Cardiovascular disease is the leading cause of death in the diabetic population. However, molecular mechanisms underlying diabetic cardiomyopathy remain unclear. We analyzed Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and excitation-contraction coupling in db/db obese type 2 diabetic mice and their control littermates. Echocardiography showed a systolic dysfunction in db/db mice. Two-photon microscopy identified intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) transient decrease in cardiomyocytes within the whole heart, which was also found in isolated myocytes by confocal microscopy. Global [Ca\textsuperscript{2+}]\textsubscript{i} transients are constituted of individual Ca\textsuperscript{2+} sparks. Ca\textsuperscript{2+} sparks in db/db cardiomyocytes were less frequent than in +/+ myocytes, partly because of a depression in sarcoplasmic reticulum Ca\textsuperscript{2+} load but also because of a reduced expression of ryanodine receptor Ca\textsuperscript{2+} channels (RyRs), revealed by \textsuperscript{3}Hryanodine binding assay. Ca\textsuperscript{2+} eflux through Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was increased in db/db myocytes. Calcium current, \(I_{\text{Ca}}\textsuperscript{\text{Ca}}\), triggers sarcoplasmic reticulum Ca\textsuperscript{2+} release and is also involved in sarcoplasmic reticulum Ca\textsuperscript{2+} refilling. Macroscopic \(I_{\text{Ca}}\textsuperscript{\text{Ca}}\) was reduced in db/db cells, but single Ca\textsuperscript{2+} channel activity was similar, suggesting that diabetic myocytes express fewer functional Ca\textsuperscript{2+} channels, which was confirmed by Western blots. These results demonstrate that db/db mice depressed cardiac function, at least in part, because of a general reduction in the membrane permeability to Ca\textsuperscript{2+}. As less Ca\textsuperscript{2+} enters the cell through \(I_{\text{Ca}}\textsuperscript{\text{Ca}}\), less Ca\textsuperscript{2+} is released through RyRs. Diabetes 55:608–615, 2006

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\textsuperscript{[Ca\textsuperscript{2+}]}\textsubscript{i}, intracellular calcium concentration; NCX, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; RyR, ryanodine receptor Ca\textsuperscript{2+} channel; SERCA, sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase.

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RESEARCH DESIGN AND METHODS

M-mode echocardiography. Fifteen-week-old male and female C57BL/KsJ-db (db/db) mice and their control littermates (+/+ ) were used. All experiments were carried out according to the ethical principles laid down by the French Ministry of Agriculture and European Union Council Directives for

Diasinet prevalence is rising in Western countries and affects ~143 million patients worldwide, thus approximately five times surpassing estimations made a decade ago (1). About 90% of diabetic patients exhibit non-insulin-dependent (or insulin-resistant) type 2 diabetes, and at least 80% of type 2 diabetic patients in Western countries are obese. These patients usually exhibit severe glucose intolerance compared with lean type 2 diabetic patients (2). Cardiovascular disease is the leading cause of death in the diabetic population (3–5). Although cardiovascular problems could be the result of associated comorbidities, a defect in cardiac performance has been detected in diabetic patients in the absence of vascular injury, supporting the existence of a specific diabetic cardiomyopathy. Reductions in diastolic compliance, contractility, and rate of myocardial relaxation characterize diabetic cardiomyopathy (6–8). However, cellular and molecular bases of cardiac dysfunction are largely unknown in type 2 diabetes.

Because Ca\textsuperscript{2+} release and uptake are at the root of contraction and relaxation, alteration of Ca\textsuperscript{2+} handling has been suspected to drive the progression of functional abnormalities in diseased hearts. However, data regarding Ca\textsuperscript{2+} handling in type 2 diabetes are limited and ambiguous. For example, a rat model of insulin-resistant obese type 2 diabetes (cp/cp) shows enhanced Ca\textsuperscript{2+} uptake into sarcoplasmic reticulum, but sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) levels and sarcoplasmic reticulum Ca\textsuperscript{2+} load are unaltered (9). By contrast, other well-known models of type 2 diabetes such as Otsuka Long Evans Tokushima Fatty rats (10) and db/db mice (11) present defects in both function and expression of SERCA. Strep-tozotocin injection in neonatal rats induces adult non-insulin-dependent diabetes, and the activities of plasmalemmal Ca\textsuperscript{2+} ATPase (12,13) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) (13) are both decreased in this model. Thus, a distinct Ca\textsuperscript{2+} transporter malfunction has been identified in different diabetic models, but an integrated mechanism of altered Ca\textsuperscript{2+} homeostasis is lacking.

We characterized Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and excitation-contraction coupling in hearts of db/db mice, an obesity-linked mouse model of type 2 diabetes. Like humans with diabetic cardiomyopathy, db/db mice display increased left ventricular end-diastolic pressure, decreased left ventricular systolic pressure at high preload, and lowered cardiac output (14). We found impaired intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) cycling that may explain these functional changes: a reduction in L-type calcium current \(I_{\text{Ca,L}}\) underlies a reduction in [Ca\textsuperscript{2+}]\textsubscript{i} transient and contraction, both of which are exacerbated by a decrease in ryanodine receptor Ca\textsuperscript{2+} channel (RyR) density and sarcoplasmic reticulum Ca\textsuperscript{2+} load.
the care of laboratory animals. Transthoracic echocardiography was performed in anesthetized animals (35 mg/kg sodium pentobarbital) using an ultrasound machine (Esaote “Mylab”, Florence, Italy) and a 10-MHz phased-array transducer. Parameters were determined using the leading-edge method of the American Society of Echocardiography. End-diastole was determined at the maximal left ventricular diastolic dimension, and end-systole was taken at the peak of posterior wall motion. Systolic function was calculated as fractional shortening; % fractional shortening = (left ventricular end-diastolic dimension – left ventricular end-systolic dimension)/left ventricular end-diastolic dimension × 100. Heart rate was determined from measurements of cardiac cycle length on spectral Doppler tracings of the aortic outflow. All data are the average of at least three separate scans, each scan representing the average of three consecutive beats. The average of at least three separate scans, each scan representing the average of three consecutive beats.

Two-photon microscopy in whole hearts. At the time of the experiments, mice were weighed and anesthetized with sodium pentobarbital (100 mg/kg i.p.). The heart was quickly removed and weighed. The aorta was cannulated, and mice were weighed and anesthetized with sodium pentobarbital (100 mg/kg i.p.). The heart was perfused with a HEPES-based Tyrode solution and used for experiments. First, the heart was perfused with a solution containing 120 mmol/l potassium glutamate, 25 mmol/l KCl, 2 mmol/l Na2ATP, 0.012 mmol/l Na3HPO4, 20 mmol/l NaF, 300 mmol/l sucrose, 0.8% glutathione, and 10 mmol/l glucose (pH 7.4 with KOH). Borosilicate pipettes (5–12 MΩ) were filled with internal solution containing 50 mmol/l CaCl2 and 50 mmol/l butanedione monoxime (16) to prevent contraction. The heart was then cannulated for two-photon microscopy. After 20–30 min, the heart was placed on the stage of an upright microscope (Axioskop 2FSmot Meta LSM510, adapted for two-photon microscopy, ceramic objective IR40x, numerical aperture 0.8; Zeiss) under continuous perfusion. The hearts were electrically stimulated through a bipolar platinum electrode at 2 Hz. Ca2+ images were obtained with illumination supplied by a mode-locked Ti:Sapphire laser (Mira 900; Coherent) tuned to a center wavelength of 830 nm. Emission was collected at >560 nm wavelength. [Ca2+]i transients were recorded in the line-scan mode at 3 ms/line. To visualize cell membranes, the heart was loaded with the voltage-sensitive dye di-4 ANEPPS in a single bolus (16), and three-dimensional images were recorded with the two-photon microscope.

Isolated cells. Ventricular myocytes were isolated from adult mice by previously described (15) methods. Whole-cell patch clamp (Axopatch 200A; Axon Instruments) was used to monitor i-type Ca2+ currents (Icalc). The patch pipette contained 50 mmol/l Fluo-3 pentapotassium salt to simultaneously record the associated [Ca2+]i transient. Voltage protocol and solutions were as previously described (17). [Ca2+]i transients were recorded with the confocal microscope (MetaZeiss LSM510, objective water immersion 40x). The 488-nm line of the argon laser was used to illuminate, and emission was collected at >560 nm. The line of scan was selected parallel to the longitudinal cell axis to measure cell shortening.

Sarcoplasmic reticulum Ca2+ load was estimated by rapid caffeine application to isolated cells. Myocytes were previously depolarized to 0 mV (150-ms steps) from a holding potential of −80 mV for 1 min, then 10 mmol/l caffeine was added, and the associated fluorescence image was recorded by confocal microscopy. In some cells, the associated NCX current was recorded by patch clamp pipette. To estimate the amount of Ca2+ released by the sarcoplasmic reticulum (18), [Ca2+]i sparks were recorded in saponin-permeabilized myocytes as previously described (19) using an internal solution containing 50 mmol/l free Ca2+. Image analyses were done using homemade routines in IDL (interactive data language) (20). All experiments performed on isolated hearts or myocytes were carried out at room temperature (21–23°C).

Single-channel recording. Cardiomyocytes were placed in a depolarizing solution containing 120 mmol/l potassium glutamate, 25 mmol/l KCl, 2 mmol/l MgCl2, 10 mmol/l HEPES, 2 mmol/l EGTA, 1 mmol/l CaCl2, 1 mmol/l Na3ATP, and 10 mmol/l glucose (pH 7.4 with KOH). Borosilicate pipettes (5–12 MΩ) were coated with Sylgard and filled with a solution containing 70 mmol/l BaCl2, 90 mmol/l sucrose, and 10 mmol/l HEPES (pH 7.4 with TEA-Off). Ba2+ currents carried by single l-type Ca2+ channels were recorded using the cell-attached configuration of the patch-clamp technique as previously described (21), applying voltage steps to +20 mV from a holding potential of −100 mV, at least 180 sweeps per experiment. Data were sampled at 10 kHz and filtered at 2 kHz using an Axopatch 200A amplifier and analyzed with pClamp6 software (Axon Instruments). In case of multichannel patches, all data were corrected by the number of channels as described previously (22).

[iH]ryanodine binding assay. Hearts were homogenized in a buffer containing 50 mmol/l Na2ATP, 300 mmol/l sucrose, 0.012 mmol/l leupeptin, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 0.01 mmol/l benzamidine (pH 7 with NaOH). Insoluble material was removed by centrifugation at 1,000 rpm for 1 min (4°C). Protein quantification was done with the Bradford assay using BioRad reagents. The [1H]ryanodine binding assay was performed as described previously (23) using 20 μg heart protein homogenate/assay tube in a buffer containing 1 mol/l NaCl, 50 μmol/l CaCl2, and 20 mmol/l Na-PIPES, pH 7.2. Bmax, the maximal number of receptor sites, and Kd, the affinity constant of the receptor-ligand interaction, were obtained by fitting data points with the formula B = Bmax × [iH]ryanodine/Kd + [iH]ryanodine), where B is the specific binding. Nonspecific binding was determined in the presence of 20 μmol/l ryanodine. Western blot. Ventricular protein homogenates were made as described previously (21). Ventricles were rapidly frozen in liquid nitrogen and then placed in Tris solution (50 mmol/l, pH 7.4) containing 1 mmol/l phenylmethylsulfonyl fluoride and chelated using an Ultra-Turrax disperser. Suspension was centrifuged for 15 min (3,500 × g). Supernatant was then centrifuged for 60 min (55,000 × g). The pellet was dissolved in 50 mmol/l Tris solution (pH 8.0) containing 2% SDS, 20% glycerol, and 5 mmol/l dithiothreitol. After shaking for 30 min, probes were frozen at −80°C until subjecting to SDS-PAGE on 8% running gel and 5% stacking gel. For protein analysis, equal amounts of 15 μg per lane were used. SDS-PAGE was blotted to polyvinylidene fluoride membranes (Bio-Rad) using a semidyry Western-blot system. Ca1,2 (α1C) proteins were detected by a specific antibody (C-1603; dilution, 1:600; Sigma-Aldrich) using alkaline-phosphatase reaction. Protein bands were analyzed densitometrically.

RESULTS
db/db mice hearts are functionally defective but not hypertrophied. Echocardiography confirmed functional impairment of db/db hearts (Fig. 1A). Decreased shortening fraction in db/db mice indicated contractile dysfunction (Fig. 1B), whereas left ventricular wall thickness was similar in +/+ and db/db mice (Table 1). Heart weight was similar for both groups (Fig. 1C). To assess hypertrophy of cardiomyocytes, we measured cell dimensions in hearts loaded with the voltage-sensitive dye di-4 ANEPPS, which labels the external cell membrane, and three-dimensional images were recorded. Deconvolution was applied with Huygens (Bitplane) to correct for thickness distortion (24). A deconvoluted two-photon image of cell membranes is shown in Fig. 1D. In agreement with unchanged cell capacitance (Table 1), calculated cell volume was not altered (Fig. 1E).

Cellular [Ca2+]i transient in whole hearts. To determine whether altered cellular Ca2+ signaling underlies this cardiac dysfunction, we recorded [Ca2+]i transients in whole hearts. Freshly explanted hearts were loaded with Rhod-2 (Fig. 1F) and electrically evoked [Ca2+]i transients in individual myocytes were visualized with two-photon microscopy in the line-scan mode. Figure 1G shows representative images of a +/+ cell (top), of a db/db cell (middle), and the corresponding calculated [Ca2+]i transients (bottom). The [Ca2+]i transients recorded in db/db cells were smaller in amplitude and decayed more slowly. Averaged data (Fig. 1H) indicate a significant reduction of [Ca2+]i transient amplitude in db/db myocytes compared with +/+ myocytes. The duration of [Ca2+]i transient at half-maximum amplitude, D1/2, was significantly longer (P < 0.01) in db/db myocytes (170.9 ± 5.4 ms) than in +/+ cells (104.1 ± 2.4 ms). Thus, db/db hearts have depressed contractile properties and decreased [Ca2+]i transients but with no apparent remodeling of the heart. To characterize impairment of [Ca2+]i transients in more detail, we analyzed the function and expression of l-type Ca2+ channels and RyRs and measured sarcoplasmic reticulum Ca2+ load, thus examining critical steps in cardiac Ca2+ cycling more comprehensively.
ALTED Ca^{2+} CYCLING IN db/db CARDIOMYOCYTES

**L-type Ca^{2+} channels and excitation-contraction coupling.** Ca^{2+} release from the sarcoplasmic reticulum is graded by $I_{\text{Ca}}$, the “trigger” current carried by L-type Ca^{2+} channels. We thus recorded whole-cell $I_{\text{Ca}}$ in single myocytes. $I_{\text{Ca}}$ was significantly decreased in db/db myocytes compared with controls (+/+) (Fig. 2A and D). To determine the $I_{\text{Ca}}$-sarcoplasmic reticulum Ca^{2+} release coupling efficiency, we simultaneously recorded $I_{\text{Ca}}$ and its triggered [Ca^{2+}]_i transient by confocal microscopy. Consistent with reduced [Ca^{2+}]_i, transients observed in single cells within the whole hearts, [Ca^{2+}]_i transients were decreased and slowed in isolated db/db cells as well. Figure 2C is a plot of peak [Ca^{2+}]_i transient (P/F_p, with F_p the fluorescence signal, and F_b, the diastolic fluorescence) as a function of voltage step in db/db and +/- cells. The decay time of the [Ca^{2+}]_i transient, calculated by fitting the decay phase of the fluorescence signal to a mono-exponential function, was prolonged in db/db myocytes (decay time constant at 0 mV, 160.1 ± 8.1 ms in 38 +/- cells vs. 192.7 ± 12.4 ms in 16 db/db myocytes; $P < 0.05$). The reduction of sarcoplasmic reticulum Ca^{2+} release in db/db myocytes obviously paralleled the decrease of whole-cell $I_{\text{Ca}}$. Accordingly, cell contraction, which steeply follows the magnitude of the [Ca^{2+}]_i transient (25), was significantly reduced in db/db cells at most potentials (Fig. 2B). Cell shortening, as shown in Fig. 2A (lower traces), was also slowed in diabetic myocytes. Maximum derivative over time (dL/dt) at 0 mV (in % cell length/ms) was 0.33 ± 0.03 in +/- cells vs. 0.21 ± 0.03 in db/db cells ($P < 0.05$). To estimate excitation-contraction coupling gain, we normalized Ca^{2+} release (amplitude of [Ca^{2+}]_i transient) by total Ca^{2+} entry (integral of $I_{\text{Ca}}$) and plotted it as a function of voltage (Fig. 2E). db/db and +/- myocytes showed similar excitation-contraction coupling gain, indicating that the efficacy of $I_{\text{Ca}}$ to trigger Ca^{2+} release remains unchanged in db/db myocytes.

The decrease of macroscopic $I_{\text{Ca}}$ could be due to altered voltage dependence, impaired single-channel gating, or decreased expression of L-type Ca^{2+} channels. We first analyzed steady-state activation and inactivation of $I_{\text{Ca}}$. Figure 3A shows that steady-state inactivation curves in +/+(solid line and open symbols) and db/db (dotted line and filled symbols) were not different regarding half-inactivation potential ($V_{1/2}$: -33.1 ± 1.5 mV for +/-, $n = 17$ vs. -31.6 ± 1.1 mV for db/db, $n = 11$; $P > 0.05$) or slope factor (5.53 ± 0.31 for +/-, $n = 17$ vs. 5.44 ± 0.54 for db/db, $n = 11$; $P > 0.05$). Steady-state activation was slightly shifted toward more depolarized potentials in db/db myocytes (dotted line) compared with +/- myocytes (solid line) (-17.0 ± 0.7 mV in +/- cells, $n = 43$ vs. -13.8 ± 0.8 mV, $n = 20$; $P < 0.01$) without alteration of the slope factor (4.95 ± 0.29 in +/- cells vs. 4.81 ± 1.19 in db/db).

Reduction of $I_{\text{Ca}}$ may reflect altered single-channel properties and/or decreased number of functional channels in the membrane. Recording of single L-type Ca^{2+} channels (Fig. 3B) revealed no difference regarding fraction of active sweeps (46 ± 8 vs. 44 ± 3% in +/- vs. five db/db patches, respectively) and mean open time (0.61 ± 0.05 vs. 0.58 ± 0.02 ms in +/- vs. db/db, respectively), open probability in active sweeps (+/-, 14.3 ± 4.2%; db/db, 21.3 ± 4.5%), and peak ensemble average currents (Fig. 3B, bottom panels) (-77 ± 23 fA in +/- vs. -82 ± 17 fA in db/db) obtained with db/db ($n = 5$ patches) or +/- ($n = 6$ patches) myocytes. Thus, the activity of single L-type Ca^{2+} channels was un-
changed. These findings suggest that the 31.2% decrease in macroscopic $I_{\text{Ca}}$ may reflect lower expression of channel proteins. To test this possibility, we carried out Western-blot analysis of $\text{Ca}^{2+}$ channel 1.2, the pore-forming $\alpha_{1C}$ subunit of L-type $\text{Ca}^{2+}$ channels (Fig. 3C). In agreement with the decrease in whole-cell $I_{\text{Ca}}$, the expression of $\alpha_{1C}$ protein was reduced by $-38\%$ in $\text{db/db}$ ventricles (Fig. 3C).

FIG. 1. $\text{db/db}$ mice show cardiac dysfunction associated with decreased $[\text{Ca}^{2+}]_i$ transients but lack signs of cardiac hypertrophy. A: M-mode echocardiographic images obtained in $+/+$ (left) and $\text{db/db}$ mouse (right). B: Fractional shortening in $+/+$ ($n = 3$) and $\text{db/db}$ ($n = 3$) mice. C: $+/+$ and $\text{db/db}$ heart weight. D: Deconvoluted two-photon image in a mouse heart loaded with di-4 ANEPPS. E: Cell volumes in $+/+$ ($n = 40$) and $\text{db/db}$ mice ($n = 66$). F: Two-photon image of mouse heart loaded with Rhod-2 AM. G: Examples of two-photon line-scan images recorded in hearts loaded with Rhod-2 AM in cell from $+/+$ (top) and $\text{db/db}$ heart (middle). Corresponding $[\text{Ca}^{2+}]_i$ transients (expressed as $F/F_0$) are superimposed at the bottom. Black trace, $+/+$ cell. Blue trace, $\text{db/db}$ cell. Hearts were paced at 2 Hz. H: Averaged values of peak $F/F_0$ in $+/+$ (113 cells from five hearts) and $\text{db/db}$ (77 cells from four hearts). *$P < 0.0001$.

FIG. 2. $\text{db/db}$ mice exhibit depressed $[\text{Ca}^{2+}]_i$ transients due to $I_{\text{Ca}}$ decrease. A: Example recordings taken from $+/+$ cell (left) and $\text{db/db}$ cell (right). From top to bottom: voltage protocol, $I_{\text{Ca}}$, line-scan confocal image, fluorescence trace, and shortening trace (CL%, percentage of cell length). B: Cell shortening as a function of voltage in $+/+$ (open circle and solid line, $n = 20$) and $\text{db/db}$ cells (blue circle and dashed line, $n = 11$). C: Peak $[\text{Ca}^{2+}]_i$ transient ($F/F_0$, see Fig. 1) plotted against voltage in $+/+$ ($n = 44$) and $\text{db/db}$ ($n = 20$) myocytes. Symbols as in B. D: $I_{\text{Ca}}$ density plotted against voltage in $+/+$ and $\text{db/db}$ cells. Symbols and number of samples as in C. E: Plot of excitation-contraction coupling gain calculated for each voltage dividing the $F/F_0$ by the integral of $I_{\text{Ca}}$ (pC/pF). Open circles, $+/+$ cells; blue circles, $\text{db/db}$ cells. *$P < 0.05$; **$P < 0.005$. 

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RyR activity and abundance. The opening of a RyR cluster produces local, rapid, and brief elevations in \([Ca^{2+}]_i\) termed \(Ca^{2+}\) sparks (26). \(Ca^{2+}\) sparks occur spontaneously during diastole and are activated during systole by \(I_{Ca}\). To avoid the compounding effect of \(I_{Ca}\), we analyzed \(Ca^{2+}\) sparks in saponin-permeabilized myocytes. In the absence of external membrane control, the excitation-contraction coupling machinery is reduced to \(Ca^{2+}\) release by RyR and \(Ca^{2+}\) uptake by SERCA2a, thus allowing direct assessment of these parameters. Moreover, permeabilized cells allow easy clamping of internal \([Ca^{2+}]_i\), so that \(Ca^{2+}\) uptake and release in \(+/-\) and \(db/db\) cells may be determined under identical conditions. Figure 4A shows confocal line-scan images in a \(+/+\) (top) and a \(db/db\) (bottom) myocyte, which exhibited fewer \(Ca^{2+}\) sparks. The averaged \(Ca^{2+}\) spark frequency recorded in \(db/db\) myocytes was reduced in diabetic myocytes (Fig. 4B). An analysis of RyR expression by \(^{3}H\)ryanodine binding to cardiac homogenates (Fig. 4C) revealed that \(B_{max}\) was decreased in \(db/db\) hearts (Fig. 4D) (0.10 ± 0.01 pmol/mg protein) compared with \(+/+\) hearts (0.16 ± 0.02 pmol/mg protein), whereas \(K_d\) the RyR-\(^{3}H\)ryanodine affinity constant, was similar (3.7 ± 1.1 and 4.4 ± 0.8 nmol/l in \(+/+\) and \(db/db\) hearts, respectively). Thus, a reduction in RyR expression levels may contribute, at least in part, to the decrease in \(Ca^{2+}\) spark occurrence observed in permeabilized cells.

Sarcoplasmic reticulum \(Ca^{2+}\) load. Sarcoplasmic reticulum \(Ca^{2+}\) load is a major determinant of \(Ca^{2+}\) release and, by extension, a critical factor in \(Ca^{2+}\) spark frequency and \([Ca^{2+}]_i\), transient amplitude. It is plausible, therefore, that sarcoplasmic reticulum \(Ca^{2+}\) load contributes to the decrease in both amplitude of \([Ca^{2+}]_i\) transient (Figs. 1H and 2C) and \(Ca^{2+}\) spark frequency (Fig. 4B) observed in \(db/db\) hearts, independently or in combination with the decreased expression of \(L\)-type \(Ca^{2+}\) channels and RyRs. We thus measured sarcoplasmic reticulum \(Ca^{2+}\) content after caffeine application in conditions used to record \(Ca^{2+}\) sparks and \([Ca^{2+}]_i\), transients, i.e., in permeabilized and intact myocytes, respectively. Two examples of line-scan images recorded during caffeine application in permeabilized myocytes and the average data are displayed in Fig. 4E. Permeabilized \(db/db\) myocytes have lower sarcoplasmic reticulum \(Ca^{2+}\) content, suggesting a decreased activity of SERCA pump. We also estimated sarcoplasmic reticulum \(Ca^{2+}\) load by rapid caffeine application to non-permeabilized myocytes. Figure 5A shows examples of line-scan images recorded in a \(+/+\) (top) and a \(db/db\) (bottom) myocyte. The caffeine-evoked \([Ca^{2+}]_i\), transient was smaller in \(db/db\) myocyte, as demonstrated by the averaged data in Fig. 5B. The caffeine-evoked \([Ca^{2+}]_i\) transient activates the forward-mode of the NCX, eliciting an inward current as \(Ca^{2+}\) is extruded. Hence, the integral of the inward NCX current is a reliable index of the sarcoplasmic reticulum \(Ca^{2+}\) load (18). Figure 5C shows examples of NCX currents (left) and their running integrals (right) in a \(+/+\) (top) and a \(db/db\) (bottom) cell. Figure 5D shows the averaged data of the integral of the caffeine-evoked NCX current and confirms the reduction of sarcoplasmic reticulum \(Ca^{2+}\) load in \(db/db\) myocytes. Thus, a reduction in sarcoplasmic reticulum \(Ca^{2+}\) load may contribute, in combination with decreased expression of RyRs and \(L\)-type \(Ca^{2+}\) channels, to the decrease in \(Ca^{2+}\) spark frequency and \([Ca^{2+}]_i\) transient amplitude and may be a major determinant of the contractile dysfunction of diabetic hearts.

The diminution in sarcoplasmic reticulum \(Ca^{2+}\) load may reflect decreased \(Ca^{2+}\) entry through \(I_{Ca}\) and slowing in sarcoplasmic reticulum \(Ca^{2+}\) uptake but also increased efflux through the NCX. In many models of heart failure, the NCX current (\(I_{NCX}\)) is increased (27). We measured peak \(I_{NCX}\) (pA) activated by the increase in \([Ca^{2+}]_i\) (\(F/F_0\)) at a membrane potential of \(-80\) mV and found that \(I_{NCX}\) is increased in \(db/db\) myocytes (in pA/\(F/F_0\), 27.6 ± 2.6 in 20 \(+/+\) cells vs. 47.9 ± 7.2 in 20 \(db/db\) cells, \(P < 0.05\)). In heart failure after myocardial infarction, we found that although the \(I_{NCX}\) was increased, there was a concomitant increase in cell volume that cancels out the relative increase of \(I_{NCX}\), thus unfaltering the capacity of the cell to extrude \(Ca^{2+}\) (28). We therefore normalized \(I_{NCX}\) by \([Ca^{2+}]_i\) and by cell volume. We found that \(I_{NCX}\) (pA) normalized by \([Ca^{2+}]_i\) (\(F/F_0\)) and by cell volume (mL) was 1.0 ± 0.1 in \(+/+\) myocytes (\(n = 20\)) and 1.6 ± 0.2 in \(db/db\) (\(n = 20\)), \(P < 0.05\). So \(db/db\) myocytes show an increased \([Ca^{2+}]_i\), efflux for a given \([Ca^{2+}]_i\).
DISCUSSION

Cardiovascular alterations constitute the most frequent cause of death in diabetic patients (3–5). At present, an integrated scheme of excitation-contraction coupling alterations in type 2 diabetic hearts is incomplete. Our study of db/db mice, which experiences obesity-associated type 2 diabetes, shows that the contractile dysfunction linked with the cardiomyopathy can be explained, at least in part, by disturbed Ca\(^{2+}/\)H\(^{+}\) cycling, i.e., reduced Ca\(^{2+}\) influx via L-type Ca\(^{2+}/\)H\(^{+}\) channels, lowered Ca\(^{2+}\) release from sarcoplasmic reticulum, slowed Ca\(^{2+}\) reuptake, and increased Ca\(^{2+}\) efflux.

db/db mice exhibit cardiac failure that is evident as early as 12 weeks of age (29). Here, we used 15-week-old db/db mice and confirmed the depression of systolic function by M-mode echocardiography (Fig. 1A and B). Systolic dysfunction is accompanied by a dramatic decrease of cellular [Ca\(^{2+}\)]\(_{i}\), transients recorded in whole hearts (Fig. 1G) and isolated field-stimulated (11) and patch-clamped myocytes (Fig. 2A). This depression of Ca\(^{2+}\) release could underlie the systolic dysfunction of db/db hearts, although other factors (e.g., contractile myofibrils and elastic properties of the heart) may contribute.

The simultaneous recording of cell shortening, [Ca\(^{2+}\)]\(_{i}\) transient and \(I_{Ca}\) in isolated db/db myocytes revealed that cell contraction is decreased without an apparent decrease in excitation-contraction coupling efficiency (Fig. 2E). The cellular contractile dysfunction is consistent with the echocardiographic measurements presented in Fig. 1. Furthermore, the amplitude of the [Ca\(^{2+}\)]\(_{i}\) transient recorded in isolated cells was reduced (Fig. 2C), which is consistent with data obtained by two-photon microscopy in whole hearts (Fig. 1). Interestingly, the trigger \(I_{Ca}\) was also reduced in db/db myocytes (Fig. 2D), thereby imposing a limitation on the amount of Ca\(^{2+}\) ions entering into diabetic myocytes. This limitation is likely exacerbated by the shift of \(I_{Ca}\) activation curve toward more depolarized potentials, which results in a reduction of the “window current.” Given the fact that the activity of single L-type Ca\(^{2+}\) channels is not modified and that the reduction in the density of pore-forming subunit \(\alpha_{1c}\) is similar to the reduction in whole-cell \(I_{Ca}\), it is likely that the reduction in

![Image](https://via.placeholder.com/150)

FIG. 4. Ca\(^{2+}\) spark occurrence is decreased in db/db hearts, along with a reduction in RyR expression. A: Line-scan images showing spontaneous Ca\(^{2+}\) sparks recorded in +/- and db/db permeabilized myocytes. B: Ca\(^{2+}\) sparks per second per 100 \(\mu\)m recorded in 10 +/- and in 14 db/db myocytes. C: Specific \[^{3}H\]ryanodine binding measured in +/- (open circles) and db/db (blue circles) hearts. Lines represent fitting of the points with the equation \(B = B_{max} \times \left[^{3}H\right]\text{ryanodine/}K_{d} + \left[^{3}H\right]\text{ryanodine}\) with \(B\) as the specific binding. D: Averaged \(B_{max}\) calculated for six +/- and six db/db hearts. E: Left, line-scan images recorded in +/- (top) and db/db (bottom) permeabilized myocytes during 20 mmol/l caffeine application. Right, averaged peak fluorescence ratio (measured as \(F/F_{0}\) as in Fig. 1) obtained during caffeine application in +/- (n = 20) and db/db myocytes (n = 20). *\(P<0.05\); **\(P<0.01\).
I_{Ca} is the result of fewer L-type Ca^{2+} channels in db/db mice hearts. A decrease in [Ca^{2+}]_i transient has been found in other models of cardiac pathology and contractile dysfunction (rev. in 27), but in most cases, cardiac hypertrophy and normal I_{Ca} density accompany the depressed [Ca^{2+}]_i transient. In the case of type 2 diabetic mice, a systematic reduction of I_{Ca} (Fig. 2D) may underlie the decreased sarcoplasmic reticulum Ca^{2+} release and contraction either directly, by triggering lower Ca^{2+} release, or indirectly, by lowering the sarcoplasmic reticulum Ca^{2+} load. Under a reduced I_{Ca}, a new equilibrium may be reached in diabetic hearts where Ca^{2+} entry, release, and uptake are reduced. The reduction in sarcoplasmic reticulum Ca^{2+} load may also be due to a depression in SERCA2a activity. In fact, we found the [Ca^{2+}]_i transient to decay slower in db/db myocytes. In mice, decline of the [Ca^{2+}]_i transient is mainly (～90%) due to SERCA2a activity, with relatively minor contribution (～9%) by the NCX (30). Thus slowing of the [Ca^{2+}]_i transient in db/db is most likely caused by decreased SERCA activity. In support of this notion, others have shown a large increase in the expression of the SERCA inhibiting protein phospholamban in db/db hearts (11). Moreover, the increased Ca^{2+} efflux through the NCX can contribute to decreasing the sarcoplasmic reticulum Ca^{2+} load.

RyR activity can be determined by examining Ca^{2+} sparks. We found that db/db cardiac myocytes exhibit reduced rate of Ca^{2+} spark frequency (Fig. 4A and B). This reduction cannot be explained by changes in diastolic [Ca^{2+}]_i (11) or I_{Ca} (Fig. 3) because Ca^{2+} sparks were recorded in permeabilized myocytes, where internal [Ca^{2+}]_i is fixed and I_{Ca} is absent. However, permeabilization may wash out soluble components with capacity to modulate RyRs (Na^{+}, Mg^{2+}, sorcin...) that may be altered in db/db myocytes. The decreased Ca^{2+} spark frequency may instead be an indication of depressed expression of RyRs (Fig. 4C and D) and/or the sarcoplasmic reticulum Ca^{2+} load (Fig. 4E). The decrease in RyR abundance is consistent with data obtained in type 1 diabetic hearts (31–34), although in type 2 diabetes, no change in RyR expression was found in two previous studies (9,11). A decrease in the frequency of spontaneous Ca^{2+} sparks could have a beneficial effect by preventing Ca^{2+} leakage from the sarcoplasmic reticulum and enhancing the sarcoplasmic reticulum Ca^{2+} load (18), analogous to effects seen with FKBP12.6 overexpression (35). But because sarcoplasmic reticulum Ca^{2+} load was decreased in db/db myocytes (Fig. 4E), this suggests that defects concomitant to reduced RyR expression contribute to altered Ca^{2+} cycling in db/db mice hearts.

This study has been performed in type 2 diabetic mice with obesity, which recapitulate human pathology. However, even if animal models are a valuable resource to elucidate molecular mechanisms of human pathology, there are clear limitations to translate results from bench to bedside. For example, the heart rate in mice is higher than in humans, so the relative contribution of SERCA2 over NCX is notably different (30).

In conclusion, we have identified impaired Ca^{2+}-induced Ca^{2+} release in obesity-linked type 2 diabetes that underlies depressed cardiac function. A general reduction in the membrane permeability to Ca^{2+} was identified. Because less Ca^{2+} enters the cell through I_{Ca}, less Ca^{2+} is released through RyRs. This is due to a reduction in both sarcomlemmal Ca^{2+} channels and sarcoplasmic reticulum Ca^{2+} release channels expression.

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