Diabetic hyperglycaemia activates CaMKII and arrhythmias by O–linked glycosylation

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Ca2+/calmodulin-dependent protein kinase II (CaMKII) is an enzyme with important regulatory functions in the heart and brain, and its chronic activation can be pathological. CaMKII activation is seen in heart failure, and can directly induce pathological changes in ion channels, Ca2+ handling and gene transcription1. Here, in human, rat and mouse, we identify a novel mechanism linking CaMKII and hyperglycaemic signalling in diabetes mellitus, which is a key risk factor for heart2 and neurodegenerative diseases3,4. Acute hyperglycaemia causes covalent modification of CaMKII by O-linked N-acetylgalactosamine (O-GlcNAc). O-GlcNAc modification of CaMKII at Ser 279 activates CaMKII autonomously, creating molecular memory even after Ca2+ concentration declines. O-GlcNAc-modified CaMKII is increased in the heart and brain of diabetic humans and rats. In cardiomyocytes, increased glucose concentration significantly enhances CaMKII-dependent activation of spontaneous sarcoplasmic reticulum Ca2+ release events that can contribute to cardiac mechanical dysfunction and arrhythmias5. These effects were prevented by pharmacological inhibition of O-GlcNAc signalling or genetic ablation of CaMKII6. In intact perfused hearts, arrhythmias were aggravated by increased glucose concentration through O-GlcNAc- and CaMKII-dependent pathways. In diabetic animals, acute blockade of O-GlcNAc inhibited arrhythmogenesis. Thus, O-GlcNAc modification of CaMKII is a novel signalling event in pathways that may contribute critically to cardiac and neuronal pathophysiology in diabetes and other diseases.

Under basal conditions, CaMKII is autoinhibited by an interaction between the regulatory and catalytic subunits of each CaMKII monomer (Fig. 1a). Ca2+/calmodulin (Ca2+/CaM) binding to the regulatory domain disrupts autoinhibition, opening the structure to allow the catalytic domain to phosphorylate targets5. This conformational change is also the basis for fluorescence resonance energy transfer (FRET) changes in a CaMKII activity reporter called Camui, which uses full-length CaMKII and attached green fluorescent proteins6,7 (Fig. 1a). Open-state CaMKII is subject to post-translational modifications, including phosphorylation at T286 (ref. 8) and oxidation at the MM280/280' site (Extended Data Fig. 1b, c), ruling out involvement of those sites. Camui-dependent phosphorylation at T286 (ref. 8) and oxidation at the MM280/280' site (Extended Data Fig. 1b, c), ruling out involvement of those sites. Camui-dependent phosphorylation at T286 (ref. 8) and oxidation at the MM280/280' site (Extended Data Fig. 1b, c), ruling out involvement of those sites.

Using Camui as a direct CaMKII activity reporter, cells exposed to glucose-free or low-glucose (100 mg dl–1; 5.5 mmol l–1) conditions did not exhibit autonomous CaMKII activity (in lysates plus Ca2+/CaM and EGTA buffer) (Fig. 1b, white bars). However, glucose levels corresponding to borderline or severe diabetes (240–500 mg dl–1) induced robust autonomous CaMKII activation. The non-metabolizable sugar mannitol did not activate autonomous CaMKII activity (Extended Data Fig. 1a). Glucose-dependent CaMKII activation was still present in CaMKII mutants lacking critical autophosphorylation and oxidation sites (Extended Data Fig. 1b, c), ruling out involvement of those pathways.
Post-translational modification by O-GlcNAc (O-GlcNAcylation) can alter protein function\textsuperscript{13,14}, and such regulation is seen in heart\textsuperscript{11,12} and brain proteins\textsuperscript{13,15}. O-GlcNAcylation is enhanced by elevated glucose concentration, which raises levels of the direct substrate (uridine diphosphate (UDP)-\textsuperscript{N}-acetylglucosamine) of the enzyme O-GlcNAc transferase (OGT). O-GlcNAc groups are removed by the enzyme O-GlcNAcase. We tested whether direct O-GlcNAcylation might mediate glucose-induced autonomous CaMKII activation, analogous to autophosphorylation in the conserved CaMKII regulatory domain (Extended Data Fig. 1e). Two consensus O-GlcNAcylation sites are T286 and S279. T286A-mutant Camui only slightly limited glucose-induced autonomous activation (Extended Data Fig. 1b), but that could be indirect, through synergy between O-GlcNAcylation at another site enhancing T286 autophosphorylation.

Remarkably, S279A-mutant Camui abolished glucose-induced autonomous CaMKII activation (Fig. 1b, black bars). Importantly, S279A had no effect on either direct CaMKII activation or on autonomous activity induced by autophosphorylation or oxidation (Fig. 1c). Thus, S279 may be a specific target for O-GlcNAc-mediated CaMKII activity during hyperglycaemia.

High glucose did not alter the CaMKII activation state in cells kept in Ca\textsuperscript{2+}-free, EGTA-containing conditions (Fig. 1d). When cells were exposed to elevated glucose (and normal Ca\textsuperscript{2+}), the subsequently measured maximal Ca\textsuperscript{2+}/CaM-dependent activity was enhanced (Fig. 1d, middle bars). Pre-treatment with the CaMKII inhibitor KN-93 (which locks CaMKII in the closed high-FRET state) prevented autonomous activation by high glucose, even in the presence of Ca\textsuperscript{2+}/CaM. Rat cardiomyocytes expressing Camui and exposed to high glucose (without stimulation) for 24 h showed no significant change in baseline CaMKII activity (Extended Data Fig. 2b). The CaMKII inhibitor KN-93 blocked CaMKII activity in the closed high-FRET state and prevented the increase in Ca\textsuperscript{2+}/CaM-dependent activity (Extended Data Fig. 2b).

Although glucose-induced CaMKII activity involves S279 and an autonomous CaMKII-dependent pathway.

To confirm our Camui observations, we cultured rat myocytes for 24 h in varying glucose concentrations and measured autonomous CaMKII activity (± ISO) using a standard assay (\textsuperscript{32}P incorporation into a CaMKII substrate; Fig. 1f). CaMKII activity was increased by glucose >200 mg dl\textsuperscript{-1}, and by combined ISO and high glucose. O-GlcNAcylation is dynamic in cells and limited by glucose availability\textsuperscript{10} and the enzymatic functions of OGT\textsuperscript{16} and O-GlcNAcase\textsuperscript{17}. Specific inhibition of glutamine–fructose amidotransferase by 50 μM diazoxonoreline (DON) to prevent production of the OGT substrate (Fig. 4g), and hence O-GlcNAcylation, abolished glucose-induced autonomous CaMKII activation (Fig. 1g). Conversely, inhibition of O-GlcNAcase with 100 nM thiamet-G (Thm-G) promotes O-GlcNAc modification and enhanced myocyte CaMKII activity in conditions of elevated glucose concentration. Mutant-S279A Camui was not appreciably activated by high glucose in intact cells (Extended Data Fig. 1d). Thus, glucose-induced CaMKII activity involves S279 and an O-GlcNAc-dependent pathway.

To determine the extent of CaMKII O-GlcNAcylation in heart and brain, we used a custom-designed antibody that specifically recognizes this modification\textsuperscript{13}. The fraction of CaMKII that was O-GlcNAc-modified and autophosphorylated was increased in rat myocytes cultured in high relative to normal glucose (350 versus 150 mg dl\textsuperscript{-1}, Fig. 2a), confirming that high glucose induces O-GlcNAc modification and increased activation of CaMKII. O-GlcNAc modification of CaMKII was blocked by KN-93 and in the S279A mutant (Fig. 2a), whereas it was enhanced by treating myocytes with 100 nM ISO 20 min before lysis (Extended Data Fig. 2a). We verified that the antibody reacted specifically to O-GlcNAc by immunoblot before and after \( \beta \)-elimination reactions that specifically cleaved O-linked glycans without degrading proteins (Extended Data Fig. 2b). The O-GlcNAc antibody no longer recognized high-glucose-treated CaMKII after \( \beta \)-elimination, but CaMKII levels were unaltered. O-GlcNAc modification of CaMKII was also disrupted by 50 μM DON and enhanced by 100 nM Thm-G (Extended Data Fig. 2c).

We subjected peptides encoding the regulatory domain of CaMKII to in vitro labelling with O-GlcNAc transferase and confirmed

Figure 2 | O-GlcNAcylation of CaMKII occurs in vivo. a, Immunoblot (IB) with O-GlcNAc-specific and CaMKII-phospho-T286-specific (P-T286) antibodies show that high glucose concentration (350 mg dl\textsuperscript{-1}) increases O-GlcNAcylation and activation of CaMKII, but not in S279A-mutant Camui or after KN-93 treatment (\( n = 3 \) myocyte preparations). IB, immunoprecipitate. b, The ratio of O-GlcNAc-modified to total CaMKII is increased in heart (\( n = 6 \) hearts per group) and brain (\( n = 3 \) brains per group) from diabetic versus control non-diabetic human patients. Ctrl, control. HF, heart failure. c, O-GlcNAc modification of CaMKII is also increased in heart and brain from diabetic rats compared with wild-type controls (number of rats indicated). Data are shown as mean ± s.e.m. \( *P < 0.05, **P < 0.01 \) versus control.

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the S279 site as a target for O-GlcNAc modification using electron-transfer-dissociation mass spectrometry (ETD-MS; Extended Data Fig. 3).

CaMKII expression is increased in patients with heart failure18, and elevated CaMKII expression and activity have been implicated in the transition to heart failure19,20. Using the O-GlcNAc-specific antibody, we probed cardiac samples (holding total CaMKII constant) from patients with heart failure and diabetes (blood glucose >400 mg dl−1), alongside failing and non-failing non-diabetic hearts (blood glucose <200 mg dl−1). The fraction of CaMKII that was O-GlcNAc modified was doubled in heart failure patients and nearly tripled in heart failure patients with diabetes versus those with non-failing, non-diabetic hearts (Fig. 2b). Similarly, brain samples from people with diabetes had significantly increased O-GlcNAc-modified CaMKII relative to those from non-diabetic people. In a diabetic rat model with reduced insulin secretion and blood glucose >600 mg dl−1 (ref. 21), CaMKII O-GlcNAcylation was greatly elevated in heart and brain samples versus in control rats (Fig. 2c). Interestingly, CaMKII autophosphorylation was also enhanced in cardiac tissue from diabetic rats (Extended Data Fig. 2d), consistent with synergistic CaMKII activation through these mechanisms. Taken together, our data demonstrate that CaMKII O-GlcNAcylation and activation occur in the heart and brain of diabetic subjects.

Ryanodine receptor (RyR) phosphorylation by CaMKII enhances cardiac sarcoplasmic reticulum Ca2+ release events (Ca2+ sparks and waves)22–24. In intact isolated myocytes, GlcNAcase inhibition with Thm-G or elevated glucose concentration alone increased Ca2+ spark and wave frequency (Fig. 3a–c). The Thm-G-induced Ca2+ spark increase occurred without altered sarcoplasmic reticulum Ca2+ content (Extended Data Fig. 4a) and was prevented by the CaMKII inhibitor KN-93, but not its inactive analogue KN-92. Combining Thm-G treatment with increased glucose concentration (350 mg dl−1) markedly increased Ca2+ sparks and waves, consequently depleting sarcoplasmic reticulum Ca2+ (Extended Data Fig. 5). Thus, hyperglycaemia and reduced GlcNAcase activity synergize in activating sarcoplasmic reticulum Ca2+ release. Acute blockade of either CaMKII (+KN-93) or O-GlcNAcylation (+DON) prevented glucose-dependent Ca2+ sparks (Fig. 3c), but did not alter sarcoplasmic reticulum Ca2+ load (Extended Data Fig. 4b). Ca2+ spark frequency versus control was neither altered by DON (Fig. 3c) nor the non-metabolizable sugar mannitol (Extended Data Fig. 4c). Thus, glucose-induced arrhythmogenic Ca2+ waves occur through a CaMKII- and O-GlcNAc-dependent mechanism.

To test whether CaMKII-δ (the dominant cardiac isoform) is required for O-GlcNAc-dependent effects on sarcoplasmic reticulum Ca2+ release, we used myocytes from CaMKII-δ knockout mice. Neither Ca2+ transient amplitude nor sarcoplasmic reticulum Ca2+ load (Extended Data Fig. 4d, e) were altered by acute Thm-G exposure in wild-type or CaMKII-δ knockout mouse cells. Ca2+ spark frequency was significantly enhanced by Ca2+ in wild-type but not in CaMKII-δ knockout myocytes (Fig. 3d).

Using optical mapping in Langendorff-perfused rat hearts exposed to 400 mg dl−1 glucose, we observed a significant increase in premature ventricular complexes (PVCs) compared with baseline (Fig. 4a),
consistent with observations of enhanced PVCs in human diabetic patients\textsuperscript{39}. This effect was attenuated by inhibiting either CaMKII (using KN-93) or O-GlcNAc (using DON) (Fig. 4b). We also mapped intracellular \([\text{Ca}^{2+}]_i\) ([Ca\textsuperscript{2+}]i) and voltage simultaneously. Epicardial activation during PVCs was typified by markedly slowed conduction and activation times compared with normal activation (Fig. 4c). Additionally, spontaneous diastolic [Ca\textsuperscript{2+}]i elevation preceded the action potential upstroke in high glucose conditions (Fig. 4d), an effect prevented by blocking OG\textsuperscript{T} using DON pre-treatment (Extended Data Fig. 6a, b).

We also observed \textit{in vivo} arrhythmia susceptibility in normal and diabetic rats during challenge with caffeine and dobutamine (Fig. 4e, f). DON pre-treatment ablated arrhythmias induced by caffeine and dobutamine in diabetics, but had no effect on baseline or caffeine/dobutamine-induced arrhythmia in non-diabetic rats. We also confirmed that CaMKII activity is elevated in diabetic rat hearts, and that this effect is blunted by pre-treatment of rats with DON (Extended Data Fig. 6c), consistent with O-GlcNAc- and CaMKII-dependent hyperglycaemia-induced arrhythmogenesis.

We identified a novel mechanism for autonomous CaMKII activation by O-GlcNAc modification at CaMKII S279 (Fig. 4g). Acute extra-cellular glucose elevation, to levels that mimic those in diabetic patients, suffices to activate CaMKII through this pathway in intact cardiac myocytes and leads to arrhythmic events in intact hearts and animals. In diabetic hearts and brains CaMKII O-GlcNAcylation is elevated and this may contribute to pathological alterations in cardiac myocytes and neurons. Indeed, this pathway may synergize with autonomous CaMKII activation by phosphorylation\textsuperscript{8} and oxidation\textsuperscript{26}, which are important in signalling in many cell types. CaMKIV, related to CaMKII, is O-GlcNAcylated at S189, which inhibits its activation by Ca\textsubscript{MK}II kinase\textsuperscript{35}. O-GlcNAc-mediated activation in CaMKII is not analogous to the inhibition seen for CaMKIV.

The S279 site is highly conserved in all mammalian CaMKII isoforms (Extended Data Fig. 1e), and the robust functional effects in cardio-myocytes suggest that hyperglycaemia can readily activate CaMKII in both heart and brain, and alter phosphorylation of multiple CaMKII targets (including CaMKII itself) to exert both acute (for example, altered Ca\textsuperscript{2+} handling/arrhythmias) and chronic (for example, transcriptional regulation) effects in many tissues. CaMKII is an important nodal point in both acute and chronic modulation of ion channels in both heart and brain. Overactivation of CaMKII caused by hyperglycaemia during diabetes may lead to widespread and as yet unappreciated pathological consequences that merit exploration. It is already known that overactivation of CaMKII occurs in heart failure and neuronal excitotoxicity, and that this activated CaMKII can contribute to major dysfunction at the level of acute ion channel modulation that contributes to cardiac arrhythmias\textsuperscript{13,14}, reduced contractility, neuronal damage\textsuperscript{28} and altered gene transcription\textsuperscript{1}. In diabetes, these powerful CaMKII signalling pathways are likely to be activated by hyperglycaemia-induced O-GlcNAc modification of CaMKII, and this should be considered in future therapeutic strategies. This could also broaden the impact of CaMKII inhibitors in therapeutics in heart disease and beyond.

METHODS SUMMARY
Camui constructs were generated as previously described.\textsuperscript{40} HEK293 cells were kept in culture for 24 h and transiently transfected with expression plasmids encoding Camui. Cells were cultured for an additional 24 h after transfection. Fluorescence measurements were performed using fluorescence spectrophotometry, excited at 440 nm with emission recorded at 477 nm for cyan fluorescent protein (CFP) (FC\textsubscript{CFP}) and 527 nm for yellow fluorescent protein (YFP) (F\textsubscript{YFP}). Camui fluorescence and ratio (FC\textsubscript{YFP}/F\textsubscript{CFP}) was measured with 10 \(\mu\)M Ca\textsubscript{2+} as a maximal activity and with 1 mM EGTA to chelate Ca\textsuperscript{2+} for assessing deactivated or autonomous CaMKII activation. CaMKII activity was directly measured as incorporation of \(^{32}\text{P}\) from ATP-\(^{32}\text{P}\) into an artificial substrate as previously described.\textsuperscript{24} Ca\textsubscript{2+} transients and sparks were recorded using confocal microscopy. Pooled data are represented as mean \(\pm\) s.e.m. Statistical comparisons were made with repeated two-way analysis of variance and paired Student’s \(t\)-test where applicable. \(P < 0.05\) was considered significant.
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Author Contributions J.R.E. and D.M.B. conceived the project. J.R.E. and L.P. carried out most of the experiments. L.W. and C.M.R. conducted optical mapping and in vivo ECG experiments and analysis. G.H., R.J.C. and G.W.H. conducted ETD-MS analysis. A.F. and K.D. generated constructs, performed animal surgeries, and participated in data analysis. F.D. contributed diabetic rats and some analysis therewith. J.R.E. and D.M.B. wrote the manuscript, with assistance from the other authors.

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METHODS

Construction of adenoviral vectors encoding biosensors. The Camui construct23 was incorporated in adenoviruses using the AdEasy adenoviral vector system (Qbiogene) to ensure high infection efficiency in terminally differentiated adult ventricular myocytes. Mutant variants of Camui (T286A, CM280/281V and S279A) were generated using the commercially available QuickChange site-directed mutagenesis kit (Stratagene), and likewise incorporated into adenovirus.

HEK293-cell transfection. HEK293 cells (mycoplasma free at the time of this study) were cultured in DMEM (Invitrogen) with 5% FBS and penicillin/streptomycin for 24 h, and then transiently transfected with expression plasmids encoding Camui using a mammalian transfection kit (Stratagene). Cells were cultured for an additional 36 h after transfection. Camui expression was checked by fluorescence microscopy before experiments.

Human and rat models of diabetes. Failing hearts from type 2 diabetic and non-diabetic patients were obtained at the time of orthotopic heart transplantation as a gift from K. Margulies (University of Pennsylvania). Brain samples from human temporal cortex were obtained as a gift from L.-W. Jin and M. Melara (University of California, Davis). All human specimens, including failing heart tissues and brain samples, were obtained in accordance with Institutional Review Board approval at the respective institutions where samples were collected. All tissue was obtained with informed consent before transplantation surgery.

Inclusion in tissue-based studies was restricted on the basis of age, sex, race or ethnic status. Human cardiac tissue samples were divided into three groups: a non-failing and non-diabetic group (Fig. 2b control: 3 males, 3 females; ages 42–60), a heart failure group not under treatment for diabetes and with blood glucose < 200 mg dl\(^{-1}\) (Fig. 2b heart failure: 3 males, 3 females; ages 41–63), and a heart failure group with diagnosed diabetes and blood glucose > 200 mg dl\(^{-1}\) (Fig. 2b heart failure/diabetes: 4 males, 2 females; ages 38–66).

Human brain tissue samples were divided into two groups: a non-diabetic group (Fig. 2b control: 3 females; ages 79–89), and a diabetic group with blood glucose > 200 mg dl\(^{-1}\) (Fig. 2b diabetes: 3 females; ages 65–82). Male Sprague–Dawley (SD) rats transgenic for human amylin in the pancreatic β-cells (HIP rats) were used at age 10–12 months as previously described28.

Animal studies were not randomized or blinded for this study. Sample sizes were determined by power analysis or based on previous studies with the selected models28. Blood glucose levels in rats were measured 1 day before experiments were conducted using a OneTouch Ultra glucose meter (LifeScan; model no. AW 060-213-01A). All diabetic HIP rats had a blood glucose concentration of over 440 mg dl\(^{-1}\) (Fig. 1a). All diabetic HIP rats had a blood glucose concentration of over 440 mg dl\(^{-1}\) (Fig. 1a).

In vitro fluorescence and CamKII activity assays. Fluorescence measurements were performed using an MS SpectraMax plate reader spectrophotometer ( Molecular Devices). Excitation and emission slits were set at 4 nm. An excitation wavelength of 440 nm was used, and dual photon counting emission detectors were set at 527 nm (CFP) and 527 nm (YFP), respectively. HEK cells or rat ventricular myocytes expressing Camui were treated with 10 μM CaM and 200 μM Ca\(^{2+}\), then lysed in a buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl\(_2\), and protease inhibitors to measure ‘direct’ activation (for example, see Fig. 1b).

Intramitochondrial fluorescence measurements, Ca\(^{2+}\)/CamT treatment was performed in the presence of 100–500 mg dl\(^{-1}\) glucose (Fig. 1b, d, e, g), 100 μM ATP (Fig. 1c) or 1 μM H\(_2\)O\(_2\) (Fig. 1c). Cell lysis was then performed in a buffer containing 1 mM EGTA to chelate Ca\(^{2+}\) and isolate the kinase activity attributed to spontaneous (rather than direct) activation. In Fig. 1d, cells were pretreated with 1 mM EGTA and 10 μM KN-92/KN-93. In Fig. 1e, myocytes were not directly treated with Ca\(^{2+}\) and ATP (Fig. 1e).

Myocyte isolation and adenoviral infection. All protocols involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of California, Davis Institutional Animal Care and Use Committee. Adult rat ventricular myocytes were isolated as previously described27. Myocytes were seeded on laminin-coated coverslips in serum-free PC-1 medium (Lonza) supplemented with penicillin/streptomycin. Myocytes were infected for 2 h at a multiplicity of infection of 10–100 with adenovirus encoding Camui, followed by replacement with fresh medium. Infected cells were kept in culture for 36 h with one final replacement of fresh medium 1 h before experiments.

Confocal microscopy imaging. Coverslips were mounted on the stage of an inverted microscope (Zeiss, LSM5 Pascal) equipped with a 40 × 1.4 NA water immersion objective lens. Argon laser excitation wavelengths were 458 nm for CFP and 514 nm for YFP. CFP emission fluorescence was measured by confocal microscopy at 485 ± 15 nm, and YFP emission fluorescence was measured at ≥535 nm. Camui imaging experiments were performed as previously described27. Image software was used for image analysis.

Spark measurements. Intact ventricular myocytes were loaded with Fluo-3 AM (5 μM; Molecular Probes) and transients were recorded as previously described27. Ca\(^{2+}\) transients were obtained by field stimulation at 1 Hz. Sarcoplasmic reticulum Ca\(^{2+}\) load was evaluated by the Ca\(^{2+}\) transient upon rapid caffeine application (10 mM). Experiments were performed with confocal microscopy (BioRad, Radiance 2100, ×40 objective) using line scan mode with argon 4 laser (excitation at 488 nm, emission at >505 nm). Image analysis used ImageJ and homemade routines in interactive data language (IDL).

Langendorff-perfused rat hearts. All procedures involving animals were approved by the Animal Care and Use Committee of the University of California, Davis and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Adult male Sprague–Dawley rats (250–300 g) were anaesthetized with pentobarbital sodium (150 mg kg\(^{-1}\), intraperitoneal (IP)) containing 500 IU kg\(^{-1}\) of heparin. After a midventricular incision, hearts were rapidly excised and Langendorff perfused at 37 °C with oxygenated (95% O\(_2\), 5% CO\(_2\)) modified Tyrode’s solution of the following composition (in mM\(^{-1}\)): NaCl 128.2, CaCl\(_2\) 1.3, KCl 4.7, MgCl\(_2\) 1.05, NaH\(_2\)PO\(_4\) 1.19, NaHCO\(_3\) 20 and glucose 11 (pH 7.4). Flow rate (~6–15 ml min\(^{-1}\)) was adjusted to maintain a perfusion pressure of 60–70 mm Hg. One leaflet of the mitral valve was carefully damaged with sharp forceps inserted through the pulmonary vein to prevent solution convection in the left ventricular cavity after suppression of ventricular contraction. This also prevented acidification of the perfusate and the development of ischemia in the left ventricle. Two Ag/AcCl disc electrodes were positioned in the bath to record an ECG analogous to a lead 1 configuration. ECG was continuously recorded throughout the duration of the experiment. A bipolar pacing electrode was positioned on the base of the left ventricular epicardium for pacing, which was performed at a basic cycle length (BCL) of 200 ms using a 2 ms pulse width at twice the diastolic threshold.

Dual optical mapping of V\(_m\) and Ca\(^{2+}\). Hearts were loaded with the fluorescent intracellular Ca\(^{2+}\) indicator Rhod-2 AM (Molecular Probes; 250 μl of 1 mM) in dimethylsulphoxide (DMSO) containing 10% pluronic acid and were subsequently stained with the voltage-sensitive dye RH237 (Molecular Probes; 25 μl of 1 mM in DMSO). Blebbistatin (Tocris Bioscience; 10–20 μM) was added to the perfusate to eliminate motion artefact during optical recordings. The anterior epicardial surface was excited using LED light sources centred at 530 nm and band-pass filtered from 511–551 nm (LEX-2; Scifma) and focused directly on the surface of the preparation. The emitted fluorescence was collected through a 50 mm objective (Nikon) and split with a dichroic mirror at 630 nm (Omega). The longer wavelength moieties, containing the V\(_m\) signal, were long-pass filtered at 700 nm, and the shorter wavelength moieties, containing the Ca\(^{2+}\) signal, were band-pass filtered between 574–606 nm. The emitted fluorescence signals were recorded using two CMOS cameras (MiCam Ultima-L; SciMedia) with a sampling rate of 1 kHz and 100 × 100 pixels with a 20 × 20 mm field of view. The atrioventricular node was ablated using a fine-tip thermal cautery (Acuderm) to produce a slow intrinsic rhythm which allowed for ectopic activity and PVCs to escape. After loading the dyes, baseline electrophysiological parameters were recorded during normal rhythm as well as left ventricular epicardial pacing at a BCL of 200 ms. Hearts were then subjected to hyperglycaemia (400 mg dl\(^{-1}\)) with (n = 3) or without (n = 5) pre-treatment (10 min) with the O-GlcNAc inhibitor DON (50 μM). Optical recordings were taken every 5 min after treatment and ECG was continuously recorded.

In vivo ECG recordings. In vivo experiments were performed in anaesthetized diabetic rats (blood glucose >500 mg dl\(^{-1}\) ). Rats received an injection of caffeine (IP; 120 mg kg\(^{-1}\)) and dobutamine (intravenous; 50 μg kg\(^{-1}\) ) during in vivo experiments. The same individuals were pre-treated (30 min before caffeine/dobutamine challenge) with an IP injection of DON (5 mg kg\(^{-1}\) ). Experiments were

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done 1 week apart, and some individuals received the reverse of the described procedure (+DON in first trial, −DON in second trial) to control for compensation effects between trials. For quantification of arrhythmia scores, the severity of arrhythmias was quantified using a previously published scoring system. Each individual heart was evaluated by means of a 5-point arrhythmia score, where single PVCs were given a score of 1, bigeminy/salvos a score of 2, ventricular tachycardia a score of 3, ventricular fibrillation a score of 4, spontaneous ventricular fibrillation a score of 5, and an assigned number corresponded to the most severe type of arrhythmia observed in that heart. Scores were used for group analysis of severity of arrhythmias.

Optical mapping data analysis and statistics. Optical mapping data analysis was performed using two different commercially available analysis programs (BV_Analyze, Brainvision; and Optiq, Cairn). $V_m$ and Ca$^{2+}$ data sets were spatially aligned and processed with a Gaussian spatial filter (radius 3 pixels). For both action potentials and Ca$^{2+}$ transients (CaTs), activation time was determined as the time at 50% between diastolic and peak amplitude. Diastolic Ca$^{2+}$ elevation was measured as the percentage of diastolic Ca$^{2+}$ increase relative to the following CaT amplitude at baseline and 30 min post-treatment. The average diastolic Ca$^{2+}$ elevation was calculated for each heart by averaging all Ca$^{2+}$ signals from the entire anterior surface of the heart within the optical mapping field of view. PVC incidence was determined from the continuous ECG recording as the number of PVCs that occurred during a 15 min period of baseline activity (before initiation of treatment) and during the first 15 min of treatment.

All values are presented as mean ± s.e.m.; n values are generally biological replicates (hearts, brains, animals, myocytes, cell preparations) as indicated in legends. In addition, three technical replicates (triplicates) from three biological replicates were used for some cellular Camui experiments in Fig. 1. Comparisons between two groups of data were made using a Student’s t-test, paired where appropriate or with repeated two-way analysis of variance. $P < 0.05$ was considered statistically significant.

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Extended Data Figure 1 | O-GlcNAc effect is not abolished by T286A or CM280/281VV mutation and CaMKII regulatory domain contains consensus O-GlcNAc modification sites. 

(a) Increased glucose concentration, but not osmolarity-matched mannitol, activates CaMKII in HEK cells (n = 9).

(b) O-GlcNAc-dependent CaMKII activation is reduced but still present in T286A-mutant Camui (n = 9). WT, wild type.

(c) Glucose-dependent CaMKII activation is preserved in CM280/281VV-mutant Camui expressed in HEK-cell lysates (n = 9).

(d) Activation of Camui by increased glucose is blunted in the S279A mutant and ablated entirely by DON (n values: wild type = 100, wild type + DON = 72, S279A = 57, S279A + DON = 44 cells).

(e) These sites are conserved in all known isoforms of CaMKII and in a wide variety of mammalian species. Data are mean ± s.e.m. *P < 0.05, **P < 0.01 versus control.
Extended Data Figure 2 | O-GlcNAc modification of CaMKII is enhanced in hyperglycaemic conditions. 

a, Immunoblot with an O-GlcNAc-specific antibody shows O-GlcNAc modification of CaMKII is inducible by increased glucose availability and is enhanced by Iso treatment (n values indicated).

b, O-GlcNAc modification of CaMKII is reversed by β-elimination reaction before immunoblot.

c, O-GlcNAc modification of CaMKII is ablated by DON and enhanced by Thm-G.

d, Autophosphorylation of cardiac CaMKII is significantly increased in a rat model of diabetes. n = 3 for all immunoblots except where indicated. Data are mean ± s.e.m. *P < 0.05, **P < 0.01 versus control.
Extended Data Figure 3 | ETD-MS analysis confirms O-GlcNAc modification at S279A. A synthetic peptide encoding the regulatory domain of CaMKII was subjected to *in vitro* O-GlcNAc labelling followed by ETD-MS analysis. Examination of the 507.25 m/z peptide fragment (top right inset) indicates the presence of an O-GlcNAc modification at S279 (c6 to c7 fragmentation).
Extended Data Figure 4 | Sarcoplasmic reticulum Ca\(^{2+}\) content, sparks and twitch Ca\(^{2+}\) transients. a, b, Sarcoplasmic reticulum (SR) content is unaffected by Thm-G (a) or DON (b) in isolated rat myocytes (n values indicated). c, Mannitol does not enhance calcium spark frequency in isolated rat myocytes. d, e, Ca\(^{2+}\) transient amplitude (d, n = 13) and SR content (e, n = 13) are unaffected by Thm-G treatment in isolated myocytes from wild-type (WT) or CaMKIIδ-knockout mice. Data are mean ± s.e.m. NS, no significant difference.
Extended Data Figure 5 | Ca\textsuperscript{2+} sparks induced by glucose and Thm-G.

a, b, Simultaneous treatment with 3,500 mg dl\textsuperscript{-1} glucose and Thm-G greatly enhances spark frequency (a) and SR Ca\textsuperscript{2+} depletion (b) in isolated rat myocytes (n = 6). Data are mean ± s.e.m.
Extended Data Figure 6 | Diastolic calcium elevation under high glucose is suppressed by pre-treatment with 50 mM DON. a, Average diastolic calcium elevation at baseline and following treatment with either high glucose (HG) or DON plus high glucose. Calcium elevation was measured as the percentage increase in the diastolic calcium signal relative to the amplitude of the following transient (n = 3). b, Example transients during baseline conditions (black) and after treatment with either high glucose or DON plus high glucose (grey). Minimal diastolic calcium elevation occurs after pre-treatment with DON. n = 3–4 rats for all data points. c, CaMKII activity is enhanced in heart lysate from diabetic rats (n = 3), and this effect is blunted by treatment with DON. Data are mean ± s.e.m. *P < 0.05 versus control.