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Mechanisms of impaired calcium handling underlying subclinical diastolic dysfunction in diabetes

Véronique A. Lacombe^{1,2}, Serge Viatchenko-Karpinski^{2,3}, Dmitry Terentyev^{2,3}, Arun Sridhar^{1,2}, Sitaramesh Emani⁴, John D. Bonagura^{2,5}, David S. Feldman^{2,4}, Sandor Györke^{2,3}, and Cynthia A. Carnes^{1,2}

¹ College of Pharmacy, The Ohio State University, Columbus, Ohio

² Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio

³ Departments of Physiology and Cell Biology, The Ohio State University, Columbus, Ohio

⁴ Internal Medicine, The Ohio State University, Columbus, Ohio

⁵ Veterinary Clinical Sciences, The Ohio State University, Columbus, Ohio

Abstract

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 Ca^{2+} 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 Ca^{2+} current; however, Ca^{2+} transient amplitude was reduced and transient decay was prolonged by 38% in diabetic compared with control myocytes ($P < 0.05$). Sarcoplasmic reticulum Ca^{2+} load (estimated by measuring the integral of caffeine-evoked Na^+ - Ca^{2+} exchanger current and Ca^{2+} transient amplitudes) was reduced by ~50% in diabetic myocytes ($P < 0.05$). In permeabilized myocytes, Ca^{2+} spark amplitude and frequency were reduced by 34 and 20%, respectively, in diabetic compared with control myocytes ($P < 0.05$). Sarco(endo)plasmic reticulum Ca^{2+} -ATPase-2a protein levels were decreased during diabetes. These data suggest that in vitro impairment of Ca^{2+} reuptake during myocyte relaxation contributes to in vivo diastolic dysfunction, with preserved global systolic function, during diabetes.

Keywords

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 ventricular 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 diastolic 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 Ca^{2+} handling, may underlie early relaxation abnormalities. Indeed, chronic diabetes mellitus has been associated with altered Ca^{2+} 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 Ca^{2+} 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 dysfunction, 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 ip 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 animal strain, frequency or route of injection or preparation of 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, preejection period and ejection time of the pulmonary artery were measured by pulsed-wave Doppler recordings, and the ratio of preejection 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 global 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 aortic 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 recorded variable 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 MgCl₂, 5 KCl, 5 HEPES, 1.8 CaCl₂, 5 glucose, pH 7.4 at a temperature of 35°C. The pipette solution contained (in mM) 130 KCl, 5 MgCl₂, 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 ($I_{K_{sus}}$) was measured at the end of the 300-ms test pulse. The transient outward K⁺ current (I_{to}) was determined by subtracting the sustained outward current from the peak outward current (43).

Inward rectifier K⁺ current (I_{K1}) 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_{K1} inward conductance (mS/cm²) was determined by calculating the slope of the linear portion of the current density-voltage relationship from −140 to −100 mV. Peak outward I_{K1} density was measured as the current at −60 mV (I_{-60}) (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_2ATP_3 , 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+} ex-changer (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 60×1.4 NA oil objective). Fluo-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). To determine the coupling efficiency of L-type Ca^{2+} current (I_{Ca})-induced SR Ca^{2+} release, we simultaneously recorded I_{Ca} and the I_{Ca} -induced Ca^{2+} transient by confocal microscopy. To estimate excitation-contraction coupling gain, we normalized Ca^{2+} release (amplitude of Ca^{2+} transient) to total Ca^{2+} entry (integral of I_{Ca}) and plotted it as a function of voltage.

Ca^{2+} sparks were measured in permeabilized myocytes {at ~ 100 nM free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) } 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(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)2a, total phospholamban (PLB), and phosphorylated PLB (at serine 16), and calsequestrin (CASQ2) proteins were determined by immunoblot analysis. Cell lysate proteins (10 μg) were subjected to SDS-PAGE (4–20%), 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 (Sigmapstat 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 ± 7.6 and 386.8 ± 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 preejection period-to-ejection time ratio obtained from the pulmonary artery was not different throughout the study in either diabetic or control rats (0.154 ± 0.01 vs. 0.139 ± 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 out-flow, increased by 67.9% in rats at 8 wk of diabetes (22.5 ± 0.9 and 37.4 ± 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 ± 0.02 at baseline vs. 1.08 ± 0.02 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 (APD₅₀) in diabetic myocytes (Fig. 3). Similar results were found for APD₉₅, 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 ± 1.7 vs. -83.2 ± 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_{to} (Fig. 4A), I_{Ksus} (Fig. 4B), and outward I_{K1} (Fig. 4C) were determined in diabetic and control cardiac myocytes. I_{to} was significantly augmented between -40 to $+30$ mV in diabetic myocytes ($P < 0.05$ vs. control). In contrast, I_{Ksus} was reduced at positive voltages starting from $+20$ mV in diabetic myocytes. There was no difference in inward I_{K1} slope conductance between the two groups. Peak outward I_{K1} was reduced in the diabetic myocytes compared with controls ($P < 0.05$; Fig. 4E).

To determine whether altered cellular Ca^{2+} handling underlies the observed diastolic dysfunction and increased APD in our chronic model of diabetes, we also measured I_{Ca} - and I_{Ca} -induced intracellular Ca^{2+} transients as a function of voltage in patch-clamped myocytes (Fig. 5). There were no changes in I_{Ca} in diabetic myocytes compared with control myocytes (Fig. 5C, Table 1). The peak amplitude of I_{Ca} and the time course of I_{Ca} decay did not differ between experimental groups ($P =$ not significant, Table 1). However, the gain of calcium-induced calcium release was significantly reduced in diabetic vs. control myocytes, indicating

that the ability of I_{Ca} to trigger 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 (τ) of the 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 Ca^{2+} transient amplitude and the integral of the caffeine-induced I_{NCX} , was reduced by approximately twofold in diabetic myocytes (Fig. 5, E–G).

To further explore the effects of chronic diabetes on SR Ca^{2+} release, we performed measurements of spontaneous Ca^{2+} sparks, which assessed local Ca^{2+} release by a cluster of ryanodine channels. Spark frequency and amplitude were significantly 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-induced Ca^{2+} transients was also significantly reduced ($P < 0.05$) in permeabilized diabetic myocytes.

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 intramyofibrillar cytoskeletal proteins (e.g., microtubules and titin, respectively) have been proposed to contribute to impaired cardiomyocyte relaxation and increased stiffness (20). However, the contribution of abnormal Ca^{2+} homeostasis to isolated diastolic dysfunction in diabetes is not well defined.

In this study, we report a prolonged Ca^{2+} transient decay during diastole, associated with marked depletion of SR Ca^{2+} content in intact diabetic myocytes, which is consistent with decreased cytosolic Ca^{2+} removal through decreased SERCA2a activity. In permeabilized myocytes, where we directly assessed SR Ca^{2+} release by RyR and Ca^{2+} uptake by SERCA2a at diastolic $[\text{Ca}^{2+}]_i$, we observed decreased intra-SR Ca^{2+} stores, which resulted in decreased Ca^{2+} spark frequency and amplitude in diabetic myocytes. Thus, impaired Ca^{2+} reuptake may be a major determinant of diastolic dysfunction at the organ level in diabetic hearts, and the secondary reduction in SR Ca^{2+} load combined with decreased excitation-contraction coupling efficiency may contribute to the decreased Ca^{2+} transient amplitude and Ca^{2+} spark frequency. Alternatively, the decreased frequency and amplitude of spontaneous Ca^{2+} sparks during the diastolic phase could be a compensatory mechanism by which the reduced SR Ca^{2+} leak will enhance SR Ca^{2+} load (34). Another potential alternative hypothesis is that NCX activity is increased and contributes to reduced SR Ca^{2+} load in diabetic myocytes although the relative contribution of NCX to cytosolic Ca^{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^{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^{2+} leak during STZ-induced diabetic cardiomyopathy (8,49) as suggested by increased Ca^{2+} spark frequency in diabetic myocytes (49). This latest finding is apparently in contrast to our observations of decreased calcium spark frequency and 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_{NCX} and I_{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.

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_{to} (accelerated phase 1 repolarization), while the reductions in outward $I_{K_{sus}}$ and outward I_{K1} contributed to the observed APD₅₀ and APD₉₅ prolongation. Our results are consistent with results from transgenic mice where a reduction in $I_{K_{sus}}$ 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_{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_{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 K^+ currents, rather than decreased I_{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 $I_{K_{sus}}$), 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^{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^{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^{2+} uptake appear to underlie isolated subclinical diastolic dysfunction, an early pathological event that may progress to diastolic heart failure.

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Fig. 1.

Left ventricular (LV) contractile function is unchanged in diabetic rats. **A:** representative paired M-mode echocardiograms at baseline and at 8 wk after streptozotocin (STZ) injection. IVS, interventricular septum; LVPW, left ventricular posterior wall; RV, right ventricle. **B:** left ventricular (LV) ejection fraction, a surrogate of global systolic function, at baseline and at 8 wk after STZ or placebo injection in diabetic ($n = 8$) and age-matched controls ($n = 7$), respectively. **C:** LV Doppler myocardial performance (Tei) index at baseline and at 8 wk after STZ or placebo injection in diabetic ($n = 8$) and age-matched controls ($n = 7$), respectively. Data are expressed as means $\pm$ SE.

Fig. 2.

Diastolic dysfunction occurs in diabetic rats. **A**: representative pulsed-wave Doppler images of mitral inflow and pulmonary venous flow velocities in a diabetic rat at baseline and at 8 wk of diabetes of maximal early (E) and late (A) diastolic flow velocities. PVs, systolic flow; PVd, diastolic flow; PVa, atrial reversal flow. The scale in milliseconds is shown; sweep speed is 200 mm/s. **B**: systolic-to-diastolic (S/D) velocity ratios of the pulmonary (P) veins in diabetic ($n = 8$) and age-matched control rats ($n = 7$) at baseline and at 8 wk of diabetes. $*P < 0.05$ when comparing values at baseline vs. at 8 wk. **C**: atrial reversal wave velocity and duration at baseline ($n = 8$) and at 8 wk of diabetes ($n = 8$). $*P < 0.05$. **D**: velocities of the E and A waves, and E-to-A ratio at baseline ($n = 4$) and at 8 wk of diabetes ($n = 5$). $*P < 0.05$ when comparing values at baseline vs. at 8 wk.

Fig. 3.

Action potential (AP) prolongation and presence of afterdepolarizations in diabetic myocytes. *A*: representative AP in diabetic (solid line) and age-matched control rat (dashed line) (0.5 Hz). *B*: AP duration at 50% and 95% of repolarization (APD₅₀ and APD₉₅, respectively), in diabetic ($n = 7-10$) and control ($n = 6-11$) cardiac myocytes at 1 and 2 Hz. *C*: representative APs in a diabetic myocyte displaying early afterdepolarizations (*). *D*: percentage of diabetic and control cardiac myocytes displaying afterdepolarizations. The number on the bars corresponds to the total number of cells. $*P < 0.05$ when comparing values from control vs. diabetic myocytes.

Fig. 4.

Outward sustained K^+ currents (I_{Ksus}) and outward rectifier K^+ current (I_{K1}) are reduced in diabetes-induced diastolic dysfunction, while transient outward current (I_{to}) is increased. **A**: average current-voltage ($I-V$) relationship for I_{to} . Open and closed squares represent control ($n = 8$) and diabetic ($n = 8$) myocytes, respectively. **B**: average $I-V$ relationship for I_{Ksus} . **C**: average $I-V$ relationship for inward I_{K1} . **D**: inward slope conductance of I_{K1} measured between -140 mV and -100 mV of I_{K1} $I-V$ curve (refer to **C**) in control ($n = 5$) and in diabetic ($n = 7$) cardiac myocytes. **E**: peak outward I_{K1} measured at -60 mV (refer to **C**) in control (white bar) and in diabetic (black bars) cardiac myocytes. $*P < 0.05$ vs. control; pA/pF, picoamperes/picofarads.

Fig. 5.

Diabetes reduces L-type Ca_a current (I_{Ca})-induced Ca^{2+} transients and sarcoplasmic reticulum (SR) Ca^{2+} content when measured in intact patch-clamped myocytes. **A:** representative confocal line scan images of Ca^{2+} transients (*top*) along with their spatial averages (*middle*) and recordings of I_{Ca} (*bottom*), induced by a depolarizing step from -50 to 0 mV, in control (*left*, $n = 10$) and diabetic (*right*, $n = 6$) myocytes. F_0 , diastolic fluorescence. Average amplitudes of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)-induced Ca^{2+} transients (**B**) and peak I_{Ca} densities as functions of membrane potential (**C**) in age-matched control (black) and diabetic (red) cardiac myocytes. **D:** Excitation contraction coupling gain (amplitude of the fluorescent signal divided by the integral of Ca^{2+} current) in control and diabetic myocytes. **E:** representative raw tracing of Ca^{2+} fluorescent signals (*top*) and sodium-calcium exchanger (NCX) currents (*bottom*) in diabetic and control myocytes after application of 10 mM caffeine. **F:** caffeine-induced Ca^{2+} transient amplitudes are reduced in diabetic compared with control myocytes. **G:** integral of NCX ($\int I_{\text{NCX}}$) after caffeine application in diabetic and age-matched control myocytes. $*P < 0.05$ when comparing values from control vs. diabetic myocytes.

Fig. 6.

Ca^{2+} sparks and SR Ca^{2+} content are reduced in permeabilized myocytes from diabetic rats. **A**: representative line scan images of Ca^{2+} sparks. **B**: averaged surface plots of Ca^{2+} sparks (plots were obtained by averaging of 20% of the biggest events). **C**: pooled data for spark amplitude, $n = 928$ and $1,130$ events for normal and diabetic respectively, $*P < 0.001$ when comparing values from control vs. diabetic myocytes. **D**: pooled data for spark frequency; $n = 68$ and 138 cells for normal and diabetic respectively, $**P < 0.05$. **E**: representative time-dependent profiles of caffeine-induced Ca^{2+} transients recorded in control (black) and diabetic (red) myocytes. **F**: pooled data for amplitude of caffeine-induced transients, $n = 14$ and 28 for

normal and diabetic myocytes respectively. Experiments were performed on 8 diabetic heart preparations and 4 age-matched control Wistar rats.

Fig. 7.

Expression of diastolic SR Ca^{2+} handling proteins in normal and diabetic myocytes. **A:** representative immunoblots of sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)2a, phospholamban (PLB), serine 16 phosphorylated PLB, and calsequestrin (CASQ2) in control (Con) and diabetic (Diab) myocytes. **B:** normalized optical density (OD; relative to controls) of SERCA2a from diabetic hearts was reduced by 33% compared with controls ($n = 10$; $*P < 0.05$), whereas there were no significant changes in expression levels of other proteins (n from 6 to 10).

Table 1
Parameters of I_{Ca} and Ca^{2+} transients from control and diabetic hearts

	I_{Ca}			Ca Transients			No.
	Peak Amplitude, pA/pF	τ_{fast} , ms	τ_{slow} , ms	F/F ₀	Rise Time, ms	Decline Rate Constant, s ⁻¹	
Control	-6.4±0.9	16.2±5.2	77±32	1.82±0.11*	31±3	2.9±0.2	6
Diabetic	-5.7±1.1	15.4±5.1	89±27	1.46±0.12*	39±3*	4.1±0.4*	5

Values are means ± SE. I_{Ca} , L-type Ca^{2+} current; pA/pF, picoamperes/picofarads.

* Significantly different at $P < 0.05$ vs. control.

Table 2
Properties of spontaneous Ca²⁺ sparks in permeabilized myocytes from control and diabetic hearts

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

Values are means $\pm$ 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.