a chronic component). Notably, these effects were completely prevented in CaMKII δ-S280A and slightly attenuated in CaMKII δ-MMVV.

**Diabetes Induces Arrhythmogenic APDs Predominantly by CaMKII S280 O-GlcNAcylation**

Finally, in Figures 7 and 8, we subjected WT and diabetic myocytes to similar electrophysiological analysis as was done for acute hyperglycemia in Figures 3 and 4, informed by the in vivo proarrhythmic tendencies observed at the whole animal level in Figure 5F. Diabetes induction by streptozotocin led to pronounced AP remodeling even when measured at 100 mg/dL glucose, with APD prolonged versus vehicle controls and alternans already at physiological pacing rates (8 Hz) versus negligible alternans in vehicle controls even at 10 Hz (Figure 7A, 7E, and 7F). Raising glucose in streptozotocin myocytes to 540 mg/dL further increased APD and induced APD alternans already at 6 Hz. These arrhythmogenic AP changes were prevented by acute cell pre-treatment with a selective CaMKII inhibitor peptide AIP (autocamtide-2-related inhibitory peptide; 1 μmol/L; Figure 7B). Importantly, high glucose also failed to increase APD or alternans in diabetic CaMKII δ-S280A myocytes (Figure 7C), but in MMVV myocytes the effects were nearly as large as in WT+streptozotocin (Figure 7D). Conversely, Ang II did not induce additional APD prolongation or alternans in MMVV myocytes, although it did in the other 3 myocyte groups studied (Figure 7E and 7F). The conclusions here parallel the above for SR Ca^{2+} leak, in that CaMKII δ-S280 appears essential for the high-glucose induced effects on APD and alternans, whereas the Ang II effect requires CaMKII δ-MM281/2. Furthermore, the 4-week streptozotocin treatment alone produces effects that are incompletely reversed by simply lowering extracellular glucose from the high in vivo levels to those in the standard perfusate during cell isolation and incubation (leftmost 2 white bars in Figure 7E and 7F).
However, even the acute restoration of the diabetic extracellular glucose level can further promote these APD effects and may more closely resemble the in vivo situation in these diabetic mice.

Spontaneous diastolic DADs and sAPS were also measured in vehicle and streptozotocin-treated mice (Figure 8A through 8D and Figure IVC in the Data Supplement) and the effects qualitatively parallel

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7F). Even though the acute restoration of the diabetic extracellular glucose level can further promote these APD effects and may more closely resemble the in vivo situation in these diabetic mice.
CaMKII O-GlcNAcylation and Arrhythmias in Diabetes

Figure 6. Diabetes-induced diastolic sarcoplasmic reticulum (SR) Ca2+ leak is CaMKIIδ (Ca2+/calmodulin-dependent protein kinase II)-S280 O-GlcNAcylation-dependent.

A. Experimental protocol and representative intracellular Ca2+ signals (as changes in Fluo-4 fluorescence) showing spontaneous Ca2+ waves in wild type (WT) following 4-week of streptozotocin (STZ)-induced diabetes. B. Diabetes slightly reduced the amplitude of the intracellular Ca2+ transient and prolonged the Ca2+ transient decay without significantly altering SR Ca2+ load (n[CaT]=total number of cells/animals, WT+vehicle/normal glucose=16/7, WT+vehicle/high-glucose=9/7, WT+STZ/normal glucose=20/6, WT+STZ/high-glucose=11/6, S280A+STZ/normal glucose=13/5, S280A+STZ/high-glucose=11/5, mutated Met281Val and Met282Val [MMVV]+STZ/normal glucose=13/5, MMVV+STZ/high-glucose=10/5; n[SR load]=total number of cells/animals, WT+Vehicle=12/6, WT+STZ=12/7, S280A+STZ=9/5, MMVV+STZ=10/5). Acute effect of high-glucose within the same genotype/treatment was tested using nested t test. Effect of STZ-induced diabetes (in normal glucose) vs WT+Vehicle was tested using nested 1-way ANOVA, followed by Dunnett multiple comparisons test. C. Ca2+ sparks were increased in diabetic WT and further increased by acute high-glucose treatment. D. The increase in Ca2+ spark rate was prevented in CaMKIIδ-S280A and was attenuated in the CaMKIIδ-MMVV (n=total number of cells/animals, WT+Vehicle=12/6, WT+STZ=10/6, S280A+STZ=9/5, MMVV+STZ=10/5). Nested t test. CaSpF indicates Ca2+ spark frequency.
Hegyi et al. CaMKII O-GlcNAcylation and Arrhythmias in Diabetes

and extend those for control animals (in Figure 4A through 4D) and for APD (Figure 7A through 7F). That is, streptozotocin promoted DADs and sAPs substantially, even at baseline glucose levels, in WT and CaMKIIδ-MMVV mice and this was exacerbated at the diabetic glucose level, especially in WT mice. However, those effects were completely prevented in CaMKIIδ-S280A mice. An interesting note here is that Ang II recruited a further increase in DADs (versus high glucose) only in the S280A mice (which had the lowest value in high glucose). But that may reflect a sort of ceiling effect for DADs in the WT cells, because Ang II still promoted sAPs in all but the MMVV mice (Figure 8C and 8D).

**DISCUSSION**

CaMKII upregulation has been linked to various heart diseases including cardiac hypertrophy, HF, and arrhythmias. Altered intracellular Ca$^{2+}$ signaling can be a cause but also a consequence of pathological CaMKII activation that leads to impairment of fundamental cellular processes such as excitation-contraction coupling, transcriptional regulation, cell mechanics, and energetics. PTMs of CaMKII may lead to sustained kinase activation when the intracellular Ca$^{2+}$ transient has not yet been changed, thus promoting disease. CaMKII upregulation has been shown in diabetic cardiomyopathy, and CaMKII inhibition provided substantial benefit in...
Increased production of ROS, previously shown in diabetes, may further enhance CaMKII activation by oxidation of 2 neighboring methionines (281/2) in the regulatory domain of CaMKIIδ. Interestingly, it was previously shown that the increased ROS production and upregulation of p47phox and p67phox (NOX2 associated proteins) were attenuated following CaMKII inhibition in diabetic cardiomyocytes. However, we found that acute hyperglycemia led to CaMKII activation and its cellular consequences (RyR leak-mediated arrhythmogenic AP changes) exclusively via O-GlcNAcylation of S280 and not via MM281/2 oxidation. Even in chronic diabetes, CaMKIIδ-S280A was found to be largely protective, whereas CaMKIIδ-MMVV had only a minor role. These data suggest that CaMKII O-GlcNAcylation can be an early step in hyperglycemia-induced signaling, which then triggers kinase activation and may lead to further detrimental cellular remodeling, including increased ROS production. Moreover, a stimulated renin-Ang-aldosterone system, characteristic of type 2 diabetes and metabolic syndrome, can induce NOX-2-dependent ROS production and CaMKII oxidation. In contrast with our data here, Mesubi et al recently reported a predominant role for CaMKII oxidation in atrial fibrillation susceptibility in diabetes induced by one-time high-dose streptozotocin or repeated low-dose streptozotocin-high-fat diet. Therefore, the contribution of O-GlcNAcylation and oxidation of CaMKII may differ in different diabetes types, cardiac regions, and stages of disease. Overall, our results highlight the importance of tight blood glucose control in patients with diabetes and suggest that even significant temporary rises in blood glucose levels (impaired glucose tolerance) may lead to significant CaMKII activation by O-GlcNAcylation.

A dynamic cross-talk between O-GlcNAcylation and phosphorylation at the same or neighboring serine/threonine residues may occur in many nucleocytoplasmic proteins. In acute hyperglycemia, both S280 O-GlcNAcylation and T287 autophosphorylation of CaMKIIδ were previously found to be enhanced, suggesting a synergy between the 2 PTMs, creating molecular memory and prolonged active open state of the kinase. On the contrary,
O-GlcNAcylation of PLB at S16 was shown to inhibit the phosphorylation of the same site by PKA (protein kinase A) and inhibit SERCA2 (SR Ca\(^{2+}\) ATPase 2), where only one of these modifications is possible at a given serine.\(^{15}\) This mechanism may also affect the CaMKII-dependent phosphorylation of the neighboring T17 in PLB because the Stokes radius of an O-GlcNAc moiety is \(\approx 5\times\) larger than a phosphate. In line with this, PLB O-GlcNAcylation was increased, pS16 was decreased, and pT17 levels were unchanged in diabetes (Figure 5G and Figure VI in the Data Supplement), and the intracellular Ca\(^{2+}\) transient decay was prolonged (Figure 6) indicating slowed SERCA and higher inhibition by PLB. These results agree with literature data reporting increased PLB expression and higher inhibition by PLB. These results agree with literature data reporting increased PLB expression and higher inhibition by PLB. These results agree with literature data reporting increased PLB expression and higher inhibition by PLB.

CaMKII gets activated predominantly in the dyadic cleft where local intracellular [Ca\(^{2+}\)] may rise to 50 μmol/L during systolic SR Ca\(^{2+}\) release. From here, CaMKII may translocate to phosphorylate cytoplasmic and nuclear targets. As for Ca\(^{2+}\)-dependent CaMKII activation,\(^{27}\) we found CaMKII-S280 O-GlcNAcylation in acute hyperglycemia further enhances CaMKII\(\delta\) mobility in the cytosol.

Remodeling in several ion channels, including voltage-gated Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels have been shown in diabetes resembling the one that occurs via CaMKII signaling.\(^{10,31}\) We have recently shown that K\(^+\) channel downregulation in diabetes was markedly attenuated in CaMKII-S280 mice.\(^{26}\) Diabetes is also frequently associated with HF, which is known to induce complex ion channel remodeling\(^{26,40-42}\) and intracellular Ca\(^{2+}\) mishandling\(^{32,43,44}\) leading to impaired AP repolarization and SR Ca\(^{2+}\) leak (via RyR pS214), and subsequent increase in the susceptibility to triggered activities and arrhythmias. This is in line with a recently demonstrated 2-hit arrhythmia model in diabetic hyperglycemia.\(^{45}\) CaMKII inhibition has been shown to prevent these arrhythmogenic events in various preclinical models of diabetes and HF.\(^{10,31}\)

However, global CaMKII inhibition may have some detrimental effects in specific conditions relevant to diabetes, such as acidosis.\(^{46}\) Preventing just the O-GlcNAcylation of CaMKII and repressing its pathological autonomous activation may serve as a precision medicine approach to prevent arrhythmias in diabetic hyperglycemia.

Diastolic dysfunction is an early manifestation of diabetic cardiomyopathy and frequently reported in patients.\(^{47}\) Here, we show that CaMKII-S280 O-GlcNAcylation may contribute to diastolic dysfunction in diabetes. This effect could be mediated by the increased diastolic SR Ca\(^{2+}\) leak and also the phosphorylation of titin which increases diastolic stiffness in HF.\(^{48}\) However, assessing the contribution of CaMKII to diastolic dysfunction requires further investigation in additional animal models. Another limitation of our study is that we did not broadly assess possible phosphorylation differences attributed to CaMKII-S280 O-GlcNAcylation among the many CaMKII substrates in the diabetic hearts. HF with preserved ejection fraction is a major clinical challenge in diabetes, with very limited treatment options currently available.\(^{49}\) Empagliflozin, a SGLT2 (sodium-glucose cotransporter 2) inhibitor was demonstrated to significantly reduce cardiovascular mortality and hospitalization for HF in patients with diabetes in the EMPA-REG OUTCOME (empagliflozin cardiovascular event trial in type 2 diabetes mellitus patients).\(^{50}\) Importantly, empagliflozin was shown to reduce CaMKII activation and its cellular consequences in failing murine and human ventricular myocytes.\(^{51}\) Along those lines, the related drug exenatide induced of CASK (Ca/CaM dependent serine protein kinase) that inhibit CaMKII and could partially ameliorate hyperglycemic effects that we described here.\(^{52}\) Moreover, empagliflozin blunted postprandial glucose excursions and reduced glucose-variability in patients with diabetes, which may attenuate O-GlcNAc-dependent CaMKII activation.\(^{53}\) In line with this, dapagliflozin, another SGLT2 inhibitor was shown to reduce O-GlcNAcylated protein levels in a type 2 diabetic murine model.\(^{54}\)

Enhanced O-GlcNAcylation was found in HF also in nondiabetic patients\(^{55}\) and animals\(^{13}\) as a result of altered cardiac metabolism. CaMKII O-GlcNAcylation was also enhanced in human failing hearts and even higher in HF patients with diabetes.\(^{4}\) Therefore, it may represent a therapeutic target in a broader range of heart diseases. Moreover, CaMKII O-GlcNAcylation is not restricted to the heart, but it has also been shown in brain samples of patients with diabetes and may occur in other organs as well.\(^{4}\) Impaired O-GlcNAcylation has been implicated in the pathogenesis of Alzheimer disease,\(^{56}\) neurodegeneration,\(^{57}\) and cancer.\(^{57}\) The potential involvement of CaMKII O-GlcNAcylation in those diseases is yet to be tested. Indeed, the ability of hyperglycemia to promote autonomous activation of the widespread and multifunctional CaMKII (already implicated in pathological heart disease) suggest value in further exploration of this pathway in other diseases.

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