Exercise training during diabetes attenuates cardiac ryanodine receptor dysregulation

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Shao CH, Wehrens XH, Wyatt TA, Parbhu S, Rozanski GJ, Patel KP, Bidasee KR. Exercise training during diabetes attenuates cardiac ryanodine receptor dysregulation. J Appl Physiol 106: 1280–1292, 2009. First published January 8, 2009; doi:10.1152/japplphysiol.91280.2008.—The present study was undertaken to assess the effects of exercise training (ExT) initiated after the onset of diabetes on cardiac ryanodine receptor expression and function. Type 1 diabetes was induced in male Sprague-Dawley rats using streptozotocin (STZ). Three weeks after STZ injection, diabetic rats were divided into two groups. One group underwent ExT for 4 wk while the other group remained sedentary. After 7 wk of sedentary diabetes, cardiac fractional shortening, rate of rise of left ventricular pressure, and myocyte contractile velocity were reduced by 14, 36, 44%, respectively. Spontaneous Ca2+ release (SR) was altered. RyR2 also exhibited 1.8- and 1.5-fold increases in phosphorylation at Ser2808 and Ser2814. PKA activity was reduced by 75%, but CaMKII activity was increased by 50%. Four weeks of ExT initiated 3 wk after the onset of diabetes blunted decreases in cardiac fractional shortening and rate of left ventricular pressure development, increased the responsiveness of the myocardium to isoproterenol stimulation, attenuated the increase in Ca2+ spark frequency, and minimized dysynchronous and diastolic Ca2+ releases. ExT also normalized the responsiveness of RyR2 to Ca2+ activation, attenuated increases in RyR2 phosphorylation at Ser2808 and Ser2814, and normalized CaMKII and PKA activities. These data are the first to show that ExT during diabetes normalizes RyR2 function and Ca2+ release from the sarcoplasmic reticulum, providing insights into mechanisms by which ExT during diabetes improves cardiac function.

However, numerous studies including work from our laboratory suggest that these defects stem in part from perturbation in intracellular Ca2+ cycling (3, 11, 39, 44).

The release of Ca2+ from the internal sarcoplasmic reticulum via type 2 ryanodine receptor (RyR2) is an integral step in the cascade of events leading to cardiac muscle contraction (4). Our group (44) and others (54) recently showed that ventricular myocytes isolated from streptozotocin (STZ)-induced type 1 diabetic rat hearts exhibited increased frequency of spontaneous Ca2+ sparks. Our group (44) also showed that evoked release of Ca2+ from the sarcoplasmic reticulum (SR) was dysynchronous with increased diastolic Ca2+ release. From these and other studies, we hypothesized that alteration in the sensitivity of RyR2 to Ca2+ activation (i.e., RyR2 dysregulation) during chronic diabetes is responsible in part for slowing in rates of myocardial contractility (6, 7, 8, 39, 44, 54). In longer term or more severe experimental diabetes, reduction in steady-state levels of RyR2 (and other Ca2+ cycling proteins) also contributes (8, 11, 26, 38). To date, molecular mechanisms underlying RyR2 dysregulation during chronic diabetes remain incompletely understood. However, alterations in the sensitivity of RyR2 to Ca2+ activation could result from increases in phosphorylation at PKA (Ser2808) and CaMKII (Ser2808 and Ser2814) sites (25, 49, 50). Oxidation of RyR2 by reactive oxygen species (ROS) and/or reactive carbonyl species also may contribute (9, 20, 53). Functional uncoupling of RyR2 from L-type Ca2+ channels on the invaginated T-tubule membranes also could be responsible in part for the dysynchronous Ca2+ release (46).

Clinical as well as experimental studies have consistently demonstrated that exercise training (ExT) is one of the most effective strategies for slowing the development of cardiomyopathy and for reducing the incidence of cardiovascular morbidity and mortality during diabetes (40, 41, 43). One of the most significant benefits of ExT is its ability to maintain cardiac output by blunting diabetes-induced bradycardia and the reduction in force of myocardial contractility. These improvements have been attributed to normalization of sympathetically outflow (neurohormonal), an increase in the responsiveness of the myocardium to autonomic stimulation (13, 43), and an increase in expression and function of β-adrenergic receptor and/or signaling (5, 14). ExT also was shown to normalize circulating levels of catecholamines, angiotensin II, vasopressin, neuropeptide Y, atrial natriuretic peptides, and proinflam-
matory mediators such as soluble intracellular adhesion molecule, vascular cell adhesion molecule, monocyte chemotactic adhesion molecule-1, and tumor necrosis factor-α (2, 12, 15, 16, 30, 33, 36). Although a few studies have shown that ExT normalizes expression of Ca^{2+} cycling proteins during heart failure (18, 19, 31, 32, 42), to date the effect of ExT during diabetes on myocyte intracellular Ca^{2+} cycling and the function of SR proteins remain poorly characterized. Earlier, Witzak et al. (51) found that ExT prevented Ca^{2+} dysregulation in coronary smooth muscle from diabetic dyslipidemic Yucatan swine and attributed this in part to a reduction in diabetes-induced increase in sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2b) expression. More recently, Howarth et al. (27) found that time to peak myocyte Ca^{2+} transient was prolonged by light and moderate exercise initiated 2 mo after the onset of diabetes.

The present study was undertaken to assess whether ExT initiated 3 wk after the onset of experimental type 1 diabetes is able to blunt increases in spontaneous and diastolic Ca^{2+} releases, prevent dysynchronous Ca^{2+} release, and normalize the sensitivity of RyR2 to Ca^{2+} activation. We also investigated whether ExT is able to attenuate increases in phosphorylation of RyR2 at Ser^{2809} and Ser^{2814}.

**MATERIAL AND METHODS**

**Chemicals and Drugs**

Mouse monoclonal RyR2, SERCA2, and phospholamban (PLN) antibodies were obtained from Affinity Bioreagents (Golden, CO). Phospho-CaMKII (Thr^{286/287}, clone 22B1) was obtained from Cayman Chemicals (Ann Arbor, MI). Actin antibodies (C-11) and anti-β-adrenergic receptor antibodies (ABS-6) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phosho-RyR2(Ser^{2808}) (1:1,000) and phosho-RyR2(Ser^{2814}) (1:1,000) phosphoepitope-specific antibodies were custom generated using peptide C-RTRRI-(pS)-QTSQV, corresponding to the PKA phosphorylation site region at Ser^{2808} on RyR2, and peptide CSQTSQV-(pS)-VD, corresponding to CaMKII phosphorylation of RyR2 at Ser^{2814}. Two collagenase was obtained from Worthington Biomedical (Lakewoo, NJ). Dulbecco’s modified Eagle’s medium (DMEM) and F-12 supplement were obtained from GIBCO (Carlsbad, CA). Heptapeptide substrate (LRRASLG) for the PKA activity assay was obtained from Bachem Biosciences (Torrance, CA). P-81 phosphocellulose papers were obtained from Whatman (Hillsboro, OR). Other reagents and solvents used were of analytical grade.

**Induction and Verification of Experimental STZ-Induced Diabetes**

Animal procedures used for the study adhered to the American Physiological Society’s “Guiding Principles in the Care and Use of Animals” and were in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals (37). Animal protocols also were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Thirty-six male Sprague-Dawley rats (200–210 g) were purchased from Sasco Breeding Laboratories (Omaha, NE). Animals were housed with a 12:12-h light-dark cycle at ambient 22°C, 30–40% relative humidity, and were given laboratory chow and tap water ad libitum. After acclimatization for 1 wk, rats were assigned randomly to one of two groups: control or STZ-diabetic. STZ-diabetic rats received a single injection of STZ (55 mg/kg ip; Sigma-Aldrich, St. Louis, MO) in a 2% solution of cold 0.1 M citrate buffer (pH 4.5). Control rats were injected with a similar volume of citrate buffer only. Blood sugar levels of animals were maintained between 19.2 and 25 mmol (350–450 mg/dl) throughout the study.

**Exercise Training Protocol**

Starting 1 wk after injection of STZ (or vehicle), rats were removed from their cages and placed on a slow-moving (2 m/min) treadmill for 10–15 min/day to acclimatize them to the movement of the treadmill. This acclimatization continued for two more weeks. Twenty-one days after injection of STZ (or vehicle), control and diabetic animals were randomly subdivided into two groups each. One group of control and one group of diabetic rats continued on the treadmill for 10–15 min/day, 5 days/wk, at a speed of 2 m/min for the duration of the protocol. These rats were designated sedentary (control or diabetic). The other groups of control and diabetic animals were subjected to ExT. During week 1, rats were placed on a treadmill for 15–20 min/day at a speed of 15–20 m/min and a grade of 0%. During week 2, the speed and duration of ExT were gradually increased to a maximum rate of 25 m/min and a duration of 60 min/day. During week 3, the intensity of the ExT was gradually increased by increasing the grade of the treadmill running platform to 5% while maintaining the speed and duration. During week 4 of ExT (week 7 of protocol), the speed was maintained at 25 m/min, the grade at 5%, and the duration at 60 min.

**Assessment of Cardiac Function**

**M-mode echocardiography.** Sixteen hours after the last bout of ExT, M-mode echocardiography was performed on all rats within the four groups (sedentary control, sedentary STZ-diabetic, ExT control, and ExT STZ-diabetic). For this, rats were lightly anesthetized with ketamine-acepromazine (91 mg/kg ip ketamine and 1.8 mg/kg ip acepromazine), and an Acuson Sequoia 512C ultrasound system (Siemens) with an Acuson 15L8 probe was used to measure heart rate, left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV). Percent fractional shortening (FS) was calculated using the equation FS = [(LVEDV − LVESV)/LVEDD] × 100. Percent ejection fraction (EF) was calculated using the equation EF = [(LVEDV − LVESV)/LVEDV] × 100.

**In vivo hemodynamics.** Heart rate, peak left ventricular pressure (LVP), left ventricular end-diastolic pressure (LVEDP), and rates of change of left ventricular pressures (dP/dt) were also evaluated to ascertain the effect of ExT on cardiac hemodynamics. For this, rats were anesthetized with Inactin (20 mg/kg ip), and a Millar catheter (Millar Instruments, Houston TX) containing a pressure transducer was inserted into the left ventricle via the right carotid artery as previously described (5). Another catheter was inserted via the right femoral vein for administration of isoproterenol. Basal cardiac hemodynamic parameters were then measured for the four groups of rats. After basal parameters were assessed, a bolus dose of 0.1 μg/kg isoproterenol was administered into the right femoral vein to assess the responsiveness of the heart to β-adrenergic receptor stimulation. A PowerLab data acquisition system (ADInstuments, Colorado Springs, CO) was used for acquiring data. Microsoft Excel (Seattle, WA) and GraphPad Prism 4.0 (San Diego, CA) were used for analysis of data.

**Sample Collection**

Thirty minutes after isoproterenol injection (for in vivo hemodynamic measurements), animals were injected with a single lethal dose of Inactin (150 mg/kg ip). Abdominal cavities were opened, and blood samples were collected via left renal arteries. Chest cavities were then opened, and hearts were removed and quick-frozen, either by being dropped into liquid nitrogen or embedded in crushed dried ice, or placed in Krebs-Henseleit buffer for isolation of ventricular myocytes. Soleus muscles from hind legs were also excised, quick-frozen, and stored at −80°C until use.

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Citrate Synthase Activity

Citrate synthase activity in soleus muscle was measured spectrophotometrically, employing the method described by Srere (47). All measurements were performed in duplicate under the same experimental setting at 20–22°C. Citrate synthase activities were normalized to total protein content and are reported as micromoles per milligram of protein per minute.

Isolation of Myocytes

Ventricular myocytes were isolated as described previously (44). Briefly, 10 min before injection of Inactin (150 mg/kg ip), rats were injected with heparin (1,000 U/kg ip). Hearts were removed and perfused with collagenase, and left ventricular myocytes were isolated. Cells were used within 5–6 h after isolation.

Myocyte Contractile Kinetics

Cells were placed in a chamber mounted on the stage of an inverted microscope (Zeiss X–40; Göttingen, Germany) at room temperature (22–24°C) and field stimulated (10 V) at a frequency of 0.5 Hz for 10 ms in duration, using a pair of platinum wires. Contractile kinetics of myocytes was measured using a high-speed video edge-detection system (IonOptix, Milton, MA) (44).

Measurement of Spontaneous Ca2+ Release

Spontaneous Ca2+ releases (Ca2+ sparks) were assessed as described previously (44). Briefly, ventricular myocytes in DMEM-F12 were incubated for 1 h at 37°C in petri dishes containing glass coverslips that had been previously coated with laminin. After incubation, unbound cells were gently removed by suction, and Tyrode coverslips that had been previously coated with laminin. After incubation, unbound cells were gently removed by suction, and Tyrode solution (140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl2, 1.0 mM CaCl2, 10 mM HEPES, 0.25 mM NaH2PO4, and 5.6 mM glucose, pH 7.3) was added. Cells were then loaded with fluo-3 (5 μM) for 30 min at 37°C. Spontaneous Ca2+ sparks were recorded in line-scan mode with the use of a Zeiss LSM 410 confocal microscope equipped with an argon-krypton laser (25 mW, 5% intensity) with a ×60 objective. Spark characteristics were analyzed using analysis system (IonOptix, Milton, MA) (44).

Measurement of Evoked Ca2+ Release

Evoked Ca2+ release was assessed as described previously (44). Briefly, cells bound to laminin-coated coverslips in DMEM-F12 were loaded with fluo-3 (5 μM) for 30 min at 37°C. At the end of the incubation, cells were washed to remove extracellular fluo-3 and placed in a chamber on the stage of the confocal microscope. Cells were then field stimulated at 0.5 Hz (10 V for 10 ms), and changes in fluorescence intensities (ΔF) were determined. Fluo-3 was excited by light at 488 nm, and fluorescence was measured at wavelengths of >515 nm. LSM 5 Meta, Prism 4.0, and Microsoft Excel were used for analyzing rates of Ca2+ rise (linear regression) and decay constants (one-phase exponential decay).

Measurement of SR Ca2+ Load

Myocytes were loaded with fura-2 AM in DMEM-F12 medium containing 1.2 mM Ca2+. After loading, cells were washed to remove extracellular fura-2 AM and then pulse stimulated four times at a frequency of 0.025 Hz. Forty seconds after the last stimulation, cells were challenged with 10 mM caffeine, and the rate and amplitude of Ca2+ release were recorded using a dual-excitation fluorescence photomultiplier system (Image Master Fluorescence Microscope; Photo Technology International USA).

Assessing Ca2+ Sensitivity of RyR2

The responsiveness of RyR2 to Ca2+ activation and deactivation was assessed using [3H]ryanodine binding assays (6–9, 44). For this, SR membrane vesicles (0.1 mg/ml) from sedentary and ExT animals were incubated in binding buffer (500 mM KCl, 20 mM Tris·HCl, 5 mM reduced glutathione, and 100 μM EGTA, pH 7.4) for 2 h at 37°C with 6.7 nM [3H]ryanodine and increasing amounts of Ca2+ (0.1 nM to 5 mM). After incubation, vesicles were filtered and washed, and the amount of [3H]ryanodine bound to RyR2 was determined using liquid scintillation counting. Nonspecific binding was determined simultaneously by incubating vesicles with 1,000 nM unlabelled ryanodine.

Determination of Total and Phosphorylated RyR2 (Ser2809 and Ser2814)

Myocytes were prepared from sedentary and ExT rat hearts using the procedure described previously (6–9, 44) with the incorporation of phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich) in the isolation buffer. Western blot analyses were then used to determine relative levels of total and phosphorylated RyR2 (Ser2809 and Ser2814) in samples. Primary RyR2 antibodies were used at 1:2,500 and 1:1,000 dilutions for phosphorylated RyR2. Membranes were electrophoresed using 4–15% linear gradient polyacrylamide gels at 150 V for 2.5 h.

Determination of SERCA2 and PLN

Because SERCA2 is intimately involved in regulating the SR Ca2+ load, Western blot analyses also were performed to determine steady-state levels of SERCA2 and its intrinsic regulator, PLN. For SERCA2 assessment, membranes were electrophoresed using 4–15% linear gradient polyacrylamide gels at 150 V for 2.5 h with 1:2,000 dilution of the primary antibody. For PLN, membranes were electrophoresed using 15% linear gradient polyacrylamide gels at 150 V for 1.5 h with 2:1,000 dilution of the primary antibody.

PKA Activity

Intrinsic PKA activity was determined using a modification of procedures previously described (52). Briefly, left ventricular homogenates (20 μl) were added to 50 μl of reaction mix (130 μM PKA substrate heptapeptide (LRRASLG), 0.9 mg/ml BSA, 0.2 mM IBMX, 20 mM Mg-acetate, and 0.2 mM γ-[32P]ATP in a 40 mM Tris·HCl buffer, pH 7.5) and incubated at 30°C for 10 min. Experiments also were performed in the presence of 10 μM cAMP to assess maximum activatable PKA activity in each sample. Incubations were halted by spotting 60 μl of each sample onto P-81 phosphocellulose papers. Papers were then washed five times for 5 min each in 75 mM phosphoric acid, washed once in ethanol, dried, and analyzed by scintillation spectroscopy. Kinase activity is expressed as picomoles of phosphate incorporated per minute per milligram of protein. Experiments were conducted three separate times (n = 3), and the results are expressed as means ± SE for each data point.

CaMKII Activity

The extent of phosphorylation at Thr287 on CaMKII was measured and used as an indirect measure of total cytoplasmic CaMKII activity. For this, Western blot analyses were conducted to determine total CaMKII protein as well as phospho-CaMKII(Thr287). Phospho-CaMKII(Thr287) antibodies were used at 1:1,000 dilution.

Statistical Analysis

Differences among values from each of the four groups (control, STZ-induced, ExT control, and ExT diabetic) were evaluated using two-way ANOVA, employing Prism 4 (GraphPad Software, San Diego, CA). Data are means ± SE. Results were considered significantly different if P < 0.05.
RESULTS

General Characteristics of Animals

The general characteristics of the animals used in this study are shown in Table 1. Diabetic animals fed normally, but their body weights were significantly less ($P < 0.05$) than that of controls. Sedentary animals tended to have higher body masses than their ExT counterparts, but mean difference was not large enough to attain statistical significance. Throughout the protocol, mean blood glucose levels of sedentary and ExT diabetic animals were $\sim 22.0 \pm 2.5$ mM. Glycosylated hemoglobin levels of sedentary and ExT diabetic animals were $>8\%$. Plasma insulin levels were lower in sedentary and ExT diabetic animals ($P < 0.05$). Hearts from sedentary and ExT diabetic animals were significantly smaller than those from sedentary controls ($P < 0.05$), and this impacted on heart-to-body weight ratios (Table 1). ExT increased citrate synthase activity by $>75\%$ (Table 1) in both control and diabetic animals.

In Vivo Left Ventricular Function

M-mode echocardiography. Compared with nondiabetic controls, STZ-diabetic rats were bradycardic (416.5 ± 17.6 vs. 304.8 ± 13.1 beats/min; Fig. 1). Sedentary diabetic animals had significant reductions in EF and FS compared with sedentary nondiabetic controls. QRS intervals also were significantly greater in sedentary diabetic animals ($P < 0.05$). Mean LVEDD and LVESD also were significantly larger in sedentary diabetic animals than in sedentary control animals. Diabetes did not alter mean aortic diameter. ExT attenuated the bradycardia and blunted increases in LVEDD, LVESD, and QRS and QT intervals. ExT also blunted the decrease in percent FS. There was no significant difference in mean EF between sedentary and ExT diabetic animals. The ExT protocol used in this study did not alter cardiac parameters in control animals.

In vivo hemodynamics. Consistent with echocardiographic data, mean basal heart rate of Inactin-anesthetized sedentary diabetic animals was significantly lower than that of sedentary control animals (296 ± 14.5 vs. 348 ± 9.3 beats/min, respectively; $P < 0.05$). Mean peak LVP and $\pm dP/dt$ also were significantly lower in sedentary STZ-diabetic animals than in sedentary control animals (Fig. 2, A and B). Diabetes also increased mean LVEDP (8.1 ± 1.9 vs. 1.2 ± 0.9 mmHg, $P < 0.05$). ExT did not significantly change basal peak LVP in control animals, but it significantly blunted the reduction in peak LVP induced by diabetes and attenuated the increase in LVEDP to 4.2 ± 0.4 mmHg ($P < 0.05$). Although ExT did not attenuate the reduction in basal $+dP/dt$, it significantly increased $-dP/dt$ (Fig. 2B, bottom left).

Table 1. General characteristics of animals used in this study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sedentary Control</th>
<th>Sedentary STZ-Diabetic</th>
<th>Exercise-Trained Control</th>
<th>Exercise-Trained STZ-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>366.6 ± 17.7</td>
<td>270.6 ± 23.4*</td>
<td>340.8 ± 12.3</td>
<td>260.9 ± 14.7</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>4.9 ± 0.2</td>
<td>21.2 ± 2.4*</td>
<td>4.7 ± 0.1</td>
<td>20.5 ± 3.1</td>
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<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.08 ± 0.10</td>
<td>0.29 ± 0.05*</td>
<td>1.10 ± 0.11</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.2 ± 0.12</td>
<td>1.0 ± 0.02*</td>
<td>1.3 ± 0.04</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Heart/body weight ratio, mg/g × 1,000</td>
<td>3.3 ± 0.1</td>
<td>3.7 ± 0.1*</td>
<td>3.8 ± 0.2*</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Glycosylated hemoglobin, %</td>
<td>4.1 ± 0.2</td>
<td>8.4 ± 0.2*</td>
<td>3.9 ± 0.1</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>Citrate synthase activity, $\mu$mol·g tissue$^{-1}$·min$^{-1}$</td>
<td>10.6 ± 2.4</td>
<td>9.8 ± 1.1</td>
<td>18.6 ± 1.9*</td>
<td>18.0 ± 1.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE of parameters measured in sedentary control, sedentary streptozotocin (STZ)-diabetic, exercise-trained control, and exercise-trained STZ-diabetic rats ($n = 9$ per group). *$P < 0.05$, significantly different from sedentary control. †$P < 0.05$, significantly different from sedentary STZ-diabetic.

ExT during diabetes also significantly enhanced the responsiveness of hearts to isoproterenol stimulation (Fig. 2B). When injected with 0.1 $\mu$g/kg isoproterenol, mean peak LVP in hearts of sedentary control animals increased to 139.9 ± 5.0 mmHg, whereas that of sedentary STZ-diabetic animals was 124.6 ± 8.0 mmHg. Values for ±$dP/dt$ following isoproterenol stimulation also increased (Fig. 2B). Similar trends also were observed following injection with 0.5 $\mu$g/kg isoproterenol (data not shown). In these studies, ExT did not significantly alter ±$dP/dt$ in control animals (Fig. 2B).

Myocyte Contractile Kinetics

As reported previously (44), our laboratory found that during isolation, myocytes from sedentary diabetic rats were less tolerant to Ca$^{2+}$ reconstitution compared with myocytes from sedentary control rats. ExT minimized this Ca$^{2+}$ intolerance and increased the yield of myocytes to near that of control animals ($\sim 65\%$). There were no significant differences in the mean lengths of myocytes isolated from sedentary control, sedentary diabetic, ExT control, or ExT diabetic rat hearts (Fig. 3). We did not measure cell volume. When cells were stimulated at a frequency of 0.5 Hz, velocities of cell shortening and relengthening were significantly slower in diabetic myocytes than in sedentary control myocytes. Extent of shortening also was 26% less in myocytes isolated from sedentary STZ-diabetic animals, and times to 50% peak myocyte shortening and relengthening were longer. ExT during diabetes blunted the reduction in contractile kinetics induced by diabetes.Velocities of contraction and relaxation and extent of cell shortening in myocytes from ExT control animals were not significantly different from those of sedentary control animals.

Spontaneous Ca$^{2+}$ Release From SR

Figure 4 shows representative line-scan images of spontaneous Ca$^{2+}$ release in myocytes from sedentary control (A), sedentary STZ-diabetic (B), ExT control (C), and ExT STZ-diabetic animals (D). Greater than 80% of myocytes from sedentary control rat hearts exhibited spontaneous Ca$^{2+}$ releases, whereas $\sim 50\%$ of myocytes from sedentary diabetic rats showed spontaneous Ca$^{2+}$ sparks. Interestingly, those diabetic myocytes that did generate spontaneous Ca$^{2+}$ releases did so at a frequency that was three-fold higher than that of sedentary control myocytes (Fig. 4B). The duration and full width at half maximum of Ca$^{2+}$ sparks in sedentary diabetic myocytes were similar to those in sedentary controls, but rate of Ca$^{2+}$ rise was slower and peak Ca$^{2+}$ amplitude was less. Greater than 75% of myocytes from ExT STZ-
diabetic animals generated spontaneous Ca\(^{2+}\) sparks, albeit at a lower frequency. ExT did not change the frequency of spontaneous Ca\(^{2+}\) spark generation in control animals, but the sparks were somewhat brighter (increase Ca\(^{2+}\) amplitude).

**Evoked Ca\(^{2+}\) Release**

Representative evoked intracellular Ca\(^{2+}\) transients are shown in Fig. 5. When cells were stimulated at a frequency of 0.5 Hz, the rate of evoked Ca\(^{2+}\) release, peak Ca\(^{2+}\) transient amplitude (ΔF), and Ca\(^{2+}\) decay times were significantly (P < 0.05) slower in sedentary diabetic rat myocytes than in sedentary control myocytes. Myocytes from sedentary diabetic animals also exhibited diastolic Ca\(^{2+}\) release in between pulses (green arrows in Fig. 5B). About 50% of ventricular myocytes from sedentary diabetic rat hearts, primarily those that exhibited increased Ca\(^{2+}\) spark frequency, also showed dysynchronous (nonuniform) Ca\(^{2+}\) releases (white arrow in Fig. 5B). ExT blunted diabetes-induced slowing in rates of Ca\(^{2+}\) rise and...
decay and reduction in Ca\(^{2+}\) transient amplitude. Myocytes from ExT STZ-diabetic animals did not exhibit diastolic Ca\(^{2+}\) release in between pulses (compare Fig. 5, B and D). ExT did not significantly alter the kinetics of Ca\(^{2+}\) transients in nondiabetic animals.

SR Ca\(^{2+}\) Load

As shown in Fig. 6, basal intracellular Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_i\)) in sedentary diabetic rat myocytes were higher than in sedentary control myocytes (340 nm/380 nm fluorescence ratio = 3.13 ± 0.01 vs. 0.07 ± 0.01, basal [Ca\(^{2+}\)] in sedentary diabetic myocytes = 119.1 ± 1.3 nM, and basal [Ca\(^{2+}\)] in sedentary control myocytes = 83.1 ± 1.2 nM, \(n = 6\)). The amplitudes of depolarization-evoked Ca\(^{2+}\) transients in sedentary diabetic myocytes also were significantly smaller than in sedentary control myocytes (compare responses at small arrows in Fig. 6, A and B). When challenged with 10 mM caffeine, sedentary diabetic myocytes released 15.4 ± 3.1% less Ca\(^{2+}\) from the SR compared with sedentary control myocytes (P < 0.05). Rate of Ca\(^{2+}\) rise and time to 50% Ca\(^{2+}\) decay (T\(_{50}\) decay) also were significantly slower in sedentary control myocytes compared with sedentary diabetic myocytes (rate of Ca\(^{2+}\) rise = 0.014 ± 0.001 vs. 0.040 ± 0.002 fluorescence ratio/s and T\(_{50}\) decay = 17.31 ± 1.2 vs. 8.66 ± 0.97 s, P < 0.05). ExT blunted the rise in basal [Ca\(^{2+}\)]\(_i\), increased the amplitudes of evoked Ca\(^{2+}\) releases, increased the rate and amplitude of caffeine-releasable Ca\(^{2+}\), and increased T\(_{50}\) decay (Fig. 6D). ExT did not significantly alter the amplitude of evoked and caffeine-releasable Ca\(^{2+}\) from control myocytes (Fig. 6C).

Ca\(^{2+}\) Sensitivity of RyR2

After normalization to β-actin, no significant differences were observed in steady-state levels of RyR2 protein in hearts from sedentary control, sedentary STZ-diabetic, ExT control, and ExT STZ-diabetic rats (Fig. 7, B and C, middle autoradiograms). However, RyR2 from sedentary diabetic animals exhibited altered responsiveness to Ca\(^{2+}\) activation and bound less [\(^3\)H]ryanodine at peak [Ca\(^{2+}\)] (200–300 μM, Fig. 7A). Half-maximal Ca\(^{2+}\) activation of diabetic RyR2 also occurred at lower [Ca\(^{2+}\)] for sedentary diabetic (EC\(_{50}\) activation = 23.3 ± 5.2 μM for sedentary STZ-diabetic vs. 72.4 ± 8.1 μM for sedentary control). In this study we also found that RyR2 from sedentary diabetic animals was less sensitive to Ca\(^{2+}\) deactivation (EC\(_{50}\) deactivation = 3,102.1 ± 290.1 μM Ca\(^{2+}\) for sedentary diabetic vs. 2,091.1 ± 350.5 μM Ca\(^{2+}\) for sedentary control). ExT normalized the sensitivity of RyR2 to Ca\(^{2+}\) activation and deactivation. ExT did not significantly
Fig. 4. Representative line-scan images showing spontaneous Ca\(^{2+}\) releases in isolated myocytes from sedentary control (A), sedentary STZ-diabetic (B), ExT control (C), and ExT STZ-diabetic rat hearts (D). Graphs below each panel show the fluorescence profile of the spark highlighted above with the green arrow. A spatiotemporal profile of Ca\(^{2+}\) sparks is shown in the chart at bottom. Values are mean ± SE (n ≥ 43 cells). \((F - F_0)/F_0\), change in fluorescence intensity representing peak Ca\(^{2+}\) transient amplitude; \(T_50\) decay, time to 50% Ca\(^{2+}\) decay.*\(P < 0.05\), significantly different from sedentary and ExT control. **\(P < 0.05\), significantly different sedentary STZ-diabetic.
alter the responsiveness of RyR2 from control nondiabetic animals to Ca$^{2+}$ activation or deactivation.

**Relative Levels of RyR2 Phosphorylation at Ser$^{2808}$ and Ser$^{2814}$**

One likely mechanism for increased sensitivity of RyR2 to Ca$^{2+}$ activation is an increase in its extent of phosphorylation (25, 34, 49). In the present study we investigated phosphorylation at Ser$^{2808}$ and Ser$^{2814}$. Consistent with prior reports (44, 54), we found increased phosphorylation of RyR2 at Ser$^{2808}$ (75.2 ± 1.5% over control) in hearts of sedentary STZ-diabetic rats (Fig. 7B). In this study we also found increased phosphorylation of RyR2 at Ser$^{2814}$ (50.1 ± 8.1 over control, Fig. 7C). Increases in phosphorylation of RyR2 at Ser$^{2808}$ and Ser$^{2814}$ were attenuated with ExT. RyR2 from ExT control animals also consistently showed elevated levels of phosphorylation at Ser$^{2808}$ and Ser$^{2814}$ (Fig. 7, B and C). We also measured and found reduced levels of calstabin2 on RyR2 from sedentary STZ-diabetic animals, and ExT blunted dissociation of calstabin2 from RyR2 (Fig. 7B, bottom autoradiogram). Interestingly, the steady-state level of RyR2 protein was not significantly different between sample types.

**Relative Levels of SERCA2 and PLN**

As shown above, reuptake of Ca$^{2+}$ into SR following electrical and caffeine stimulations was slower in myocytes from diabetic rat hearts than in myocytes from control animals. These data prompted us to investigate steady-state levels of SERCA2 and its regulator protein, PLN. As shown in Fig. 7D,
although there was a trend toward a reduction in steady-state levels of SERCA2 in sedentary diabetic rat hearts (using low-dose STZ), the difference was not large enough to attain statistical significance (86.2 ± 12.3 vs. 100.0 ± 4.0, n = 7 animals). We did not detect any significant change in either monomeric (unphosphorylated) or pentameric (phosphorylated) PLN levels in hearts of control and STZ-diabetic animals. Interestingly, hearts from ExT control animals consist-
tently expressed significantly higher levels of SERCA2 (Fig. 7D, graph), and pentameric PLN levels (128.2 ± 6.4% over sedentary controls) also were higher. Monomeric PLN levels, however, remained unchanged. ExT during diabetes did not alter steady-state levels of SERCA2 and PLN (monomeric or pentameric).

**PKA and CaMKII Activities**

Maximum activatable as well as intrinsic PKA activities were assessed in the presence and absence of exogenous cAMP, as described above. In the presence of 10 μM cAMP, there were no significant differences in maximum cAMP-activatable PKA in ventricular homogenates from sedentary control, sedentary STZ-diabetic, ExT control, and ExT STZ-diabetic rat hearts (Fig. 8A). Interestingly, in the absence of exogenous cAMP, homogenates from sedentary STZ-diabetic rat hearts exhibited lower intrinsic PKA activity than those from sedentary control animals (Fig. 8B; P < 0.05). ExT blunted the extent of reduction of intrinsic PKA activity induced by diabetes. ExT did not alter maximum or intrinsic PKA activities in hearts from control nondiabetic rats.

Phosphorylation of CaMKII at Thr287 also was assessed as an indirect measure of CaMKII activity. As shown in Fig. 8C, homogenates from sedentary STZ-diabetic rat hearts exhibited a 50.1 ± 8.1% increased phosphorylation of CaMKII at Thr287, and this increase was blunted with ExT. Interestingly, hearts from ExT control animals also showed an increase in Thr287 phosphorylation.

**DISCUSSION**

Clinical studies have repeatedly demonstrated that ExT slows and/or delays the progression of myocardial contractility loss induced by both type 1 and type 2 diabetes. However, molecular mechanisms underlying this beneficial effect remain incompletely characterized. In the present study a multifaceted approach was used to reveal for the first time that ExT during diabetes minimizes dysregulation of RyR2 by a mechanism that involves, at least in part, reductions in phosphorylation at Ser2808 and Ser2814. The present study is unique in that ExT would improve this hemodynamic parameter. In in vivo hemodynamics studies, ExT did not increase basal +dP/dt but did significantly increase +dP/dt in response to isoproterenol stimulation to near control values. These data are consistent with our earlier findings showing that ExT selectively preserves β1-adrenoceptor expression and signaling (5). ExT also increased basal −dP/dt as well as −dP/dt in response to isoproterenol. The latter finding suggests that ExT during diabetes is enhancing and/or increasing the activity of SERCA2. This is especially important because diastolic heart

![Fig. 8](https://example.com/fig8.png)

**Fig. 8.** Maximum PKA activity (A) and intrinsic or basal PKA activity (B) in homogenates prepared from sedentary control, sedentary STZ-diabetic, ExT control, and ExT STZ-diabetic rat hearts. Data are means ± SE from 4 analyses. C: representative Western blots of total CaMKII (tCaMKII) and phospho-CaMKII (Thr286/287) (pCaMKII) levels. Graph at bottom shows means ± SE of pCaMKII(Thr286/287) levels obtained from 5 separate preparations. *P < 0.05, significantly different from sedentary control.
failure, which accounts for ~50% of all heart failures, stems in part from a loss of SERCA2 activity (17). Consistent with echocardiographic and in vivo hemodynamics data, we found that ExT also preserved myocyte contraction and relaxation velocities and extent of cell shortening. To our knowledge, these data are the first to show in an experimental model that ExT initiated after the onset of diabetes blunts myocyte contractility loss.

Having established that ExT is effective in slowing the development of myocardial contractility, we then investigated Ca2+ released from the SR. Consistent with earlier work (44, 54), in the present study we found that spontaneous Ca2+ sparks, which arise from aberrant activation of RyR2, increased in frequency during diabetes, and this increase was attenuated with ExT. ExT also attenuated decreases in Ca2+ transient amplitude as well as decreases in the rate of Ca2+ rise, parameters that are dependent in part on the activity of RyR2. The activity of SERCA2 that dictates the SR Ca2+ load also is increased with ExT (4). The amplitudes of Ca2+ sparks in ExT control animals were brighter than those in sedentary control animals, but their frequencies were similar. This may be due in part to an increase in expression and/or activity of SERCA2 and the resultant increase in SR Ca2+ load. RyR2 opening for a longer duration of time also may contribute.

Previously, we (44) reported that the increase in Ca2+ spark frequency seen during diabetes maybe as a result of dissociation of calstabin2 from the RyR2 complex. Consistent with this hypothesis, in the present study we found that SR membranes prepared from ExT diabetic rat hearts (which showed normalized Ca2+ spark frequency) contained higher levels of calstabin2 bound to RyR2. Myocytes from ExT control hearts that exhibited a low frequency of spontaneous Ca2+ sparks also had higher levels of calstabin2 on RyR2.

In addition to spontaneous Ca2+ release, we also investigated the effect of ExT on evoked Ca2+ transients. Similar to our previous report (44), in the present study ~50% of myocytes from sedentary diabetic rat hearts exhibited dysynchronous Ca2+ release with diastolic Ca2+ release in between pulses. ExT initiated after the onset of diabetes also attenuated dysynchronous and diastolic Ca2+ releases. Although the increase in diastolic Ca2+ release is directly attributed to dysregulation of RyR2 (gain of function), the mechanism(s) underlying dysynchronous Ca2+ release from the SR remains poorly characterized. Previously, we postulated that this defect could be due to alterations in the sensitivity of RyR2 to Ca2+ stimulation, since our own and the majority of studies found no changes in the activity of L-type Ca2+ current during diabetes (10, 11, 29 44). However, dysynchronous Ca2+ release also could arise from a disruption of the dyad junction architecture (46). In unpublished studies done in collaboration with Dr. Clara Franzini-Armstrong (University of Pennsylvania), we found no change in the dyad junction architecture in hearts from control and sedentary diabetic rats.

Another major finding of the present study is that ExT during diabetes blunts alterations in Ca2+ sensitivity of RyR2 induced by diabetes. Similar to earlier reports (8, 44), we found that at equivalent amounts, RyR2 protein from sedentary diabetic rat hearts bound less [3H]ryanodine at peak [Ca2+] compared with RyR2 protein from sedentary control animals. Further studies are needed to determine whether this is due to a reduction in channel gating or conductance. Diabetic RyR2 also was more sensitive to Ca2+ activation, and a higher [Ca2+] was needed for channel deactivation. ExT blunted these Ca2+ changes. To date, mechanisms underlying altered sensitivity of RyR2 to Ca2+ activation and/or deactivation remain poorly defined. However, studies indicate that this could be due in part to an increase in extent of phosphorylation of RyR2 by PKA and CaMKII (25, 34, 48, 49, 50).

To address this, we used phosphospecific antibodies to assess phosphorylation of RyR2 at Ser2808 (PKA and CaMKII sites) and Ser2814 (CaMKII site). In the present study we found that although total RyR2 remained unchanged, phosphorylation of RyR2 at Ser2808 and Ser2814 increased significantly during diabetes, and these increases were attenuated with ExT. To our knowledge, these data are the first to directly show that phosphorylation of RyR2 at Ser2814 increases during diabetes and that ExT attenuates increases in RyR2 phosphorylation at both Ser2808 and Ser2814.

To discern which kinase is responsible for the increased phosphorylation of RyR2 during diabetes, we assessed PKA and CaMKII activity. Consistent with an earlier study (38), we also found increased endogenous CaMKII activity (assessed from phospho-Thr287). CaMKII is a multimeric holoenzyme composed of 6–12 subunits, and the δ isoform predominates in the heart (35). A spliced variant of this isoform, δε, is present in the cytoplasm, and this isoform is responsible for phosphorylation of several Ca2+ proteins, including RyR2. A rise in intracellular Ca2+ causes Ca2+-bound calmodulin to bind to and activate this kinase. Binding of Ca2+-bound calmodulin results in rapid autophosphorylation of Thr287. This autophosphorylation renders CaMKII autonomous (i.e., active) in the absence of Ca2+-calmodulin (35, 55). The increase in CaMKII activity, independently of changes in protein levels, could result from the increase in myocyte basal Ca2+ induced by diabetes (35). Lowering of cytosolic pH as a result of diabetes-induced ischemia also may be a contributing factor. Activation of the guanine nucleotide exchange factor activated by cAMP (Epac) as a result increased sympathetic activity, persistent stimulation of β-adreceptors, and elevation in myocyte cAMP levels also could increase CaMKII activity (45).

Interestingly, in the present study no significant change in maximum activatable PKA activity was detected (in the presence of 10 μM cAMP), but intrinsic or basal PKA activity was reduced. The reason for this is not clear at this time, but one possibility is that during diabetes, a greater amount of catalytic and regulatory subunits of PKA remain bound together as a result of reduction in availability of cAMP for activation. Taken at face value, our present data suggest that the increase in phosphorylation of RyR2 at Ser2808 and Ser2814 seen during diabetes stems principally from an increase in CaMKII. However, we have yet to investigate whether the PKA activity within the RyR2 macromolecular complex is higher than the cellular average during diabetes to cement this conclusion.

In the present study we found that RyR2 from ExT control animals also exhibited increased phosphorylation at Ser2808 and Ser2814. However, ExT RyR2 did not exhibit altered Ca2+ sensitivity as assessed using [3H]ryanodine binding assays. Myocytes from ExT control animals also did not exhibit an increase in Ca2+ spark frequency. From these data it appears that although increases in phosphorylation of RyR2 at Ser2808 and Ser2814 are likely to contribute to the increase in Ca2+ spark frequency (24), they are not likely to be the only
mechanism responsible for the altered Ca\textsuperscript{2+} sensitivity of RyR2 during diabetes. Posttranslational modifications resulting from increased production or availability of reactive oxygen and carbonyl species also may be contributing (8, 10, 20, 53). This is especially likely given that recent studies have shown that ExT upregulates endogenous antioxidant defenses that are capable of scavenging or reducing reactive oxygen and carbonyl species (2, 12, 22). It should be pointed out that these data also suggest that the association of calstabin2 to RyR2 may not be solely dependent on the phosphorylation state of RyR2, at least in the context of diabetic cardiomyopathy.

In the present study and in a very recent study (44), we observed an apparent discrepancy between the increase in Ca\textsuperscript{2+} spark frequency and the reduction in [\textsuperscript{3}H]ryanodine binding. One would usually expect that if there were an increase in activity of RyR2, then there would be an increase in [\textsuperscript{3}H]ryanodine binding. However, this was not the case. We speculated that this apparent discrepancy might be due to the presence of two distinct populations of RyR2 in diabetic myocytes: one population with increased responsiveness to Ca\textsuperscript{2+} and another population with reduced or little responsiveness to Ca\textsuperscript{2+} activation. The nonresponsive channels could account for the dysynchronous evoked Ca\textsuperscript{2+} release from the SR seen in the line scan mode. Additional studies need to be conducted to further characterize Ca\textsuperscript{2+}-sensitive and Ca\textsuperscript{2+}-insensitive RyR2.

In conclusion, we have shown for the first time in a comprehensive way that ExT initiated after the onset of diabetes preserves RyR2 function by reducing phosphorylation at Ser\textsuperscript{2086} and Ser\textsuperscript{2814}. These data also provided additional insights into molecular mechanisms by which ExT improves myocardial function during diabetes.

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