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Frank Christopher Howarth
UAE University

Manal M. A. Smail
UAE University

Muhammad Anwar Qureshi
UAE University

Anatoliy Shmygol
UAE University

Jaipaul Singh
UAE University

See next page for additional authors

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Frank Christopher Howarth, Manal M. A. Smail, Muhammad Anwar Qureshi, Anatoliy Shmygol, Jaipaul Singh, and Lina Al Kury

Contraction and Intracellular Calcium Transport in Epicardial and Endocardial Ventricular Myocytes from Streptozotocin-Induced Diabetic Rat

Frank Christopher Howarth, Manal M. A. Smail, Muhammad Anwar Qureshi, Anatoliy Shmygol, Jaipaul Singh¹, Lina Al Kury²

Department of Physiology, College of Medicine and Health Sciences, UAE University, Al Ain, ²Department of Health Sciences, College of Natural and Health Sciences, Zayed University, Abu Dhabi, UAE, ¹School of Forensic and Applied Sciences, University of Central Lancashire, Preston, England, UK

Abstract

Introduction: Diabetes mellitus (DM) is a global health problem. According to the International Diabetes Federation, 424.9 million people suffered from DM in 2017 and this number is expected to rise to 628.6 million by 2045. Although diabetes can affect every organ in the body, cardiovascular disease is a major cause of death and disability in people with diabetes. Diabetic patients frequently suffer from systolic and diastolic dysfunction. Within the ventricles, the electromechanical properties of cardiac myocytes vary transmurally. **Aims and Objectives:** The aim of this study was to investigate contraction and Ca^{2+} transport in epicardial (EPI) and endocardial (ENDO) myocytes from the left ventricle in the streptozotocin (STZ) – induced diabetic rat heart. **Materials and Methods:** Experiments were performed 5-6 months after STZ treatment. Ventricular myocytes were isolated by enzymic and mechanical dispersal techniques from EPI and ENDO regions of the left ventricle. Contraction and free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ were measured by video edge detection and fluorescence photometry techniques, respectively. **Results:** Myocyte length and calculated surface area were smaller in EPI-STZ compared to EPI-CON. Time to peak (TPK) shortening was prolonged in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to ENDO-CON myocytes. Time to half (THALF) relaxation of shortening was prolonged in EPI-STZ compared to EPI-CON. TPK Ca^{2+} transient was prolonged in EPI-STZ compared to EPI-CON, ENDO-STZ compared to ENDO-CON, ENDO-STZ compared to EPI-STZ and in ENDO-CON compared to EPI-CON myocytes. THALF decay of the Ca^{2+} transient was prolonged in ENDO-STZ compared to ENDO-CON. Fractional release of Ca^{2+} was increased in ENDO-STZ compared to ENDO-CON and in ENDO-STZ compared to EPI-STZ. Recovery of the Ca^{2+} transient was prolonged in ENDO-STZ compared to ENDO-CON. **Conclusion:** In conclusion the kinetics of contraction and Ca^{2+} transient and fractional release of Ca^{2+} from the sarcoplasmic reticulum are altered to different extents in EPI and ENDO myocytes from STZ-induced diabetic rat.

Keywords: Epicardial and endocardial myocytes, intracellular Ca^{2+} , myocyte contraction, rat heart ventricle, streptozotocin-induced diabetes

INTRODUCTION

Diabetes mellitus (DM) is a global health problem. According to the International Diabetes Federation, 424.9 million people suffered from DM in 2017 and this number is expected to rise to 628.6 million by 2045 (<http://www.diabetesatlas.org/resources/2017-atlas.html>). Although DM can affect every organ in the body, cardiovascular disease is a major cause of death and disability in people with diabetes.^[1-3] Diabetic patients frequently suffer from systolic and diastolic dysfunction.^[4-6] The streptozotocin (STZ)-induced diabetic rat is a widely used experimental model of DM. STZ causes damage to the pancreatic β -cells, which in turn leads to a reduction in insulin synthesis and release and a

consequent rise in blood glucose.^[7,8] Abnormalities in a variety of haemodynamic indices including stroke volume, ejection fraction, cardiac output, rate of pressure development and relaxation have been widely demonstrated in the STZ-induced diabetic heart.^[9-12] At the level of the individual ventricular myocyte, many studies have demonstrated prolonged time course of contraction and relaxation^[13,14] and either

Address for correspondence: Prof. Frank Christopher Howarth, Department of Physiology, College of Medicine and Health Sciences, UAE University, P.O. Box 17666, Al Ain, UAE.
E-mail: chris.howarth@uaeu.ac.ae

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unaltered or reduced amplitude of shortening.^[15,16] These alterations in contraction are attributed, at least in part, to disturbances in Ca^{2+} transport.^[9,10,14,17] Within the ventricles, the electromechanical properties of cardiac myocytes vary transmurally and this may be related to the gradients of stress and strain experienced *in vivo* across the ventricular walls. Electrophysiological heterogeneity across the ventricular wall is a result of differential transmural expression of various ion channel proteins that underlie the different action potential waveforms observed in epicardial (EPI) and endocardial (ENDO) regions.^[18-20] To date, many of the single-cell studies have been performed in ventricular myocytes obtained from whole ventricle. Very little is known about the regional effects of STZ-induced diabetes across the ventricles. The aim of the current study was to investigate the effects of DM after 5–6 months of STZ-treatment on contraction and Ca^{2+} transport in EPI and ENDO myocytes from the left ventricle of rat heart compared to healthy controls.

METHODS

Experimental model

Experiments were performed in the STZ-induced diabetic rat, a well-characterised animal model of DM.^[7,8] Diabetes was induced in young adult (220–250 g) male Wistar rats with a single intraperitoneal injection of STZ (60 mg/kg body weight) in citrate buffer. Age-matched control rats received an injection of citrate buffer alone. Body weight, heart weight and non-fasting blood glucose (OneTouch Ultra 2, LifeScan) were measured immediately before experiments. Experiments were performed in EPI and ENDO myocytes, 5–6 months after STZ treatment. Ethical approval for this project was obtained from the UAE University Animal Research Ethics Committee and experiments were performed in accordance with institutional guidelines.

Isolation of ventricular myocytes

Ventricular myocytes were isolated by enzymatic and mechanical dispersal techniques according to previously described techniques.^[21] After rats were euthanised with a guillotine hearts were rapidly removed and mounted on a Langendorff perfusion system. Hearts were perfused with cell isolation solution at a flow rate of 8 ml.g heart⁻¹ min⁻¹ at a temperature of 36–37°C. The cell isolation solution contained in mmol/l: 130.0 NaCl, 5.4 KCl, 1.4 MgCl₂, 0.75 CaCl₂, 0.4 NaH₂PO₄, 5.0 HEPES, 10.0 glucose, 20.0 taurine and 10.0 creatine (pH adjusted to 7.3 with NaOH). When contraction of the heart had stabilised, perfusion was switched for 4 min to Ca^{2+} -free cell isolation solution containing 0.1 mmol/l EGTA, and then for 6 min to cell isolation solution containing 0.05 mmol/l Ca^{2+} , 0.60 mg/ml Type 1 collagenase (Worthington Biochemical Corp, Lakewood, NJ, USA) and 0.075 mg/ml Type XIV protease (Sigma, Taufkirchen, Germany). After enzyme treatment, the heart was removed from the perfusion system and the left ventricle was carefully dissected according to previously described techniques.^[21] Using fine scissors, thin sections were dissected from the outermost layer

of the left ventricle (EPI) and innermost layer of the left ventricle (ENDO). The sections were carefully minced and gently shaken in collagenase-containing isolation solution supplemented with 1% BSA. Cells were filtered from this solution at 4-min intervals and resuspended in cell isolation solution containing 0.75 mmol/l Ca^{2+} .

Ventricular myocyte shortening

Ventricular myocyte shortening was measured according to previously described techniques.^[21] Cells were superfused (3–5 ml/min) with normal Tyrode containing the following in mmol/l: 140.0 NaCl, 5.0 KCl, 1.0 MgCl₂, 10.0 glucose, 5.0 HEPES and 1.8 CaCl₂ (pH 7.4). Unloaded EPI and ENDO myocyte shortening were recorded using a video edge detection system (VED-114, Crystal Biotech, Northborough, MA, USA). Resting cell length, time to peak (TPK) shortening, time to half (THALF) relaxation and amplitude of shortening (expressed as a % of resting cell length) were measured in electrically stimulated (1 Hz) myocytes maintained at 35°C–36°C. Data were acquired and analysed with Signal Averager software v 6.37 (Cambridge Electronic Design, Cambridge, UK).

Intracellular Ca^{2+}

Intracellular (Ca^{2+}) was measured in Fura-2/AM-loaded myocytes according to previously described techniques.^[21] Myocytes were alternately illuminated by 340 nm and 380 nm light using a monochromator (Cairn Research, Faversham, UK) which changed the excitation light every 2 ms. The resulting fluorescence, emitted at 510 nm, was recorded by a photomultiplier tube and the ratio of the emitted fluorescence at the two excitation wavelengths (340/380 ratio) provided an index of intracellular Ca^{2+} concentration. Resting Fura-2 ratio, TPK Ca^{2+} transient, THALF decay of the Ca^{2+} transient and the amplitude of the Ca^{2+} transient were measured in electrically stimulated (1 Hz) myocytes maintained at 35–36°C. Data were acquired and analysed with Signal Averager software v 6.37 (Cambridge Electronic Design, Cambridge, UK).

Measurement of sarcoplasmic reticulum Ca^{2+} transport

Sarcoplasmic reticulum (SR) Ca^{2+} was assessed using previously described techniques.^[21] Fura-2/AM-loaded myocytes were stimulated electrically (1 Hz) and maintained at 35–36°C. When the Ca^{2+} transients had reached a steady state, electrical stimulation was paused for 5 s. Caffeine (20 mM) was then applied for 10 s using a rapid solution switching device.^[22] Electrical stimulation was then restarted and the Ca^{2+} transients were allowed to recover to steady state. Fractional release of SR Ca^{2+} was calculated by comparing the amplitude of the electrically-evoked steady state Ca^{2+} transients with that of the caffeine-evoked Ca^{2+} transient. Ca^{2+} refilling of the SR was assessed by measuring the rate of recovery of electrically-evoked Ca^{2+} transients following application of caffeine.

Assessment of myofilament sensitivity to Ca^{2+}

In some cells shortening and Fura-2 ratio were recorded simultaneously as previously described.^[23] Myofilament

sensitivity to Ca^{2+} was assessed from phase-plane diagrams of Fura-2 ratio versus cell length by measuring the gradient of the Fura-2-cell length trajectory during late relaxation of the twitch contraction. The position of the trajectory reflects the relative myofilament response to Ca^{2+} and hence can be used as a measure of myofilament sensitivity to Ca^{2+} .^[24,25]

Statistics

The results were expressed as the mean \pm standard error of mean of “*n*” observations. Statistical comparisons were performed using the Independent samples *t*-test or one-way ANOVA followed by Bonferroni-corrected *t*-tests for multiple comparisons, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

General characteristics

Body weight and heart weight were reduced, while heart weight/body weight and non-fasting blood glucose were increased in STZ-induced diabetic rats compared to age-matched controls [Table 1].

Ventricular myocyte shortening

Cell width was not significantly ($P > 0.05$) altered in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to

ENDO-CON [Figure 1a]. Cell length was significantly ($P < 0.05$) shorter [Figure 1b] and calculated surface area [Figure 1c] was significantly ($P < 0.05$) smaller in EPI-STZ compared to EPI-CON ($n = 20-53$ cells from 6 hearts). Typical recordings of myocyte shortening in ENDO-CON and ENDO-STZ myocytes are shown in Figure 2a. TPK shortening was significantly prolonged in EPI-STZ (102.4 ± 4.7 ms) compared to EPI-CON (77.0 ± 1.8 ms) and in ENDO-STZ (100.2 ± 4.1 ms) compared to ENDO-CON (82.2 ± 2.7 ms) myocytes ($n = 33-52$ cells from 13 hearts) [Figure 2b]. THALF relaxation of shortening was significantly prolonged in EPI-STZ (67.0 ± 6.4 ms) compared to EPI-CON (46.5 ± 2.2 ms) and was not significantly altered in ENDO-STZ (56.6 ± 4.4 ms) compared to ENDO-CON (48.4 ± 2.8 ms) myocytes ($n = 33-52$ cells from 13 hearts) [Figure 2c]. Amplitude of shortening was not significantly

Table 1: General characteristics of streptozotocin-induced diabetic rats compared to controls

	Control	Streptozotocin
Body weight (g)	433.29 \pm 19.46	285.57 \pm 10.80**
Heart weight (g)	1.33 \pm 0.05	1.17 \pm 0.07*
Heart weight/body weight (mg/g)	3.08 \pm 0.18	4.07 \pm 0.18**
Non-fasting blood glucose (mg/dl)	89.14 \pm 2.83	448.71 \pm 11.55**

Data are mean \pm SEM, $n=7$ rats, * $P<0.05$, ** $P<0.01$. SEM: Standard error of mean

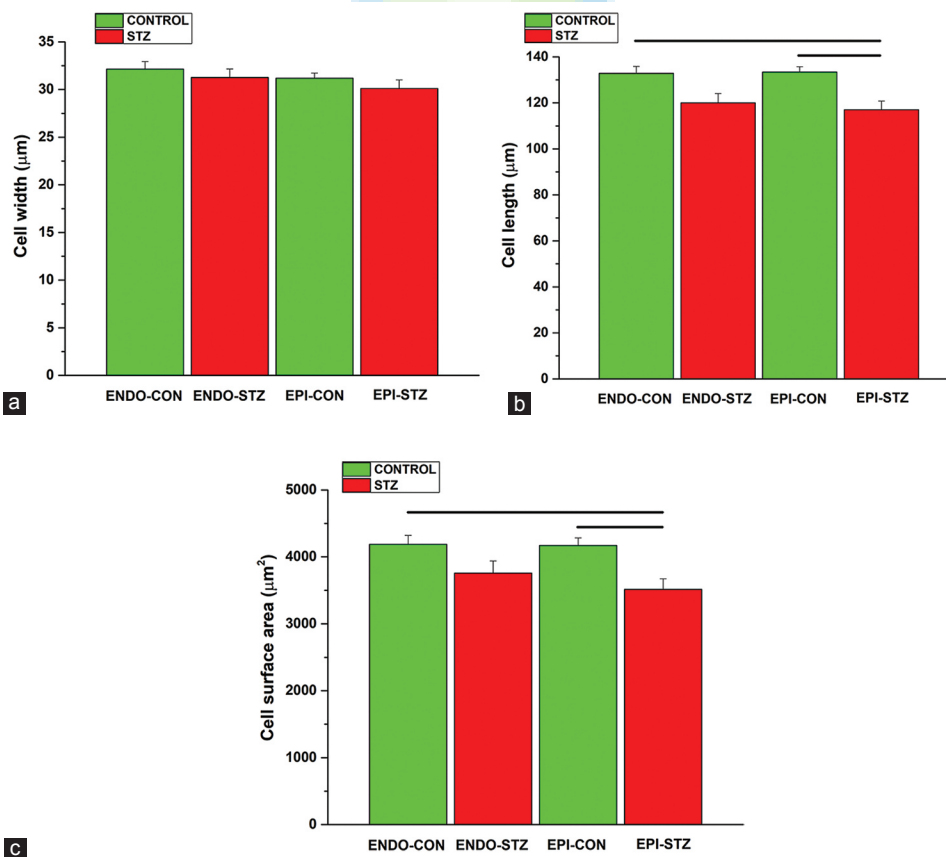


Figure 1: Cell width (a), cell length (b) and calculated cell surface area (c) in epicardial and endocardial left ventricular myocytes from streptozotocin and control rats. Data are mean \pm standard error of mean, $n = 20-53$ cells from 6 hearts

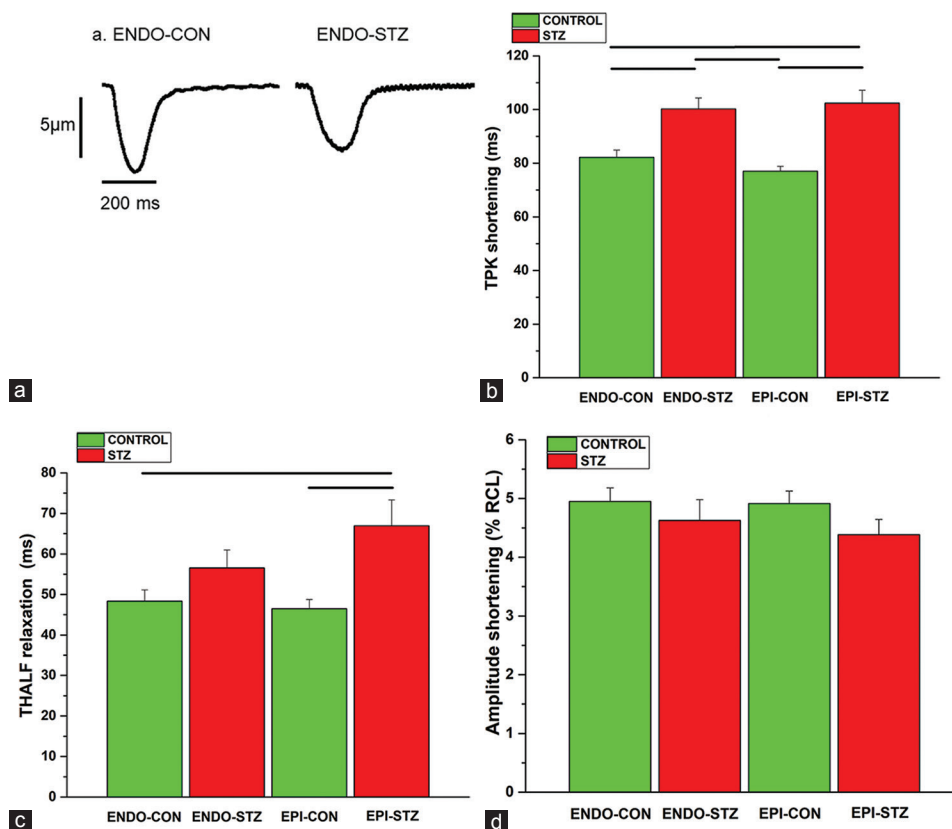


Figure 2: Typical recordings of shortening in ENDO-CON and ENDO-STZ myocytes (a), time to peak shortening (b), time to half relaxation of shortening (c) and amplitude of shortening (d) in epicardial and endocardial left ventricular myocytes from streptozotocin and control rats. Data are mean \pm standard error of mean, $n = 33$ –52 cells from 13 hearts

altered in EPI-STZ compared to EPI-CON or in ENDO-STZ compared to ENDO-CON myocytes [$n = 33$ –52 cells from 13 hearts; Figure 2d].

Intracellular Ca^{2+} transients

Typical recordings of Ca^{2+} transients in ENDO-CON and ENDO-STZ myocytes are shown in Figure 3a. Resting Fura-2 ratio was not significantly altered in EPI-STZ compared to EPI-CON or in ENDO-STZ compared to ENDO-CON myocytes ($n = 53$ –59 cells from 11 to 12 hearts) [Figure 3b]. TPK Ca^{2+} transient was significantly prolonged in EPI-STZ (62.6 ± 2.0 ms) compared to EPI-CON (53.0 ± 0.8 ms) and in ENDO-STZ (68.8 ± 1.9 ms) compared to ENDO-CON (59.8 ± 1.4 ms); myocytes TPK Ca^{2+} transient was also significantly prolonged in ENDO-STZ compared to EPI-STZ and in ENDO-CON compared to EPI-CON myocytes ($n = 53$ –59 cells from 11 to 12 hearts) [Figure 3c]. THALF decay of the Ca^{2+} transient was not significantly altered in EPI-STZ (210.2 ± 9.1 ms) compared to EPI-CON (190.0 ± 9.6 ms) and was significantly prolonged in ENDO-STZ (210.4 ± 7.0 ms) compared to ENDO-CON (165.3 ± 6.3 ms) myocytes ($n = 53$ –59 cells from 11 to 12 hearts) [Figure 3d]. Amplitude of the Ca^{2+} transient was not significantly altered in EPI-STZ compared to EPI-CON or in ENDO-STZ compared to ENDO-CON myocytes [$n = 53$ –59 cells from 11 to 12 hearts; Figure 3e].

Sarcoplasmic reticulum Ca^{2+} transport

A typical recording of electrically-evoked Ca^{2+} transients followed, after a brief pause, by a caffeine-evoked Ca^{2+} transient, followed by recovery of Ca^{2+} transients during electrical stimulation in an ENDO-CON myocyte is shown in Figure 4a. Amplitude of the electrically-evoked Ca^{2+} transient was significantly larger in ENDO-STZ compared to EPI-CON [Figure 4b], amplitude of the caffeine-evoked Ca^{2+} transient [Figure 4c] and area under the curve of the caffeine-evoked Ca^{2+} transient [Figure 4d] were not significantly altered in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to ENDO-CON myocytes ($n = 16$ –22 cells from 4 to 6 hearts). Fractional release of Ca^{2+} was not significantly altered in EPI-STZ (0.72 ± 0.04) compared to EPI-CON (0.69 ± 0.03) and was increased in ENDO-STZ (0.88 ± 0.02) compared to ENDO-CON (0.63 ± 0.07) myocytes. Fractional release was also significantly increased in ENDO-STZ compared to EPI-STZ myocytes ($n = 16$ –22 cells from 4 to 6 hearts) [Figure 4e]. Amplitude of Ca^{2+} transient data presented in Figure 3e and 4b were acquired in different sets of experiments. It was interesting to note that in Figure 3e the amplitude of Ca^{2+} transient was unaltered in EPI-STZ and ENDO-STZ compared to respective controls however, in Figure 4b the amplitude of the Ca^{2+} transient was significantly ($P < 0.05$) increased in ENDO-STZ compared to ENDO-CON

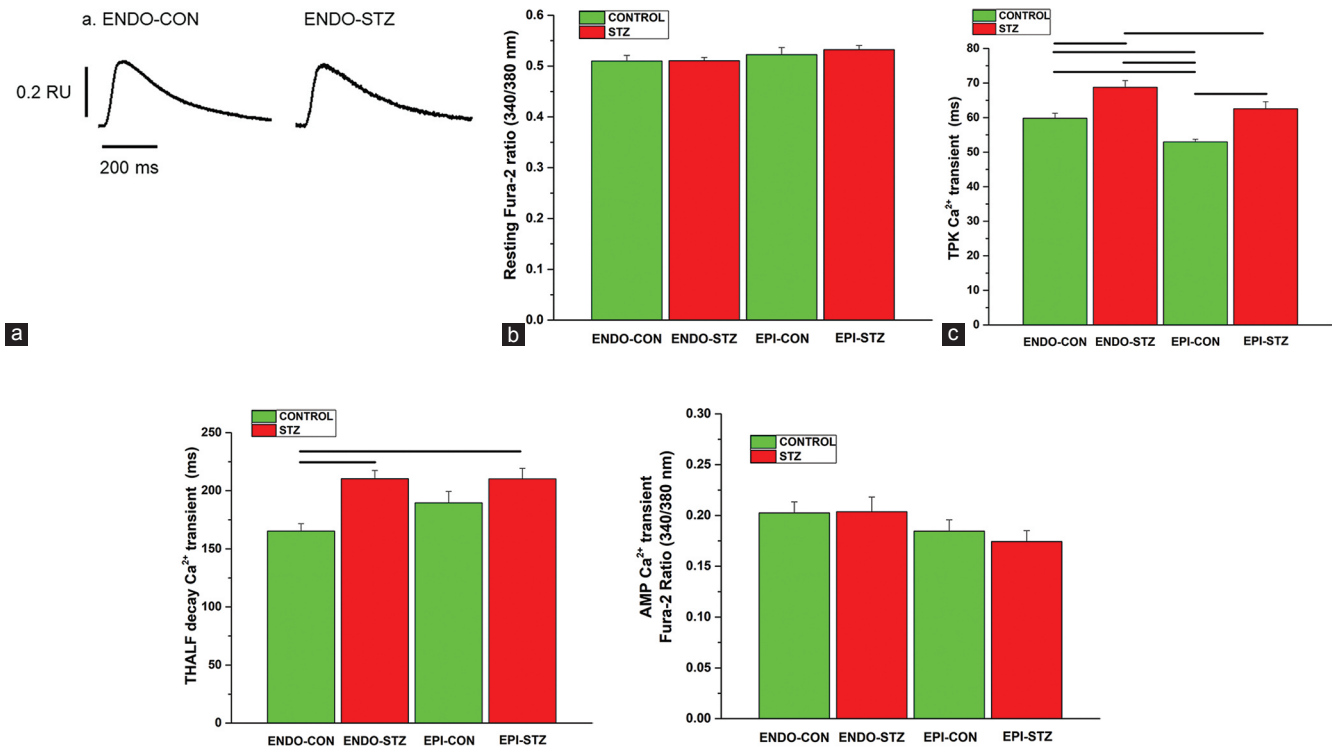


Figure 3: Typical recordings of Ca^{2+} transients in ENDO-CON and ENDO-STZ myocytes (a), resting Fura-2 ratio (b), time to peak Ca^{2+} transient (c), time to half decay of the Ca^{2+} transient (d) and amplitude of the Ca^{2+} transient (e) in epicardial and endocardial left ventricular myocytes from streptozotocin and control rats. Data are mean \pm standard error of mean, $n = 53-59$ cells from 11 to 12 hearts

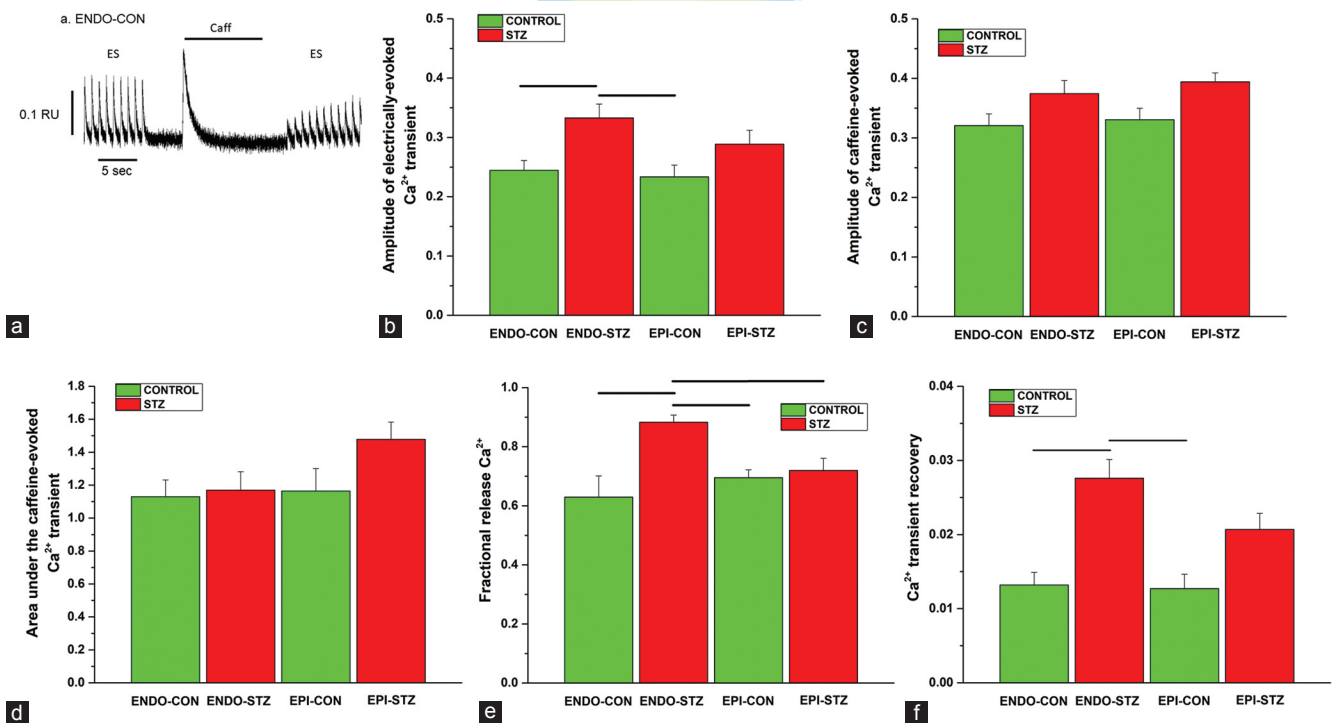


Figure 4: Typical recording of electrically-evoked Ca^{2+} transients and a caffeine-evoked Ca^{2+} transient in an ENDO-CON myocyte (a), amplitude of the electrically-evoked Ca^{2+} transient (b), amplitude of caffeine-evoked Ca^{2+} transient (c), area under the curve of the caffeine-evoked Ca^{2+} transient (d), fractional release of Ca^{2+} (e) and recovery of the Ca^{2+} transient after caffeine application and resumption of electrical stimulation (f). Data are mean \pm standard error of mean, $n = 16-22$ cells from 4 to 6 hearts. ES = Electrical stimulation

myocytes. These results show that in this model of DM there may be variability in results between sets of experiments. The rate of recovery of the electrically-evoked Ca^{2+} transient, following application of caffeine, was not significantly altered in EPI-STZ compared to EPI-CON but was increased in ENDO-STZ compared to ENDO-CON myocytes [$n = 16$ –22 cells from 4 to 6 hearts; Figure 4f].

Myofilament sensitivity to Ca^{2+}

A typical simultaneous recording of shortening and Ca^{2+} transient and of Fura-2 ratio plotted against cell length are shown in Figure 5a. Myofilament sensitivity to Ca^{2+} was not significantly altered in EPI-STZ compared to EPI-CON or in ENDO-STZ compared to ENDO-CON [Figure 5b].

DISCUSSION

The main findings of this study were as follows: (1) Cell length was shorter in EPI-STZ compared to EPI-CON; (2) TPK shortening was prolonged in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to ENDO-CON; (3) THALF relaxation of shortening was prolonged in EPI-STZ compared to EPI-CON; (4) TPK Ca^{2+} transient was prolonged in EPI-STZ compared to EPI-CON, ENDO-STZ compared to ENDO-CON, ENDO-STZ compared to EPI-STZ and in ENDO-CON compared to EPI-CON; (5) THALF decay of the Ca^{2+} transient was prolonged in ENDO-STZ compared to ENDO-CON; (6) Fractional release of Ca^{2+} was increased in ENDO-STZ compared to ENDO-CON and in ENDO-STZ compared to EPI-STZ and (7) Ca^{2+} transient recovery was prolonged in ENDO-STZ compared to ENDO-CON.

The results show that blood glucose was 5-fold higher in STZ-induced diabetic rats compared to controls. STZ causes damage to β -cells which in turn leads to a reduction in synthesis and release of insulin and consequent elevation of blood glucose.^[7,8] Consistent with many previous studies STZ rats had reduced body weight and reduced heart weight, yet their heart weight/body weight ratio was larger compared to controls suggesting cardiac hypertrophy.^[21,26,27] After 3 months of STZ

treatment, a previous study has shown that EPI and ENDO myocyte lengths were unaltered.^[21] However, in the current study, after 5–6 months of STZ treatment, the lengths of EPI and ENDO myocytes were smaller compared to respective controls.

TPK shortening was prolonged, and to similar extents, in EPI-STZ and ENDO-STZ compared to respective controls. THALF relaxation of shortening was prolonged only in EPI-STZ compared to EPI-CON. Amplitude of shortening was not altered in EPI and ENDO myocytes from STZ rat compared to controls. Previous studies have also reported prolonged TPK in myocytes isolated from whole ventricle and EPI and ENDO myocytes after 3 months of STZ treatment.^[14,21,28,29] Interestingly, THALF relaxation was only significantly prolonged in EPI-STZ compared to EPI-CON myocytes suggesting regional differences in the effects of DM on the kinetics of contraction. A previous study reported prolonged THALF relaxation in ENDO-STZ compared to ENDO-CON after 3 months of STZ treatment suggesting that the effects on dynamics of contraction alter with duration of DM.^[21] Although the kinetics of contraction were altered the amplitude of contraction was not altered in EPI and ENDO myocytes from diabetic rat compared to respective controls and this was also previously the case after 3 months of STZ treatment.^[21]

TPK Ca^{2+} transient was prolonged in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to ENDO-CON. Previous studies in myocytes from whole ventricle have also demonstrated prolonged TPK Ca^{2+} transient in STZ-induced diabetic rat.^[26,29-31] It has also been previously reported that after 3 months of STZ treatment TPK Ca^{2+} transient was only prolonged in ENDO-STZ and not in EPI-STZ myocytes compared to respective controls.^[21] It was interesting to note that TPK Ca^{2+} transient was also prolonged in ENDO-STZ compared to EPI-STZ and in ENDO-CON compared to EPI-CON suggesting regional differences in kinetics of the Ca^{2+} transient within control and diabetic hearts. THALF decay of the Ca^{2+} transient was only prolonged in ENDO-STZ compared

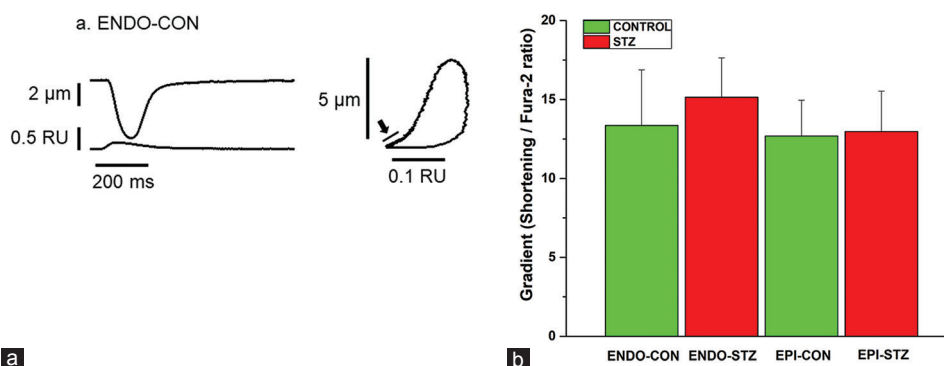


Figure 5: Typical simultaneous recording of shortening and Ca^{2+} in an ENDO-CON myocyte (left panel) and typical phase plane diagram of Fura-2 ratio unit versus cell length (right panel) in an ENDO-CON myocyte. The line and arrow indicate where measurements were made (a), Graph showing mean gradient of the Fura-2-cell length trajectory during late relaxation of the twitch contraction during the period 500–800 ms (b). Data are mean \pm standard error of mean, $n = 26$ –29 cells from 5 hearts

to ENDO-CON myocytes. Previous studies have demonstrated prolonged THALF Ca^{2+} transient in myocytes from whole ventricle and ENDO-STZ compared to ENDO-CON myocytes after 3 months of STZ treatment.^[21,28,32] Although the kinetics of the Ca^{2+} transient were altered the amplitude of contraction was not altered in EPI and ENDO myocytes from diabetic rat compared to respective controls and this was also previously the case after 3 months of STZ treatment.^[21]

Fractional release of Ca^{2+} was increased in ENDO-STZ compared to ENDO-CON and in ENDO-STZ compared to EPI-STZ. The fractional release of Ca^{2+} provides a measure of the amount of Ca^{2+} that is released during electrical stimulation compared to the amount that is releasable during application of caffeine. The results suggest that the fractional release of Ca^{2+} is larger in ENDO-STZ and is not altered in EPI-STZ compared to respective controls. Previously, it has been reported that after 3 months of STZ treatment, fractional release was reduced in EPI-STZ and not altered in ENDO-STZ compared to respective controls.^[21] These results provide further evidence of changes in Ca^{2+} handling with the duration of DM. The amplitude of the caffeine-evoked Ca^{2+} transient and area under the curve of the caffeine-evoked Ca^{2+} transient were not altered in EPI and ENDO myocytes from diabetic heart compared to respective controls. Suggesting that the changed fractional release is more likely to be associated with the generation of the electrically-evoked Ca^{2+} transient.

Ca^{2+} transient recovery was prolonged in ENDO-STZ compared to ENDO-CON. During caffeine application, there was a rapid rise in intracellular (Ca^{2+}) as Ca^{2+} was released from the SR. This was followed, under the continued presence of caffeine, by a fall in Ca^{2+} to resting levels, as Ca^{2+} was extruded from the cell, primarily through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. When caffeine was stopped and electrical stimulation was restarted, the L-Type Ca^{2+} channels were activated allowing Ca^{2+} to re-enter the cell and refill the SR. Over several beats, the amplitude of the Ca^{2+} transient was restored. Regional defects in L-Type Ca^{2+} current, $\text{Na}^+/\text{Ca}^{2+}$ exchange current or SR Ca^{2+} ATPase activity may underlie the prolonged recovery of the Ca^{2+} transient in ENDO-STZ myocytes.^[33-36] It has been previously reported that there were no alterations in Ca^{2+} transient recovery in EPI and ENDO myocytes from STZ compared to respective controls after 3 months of STZ treatment.^[21]

Myofilament sensitivity to Ca^{2+} was unaltered in EPI and ENDO myocytes from STZ compared to respective controls. Previous studies have also reported no alterations in myofilament sensitivity to Ca^{2+} in myocytes from whole ventricle of STZ-induced diabetic rat.^[37] These data suggest that myofilament sensitivity is unaltered in the STZ-induced diabetic rat.

CONCLUSION

The kinetics of contraction and Ca^{2+} transient and fractional release of Ca^{2+} from the SR are altered to different extents in EPI and ENDO myocytes from STZ-induced diabetic rat.

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Conflicts of interest

There are no conflicts of interest.

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