Am J Physiol Heart Circ Physiol 293: H3584–H3592, 2007.
First published September 21, 2007; doi:10.1152/ajpheart.00619.2007.

Sex-related effects on diabetes-induced alterations in calcium release in the rat heart

Nazmi Yaras,[1] Erkan Tuncay,[1] Nuhan Purali,[2] Babur Sahinoglu,[2] Guy Vassort,[3] and Belma Turan[1]

[1]*Departments of Biophysics, Faculty of Medicine, Ankara University, and* [2]*Hacettepe University, Ankara, Turkey; and* [3]*Institut National de la Santé et de la Recherche Médicale U637, Physiopathologie Cardiovasculaire, CHU Arnaud de Villeneuve, Montpellier, France*

Submitted 28 May 2007; accepted in final form 19 September 2007

Yaras N, Tuncay E, Purali N, Sahinoglu B, Vassort G, Turan B. Sex-related effects on diabetes-induced alterations in calcium release in the rat heart. *Am J Physiol Heart Circ Physiol* 293: H3584–H3592, 2007. First published September 21, 2007; doi:10.1152/ajpheart.00619.2007.—The present study was designed to determine whether the properties of local Ca^{2+} release and its related regulatory mechanisms might provide insight into the role of sex differences in heart functions of control and streptozotocin-induced diabetic adult rats. Left ventricular developed pressure, the rates of pressure development and decay ($\pm dP/dt$), basal intracellular Ca^{2+} level ($[Ca^{2+}]_i$), and spatiotemporal parameters of $[Ca^{2+}]_i$ transients were found to be similar in male and female control rats. However, spatiotemporal parameters of Ca^{2+} sparks in cardiomyocytes isolated from control females were significantly larger and slower than those in control males. Diabetes reduced left ventricular developed pressure to a lower extent in females than in males, and the diabetes-induced depressions in both $+dP/dt$ and $-dP/dt$ were less in females than in males. Diabetes elicited a smaller reduction in the amplitude of $[Ca^{2+}]_i$ transients in females than in males, a smaller reduction in sarcoplasmic reticulum-Ca^{2+} load, and less increase in basal $[Ca^{2+}]_i$. Similarly, the elementary Ca^{2+} events and their control proteins were clearly different in both sexes, and these differences were more marked in diabetes. Diabetes-induced depression of the Ca^{2+} spark amplitude was significantly less in females than in matched males. Levels of cardiac ryanodine receptors (RyR2) and FK506-binding protein 12.6 in control females were significantly higher than those shown in control males. Diabetes induced less RyR2 phosphorylation and FK506-binding protein 12.6 unbinding in females. Moreover, total and free sulfhydryl groups were significantly less reduced, and PKC levels were less increased, in diabetic females than in diabetic males. The present data related to local Ca^{2+} release and its related proteins describe some of the mechanisms that may underlie sex-related differences accounting for females to have less frequent development of cardiac diseases.

calcium sparks; calcium transient; ryanodine receptors; Type 1 diabetes; excitation-contraction coupling

IT HAS LONG BEEN RECOGNIZED that incidence and prevalence of certain diseases vary with sex. Differences exist between women and men in the impact of risk factors, symptoms, and therapeutic responses (1). Cardiovascular diseases are the leading cause of death in adult women in developed countries (1, 12). Results of animal studies have also revealed that there are sex differences in cardiac performance and responses to pathological conditions (3, 5, 8, 11, 22). However, differences in age, heart size, physiological status, and other factors confound comparisons, leading to variable and conflicting conclusions (20).

Influences of sex on intrinsic contractile performance in control and in diabetes-induced myocardial mechanical and electrical dysfunctions were studied by several authors (3, 4, 8, 21). Under various conditions, increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were smaller in cardiomyocytes isolated from control female rats than in male rats (8), and Schwertz et al. (21) reported significant sex-dependent variations in myocardial Ca^{2+} regulation. Furthermore, it has been demonstrated that myocardium from female rats is more resistant to diabetes-induced dysfunction than myocardium from male rats (3). In view of the important role played $[Ca^{2+}]_i$ in the regulation of heart function (16), several studies have been carried out to investigate changes in $[Ca^{2+}]_i$ in hearts subjected to pathological conditions.

A change in Ca^{2+} signaling is a hallmark of cardiomyopathy (16). A comparison of the properties of Ca^{2+} sparks that report ryanodine-sensitive Ca^{2+}-release channel receptor (RyR2) behavior in a cluster and of the status of RyR2 in male and female rat hearts may provide insight into the possible role of these factors in altered Ca^{2+} signaling, as well as in the mechanisms responsible for sex differences in cardiac contractile function, particularly under pathological conditions.

Evidence from animal models, as mentioned above, strongly suggests that basic intrinsic mechanisms may play an important role in the development of sex-related cardiac dysfunction. Therefore, this study aimed to investigate two important events. First, to examine whether there are sex-related differences in cardiomyocyte $[Ca^{2+}]_i$ metabolism and local Ca^{2+} release by RyR2 (Ca^{2+} sparks) in cardiomyocytes isolated from control male and female rats. In addition, the present study was designed to determine whether there are more sex-dependent differences in the alterations in $[Ca^{2+}]_i$ homeostasis in cardiomyocytes isolated from diabetic animals than previously reported for male rats (32). Our results demonstrated that the levels of FK506-binding protein 12.6 (FKBP12.6) and RyR2 in control female rat hearts are significantly higher than levels in males, whereas diabetes-induced RyR2 hyperphosphorylation is markedly less (~50%) in the females than in corresponding males. In addition, a lower increase in basal $[Ca^{2+}]_i$ level and a lower depression in sarcoplasmic reticulum (SR)-Ca^{2+} load in diabetic female with respect to diabetic male rats indicate that RyR2 and FKBP12.6 levels in females may have an important role in the protection

Address for reprint requests and other correspondence: B. Turan, Dept. of Biophysics, School of Medicine, Ankara Univ., Ankara, Turkey (e-mail: belma.turan@medicine.ankara.edu.tr).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

0363-6135/07 $8.00 Copyright © 2007 the American Physiological Society

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

of heart against pathological conditions such as diabetes mellitus. These observations are associated with a smaller decrease in free thiol protein level and a smaller increase in PKC translocation.

MATERIALS AND METHODS

Experimental model. Wistar rats (200–250 g) of both sexes were used in this study. Diabetes was induced by a single injection of streptozotocin (STZ; 50 mg/kg body wt). One week after STZ injection, rats having at least threefold higher blood glucose level than the preinjection level were designated as the diabetic group. All rats had free access to standard rat chow and water and were kept for 5 wk.

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (publication 85-23, revised 1996) and were approved by Ankara University School of Medicine Institutional Animal Care and Use Committee (no. 2006/143).

Langendorff-perfused hearts. Rats were anesthetized with pentobarbital sodium (30 mg/kg body wt) before they were killed. The hearts were rapidly excised and perfused according to the Langendorff procedure. The perfusion medium, containing (in mM) 119 NaCl, 4.8 KCl, 1.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 20 $NaHCO_3$, and 10 glucose, was gassed with 95% O_2-5% CO_2 and maintained at pH 7.4 at 37°C. The hearts were paced at 300 beats/min (by a square wave of twice the threshold voltage for 1.5 ms) by an electrical stimulator (DCS, Harvard), and the coronary flow rate was maintained at 10 ml/min. To measure the left ventricular pressure, the left atrium was removed, and a latex balloon connected to a pressure transducer (Spectramed model P23XL) was inserted through the mitral valve into the left ventricle. The balloon was filled with water and adjusted to the left ventricular end-diastolic pressure of 20–30 mmHg depending on the heart. Values of the left ventricular developed pressure (LVDP), rates of pressure development ($+dP/dt$), and rates of pressure decay ($-dP/dt$) were recorded on-line by a Biopac data-acquisition system (Biopac Systems, Goleta, CA). The balloon technique employed here allowed stable recording of the hemodynamic parameters over a period of more that 60 min. All hearts were stabilized for 30–40 min by perfusion with the oxygenated medium. The time to peak (TP) of the LVDP and its half-relaxation time (DT_{50}) were also measured from each pressure trace.

Isolation of cardiomyocytes. Cell isolation was performed as described previously (32). Briefly, the heart was first perfused with a Ca^{2+}-free HEPES-buffered solution (in mM: 145 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.4 Na_2HPO_4, 0.4 NaH_2PO_4, 5 HEPES, and 10 glucose at pH 7.4, bubbled with 100% O_2) for 3–5 min at 37°C. After 5 min, fresh buffer supplemented with 1 mg/ml collagenase (collagenase A; Boehringer) was recirculated (8 ml/min) for 30–35 min. At the end of the collagenase perfusion, the ventricles were cut off and stirred to disperse the myocytes. Cardiomyocytes were then washed with the collagenase-free solution to which Ca^{2+} was added stepwise up to 1 mM. The cells were kept suspended at 37°C in the HEPES-buffered solution that also contained 1 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH). The yield of Ca^{2+} tolerant cells was ~60%.

Simultaneous measurements of $[Ca^{2+}]_i$ transient and L-type Ca^{2+} current. Isolated cardiomyocytes were whole cell patch clamped in an external solution containing (in mM) 135 NaCl, 1 $MgCl_2$, 20 CsCl, 1.8, $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4. Ca^{2+} currents (I_{Ca}) were recorded at room temperature (22 ± 2°C) in the presence of cesium to inhibit K^+ currents and after a voltage ramp to −50 mV from a holding potential of −80 mV to inhibit Na^+ current, by using a patch-clamp amplifier (model HEKA EPC8), and filtered at 3 kHz. Patch pipettes (1.0–1.2 MΩ) were filled with a solution that contained (in mM) 130 CsCl, 0.4 $MgC1_2$, 20 TEA-Cl, 4 MgATP, and 10 HEPES (pH 7.2) and 50 μM fura 2 (pentapotassium salt). Cells were held at −80 mV and submitted to five sequential step-depolarizations to 0 mV to keep the SR-Ca^{2+} load constant and to allow penetration of dye into cells. Current amplitude was estimated as the difference between peak inward current and the current level at the end of the 250-ms pulse. Fluorescence was recorded using microspectrophotometer and FELIX software (Photon Technology International). Cells were sequentially excited at 340 and 380 nm at 10 Hz, and emission was measured at 510 nm. The ratio of the fluorescence emitted at 340- and 380-nm excitation ($F_{340/380}$) was calculated and used as an indicator of $[Ca^{2+}]_i$.

In a set of experiments, caffeine-induced $[Ca^{2+}]_i$ transients were recorded in fura 2-loaded cells. Cells were first field stimulated through two parallel platinum electrodes at 0.2 Hz during 1–2 min to allow for steady-state $[Ca^{2+}]_i$ transients. Stimulation was then stopped, and $[Ca^{2+}]_i$ transients were recorded. After this, cells were field-stimulated again, and caffeine (10 mM) was rapidly applied for 30 s after cessation of electrical stimulation to estimate SR-Ca^{2+} load.

Ca^{2+} spark measurement. Ca^{2+} sparks were measured as described previously (32). Briefly, a ×40 (numerical aperture of 1.3) oil immersion objective has been used for imaging the cardiomyocytes (Axiovert 200, LSM-Pascal, Zeiss). The 488-nm laser line from an argon laser (25 mW) was used to excite fluo 3, and the emitted fluorescence was collected with a long-pass filter set at 505 nm. The value for fluorescence intensity of the images was calculated by averaging pixels other than potential spark areas. Then, an image of change in fluorescence intensity-to-fluorescence intensity ratio was created by using this value for fluorescence intensity. Ca^{2+} sparks were manually detected and converted to temporal lines by averaging fluorescence intensity of 2 or 3 pixels and aligning the peak of fluorescence intensity over time. The signals were filtered with a Butterworth digital filter, and the temporal profiles were fitted to gamma function to analyze TP, peak amplitude of maximum fluorescence intensity, and DT_{50}.

Western blot analysis. Western blotting from cardiomyocytes was performed as described previously (32). Briefly, cardiomyocytes were homogenized in cold Tris·HCl buffer containing (in mM) 50 Tris·HCl (pH 7.4), 200 NaCl, 20 NaF, 1.0 Na_3VO_4, 1 DTT, and protease inhibitors (complete tablet from Roche). The pellet was suspended with the homogenization buffer, and protein amount was measured as described previously (32). The samples (100 μg of protein) were subjected to 5% SDS-PAGE for RyR2 assay and 10% SDS-PAGE for FKBP12.6 assay and then transferred electrophoretically to nitrocellulose membrane. Immunoblotting was performed with antibodies against RyR2 and phosphorylated RyR2 (kind gift of Drs A. Marks and X. Wehrens) and FKBP12.6 (Santa Cruz). Blots were washed several times with PBS and incubated with horseradish peroxidase-labeled anti-rabbit IgG (Santa Cruz) for 1 h at room temperature. Blots were then washed with PBS and then incubated with enhanced chemiluminescence Western blotting reagent (Amersham) for 1 min and exposed to X-ray film for 45–90 s.

Determination of oxidation level of sulfhydryl groups in isolated cardiomyocytes. Oxidation levels of protein sulfhydryl (SH) groups were measured as described previously (31). Briefly, cardiomyocytes were first washed with a HEPES-buffered solution containing (in mM) 123 NaCl, 5.4 KCl, 1.7 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4) and then used for estimation of total and free SH groups. These estimations were done with Ellman's reagent. For the determination of total SH groups, 0.05-ml aliquots of cell lysates were mixed with 0.8 ml of distilled water and 0.1 ml of 2 mM DTNB, with 20 min allowed for color development. Absorbance of the supernatants was read at 412 nm. To determine the free SH groups, 0.7-ml aliquots of cell lysates were mixed with 0.35 ml of 20% TCA and then centrifuged at 13,000 *g* for 10 min. Precipitates were further washed with 0.2 ml of 20% TCA, and the supernatants were adjusted to pH 8.0 with NaOH. The SH content of the supernatants was measured as described for total SH measurements.

Chemicals and statistical evaluation of data. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) except for col-

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

lagenase A (Boehringer Manheim, Roche Diagnostics, Manheim, Germany) and fura 2-AM and fluo 3 (Molecular Probes, Eugene, OR).

Between-group differences were tested and compared by two-way ANOVA followed by the Bonferroni multiple-comparison posttest and Student's *t*-test where stated. *P* values <0.05 were considered significant. Data are presented as averages ± SE.

RESULTS

General characteristics of the rats. The ratios of heart weight to body weight were similar in females and males from both control and diabetic groups (data not shown). Diabetic rats stopped gaining weight, whereas control rats gained weight within the 5-wk experimental period (Fig. 1). Blood glucose levels were significantly higher in the diabetic compared with the control group. There were no significant differences in either body weight changes or blood glucose level changes in rats of both sexes from both groups. In both groups, there were no significant sex-related differences in either body weight or blood glucose level changes.

Because there are two factors (sex and diabetes), a two-way ANOVA was used, revealing no interaction between these two measured parameters.

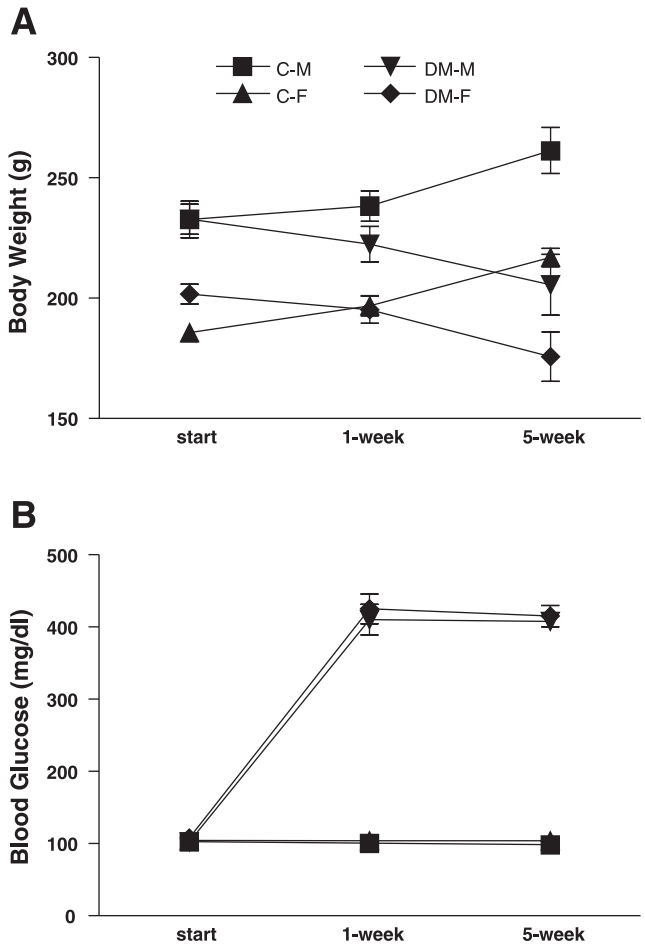


Fig. 1. Distribution of body weight and blood glucose levels between sexes during the 5-wk experimental period. *A*: body weight of control and streptozotocin (STZ)-injected male and female rats during the 5-wk experimental period. *B*: blood glucose levels in the same animals. C-M, C-F, DM-M, DM-F designate, respectively, control male and female and diabetic male and female rats; $n > 30$ in each group.

Mechanical activity of the isolated heart. Mean values of LVDP and left ventricular end-diastolic pressure and the rates of pressure development and decay ($\pm dP/dt$), as well as TP of the LVDP and DT_{50}, were not significantly different in female and male animals of the control group (Fig. 2). Diabetes-induced percent decrease in LVDP of females (37% compared with control) was significantly less than in the diabetic males (52% compared with control). Diabetes also caused significant prolongations in TP and DT_{50} of LVDP in both female and male rat hearts with respect to the controls without significant sex dependency (Fig. 2*B*). The diabetes-induced depressions in both $+dP/dt$ and $-dP/dt$ were significantly less pronounced in females (49% compared with control) than in males (69% compared with control) (Fig. 2*C*).

$[Ca^{2+}]_i$ transients and I_{Ca} in isolated ventricular cardiomyocytes. To determine whether altered cellular Ca^{2+} signaling underlies sex differences in diabetes-induced cardiac dysfunction, we recorded I_{Ca} and $[Ca^{2+}]_i$ transients simultaneously in isolated cardiomyocytes. I_{Ca} results recorded at various membrane potentials were similar in males and females and did not significantly change in the diabetic cardiomyocytes (Fig. 3). Neither the steady-state inactivation nor the steady-state activation was significantly different between sexes in control and diabetic groups.

The $[Ca^{2+}]_i$ transients recorded in both male and female diabetic animals were significantly smaller in amplitude and decayed more slowly with respect to the corresponding controls (Fig. 3). Moreover, the diabetes-induced percent reductions in the maximum amplitude of $[Ca^{2+}]_i$ transients $\Delta F_{340/380}$ ($\Delta F_{340/380} = F_{340/380}$ peak $- F_{340/380}$ basal) at 0 mV were significantly different in females than in males, demonstrating less effect of diabetes on female myocyte $[Ca^{2+}]_i$ transients (Fig. 3*C*). The DT_{50} was significantly higher in diabetic than in control animals; however, the diabetes-induced increase in DT_{50} was not significantly different in males and females (28% and 30%, respectively). The TP was significantly larger in diabetic than in control animals, also with no sex difference. Thus hearts from diabetic animals demonstrated statistically less significant decrease in $[Ca^{2+}]_i$ transients and depressed contractile properties in females than in males with no significant change in kinetics.

To characterize impairment of $[Ca^{2+}]_i$ transients in more details, we measured basal $[Ca^{2+}]_i$ levels and SR-Ca^{2+} load, thus examining critical steps in cardiac Ca^{2+} homeostasis. SR-Ca^{2+} load is a major determinant of Ca^{2+} release and, moreover, a critical factor in $[Ca^{2+}]_i$ transient amplitude and Ca^{2+} spark frequency (16, 32). The caffeine-evoked $[Ca^{2+}]_i$ transients were significantly larger in diabetic females and males than in their matching controls, with ~10% more responses in both control and diabetic females (Fig. 4*A*). Basal $[Ca^{2+}]_i$ levels, similar in control of both sexes, were significantly increased under diabetes and significantly less in cardiomyocytes of female than in cardiomyocytes of male animals (Fig. 4*B*).

Differences in local Ca^{2+} release support sex-dependent effects of diabetes in heart function. To get further insight on possible sex effect on Ca^{2+} handling, we recorded Ca^{2+} sparks in male and female cardiomyocytes from control and diabetic animals. Figure 5*A* shows examples of line-scan images recorded in the four groups. In control groups, values for peak amplitudes of maximum fluorescence intensity (0.58 ± 0.02

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

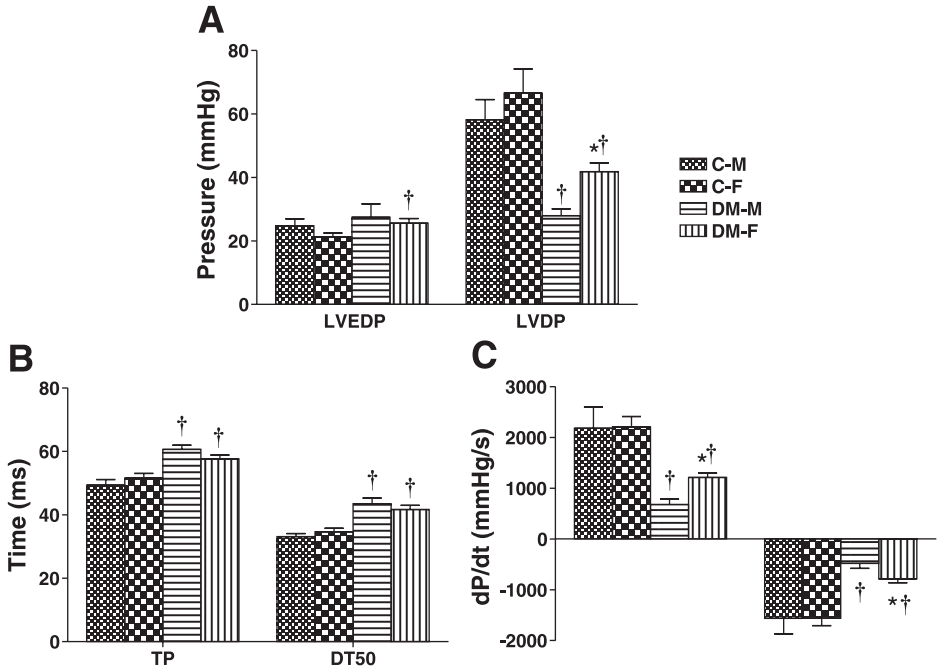


Fig. 2. Effect of sex in diabetes-induced mechanical dysfunction. Left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP) (*A*), time to peak (TP) of the LVDP and its half-relaxation time (DT_{50}) (*B*), and rates of pressure development and decay (±dP/dt; *C*) were measured in isolated hearts from both control and diabetic male and female rats (n = 12–16). Bar graphs represent means ± SE. Bonferroni multiple comparison test: $^*P < 0.05$ males vs. females; †$P < 0.001$ diabetics vs. corresponding controls; the interaction was found to be not significant.

vs. 0.49 ± 0.02), TP (11.65 ± 0.40 vs. 8.98 ± 0.48 ms), and DT_{50} (28.87 ± 1.25 vs. 21.52 ± 1.65 ms) of Ca^{2+} sparks were significantly larger in females than in males (Fig. 5*B*). The frequency and width at half-maximal amplitude of Ca^{2+} sparks were not sex dependent (Fig. 5, *C* and *D*).

Diabetes significantly depressed the amplitude of Ca^{2+} sparks in females (33% compared with control), whereas the effect was less pronounced in males (8% compared with control) (Fig. 5*B*). Mean Ca^{2+} spark frequency (0.051 ± 0.006 vs. 0.046 ± 0.007 $s^{-1}\cdot\mu m^{-1}$ male vs. female) and the spatial spread of the sparks (3.16 ± 0.20 vs. 2.99 ± 0.09 μm, male vs. female) were significantly increased in both male and female diabetic animals compared with results shown in controls (frequency: 0.032 ± 0.003 vs. 0.028 ± 0.002 $s^{-1}\cdot\mu m^{-1}$ for male vs. female; spatial spread of the sparks: 2.43 ± 0.04 vs. 2.60 ± 0.04 μm for male vs. female). In summary, diabetes induced alterations in the spatial characteristics of Ca^{2+} sparks in both sexes; however, the diabetes-induced reduction in spark amplitude in females was greater than in males.

Biochemical analysis of RyR2 in diabetic males and females. Under pathological conditions including diabetes, alterations in the characteristics of $[Ca^{2+}]_i$ transients and Ca^{2+} sparks might occur in combination with altered control and phosphorylated levels of RyRs (28, 32). Therefore, we evaluated the phosphorylation level of RyR2 in cardiomyocytes from control and diabetic rats of both sexes using specific antibodies directed against RyR2 and its phosphorylated form. In control animals, total RyR2 was significantly higher in females than in males as estimated from the Western blot bands (Fig. 6, *top*). In both sexes, diabetes depressed total RyR2. This effect was less marked in females than in males. In our previous study (32), our group had shown that RyR2 were hyperphosphorylated in diabetic male heart tissue from left ventricle compared with that shown in control. In this study, similar measurements on cardiomyocytes isolated from left ventricles of both males and females demonstrated marked hyperphosphorylation of RyR2 in diabetic cardiomyocytes without significant phosphorylation in cardiomyocytes in control groups of both sexes. The phosphorylation level of RyR2 in diabetic females was almost half that shown in diabetic males (Fig. 6, *middle*). Correspondingly, the control amounts of FKBP12.6, higher in females than in males, decreased significantly in both diabetic females and males, but the decrease was significantly less in females (Fig. 6, *bottom*).

Sex differences in diabetes-induced oxidative stress and PKC content. It is well known that metabolic shifts brought about by diabetes increased the production of reactive oxygen species (ROS). In a previous study, our group, like others, showed that oxidative stress is involved in the etiology of diabetes-induced downregulation of transient outward K^+ currents (22, 24). Moreover, in diabetic rats, Shimoni and Liu (24) reported the role of sex in the modulation of K^+ currents. In this regard, we aimed to test whether different levels of intracellular oxidants could contribute to the sex-related differences in cardiac function under control and diabetic conditions. Total and free SH levels measured in isolated cardiomyocytes of diabetic rats were significantly less than levels in controls, with less reduction in females (27%) than in males (45%) (Table 1). In addition, in the control groups, free SH levels in females were slightly, although significantly, higher than levels in males. The present data suggest that sex-dependent differences in diabetes-induced electrophysiological changes of the hearts could in part be due to sex-dependent generation of oxidative stress in diabetes.

Increased PKC activity has an important role in the pathogenesis of the heart complications in diabetes. To determine the sex dependence of these effects, we estimated total PKC protein content in the cytosolic and membrane fractions of cardiomyocytes isolated from males and females of both control and diabetic rats. The ratio of total PKC in the membrane to that in the cytosolic fraction increased significantly in both

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

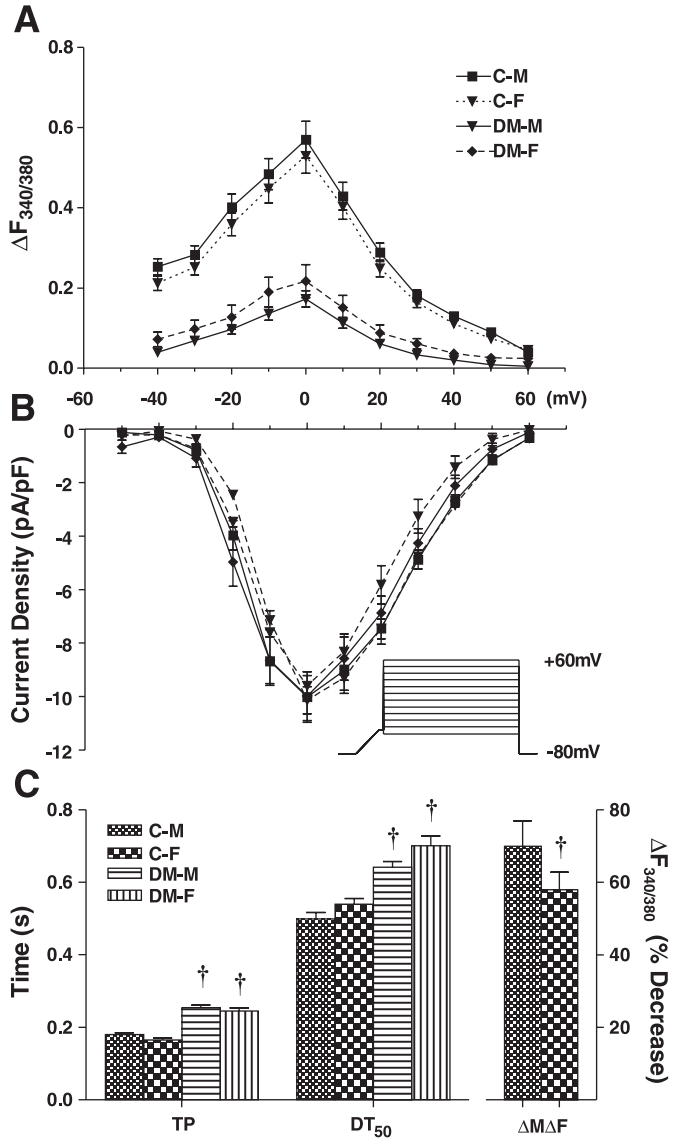


Fig. 3. Diabetic rats of both sexes exhibit depressed intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients despite unchanged Ca^{2+} current (I_{Ca}). Changes in peak amplitude (PA) of $[Ca^{2+}]_i$ transients as a function of membrane potential (*A*) and I_{Ca} densities estimated at different depolarizing potentials in cardiomyocytes (*B*) from control and diabetic male and female rats are shown. $F_{340/380}$, ratio of fluorescence emitted at 340- and 380-nm excitation. $P < 0.001$ in diabetic with respect to corresponding control animals. Two-way ANOVA revealed the significance level in diabetes as $P < 0.001$; no significant difference was found in the interaction of sex and diabetes. *C*: relative diabetes-induced reduction in peak amplitude of $[Ca^{2+}]_i$ transients (at 0 mV): change in (Δ) male control vs. diabetic male rats and change in female control vs. diabetic female ($P < 0.05$). DT_{50} and TP (means ± SE) are given in *left* of *C*. Numbers of animals and cardiomyocytes are as follows: 9 and 30 vs. 7 and 19 for males vs. males in control group and 7 and 15 vs. 8 and 26 for males vs. females in diabetic group, respectively. In Bonferroni multiple comparison test, †$P < 0.001$ diabetics vs. corresponding controls; interaction between factors was not significant.

male and female diabetic hearts; however, this increase was 22% less in females than in males (Table 1).

DISCUSSION

The present investigation demonstrates sex-dependent differences in the diabetes-induced cardiac dysfunction and emphasizes some underlying mechanisms. At first, the defective intracellular Ca^{2+} signaling with depressed amplitude and reduced kinetics of the Ca^{2+} transients and the decreased SR-Ca^{2+} loads in cardiomyocytes reported in STZ-induced diabetic male rats (32) were also observed in females. These defects, in both males and females, are attributable to an anomalous RyR2 behavior suggested by the spatiotemporal properties of the Ca^{2+} sparks that especially exhibited depressed amplitude and slowed down kinetics. In particular, the present data show, for the first time, that there are sex-dependent differences in the characteristics of local Ca^{2+} sparks and related proteins such as RyR2 and FKBP12.6 in ventricular cardiomyocytes from sexually mature normal rats. Our data also demonstrated that the levels of protein thiol oxidation and hyperphosphorylation of RyR2, as well as up-regulation of PKC, are significantly smaller in cardiomyocytes from diabetic female than in cardiomyocytes from diabetic male animals. Altogether, these findings bring novel information that can account for a lower risk in female rats with respect to male rats.

Reports on sex difference in both electrical and mechanical cardiac activities in control conditions are contradictory (3, 4, 29). Taken as a whole, the main differences observed between sexes are rather subtle but suggest a tendency for greater Ca^{2+} fluxes in females; they may also arise from either animal and sample differences or experimental protocols (7, 8, 29, 30). In female rats, we observed a weak enhanced contractile activity that could be, in most part, accounted for by changes in intracellular Ca^{2+} movements; namely, the time course of decay of the $[Ca^{2+}]_i$ transient was significantly slower in females than in males (8). Under our experimental conditions, there was no significant difference in I_{Ca} amplitude in contrast to the reports of Vizgirda et al. (27) in Sprague-Dawley rats and of Xiao et al. (29), which, in dog heart, demonstrated significant sex- and region-based differences in the electrophysiological activities. Ca^{2+} responses to caffeine application were not significantly different in rats of both sexes, indicating a similar Ca^{2+} load. However, despite similar amplitudes of the Ca^{2+} transient in both sexes (8), Ca^{2+} spark characteristics, except spark frequency and spatial spread of the sparks, were significantly larger in females than in males. This was associated with a larger content in both RyR2 and FKBP12.6 proteins. The present data are in line with the study of Chu et al. (7) that showed higher RyR2 protein and mRNA levels as well as higher Na^+/Ca^{2+} exchanger levels in females than matched males. Furthermore, we observed slight but significantly larger total and free SH levels in females.

Recently, our group (31) reported that both PKA and PKC could play important roles in the diabetes-induced alterations in the kinetic parameters of Ca^{2+} transients, Ca^{2+} loading of SR, basal Ca^{2+} level, and spatiotemporal properties of the Ca^{2+} sparks. Our group (31) also showed increased PKC and oxidized protein SH levels in cardiomyocytes from diabetic male rats. The present results on females extend the latter findings and are in line with the earlier findings suggesting sex differences in either generation of ROS (2, 13) or in the response to oxidative stress (11, 23). Furthermore, diabetes-induced alterations of free SH levels in heart have also been reported (19). In that earlier study, it was shown that ATPase activities of myofibrils and myofibrillar SH reactivity to DTNB of isolated cardiomyocytes from diabetic male rat hearts were

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

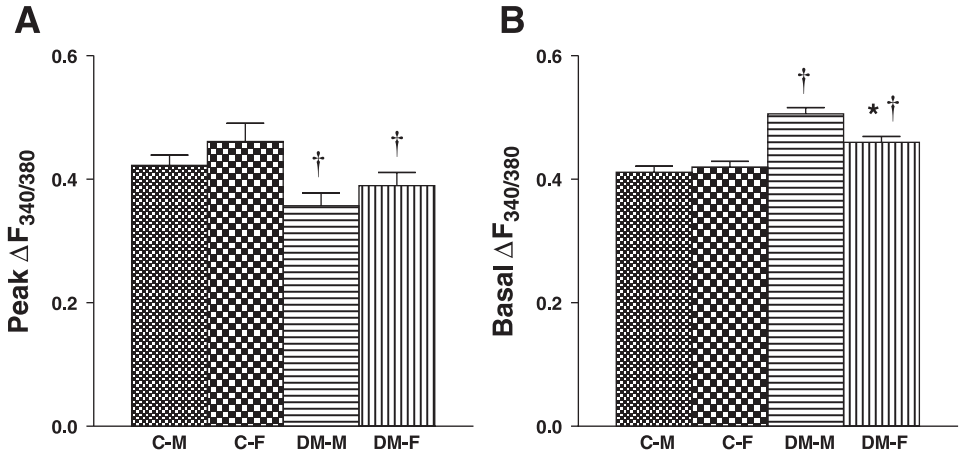


Fig. 4. Effects of sex on caffeine-induced responses and basal $[Ca^{2+}]_i$ in control and diabetic cardiomyocytes. *A*: cells were stimulated to ensure stable sarcoplasmic reticulum (SR)-Ca^{2+} load, and then caffeine (10 mM) was applied 30 s after cessation of electrical stimulation. Caffeine responses were similar in both sexes but significantly less in diabetic animals with respect to the controls ($P < 0.05$). *B*: basal $[Ca^{2+}]_i$ in diabetic compared with control animals. Values are means ± SE of 12–17 cells from at least 5 animals in each group. In Bonferroni multiple comparison test, $*P < 0.05$ males vs. females and $\dagger P < 0.001$ diabetic vs. corresponding control animals; no significant interaction was found.

depressed. Our data demonstrated greater free SH levels in females from the control group ($P < 0.05$) compared with the counterpart males. This corresponds to a lower oxidation capacity in diabetic female than in male animals. Therefore, the novel aspect of the present study lies in the finding that the induction of a specific cardiac pathology such as diabetes in female animals leads to a greater Ca^{2+} flux via RyR2, a less hyperphosphorylation level of RyR2, and a lower level of

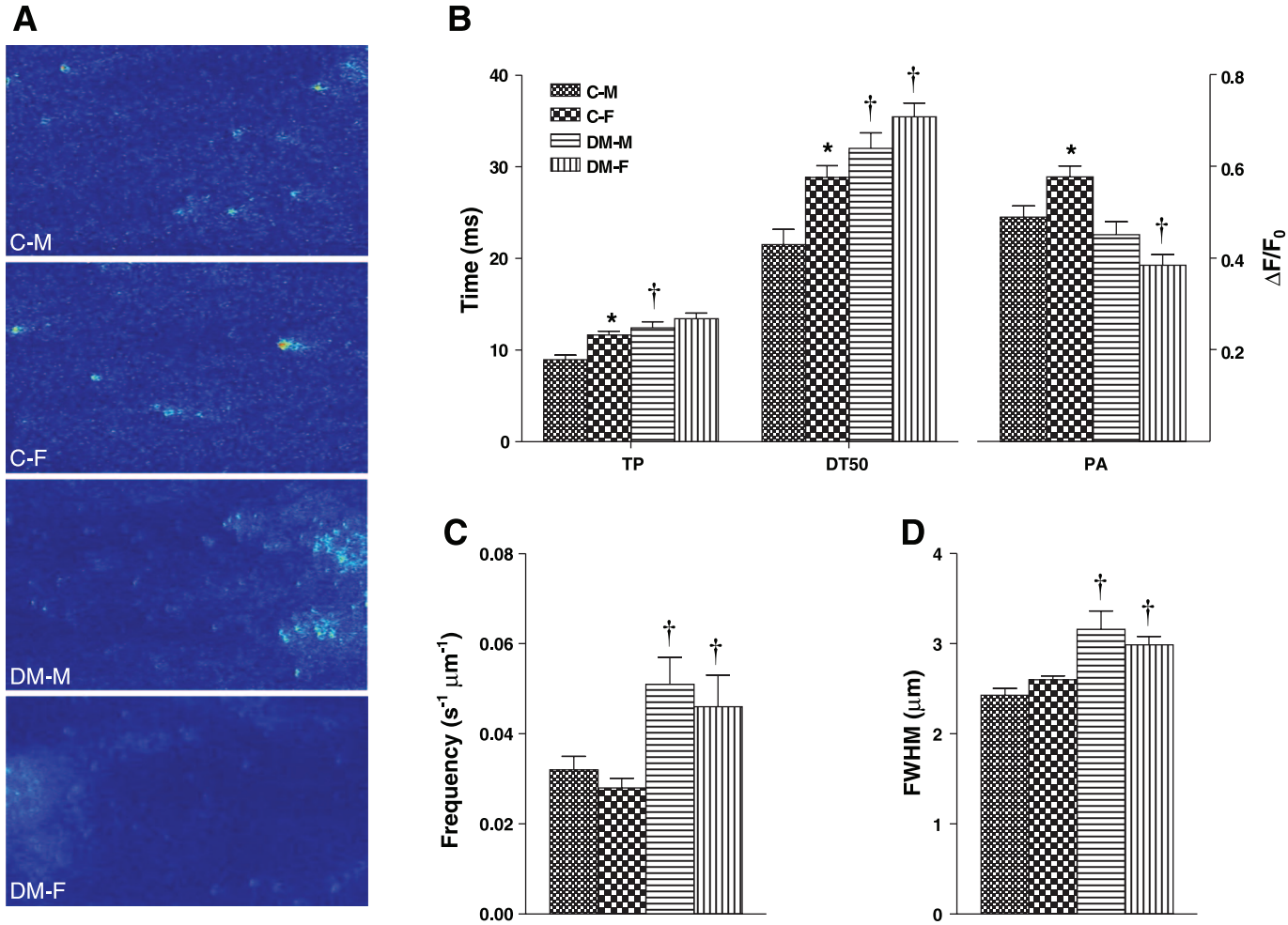


Fig. 5. Local Ca^{2+} releases in control and diabetic rat cardiomyocytes. *A*: representative line-scan images of cardiomyocytes isolated from control and diabetic male and female rats. *B*: Ca^{2+} spark characteristics: TP, DT_{50}, and peak amplitude (PA) measured in the 4 groups; $n_{rat} = 5$ or 6, $n_{cell} = 40–45$, $n_{spark} = 250–350$ vs. $n_{rat} = 5$ or 6, $n_{cell} = 35–40$, and $n_{spark} = 245–300$ in diabetic vs. control for males and females. *C* and *D*: bar graphs showing the frequency of Ca^{2+} sparks (*C*) and spatial spread of the sparks (FWHM; *D*) in the same groups of cells. In Bonferroni multiple comparison test, $*P < 0.05$ males vs. females and $\dagger P < 0.001$ diabetics vs. corresponding controls; no significant interaction was found.

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

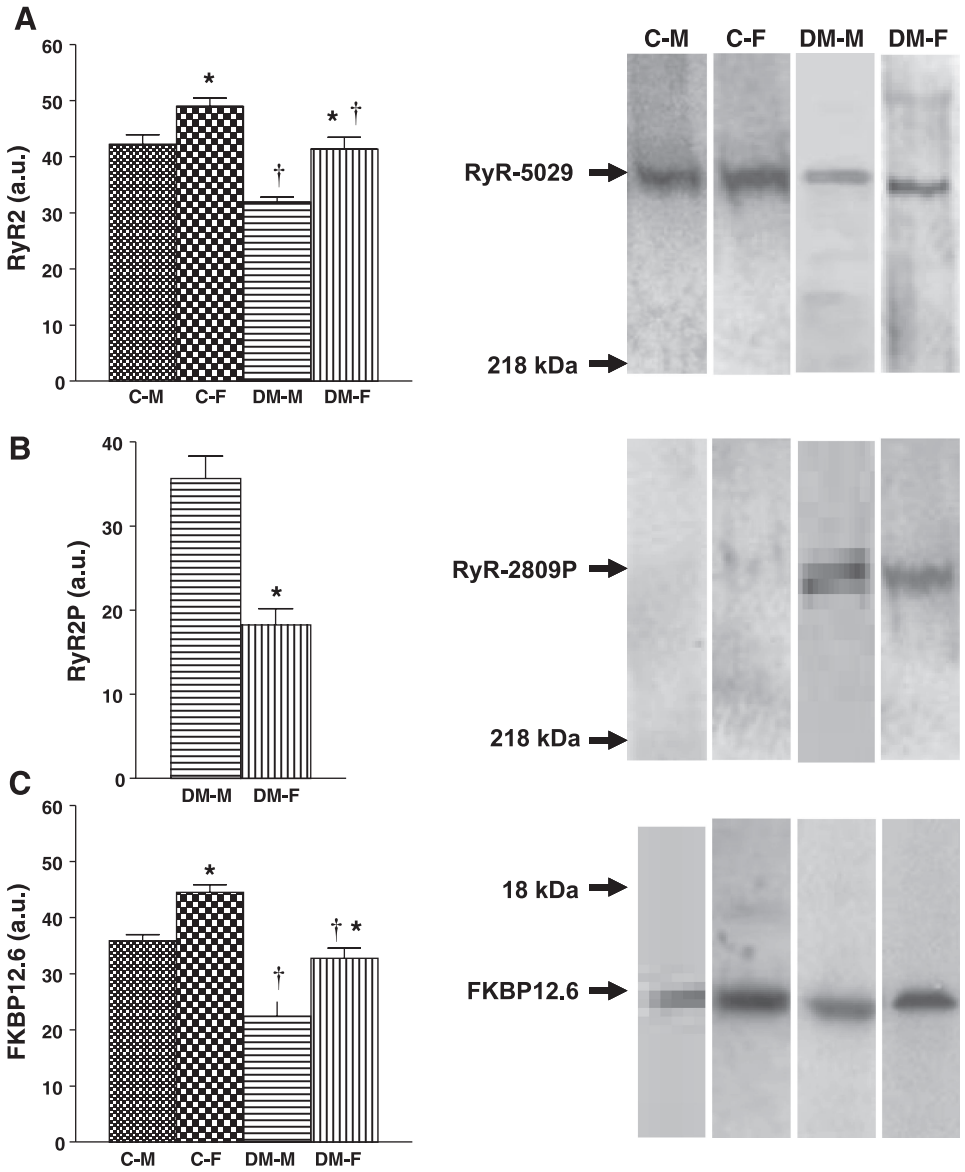


Fig. 6. Amount and phosphorylated level of cardiac ryanodine receptors (RyR2). Shown are representative Western blots (*right*) and pooled data (*left*) of RyR2 (*A*), phosphorylated RyR2 (RyR2809P; *B*), and FK506-binding protein 12.6 (FKBP12.6; *C*) protein levels in control and diabetic male and female rat cardiomyocytes. Cardiomyocytes were isolated from 4 or 5 animals and were used with double assays in each sample for measurement. AU, arbitrary unit. In Bonferroni multiple comparison test, $*P < 0.05$ males vs. females and $\dagger P < 0.001$ diabetics vs. corresponding controls; no significant interaction was found. Student's *t*-test in *B* was $*P < 0.001$.

oxidative stress than in diabetic males. Consequently, there are functional sex differences, observed as differential levels of RyR2, FKBP12.6, and total and free SH levels under both control and pathological conditions.

Depressions in contraction and relaxation of cardiomyocytes from animals with diabetes occur in parallel with reduced rates of rise and decline of $[Ca^{2+}]_i$ transients elicited by electrical stimulation. Our group (32) recently demonstrated that Ca^{2+} sparks in cardiomyocytes from diabetic rats are slower and have higher frequency with respect to the controls, consistent with alterations in Ca^{2+} handling and cardiac dysfunction. The changes in RyR2 opening kinetics could be related to hyperphosphorylation of RyR2 and subsequent FKBP12.6 release (32). However, a less severe depression of cardiac function is observed in diabetic female rats than in their male counterparts (3, 4). To account for these sex differences in contractile

Table 1. *Effects of sex and diabetes on SH and PKC contents of isolated cardiomyocytes*

	Male		Female	
	Control	Diabetes	Control	Diabetes
Free SH, μmol/mg wet wt	1.74±0.02	0.95±0.15†	1.97±0.07*	1.44±0.17*†
Total SH, μmol/mg wet wt	3.30±0.46	1.56±0.06†	3.40±0.21	2.54±0.29†
PKC ratio	1.8±0.2	2.7±0.2†	1.8±0.2	2.3±0.3*†

Values are means ± SE. SH, sulfhydryl. Cardiomyocytes were isolated from 6–8 male and female rats from control and diabetic groups. PKC ratio represents the ratio of total PKC content in the membrane fraction to cytosolic fraction. In Bonferroni multiple comparison test, $*P < 0.05$ males vs. females and $\dagger P < 0.05$ diabetic group vs. corresponding control group; no significant interaction was found.

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

activity and Ca^{2+} homeostasis, a recent study emphasized a lower expression of myosin heavy chain-β and a less phosphorylated phospholamban in female than in male diabetic rat hearts (33). In the present work, we show that most diabetes-induced alterations are indeed lower in females. Thus the diabetes-induced decrease in LVDP and the depressions in $\pm dP/dt$ were significantly less in females than in males. Despite no significant changes in I_{Ca} during diabetes in both sexes, the Ca^{2+} transient was less reduced in diabetic females. This effect was associated with less marked reductions in both RyR2 and FKBP12.6 protein contents and with less RyR2 hyperphosphorylation. These observations, together with the slightly lower Ca^{2+} spark frequency, could also account for a lower basal $[Ca^{2+}]_i$ and a slightly higher SR-Ca^{2+} load in control female rats.

It is noteworthy that diabetes-induced Ca^{2+} signaling alterations in male rats were shown to be related to the angiotensin activation pathway. Furthermore, AT_1-receptor stimulation induces the generation of ROS, which can have detrimental effects on heart function (9, 15, 17). Recent published data support this idea (14, 18), which implies a role of ROS in the amplification of PKC signaling in high-glucose-induced cell dysfunction. With these findings in mind, it can be suggested that PKC can play important roles in the alterations of both local and global Ca^{2+} releases in cardiomyocytes from diabetic rat hearts. In addition, in a recent work, we had shown that diabetes-induced Ca^{2+} signaling alterations in male rats are, in part, antagonized by the AT_1-receptor inhibitor candesartan that also reduced the diabetes-induced increase in PKC activity and oxidized thiol protein level (31). In the present study, we demonstrated that the changes in these two factors are smaller in females than in males, supporting their major involvement in the modulation of Ca^{2+} signaling. Besides, ryanodine receptors are known to be phosphorylable by PKC (26), but the physiological consequences have not yet been clarified.

That thiol oxidation was less in diabetic cardiomyocytes of female animals vs. that shown in males has already been proposed by Shimoni and Liu (22) to account for lesser variations in K^+ currents. These authors reported that the activation of autocrine/paracrine mechanisms is absent or less pronounced in cardiac cells from Type 1 diabetic females due to a protective action of estrogen (22, 24). Some of the cytoprotective effects of estrogen are related to its antioxidative properties (6, 25). Shimoni and Liu (24) recently demonstrated that induction of a specific cardiac pathology, such as diabetes, leads in females to a lower level of oxidative stress expressed as lower superoxide ion generation. Furthermore, Xin et al. (30) demonstrated that FKBP12.6 knockout male and female mice display similar increased probability of prolongation of channel opening, despite disruption of the FKBP12.6 gene, and results in myocardial hypertrophy only in male but not in female mice. They concluded that FKBP12.6 modulates excitation-contraction coupling and that estrogen, having an effect on FKBP12.6, plays a protective role in the response of the heart to the Ca^{2+} dysregulation under any pathological condition.

Although the benefit and risk of estrogen replacement therapy for cardiovascular disease remains controversial, 17β-estradiol treatment of isolated ventricular cardiomyocytes from adult rats induced a direct cardiac stimulatory action on cardiac mechanical function, likely mediated through enhanced intracellular Ca^{2+} release (10). Interestingly, supporting the above data, it has been shown that 17β-estradiol prevents oxidative stress in ovariectomized rats and has an important role in modulation of antioxidant enzyme expression and function (11, 25). Although we do not have direct data with 17β-estradiol in the present study, our results link these sex differences to the different levels of Ca^{2+} flux via RyR2, different protein levels of RyR2 and FKBP12.6, and total and free SH levels in the female compared with the male animals. Consequently, diabetes-induced cardiac dysfunction in females is significantly reduced compared with the matched-males due, at least in part, to lower levels of both RyR2 phosphorylation and oxidative stress.

In summary, by being less susceptible to Ca^{2+} overload, female rats have an "advantage" over the males. The present data indicate that this is in part related to different protein levels and/or functioning of the Ca^{2+}-handling systems, as well as different soluble SH levels under control conditions, with an higher estrogen level in female rats. These sex-dependent differences are much more marked in diabetic animals.

ACKNOWLEDGMENTS

We thank Drs. A. M. Marks and X. Wehrens for generous gift of RyR2 and phosphorylated RyR2 antibodies and A. Lacampagne and M. Ugur for helpful discussion.

GRANTS

This work has been supported by Ankara University Project Grants 2006-080-9233, DPT 2003K120-9025-6, TUBITAK-SBAG-PIA-10 (105S149), and TUBITAK-SBAG-3056 (104S591).

REFERENCES

1. **Bedinghaus J, Leshan L, Diehr S.** Coronary artery disease prevention: what's different for women? *Am Fam Physician* 63: 1393–1400, 2001.
2. **Brandes RP, Mugge A.** Gender differences in the generation of superoxide anions in the rat aorta. *Life Sci* 60: 391–406, 1997.
3. **Brown RA, Filipovich P, Walsh MF, Sowers JR.** Influence of sex, diabetes and ethanol on intrinsic contractile performance of isolated rat myocardium. *Basic Res Cardiol* 9: 353–360, 1996.
4. **Brown RA, Walsh MF, Ren J.** Influence of gender and diabetes on vascular and myocardial contractile function. *Endocr Res* 27: 399–408, 2001.
5. **Capasso JM, Remily RM, Smith RH, Sonnenblick EH.** Sex differences in myocardial contractility in the rat. *Basic Res Cardiol* 78: 156–171, 1983.
6. **Ceriello A.** New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diabetes Care* 26: 1589–1596, 2003.
7. **Chu SH, Sutherland K, Beck J, Kowalski J, Goldspink P, Schwertz D.** Sex differences in expression of calcium-handling proteins and beta-adrenergic receptors in rat heart ventricle. *Life Sci* 76: 2735–2749, 2005.
8. **Curl CL, Wendt IR, Kotsanas G.** Effects of gender on intracellular $[Ca^{2+}]$ in rat cardiac myocytes. *Pflügers Arch* 441: 709–716, 2001.
9. **Davidoff AJ, Davidson MB, Carmody MW, Davis ME, Ren J.** Diabetic cardiomyocyte dysfunction and myocyte insulin resistance: role of glucose-induced PKC activity. *Mol Cell Biochem* 262: 155–163, 2004.
10. **Duan J, Esberg LB, Dai S, Aberle NS, Lopez FL, Ren J.** Comparison of cardiac contractile and intracellular Ca^{2+} response between estrogen and phytoestrogen α-zearalanol in ventricular myocytes. *Endocrine* 24: 33–38, 2004.
11. **Hernández I, Delgado JL, Diaz J, Quesada T, Teruel MJ, Llanos MC, Carbonell LF.** 17β-Estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1599–R1605, 2000.
12. **Johnson BE, Johnson CA.** Cardiovascular disease and differences between the sexes. *Am Fam Physician* 63: 1290–1292, 2001.

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.

13. **Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, Parmer RJ.** Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. *Hypertension* 36: 878–884, 2000.
14. **Lee HB, Yu MR, Song JS, Ha H.** Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by human peritoneal mesothelial cells. *Kidney Int* 65: 1170–1179, 2004.
15. **Malhotra A, Kang BPS, Cheung S, Opawumi D, Meggs LG.** Angiotensin II promotes glucose-induced activation of cardiac protein kinase C isozymes and phosphorylation of troponin I. *Diabetes* 50: 1918–1926, 2001.
16. **Morgan J, Erny R, Allen P, Grossman W, Gwathmey J.** Abnormal intracellular calcium handling, a major cause of systolic and diastolic dysfunction in ventricular myocardium from patients with heart failure. *Circulation* 81, *Suppl* 2: III21–III32, 1990.
17. **Nickenig G, Harrison DG.** The AT(1)-type angiotensin receptor in oxidative stress and atherogenesis. II. AT_1 receptor regulation. *Circulation* 105: 530–536, 2000.
18. **Palomeque J, Sapia L, Hajjar RJ, Mattiazzi A, Vila Petroff M.** Angiotensin II-induced negative inotropy in rat ventricular myocytes: role of reactive oxygen species and p38 MAPK. *Am J Physiol Heart Circ Physiol* 290: H96–H106, 2006.
19. **Pierce GN, Dhalla NS.** Mechanisms of the defect in cardiac myofibrillar function during diabetes. *Am J Physiol Endocrinol Metab* 248: E170–E175, 1985.
20. **Schwertz D, Buttrick P.** Gender influences on myocardial structure and function. In: *Cardiovascular Health and Disease in Women* (2nd ed.), edited by Douglas P. Philadelphia, PA: Saunders, 2002, p. 426–444.
21. **Schwertz DW, Beck JM, Kowalski JM, Ross JD.** Sex differences in the response of rat heart ventricle to calcium. *Biol Res Nurs* 5: 286–298, 2004.
22. **Shimoni Y, Liu XF.** Sex differences in the modulation of K^+ currents in diabetic rat cardiac myocytes. *J Physiol* 550: 401–412, 2003.
23. **Shimoni Y, Hunt D, Chuang M, Chen KY, Kargacin G, Severson DL.** Modulation of potassium currents by angiotensin and oxidative stress in cardiac cells from the diabetic rat. *J Physiol* 567: 177–190, 2005.
24. **Shimoni Y, Liu XF.** Gender differences in the levels of angiotensin II and in its action on multiple pathways of K^+ current modulation in diabetic rats. *Am J Physiol Heart Circ Physiol* 287: H311–H319, 2004.
25. **Strehlow K, Rotter S, Wassmann S, Adam O, Grohe C, Laufs K, Bohm M, Nickenig G.** Modulation of antioxidant enzyme expression, and function by estrogen. *Circ Res* 93: 170–177, 2003.
26. **Takasago T, Imagawa T, Furukawa K, Ogurusu T, Shigekawa M.** Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. *J Biochem (Tokyo)* 109: 163–170, 1991.
27. **Vizgirda VM, Wahler GM, Sondgeroth KL, Ziolo MT, Schwertz DW.** Mechanisms of sex differences in rat cardiac myocyte response to β-adrenergic stimulation. *Am J Physiol Heart Circ Physiol* 282: H256–H263, 2002.
28. **Wehrens XH, Marks AR.** Altered function and regulation of cardiac ryanodine receptors in cardiac disease. *Trends Biochem Sci* 28: 671–678, 2003.
29. **Xiao L, Zhang L, Han W, Wang Z, Nattel S.** Sex-based transmural differences in cardiac repolarization and ionic-current properties in canine left ventricle. *Am J Physiol Heart Circ Physiol* 291: H570–H580, 2006.
30. **Xin HB, Senbonmatsu T, Cheng DS, Wang YX, Copello JA, Ji GJ, Colliers ML, Deng KY, Jeyakumar LH, Magnuson MA, Inagami T, Kotlikoff MI, Fleischer S.** Oestrogen protects FKBP12.6 null mice from cardiac hypertrophy. *Nature* 416: 334–337, 2002.
31. **Yaras N, Bilginoglu A, Vassort G, Turan B.** Restoration of diabetes-induced abnormal local Ca^{2+} release in cardiomyocytes by angiotensin II receptor blockade. *Am J Physiol Heart Circ Physiol* 292: H912–H920, 2007.
32. **Yaras N, Ugur M, Ozdemir S, Gurdal H, Purali N, Lacampagne A, Vassort G, Turan B.** Effects of diabetes on ryanodine receptor Ca release channel (RyR2) and Ca^{2+} homeostasis in rat heart. *Diabetes* 54: 3082–2088, 2005.
33. **Zhong Y, Reiser PJ, Matlib MA.** Gender differences in myosin heavy chain-β and phosphorylated phospholamban in diabetic rat hearts. *Am J Physiol Heart Circ Physiol* 285: H2688–H2693, 2003.

Downloaded from journals.physiology.org/journal/ajpheart (193.140.039.039) on January 26, 2022.