Mechanisms of impaired calcium handling underlying subclinical diastolic dysfunction in diabetes

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Lacombe VA, Viatchenko-Karpinski S, Terentyev D, Sridhar A, Emani S, Bonagura JD, Feldman DS, Gyorke S, Carnes CA. Mechanisms of impaired calcium handling underlying subclinical diastolic dysfunction in diabetes. Am J Physiol Regul Integr Comp Physiol 293: R1787–R1797, 2007. First published August 29, 2007; doi:10.1152/ajpregu.00059.2007.—Isolated diastolic dysfunction is found in almost half of asymptomatic patients with well-controlled diabetes and may precede diastolic heart failure. However, mechanisms that underlie diastolic dysfunction during diabetes are not well understood. We tested the hypothesis that isolated diastolic dysfunction is associated with impaired myocardial Ca2+ handling during type 1 diabetes. Streptozotocin-induced diabetic rats were compared with age-matched placebo-treated rats. Global left ventricular myocardial performance and systolic function were preserved in diabetic animals. Diabetes-induced diastolic dysfunction was evident on Doppler flow imaging, based on the altered patterns of mitral inflow and pulmonary venous flows. In isolated ventricular myocytes, diabetes resulted in significant prolongation of action potential duration compared with controls, with afterdepolarizations occurring in diabetic myocytes (P < 0.05). Sustained outward K+ current and peak outward component of the inward rectifier were reduced in diabetic myocytes, while transient outward current was increased. There was no significant change in L-type Ca2+ current; however, Ca2+ transient amplitude was reduced and transient decay was prolonged by 38% in diabetic compared with control myocytes (P < 0.05). Sarcoplasmic reticulum Ca2+ load (estimated by measuring the integral of caffeine-evoked Na+-Ca2+ exchanger current and Ca2+ transient amplitudes) was reduced by ~50% in diabetic myocytes (P < 0.05). In permeabilized myocytes, Ca2+ spark amplitude and frequency were reduced by 34 and 20%, respectively, in diabetic compared with control myocytes (P < 0.05). Sarco(endo)plasmic reticulum Ca2+-ATPase-2a protein levels were decreased during diabetes. These data suggest that in vitro impairment of Ca2+ reuptake during myocyte relaxation contributes to in vivo diastolic dysfunction, with preserved global systolic function, during diabetes.

diastole; diabetes mellitus; cardiomyopathy; echocardiography; electrophysiology

DIASTOLIC DYSFUNCTION REFERS to mechanical and functional abnormalities such as impairment of diastolic distensibility, filling, or relaxation of the left ventricle (LV) (4). Diastolic dysfunction is most commonly associated with diabetes, aging, hypertension, coronary artery disease, and atrial fibrillation (33, 35). Diabetic heart disease has been identified as a distinct clinical entity, independent of coronary artery disease and hypertension (35). Patients with diabetic cardiomyopathy initially have diastolic abnormalities, which often progresses to heart failure and sudden death (15, 35). Since quantification of diastolic dysfunction has traditionally been difficult without direct invasive measurements of diastolic vascular pressure and volume (15, 35), the incidence of diastolic dysfunction was underestimated until recently. More widespread application of noninvasive imaging, particularly Doppler flow imaging and more recently tissue Doppler imaging, has facilitated the recognition of diastolic dysfunction (31, 38). Diastolic dysfunction reportedly occurs in 50–100% of asymptomatic, normotensive patients with well-controlled diabetes mellitus and a preserved LV ejection fraction (35, 38, 39, 50). Although diastolic dysfunction may be asymptomatic, this myocardial manifestation of diabetes has prognostic significance, since subclinical diastolic dysfunction contributes to a four- to eightfold increase in the risk of heart failure in diabetic patients, despite preserved LV systolic function (i.e., ejection fraction >50%); this is referred to as diastolic heart failure (4, 7, 9, 20, 33, 35, 45). In addition, the prevalence of diabetes among patients with diastolic heart failure also appears to be increasing and accounts for 30 to 50% of patients with diabetic heart failure (7, 9, 33, 45).

Despite the high prevalence of isolated diastolic dysfunction among diabetic patients, specific therapeutic strategies for this patient population are currently undefined and the mechanisms that result in diastolic dysfunction are not well known. While it has been thought that changes in myocardial extracellular matrix, such as fibrosis, contribute to diabetic cardiomyopathy and in particular to diastolic dysfunction (20, 28), some studies in type 1 diabetic models exhibiting only diastolic dysfunction have not reported significant increases in myocardial collagen content (13, 15). This suggests that other mechanisms, such as impaired Ca2+ handling, may underlie early relaxation abnormalities. Indeed, chronic diabetes mellitus has been associated with altered Ca2+ homeostasis in rodent models of diabetes displaying impaired cardiac contractility (8, 10, 24, 34, 51). Since diastolic dysfunction is an early manifestation of diabetic cardiomyopathy, we anticipated that the underlying mechanisms might be different compared with those at a later stage, where contractile function is markedly depressed (39). However, the contribution of abnormal Ca2+ homeostasis to diastolic dysfunction in type 1 diabetes (e.g., insulin-dependent) is poorly defined.

Our goal was to characterize some of the cellular and molecular events associated with isolated diastolic dysfunc-

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tion, which often precedes diastolic heart failure. Our hypothesis was that diabetes-induced diastolic dysfunction at the organ level would be associated with impaired calcium homeostasis during the relaxation phase at the cellular and molecular level. A chronic model of mild diabetes (type 1) with subclinical diastolic dysfunction and preserved global systolic function was used to examine diastolic Ca$^{2+}$ handling in isolated ventricular myocytes.

**MATERIALS AND METHODS**

**Animals.** All animal protocols were reviewed and approved by The Ohio State University Institutional Animal Care and Use Committee. Eleven-week-old male Wistar rats were randomly assigned to either a control or a diabetic group. Diabetes was induced by a single injection of streptozotocin (STZ, 50 mg/kg iv diluted in citrate buffer). Since a close relationship between the STZ dose and the severity of diabetes has been demonstrated and since other parameters (such as strain, sex, age, route of injection or pretreatment with STZ, and duration of diabetes) all significantly influence the severity of the model (36), a preliminary study was conducted to identify an experimental protocol to induce a mild form of diabetes, characterized by subclinical diastolic dysfunction. The placebo-treated control group was given matched volumes of vehicle. Nonfasting venous blood samples were collected from the tail of each animal at baseline and every 2 wk during the experimental period for measurement of blood glucose (glucometer; BD Logic, Oakville, Canada).

**Echocardiographic examinations.** During anesthesia with isoflurane (minimum effective concentration delivered via mask), cardiac contraction and relaxation were assessed noninvasively by transthoracic echocardiography using parasternal long- and short-axis images at baseline and 8 wk after STZ or placebo injection. Two-dimensional, M-mode echocardiographic images and color-guided pulsed-wave Doppler images were obtained by standard echocardiographic techniques (32) using a GE Vivid-7 echocardiograph system (11.5 MHz pediatric sectorial scan transducer). Systolic function was assessed by measuring LV ejection fraction. To evaluate systolic function of the right ventricle, prejection period and ejection time of the pulmonary artery were measured by pulsed-wave Doppler recordings, and the ratio of prejection to ejection time was calculated. Diastolic dysfunction was assessed by color flow-guided, pulsed-wave Doppler recordings of the maximal early (E) and late (A) diastolic transmitral flow velocities. Additionally, Doppler flow across the pulmonary veins was recorded to measure systolic (S), diastolic (D), atrial reversal wave flow velocities and calculated S-to-D ratios (26, 29, 32). To reliably assess left ventricular function, we computed the Doppler myocardial performance (Tei) index of the LV, which was defined as the sum of isovolumic contraction time and isovolumic relaxation time (IVRT), divided by the ejection time. This index was calculated by measuring, in the same cardiac cycle, the interval between the cessation and onset of mitral flow (A), and the LV ejection time (B) recorded by Doppler echocardiography with the sample volume placed between mitral inflow and atrial outflow, and calculated as a ratio: (A – B)/B (23). All echocardiographic examinations were conducted by the same investigator (V. A. Lacombe), and data was stored digitally for offline measurements using the integrated workstation of the GE Vivid-7. Five measurements for each time point of the pulmonary artery were made and averaged by a blinded observer (S. Emani).

**Action potential recordings.** Ventricular myocytes were isolated by enzymatic dissociation as previously described (43). Amphotericin B perforated whole cell patch-clamp recording techniques were used to minimize any alterations in the intracellular milieu (43). Action potentials (APs) were elicited and recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments) and pClamp software (version 8, Axon Instruments). Cells were superfused with (in mM): 134 NaCl, 1 MgCl$_2$, 5 KCl, 5 HEPES, 1.8 CaCl$_2$, 5 glucose, pH 7.4 at a temperature of 35°C. The pipette solution contained (in mM) 130 KCl, 5 MgCl$_2$, 5 HEPES, 5 EGTA, pH 7.2. Myocytes were stimulated at 0.5, 1, or 2 Hz for 25 beats. AP durations at 50 and 95% of repolarization were calculated from an average of the last 10 AP traces.

**Measurement of K$^+$ currents.** Outward potassium currents were elicited by a series of 300-ms test potentials from −50 to +50 mV from a holding potential of −60 mV. The sustained K$^+$ current (I_{K_{sust}}) was measured at the end of the 300-ms test pulse. The transient outward K$^+$ current (I_{K_{trans}}) was determined by subtracting the sustained outward current from the peak outward current (43).

Inward rectifier K$^+$ current (I_{K_{irr}}) was elicited by voltage steps from −140 to −40 mV from a holding potential of −40 mV. The current was measured at the end of each 100-ms test pulse. I_{K_{irr}} inward conductance (ms/cm$^2$) was determined by calculating the slope of the linear portion of the current density-voltage relationship from −140 to −100 mV. Peak outward I_{K_{irr}} density was measured as the current at −60 mV (I_{K_{irr}}) (43). All currents [in picoamperes (pA)] were normalized to the cell capacitance [measured in picofarads (pF)] and expressed as pA/pF.

**Measurement of L-type Ca$^{2+}$ current, Ca$^{2+}$ transient, and Ca$^{2+}$ sparks.** Confocal Ca$^{2+}$ imaging was performed in conjunction with recordings of inward Ca$^{2+}$ current in fluo-3-loaded patch-clamped myocytes. The myocytes were stimulated by application of 400-ms-long voltage pulses to specified membrane potentials from a holding potential of −50 mV at 1-min intervals. The external solution contained (in mmol/l) 140 NaCl, 5.4 KCl, 1.0 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5.6 glucose; pH 7.3. Patch pipettes were filled with a solution that contained (in mmol/l) 90 Cs-aspartate, 50 CsCl, 3 Na$_2$ATP$_2$, 3.5 MgCl$_2$, 10 HEPES, and 0.05 Fluo-3 K-salt, pH 7.3. Rapid applications of caffeine (10 mmol/l) were used to measure sarcoplasmic reticulum (SR) Ca$^{2+}$ content. SR Ca$^{2+}$ content was assessed by both integration of the caffeine-induced Na$^+$/Ca$^{2+}$ exchanger (NCX) current (I_{NCX}) and the peak amplitude of the caffeine-induced Ca$^{2+}$ transients (22).

**Intracellular Ca$^{2+}$ imaging** was performed using a Laser Scanning Confocal System (Olympus Fluoview 1000 confocal microscope interfaced to an Olympus IX-81 inverted microscope and equipped with an Olympus 60X/1.4 NA oil objective). Fluor-3 was excited by the 488-nm beam of an argon-ion laser, and fluorescence was acquired at wavelengths > 515 nm in the line scan mode of the confocal system at rate of 2 or 6 ms per scan. The magnitude of fluorescent signals was quantified in terms of F/F$_0$, where F$_0$ is the diastolic fluorescence (22). Measurement of K$^+$ current, Ca$^{2+}$ transient, and Ca$^{2+}$ sparks. Confocal Ca$^{2+}$ imaging was performed in conjunction with recordings of inward Ca$^{2+}$ current in fluo-3-loaded patch-clamped myocytes. The myocytes were stimulated by application of 400-ms-long voltage pulses to specified membrane potentials from a holding potential of −50 mV at 1-min intervals. The external solution contained (in mmol/l) 140 NaCl, 5.4 KCl, 1.0 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5.6 glucose; pH 7.3. Patch pipettes were filled with a solution that contained (in mmol/l) 90 Cs-aspartate, 50 CsCl, 3 Na$_2$ATP$_2$, 3.5 MgCl$_2$, 10 HEPES, and 0.05 Fluo-3 K-salt, pH 7.3. Rapid applications of caffeine (10 mmol/l) were used to measure sarcoplasmic reticulum (SR) Ca$^{2+}$ content. SR Ca$^{2+}$ content was assessed by both integration of the caffeine-induced Na$^+$/Ca$^{2+}$ exchanger (NCX) current (I_{NCX}) and the peak amplitude of the caffeine-induced Ca$^{2+}$ transients (22).

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Ca$^{2+}$ sparks were measured in permeabilized myocytes [at ~100 nM free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{i}$)] by using previously described methods and were quantified by using a detection and analysis computer algorithm implemented in IDL (Research Systems) (22).

**Western blot analysis.** The levels of sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA)$_{2a}$, total phospholamban (PLB), and phosphorylated phospholamban (PLB) (at serine 16) were determined by immunoblot analysis. Cell lysate proteins (10 μg) were subjected to SDS-PAGE (4–20%) and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and probed with antibodies specific for these proteins. Anti-CASQ2, and -RyR2 antibodies were from Affinity BioReagents; anti-PLB antibodies were from Upstate Biotechnology. Anti-rat SERCA antibody was a generous gift from Dr. M. Periasamy (The Ohio State University, Columbus, Ohio). Blots were developed with Super Signal West Pico (Pierce, Rockford IL) and quantified by using a Visage 2000 blot scanning and analysis system (BioImage Systems, Jackson, MI).
Statistical analysis. Comparisons were performed by using two-tailed paired t-test, Student’s t-test, or ANOVA, as appropriate (Sigmapastat 2.03; Jandel Scientific). When a significant difference was identified by ANOVA, post hoc tests were performed using the Student-Newman-Keuls test. Statistical significance was defined at $P \leq 0.05$. All results are presented as means $\pm$ SE.

RESULTS

As expected, STZ-treated rats displayed hyperglycemia within 96 h after injection, which persisted during the 8-wk observation period (116.6 $\pm$ 7.6 and 386.8 $\pm$ 28.2 mg/dl at baseline and 8 wk after STZ injection, respectively; $n = 8$, $P < 0.05$). In contrast with controls, STZ-treated rats did not gain weight. Although STZ-treated rats were insulin deficient, they did not require insulin injections to survive; they remained alert and responsive up to 8 wk after the onset of diabetes without any clinical signs of heart failure.

Echocardiographic examination. There was no evidence of impaired global myocardial performance or systolic dysfunction in diabetic or control rats, as evident by the preserved Tei index and ejection fraction, respectively (Fig. 1). The right ventricular pre-ejection period-to-ejection time ratio obtained from the pulmonary artery was not different throughout the study in either diabetic or control rats (0.154 $\pm$ 0.01 vs. 0.139 $\pm$ 0.01 at baseline and 8 wk of diabetes, respectively; $P =$ not significant). Pulsed-wave Doppler flow imaging revealed LV diastolic dysfunction in diabetic rats (Fig. 2). The velocities of the transmitral E wave and A wave were significantly ($P < 0.05$) decreased by 22% and 30%, respectively at 8 wk after the onset of diabetes without a change in the E-to-A ratio. To better detect LV diastolic dysfunction, we also performed an analysis of the pulmonary venous flow patterns. We observed a significant increase in the systolic-to-diastolic ratio of the pulmonary venous velocities in diabetic rats at 8 wk of diabetes (due to a decrease in diastolic wave velocity by 22%, $P < 0.05$; without a significant change in systolic wave velocity). Furthermore, the duration of the atrial reversal wave was prolonged by 36% ($P < 0.05$), and the velocity of the atrial reversal wave was significantly decreased in diabetic rats at 8 wk after diabetes compared with baseline values ($P < 0.05$, Fig. 2). The absolute reductions in the magnitude of the mitral E wave and pulmonary venous D wave are consistent with a relaxation abnormality. Since the magnitudes of the mitral A wave along with the magnitude of the pulmonary venous atrial reversal wave were also decreased, the ratio of E/A was not significantly different at 8 wk of diabetes compared with baseline values. Finally, the IVRT, measured from simultaneous Doppler recordings of transmitral flow and aortic outflow, increased by 67.9% in rats at 8 wk of diabetes (22.5 $\pm$ 0.9 and 37.4 $\pm$ 1.5 ms at baseline and at 8 wk, respectively, $P < 0.05$). In control rats, there was no significant change in any parameter of mitral inflow or pulmonary vein flows, except a modest decrease in E wave velocity (1.15 $\pm$ 0.20 at baseline vs. 1.08 $\pm$ 0.20 m/s at 8 wk, $P < 0.05$).

Electrophysiological recordings, intracellular Ca$^{2+}$ transient, and Ca$^{2+}$ sparks in diabetic myocytes. In isolated ventricular myocytes, there was a significant ($P < 0.05$) increase in APD at 50% repolarization (APD$_{50}$) in diabetic myocytes (Fig. 3). Similar results were found for APD$_{55}$, which increased ($P < 0.05$) by 76 and 93% at stimulation frequencies of 1 and 2 Hz, respectively. The diabetic myocytes also exhibited an enhanced propensity to develop afterdepolarizations (Fig. 3). There was no significant difference in resting membrane potentials between the two groups ($-81.4 \pm 1.7$ vs. $-83.2 \pm 1.2$ mV for diabetic and control rats, respectively; $P =$ not significant).

To determine the electrophysiological characteristics underlying the increased APD and the propensity for afterdepolarizations, the average current density-voltage ($I$-$V$) relationships...
of $I_{\text{to}}$ (Fig. 4A), $I_{\text{Ksus}}$ (Fig. 4B), and outward $I_{\text{K1}}$ (Fig. 4C) were determined in diabetic and control cardiac myocytes. $I_{\text{to}}$ was significantly augmented between $-40$ to $+30$ mV in diabetic myocytes ($P < 0.05$ vs. control). In contrast, $I_{\text{Ksus}}$ was reduced at positive voltages starting from $+20$ mV in diabetic myocytes. There was no difference in inward $I_{\text{K1}}$ slope conductance between the two groups. Peak outward $I_{\text{K1}}$ was reduced in the diabetic myocytes compared with controls ($P < 0.05$; Table 1). However, the gain of calcium-induced calcium release was significantly reduced in diabetic vs. control myocytes, indicating that the ability of $I_{\text{Ca}}$ to trigger $\text{Ca}^{2+}$ release is decreased in diabetic myocytes (Fig. 5D). Furthermore, the calcium transient amplitude in diabetic myocytes was significantly smaller than in controls (Fig. 5B). The time constant ($\tau$) of the $\text{Ca}^{2+}$ transient decay during the diastolic phase was significantly prolonged in diabetic compared with control myocytes (Table 1). SR calcium content, measured by caffeine-induced $\text{Ca}^{2+}$ transient amplitude and the integral of the caffeine-induced $I_{\text{NCX}}$, was reduced by approximately twofold in diabetic myocytes (Fig. 5, E–G).

To further explore the effects of chronic diabetes on SR $\text{Ca}^{2+}$ release, we performed measurements of spontaneous $\text{Ca}^{2+}$ sparks, which assessed local $\text{Ca}^{2+}$ release by a cluster of ryanodine channels. Spark frequency and amplitude were sig-
nificantly reduced ($P < 0.05$) in diabetic compared with aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2). As observed in the intact myocytes, the amplitude of 10 mM caffeine-aged-matched control myocytes (Fig. 6, Table 2).

Biochemical analysis of diastolic SR Ca$^{2+}$ handling proteins in diabetes. To further elucidate the mechanisms underlying the impaired diastolic SR Ca$^{2+}$ handling, we performed immunoblot analyses to determine the contents of SERCA pump and its regulatory proteins in cell lysates from control and diabetic hearts (Fig. 7). We did not detect changes in the expression of CASQ2 (internal control), or PLB (total and phosphorylated), whereas SERCA2a content was significantly reduced in homogenates from diabetic hearts. Furthermore, altered stoichiometry between SERCA and PLB was found, as evident by the reduction of SERCA/PLB ratio by 28%.

DISCUSSION

The major finding of this study is that diabetes-induced subclinical diastolic dysfunction with preserved global systolic performance is associated with abnormalities of intramyocyte calcium regulation. We observed prolonged Ca$^{2+}$ transient decay, reduced intra-SR Ca$^{2+}$ stores, reduced Ca$^{2+}$ sparks, and decreased SERCA2a protein content, which are all consistent with decreased SR Ca$^{2+}$ reuptake during the relaxation phase of cardiac myocytes.

LV diastolic dysfunction during diabetes. Diabetic cardiomyopathy has been recognized as a distinct clinical entity, independent of coronary diseases and hypertension, and is typically initially manifested as LV diastolic dysfunction with preserved LV ejection fraction (35, 38, 39, 50). However, most experimental investigations of type 1 and type 2 diabetes in rodent models (STZ-treated rat and db/db mice) have reported marked contractile impairment with less consistent observations of diastolic dysfunction (3, 10, 18, 27, 34, 41, 44). Therefore, the pathological changes reported in these models may not apply to the majority of the diabetic patients, who exhibit diastolic dysfunction and preserved systolic function (35, 38, 39, 50). In our study, diabetic rats displayed preserved global LV myocardial performance (Tei index) and preserved LV and RV systolic function. The Tei index has been shown to accurately assess global ventricular function when compared with invasive measurements of ventricular function, such as pressure volume loops (23). However, it is worth noting that while the Tei index is a reliable indicator of all changes in LV systolic dysfunction, it may not reliably detect all forms of diastolic dysfunction (30). Therefore, we used combined color-guided Doppler of the pulmonary vein and mitral inflows to improve the sensitivity of our measures of diastolic dysfunction, as discussed below.

Few previous studies have reported isolated diastolic dysfunction in diabetic rats (both type I and II diabetes) (1, 13, 16). Using an animal model similar to ours, Dent et al. (13) described a restrictive flow pattern in diabetic rats, based on the E-to-A peak diastolic mitral inflow velocity ratio, with a normal LV shortening fraction. They examined myocardial collagen content 6 mo after the onset of diabetes as a potential contributor to the restrictive flow pattern. However, they did not observe a change in interstitial fibrosis, which suggests that impaired excitation-contraction coupling, rather than increased myocardial stiffness may occur in the early stages of diabetic diastolic dysfunction (13).

Ultrasonography has revolutionized diagnostic cardiology and is a suitable alternative to cardiac catheterizations in many instances (3). Previous studies in diabetes models have relied on transmitral flow patterns as the sole measure of diastolic dysfunction; however, results from these Doppler measures are somewhat discrepant (3, 13, 18, 27). Evaluation of LV diastolic function by transmitral flow velocity is a standard clinical method but may be confounded by normal variations in ventricular filling related to respiration, age, and heart rate (27). Notably, this method may have suboptimal sensitivity for the...
detection of diastolic dysfunction (29). For instance, Akula et al. (3) reported normal mitral inflow in diabetic rats at 8 (but not 12) wk after STZ injection. The predictive accuracy for detection of LV diastolic function can be improved if both mitral and pulmonary venous flow velocity patterns are analyzed (26, 29); therefore, we used these techniques combined to identify diastolic dysfunction in our animal model of chronic diabetes, which is to our knowledge the first report using this approach in a diabetic model. The reductions in the early left ventricular filling velocity (E wave) combined with the reduction in diastolic pulmonary venous flow velocity (D wave) in the diabetic rats, are consistent with a ventricular relaxation abnormality. Furthermore, we observed an increase in the systolic-to-diastolic velocity ratios (PVs/PVd) of the pulmonary veins and an increase in the duration of the atrial reversal wave in diabetic rats. When diastolic dysfunction is characterized by a relaxation abnormality, S/D increases, as the D is a direct determinant of transmitral E flow (32). Finally, the prolongation of IVRT observed in diabetic animals is suggestive of delayed or incomplete relaxation with relatively normal left ventricular filling pressure (32). Thus, these parameters in the diabetic animals are all early signs of LV diastolic dysfunction (e.g., impaired relaxation).

In human patients with cardiomyopathy, the increase in the PVs-to-PVd ratio is generally accompanied by an increase in the velocity of the atrial contraction wave (A wave). However, in the present study, the velocity of the mitral A wave, in concert with the velocity of pulmonary venous atrial reversal wave, decreased in magnitude. For these reasons, the ratio of E/A was unchanged. Atrial emptying velocities depend on atrial mechanical function and are modulated by ventricular compliance and autonomic activity (40). Since heart rate was decreased in diabetic compared with controls rats (230.5 ± 12.1 and 312.8 ± 8.8 beats/min, respectively), it is possible that the reduction in atrial velocities were due to atrial mechanical dysfunction and/or increased parasympathetic stimulation; both of which are known to be altered in diabetes. Indeed, decreased mRNA levels of calcium-release channels have been reported in human diabetic atrial appendage (17). Future studies that employ additional methods for assessing atrial mechanical and electrophysiological function in this model seem warranted. Considered collectively, the mitral and pulmonary Doppler flows we observed in our model of diabetes are consistent with LV diastolic dysfunction, primarily characterized by impaired ventricular relaxation.

**Impaired diastolic Ca^{2+} handling.** Ventricular relaxation is an active process requiring ATP hydrolysis for Ca^{2+} reuptake from the cytosol to the SR (20). Several factors such as changes in Ca^{2+} homeostasis, energetics, extra- and intramyofilament cytoskeletal proteins (e.g., microtubules and titin, respectively) have been proposed to contribute to impaired cardiomyocyte relaxation and increased stiffness (20). How-
ever, the contribution of abnormal Ca\textsuperscript{2+} homeostasis to isolated diastolic dysfunction in diabetes is not well defined.

In this study, we report a prolonged Ca\textsuperscript{2+} transient decay during diastole, associated with marked depletion of SR Ca\textsuperscript{2+} content in intact diabetic myocytes, which is consistent with decreased cytosolic Ca\textsuperscript{2+} removal through decreased SERCA2a activity. In permeabilized myocytes, where we directly assessed SR Ca\textsuperscript{2+} release by RyR and Ca\textsuperscript{2+} uptake by SERCA2a at diastolic [Ca\textsuperscript{2+}]	extsubscript{i}, we observed decreased intra-SR Ca\textsuperscript{2+} stores, which resulted in decreased Ca\textsuperscript{2+} spark frequency and amplitude in diabetic myocytes. Thus, impaired Ca\textsuperscript{2+} reuptake may be a major determinant of diastolic dysfunction at the organ level in diabetic hearts, and the secondary reduction in SR Ca\textsuperscript{2+} load combined with decreased excitation-contraction coupling efficiency may contribute to the decreased Ca\textsuperscript{2+} transient amplitude and Ca\textsuperscript{2+} spark frequency. Alternatively, the decreased frequency and amplitude of spontaneous Ca\textsuperscript{2+} sparks during the diastolic phase could be a compensatory mechanism by which the reduced SR Ca\textsuperscript{2+} leak will enhance SR Ca\textsuperscript{2+} load (34). Another potential alternative hypothesis is that NCX activity is increased and contributes to reduced SR Ca\textsuperscript{2+} load in diabetic myocytes although the relative contribution of NCX to cytosolic Ca\textsuperscript{2+} removal is small in rodents (<10%). This phenomenon has been observed during heart failure (34, 47), but remains somewhat controversial in diabetic hearts with reports of increased or unchanged NCX activity or decreased NCX expression (6, 10, 34).

Our major findings are in accordance with other studies in animal models of type 1 or 2 diabetes, which suggest a decrease in SERCA2a activity, although the animals in these reports displayed marked systolic impairment, with or without accompanying diastolic dysfunction (10, 34, 44). In one report, normal adult rat ventricular myocytes were incubated for 1 day in high glucose to mimic the early onset of diastolic dysfunction and the most prominent effects were slowed cytosolic Ca\textsuperscript{2+} removal, suggesting a reduction in SERCA2a activity as a contributing factor to impaired relaxation (11, 12). Furthermore, while the overexpression of SERCA2a improved myocardial contractility in diabetic rats (37, 44), the effects of SERCA2a overexpression in diabetic animals with isolated diastolic dysfunction have not been reported. Furthermore, the role of abnormal SERCA2a expression has been somewhat controversial, with some studies showing no change or decreased protein level expression in diabetic rodents (1, 5, 44).

Some investigators have reported altered function and expression of SR calcium-release channels with increased SR Ca\textsuperscript{2+} leak during STZ-induced diabetic cardiomyopathy (8, 49) as suggested by increased Ca\textsuperscript{2+} spark frequency in diabetic myocytes (49). This latest finding is apparently in contrast to our observations of decreased calcium spark frequency and

Table 1. Parameters of I\textsubscript{Ca} and Ca\textsuperscript{2+} transients from control and diabetic hearts

<table>
<thead>
<tr>
<th>Control</th>
<th>Diabetic</th>
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<tr>
<td>Peak Amplitude, pA/pF</td>
<td>1.2±0.11</td>
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<tr>
<td>(\tau\text{trans, ms})</td>
<td>16.2±5.2</td>
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<tr>
<td>(\tau\text{diss, ms})</td>
<td>77±32</td>
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<td>FF/F0</td>
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<td>Rise Time, ms</td>
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<td>Decline Rate Constant, s\textsuperscript{-1}</td>
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<tr>
<td>No.</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. I\textsubscript{Ca}, L-type Ca\textsuperscript{2+} current; pA/pF, picoamperes/picofarads. *Significantly different at \(P < 0.05\) vs. control.

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amplitude. The apparent discrepancy between these previous reports and our observations may result from the severe systolic dysfunction or the use of intact myocytes in the previous reports (8, 10, 49). In contrast, we used permeabilized myocytes to remove the confounding factors of $I_{\text{NCX}}$ and $I_{\text{Ca}}$, and also to clamp the cytosolic concentration of Ca$^{2+}$, allowing us to study diastolic Ca$^{2+}$ leak under identical conditions in diabetic and control myocytes (34). Notably, Ca$^{2+}$ spark occurrence was also reportedly decreased in hearts from insulin-resistant db/db mice, exhibiting both systolic and diastolic dysfunction, using similar experimental conditions (29). However, these results were also in contradiction with a previous study, which reported increased SR calcium leakage (measured using tetracaine) in intact diabetic myocytes from db/db mice (6). In this present study, by removing confounding factors using permeabilized myocytes, our data suggest that the primary mechanism responsible for the impaired Ca$^{2+}$ homeostasis during diabetes is a significant impairment in SERCA function.

In conclusion, our findings of 1) a prolonged Ca$^{2+}$ transient decay rate, 2) decreased SR Ca$^{2+}$ content, 3) decreased spontaneous local Ca$^{2+}$ release, 4) decreased SERCA2a protein content in diabetic myocytes all indicate impaired calcium reuptake during the diastolic phase, which results from impaired SERCA function.

Table 2. Properties of spontaneous Ca$^{2+}$ sparks in permeabilized myocytes from control and diabetic hearts

<table>
<thead>
<tr>
<th></th>
<th>Amplitude $\Delta F/F_0$</th>
<th>Rise Time, ms</th>
<th>Length, ms</th>
<th>Width, $\mu$m</th>
<th>Frequency, 100 $\mu$m/s$^{-1}$</th>
<th>No. Cells</th>
<th>No. Sparks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$0.87 \pm 0.01$</td>
<td>$8.2 \pm 0.1$</td>
<td>$16.1 \pm 0.1$</td>
<td>$2.17 \pm 0.01$</td>
<td>$8.5 \pm 0.5$</td>
<td>68</td>
<td>928</td>
</tr>
<tr>
<td>Diabetic</td>
<td>$0.71 \pm 0.01^*$</td>
<td>$7.8 \pm 0.1$</td>
<td>$16.3 \pm 0.1$</td>
<td>$2.11 \pm 0.01^*$</td>
<td>$5.6 \pm 0.5^*$</td>
<td>138</td>
<td>1130</td>
</tr>
</tbody>
</table>

Values are means ± SE for control (n = 4) and diabetic (n = 8) hearts. *Significantly different at $P < 0.001$ vs. control; †Significantly different at $P < 0.05$ vs. control.
Myocyte electrophysiology. At the cellular level, we observed a significant diabetes-induced increase in AP duration. In myocytes from diabetic animals, we observed an increase in $I_{\text{to}}$ (accelerated phase 1 repolarization), while the reductions in outward $I_{\text{Ks}}$ and outward $I_{\text{K1}}$ contributed to the observed APD50 and APD95 prolongation. Our results are consistent with results from transgenic mice where a reduction in $I_{\text{Ks}}$ results in APD prolongation and afterdepolarizations (21, 25). In addition, we have shown in a rat hypertensive heart failure model that reductions in outward $I_{\text{K1}}$ may predispose to afterdepolarizations (43). These reductions in the currents mentioned above serve to prolong APD and potentially form a substrate for the observed diabetes-induced afterdepolarizations, particularly in light of the abnormalities in intracellular calcium handling (46). Previous studies have documented a reduction in $I_{\text{to}}$ as a potential mechanism for APD prolongation in other diabetic rat models (19, 48). However, in our diabetic model with impaired ventricular relaxation (early stage of diabetic cardiomyopathy), decreased sustained outward $I_{\text{Ks}}$ currents, rather than decreased $I_{\text{to}}$, underlies the observed APD prolongation and afterdepolarizations. Indeed, in our study, the majority of diabetic myocytes displayed afterdepolarizations, which are quantitatively similar to the ones described in Kv1.5/Kv2.1 dominant negative mice with (reduced the encoded outward $I_{\text{Ks}}$), suggesting an important role for the repolarizing $K^+$ currents in the genesis of afterdepolarizations (21).

Systolic $Ca^{2+}$ handling. One surprising finding in our study was the decrease in $Ca^{2+}$ transient amplitude in ventricular myocytes from diabetic animals with preserved global myocardial performance and global systolic function. This seeming paradox has also been reported in a chronic canine model of severe but compensated left ventricular hypertrophy where calcium transient amplitude was reduced (42). One possible explanation for the apparent discrepancies between calcium transient amplitude and in vivo systolic function could be related to the increase in AP prolongation duration observed in diabetic myocytes. Indeed, AP prolongation could allow more entry of $Ca^{2+}$ through voltage-dependent $Ca^{2+}$ channels and enhance $Ca^{2+}$-induced $Ca^{2+}$ release. This increase in trigger could compensate for the observed decreased excitation-contraction gain, and may at least partially preserve in vivo cardiac contractility. Another explanation for the discrepancy between in vivo and in vitro observations in our animal model could be related to the lack of adrenergic stimulation secondary to the absence of circulating catecholamines in isolated cardiac myocytes. Adrenergic stimulation would help maintain in vivo contractile function during diabetes and enhanced adrenergic sensitivity has been reported in diabetic rats (14). Indeed, augmentation of sympathetic activity, in concert with augmentation of parasympathetic activity, has been reported in the early stage of STZ-induced diabetes, as evident by increased cardiac concentration, turnover, release, uptake, synthesis, and...
metabolism of norepinephrine (2). These possibilities may explain the observed paradoxical cellular systolic Ca\textsuperscript{2+} handling in diabetic animals displaying normal systolic function, although further studies are required to define the underlying mechanisms.

**Conclusions.** In a chronic model of diabetes, we observed depressed SR Ca\textsuperscript{2+} reuptake during the relaxation phase of cardiac myocytes, which was associated with prolonged APs and afterdepolarizations at the cellular level and with impaired ventricular relaxation at the organ level. These mechanisms of impaired SR Ca\textsuperscript{2+} uptake appear to underlie isolated subclinical diastolic dysfunction, an early pathological event that may progress to diastolic heart failure.

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