Myofilament Ca^{2+} sensitivity in ventricular myocytes from streptozotocin-induced diabetic rat

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Abstract

Contractile dysfunction is a frequently reported complication of diabetic cardiomyopathy and many of the defects observed in the clinical setting have also been reported in experimentally-induced diabetes. We have investigated the relationship between intracellular Ca^{2+} concentration and cell length during the relaxation phase of contraction in ventricular myocytes from streptozotocin (STZ) – induced diabetic rats. Cell length and intracellular Ca^{2+} concentration were measured simultaneously in electrically stimulated (1 Hz) myocytes loaded with fura-2 and maintained at 35-36 °C. The amplitude and time to peak shortening and Ca^{2+} transient were similar, however, the relaxation of contraction and the Ca^{2+} transient were significantly prolonged in myocytes from STZ-treated rats compared to controls. Myofilament Ca^{2+} sensitivity, which was assessed by plotting cell length against fura-2 fluorescence ratio during the relaxation phase of a contraction, was significantly increased in myocytes from STZ-treated (9.23 ± 0.77 μm/fura-2 fluorescence unit) compare to controls (4.84 ± 0.78 μm/fura-2 fluorescence unit). The slower time course of relaxation of contraction and Ca^{2+} transient may be explained by defective sarcoplasmic reticulum Ca^{2+} uptake and to a lesser extent mechanisms of plasma membrane Ca^{2+} efflux including Na^{+}/Ca^{2+} exchange and Ca^{2+}ATPase. In conclusion, the apparent increase in myofilament Ca^{2+} sensitivity may be attributed to slower cross-bridge cycling rate which in turn may be related to the alteration of expression of different myofilament myosin isoforms.

Keywords : *Diabetes, calcium transport, myofilament Ca^{2+} sensitivity, streptozotocin, ventricular myocytes*

Introduction

Contractile dysfunction can occur in the diabetic heart in the absence of major blood vessel disease [1-4] and many of the contractile defects observed in the clinical setting have also been reported in experimentally-induced diabetes.[1] The streptozotocin– induced diabetic rat is a widely used animal model for the study of diabetes.[1] Administration of STZ (60 mg kg^{-1}) to young adult rats causes selective pancreatic beta cell necrosis and as a consequence these animals are characteristically hyperglycaemic and hypoinsulinaemic.[1,5-7] Reduced rates of shortening, re-lengthening and the extent of shortening have been variously reported in electrically stimulated myocytes from STZ–induced diabetic rats.[5,8-10] The characteristics of contractile defects depend partly on the duration of STZ – treatment.[5,7] We have investigated the relationship between intracellular Ca^{2+} concentration and cell length during the early and late stages of relaxation of contraction in ventricular myocytes from STZ – induced diabetic rats.

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Materials & Methods

Induction of diabetes:

Diabetes was induced by a single injection of STZ administered to young male rats (230-270 g) according to previously described techniques [5,7,10]. Briefly, male Wistar rats (230-270 g) received a single intraperitoneal injection of STZ (60 mg kg^{-1}; Sigma, S-0130).[7] STZ was dissolved in a citrate buffer solution (0.1 M citric acid and 0.1 M sodium citrate, pH 4.5). Age-matched controls received an equivalent volume of citrate acid buffer solution alone. Control and diabetic rats were caged separately but housed under similar conditions. Both groups of animals were fed the same diet and water *ad libitum* until they were used 8 to 10 weeks after treatment. Blood glucose was measured with a glucose meter (One Touch II glucose meter, Lifescan Inc, USA) 3-5 days after administration of STZ and immediately prior to experiments to confirm diabetic state. Principles of laboratory animal care were followed throughout. Approval for this project was obtained from the Faculty of Medicine & Health Sciences Ethics Committee.

Ventricular myocyte isolation

Ventricular myocytes were isolated according to previously described techniques with minor modifications.[7,11] Rats were killed by decapitation using a guillotine, hearts were rapidly removed and perfused by the Langendorff's method with a physiological salt solution (isolation solution – see below) containing 0.75 mM Ca^{2+} at 37 °C. Perfusion flow-rate was adjusted to 8 ml g $heart^{-1}$ min^{-1} to allow for differences in heart weight between diabetic and control animals. When the preparation appeared stable, perfusion was switched to an isolation solution containing 0.1 mM EGTA for 4 min. The heart was then perfused with the isolation solution containing 0.05 mM Ca^{2+}, 0.75 mg ml^{-1} collagenase (Worthington, LS004196) and 0.075 mg ml^{-1} protease (Sigma, P-5147). The enzyme solution was recirculated to give a total exposure time of 6 min. At the end of the enzyme perfusion, the heart was cut down and the ventricles dissected free and cut into small slices.

The ventricular tissue was shaken in 5 ml of enzyme solution containing 1% bovine serum albumin for 4 min at 37°C and then filtered through gauze (300 μm aperture). After addition of 5 ml isolation solution containing 0.75 mM Ca^{2+}, the cell filtrate was centrifuged (400 rpm, 40 sec). The supernatant was removed and the cell pellet was resuspended in 10 ml of isolation solution containing 0.75 mM Ca^{2+}. The shaking process was repeated a total of four times. Myocytes from shakes 2 and 3 were accumulated and stored at 4 °C prior to use. Cell viability, defined by the rod-shape, was compared in ventricular myocytes from diabetic or control rat heart within 1 h after completion of the cell isolation. Cells were used during a period of 1-8 h after isolation.

Measurement of shortening and intracellular Ca^{2+} concentration

Myocytes were loaded with the fluorescent indicator fura-2 AM (Molecular Probes, USA, F-1221) as described previously.[12] In brief, 6.25 μl of a 1.0 mM stock solution of fura-2 dissolved in dimethylsulphoxide (Sigma, D-5879) was added to 2.5 ml of cells to give a final fura-2 concentration of 2.5

μM. Myocytes were shaken gently for 10 min at room temperature and then centrifuged (200-500 rpm). The supernatant was then removed and replaced with 10 ml of fresh experimental salt solution. Myocytes were left for 30 min to ensure complete hydrolysis of the intracellular ester.

Shortening and intracellular Ca^{2+} concentration were measured simultaneously using video edge detection (Crystal Biotech USA, VED-114) and fluorescence photometry (Cairn Research, England) systems, respectively. Electrically stimulated (1 Hz) myocytes were superfused (3-5 ml min^{-1}) with a physiological salt solution (experimental solution – see below) containing 1 mM Ca^{2+} at 35-36°C. The amplitude of shortening (expressed as a % of resting cell length), the time to peak (TPK) shortening and time to 50 % (T50), 70 % (T70) and 90 % (T90) relaxation were recorded for each contraction.

To measure intracellular Ca^{2+} concentration, myocytes were alternately illuminated by 340 nm and 380 nm light using a monochromator (Cairn Research, England), which changed the excitation light every 2 ms. The resultant fluorescent emission at 510 nm was recorded by a photomultiplier tube and the ratio of the emitted fluorescence at the two excitation wavelengths (340/380 ratio) was calculated to provide an index of intracellular Ca^{2+} concentration. The amplitude of the Ca^{2+} transient and TPK and T50 %, T70 % and T90 % relaxation of the Ca^{2+} transient were recorded for each contraction.

Spurgeon *et al*[13] have argued that during the final relaxation phase of contraction, the myofilaments come into quasi-equilibrium with the intracellular Ca^{2+} concentration. We have plotted cell length against fura-2 fluorescence ratio during the relaxation phase of a contraction to obtain an index of Ca^{2+} sensitivity of the myofilaments.

Solutions: The composition of the basic isolation solution was (in mM) 130.0 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 0.4 NaH_2PO_4, 5 HEPES, 10 glucose, 20 taurine and 10 creatine set to pH 7.3 with NaOH. The experimental salt solution contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 Glucose, 5 HEPES and 1 $CaCl_2$ set to pH 7.4 with NaOH.

Data analysis and statistics

Signal Averager v 6.37 (Cambridge Electronic Design, England) was used to acquire and analyse the experimental data. Results are presented as mean ± SEM from (n) cells. Statistical comparisons were made using the independent samples t-test. Significance values of less than 0.05 were considered significant.

Results

General characteristics of STZ-treated and control rats: Diabetes was confirmed in STZ-treated rats by a 4.5-fold elevation of blood glucose and a significant ($p < 0.05$) reduction of non-fasting plasma insulin (Table 1). Body and heart weights were significantly ($p < 0.01$) lower in diabetic rats compared to age-matched controls.

Characteristics of ventricular myocytes from STZ-treated and control rats: The percentage of viable myocytes (defined by the rod shape of the cell) isolated from STZ-treated rats was lower than that

from the controls despite compensating for the differences in the size of hearts by adjusting perfusion flow rates during cell isolation (Table 2). Using light microscopy, there was no obvious visual difference between rod shaped myocytes from STZ-treated compared to control rats.

Table 1: Characteristics of experimental animals

	Control	**Diabetic**
Body weight (g)	291.20 ± 11.09 (5)	199.67 ± 7.67 (6)**
Heart weight (g)	1.24 ± 0.04 (5)	1.00 ± 0.00 (6) **
Blood glucose (mg dl^{-1})	84.00 ± 4.37 (5)	321.83 ± 25.84 (6)**
Plasma insulin (ng ml^{-1})	1.98 ± 0.35 (9)	0.82 ± 0.23 (9)*

Data are mean ± SEM, (n animals), ** P<0.01, * P<0.05.

Table 2 : Characteristics of ventricular myocytes

	Control	**Diabetic**
Cell viability (% rod shaped cells)	68.80 ± 2.27 (5)	37.50 ± 2.09 (6)**
Resting cell length (μm)	96.86 ± 2.40 (28)	101.47 ± 2.27 (33)
Peak amplitude shortening (% resting cell length)	6.61 ± 0.66 (17)	5.74 ± 0.99 (20)
Time to peak shortening (ms)	119.65 ± 4.49 (17)	126.05 ± 4.27 (20)
Resting fura-2 (Fluorescence ratio units)	1.36 ± 0.06 (18)	1.27 ± 0.04 (20)
Ca^{2+} transient amplitude (Fluorescence ratio units)	0.34 ± 0.03 (18)	0.32 ± 0.03 (20)
Time to peak Ca^{2+} transient (ms)	69.42 ±4.74 (18)	67.50 ± 3.35 (20)

Data are mean ± SEM, (n cells), ** P < 0.01.

Resting cell length and fura-2 ratio were not significantly (p > 0.05) altered by STZ-treatment (Table 2).

Effects of STZ-induced diabetes on myocyte contraction and the Ca^{2+} transient: Averaged fast time base simultaneous recordings of cell length and fura-2 fluorescence ratio in electrically stimulated (1 Hz) myocytes from control and STZ-treated rats are shown in figures 1 (a) and (b), respectively. The peak amplitude of shortening and Ca^{2+} transient and the TPK shortening and Ca^{2+} transient were not significantly (P > 0.05) altered by STZ-treatment (Table 2). The time course of relaxation of contraction and the Ca^{2+} transient in electrically stimulated (1 Hz) myocytes from STZ-treated and control rats is shown in Figure 2. The T70 % and T90 % relaxation of contraction (a) and the T50 %, T70 % and T90 % of relaxation of the Ca^{2+} transient (b) were significantly (p < 0.05) prolonged in myocytes from STZ-treated compared to control rats.

The relationship between the magnitude of cell length and the corresponding fura-2 fluorescence ratio in myocytes from control and STZ-treated rats are shown in figures 1 (c) and (d), respectively. For a given contraction, the data proceed in an anticlockwise direction and the early and late phases of relaxation, which are indicated with arrows, were fitted with regression lines. The slope of the regression during the early phase of relaxation was not significantly (P > 0.05)

different in STZ (50.93 ± 8.95 μm/fura-2 fluorescence unit, n =9) compared to control (44.26 ± 5.28 μm/fura-2 fluorescence unit, n=10) myocytes. However, during the late phase of relaxation the slope of regression was significantly ($P < 0.01$) increased in STZ (9.23 ± 0.77 μm/fura-2 fluorescence unit, n=9) compared to

Figure 1 : Cell length (upper traces) and fura-2 fluorescence ratio (lower traces) measured simultaneously in electrically stimulated (1 Hz) myocytes from control (a) and STZ-treated (b) rats. The magnitude of cell length plotted against the corresponding fura-2 fluorescence ratio in myocytes from control and STZ-treated rats are shown in (c) and (d), respectively. The arrows show the period of the relaxation phase to which a line of regression was fitted. Experiments were repeated in 9-10 cells.

Figure 2: Time course of cell length (a) and fura-2 fluorescence ratio (b) at various stages during relaxation of contraction in electrically stimulated (1 Hz) myocytes maintained at 35-36 ℃ from control and STZ-treated rats. Data are mean ± SEM (n=17-20), * $p < 0.05$, ** $p < 0.01$.

control (4.84 ± 0.78 µm/fura-2 fluorescence unit, n=10) suggesting that myofilament Ca^{2+} sensitivity is increased in myocytes from STZ-treated rats.

Discussion

The STZ rat model of diabetes is well characterized and widely used for the study of diabetic cardiomyopathy.[1] As reported in several previous studies rats developed hyperglycaemia within 3 days after injection of STZ and at 8-10 weeks the STZ-treated rats showed significantly less body weight gain, increased plasma glucose and reduced non-fasting plasma insulin concentration in comparison with age-matched controls.[5,7,14] The cell viability, defined by the rod-shape, was significantly lower in myocytes from STZ-treated rats. It has been suggested that hearts from STZ-treated rats may be more sensitive to collagenase digestion than controls.[14] Attempts to equalise viability between STZ-treated and control preparations by adjusting isolation solution flow rates to allow for differences in heart weight did not significantly affect the outcome.

Consistent with several previous studies the resting cell length[5-8] and fura-2 fluorescence ratio[6,14] were not significantly altered by STZ-treatment. Reductions[15] and increases [16] of resting Ca^{2+} have also been previously reported in STZ myocytes.

Consistent with some previous studies the peak amplitude of shortening and the Ca^{2+} transient were not significantly altered in myocytes from STZ-treated compared to control rats.[6,14] Increases[7] or decreases[8,9] in the amplitude of shortening and decreases[10,15] in the amplitude of the Ca^{2+} transient have also previously been reported in myocytes after STZ-treatment.

The TPK shortening and the Ca^{2+} transient were not altered in myocytes from STZ-treated compared to control rats. Prolongation of TPK shortening in myocytes after STZ-treatment has also been reported in previous studies.[5,7,8,9]

These inconsistences in the amplitude and time course of shortening and Ca^{2+} transient may partly be attributable to differences in treatment protocols, isolation or experimental methodologies. Previous studies in our laboratory have shown that the kinetics of shortening alters as treatment time is increased.[7]

The T70 % and T90 % relaxation of contraction and T50 %, T70 % and T90 % relaxation of the Ca^{2+} transient were significantly prolonged in myocytes from STZ-treated compared to control rats. Prolonged relaxation of contraction[5,8,9] and decay of the Ca^{2+} transient have been previously reported.[5,15] Mechanisms underlying the prolonged time course of relaxation of contraction may include

depressed SR Ca^{2+} ATPase[17-20] or altered Na^{+}/Ca^{2+} exchange activity.[18, 21]

During the final phase of relaxation of contraction, the myofilaments come into quasi-equilibrium with the intracellular Ca^{2+} concentration[13] and by plotting cell length against fura-2 fluorescence ratio during the relaxation phase of a contraction, it is possible to obtain an index of Ca^{2+} sensitivity of the myofilaments.[13] The slope of the regression during the late (but not the early) phase of relaxation of contraction was significantly increased in myocytes from STZ-treated rats suggesting that myofilament sensitivity to Ca^{2+} is increased after STZ-treatment. However, in the absence of any alteration to peak amplitude of shortening or Ca^{2+} transient it is suggested that the apparent increase in myofilament Ca^{2+} sensitivity may be attributed to a reduction of cross-bridge cycling rate which is a feature that has been previously reported in papillary muscle.[22] The reduction in cross-bridge cycling may in turn be related to the alteration of myosin isoform from V_1 to V_3[23] a feature that has been frequently reported in STZ-induced diabetes.[24,25]

Conclusion

The slower time course of relaxation of contraction and Ca^{2+} transient may be explained by defective sarcoplasmic reticulum Ca^{2+} uptake and to a lesser extent mechanisms of plasma membrane Ca^{2+} efflux including Na^{+}/Ca^{2+} exchange and Ca^{2+} ATPase. The apparent increase in myofilament Ca^{2+} sensitivity may be attributed to slower cross-bridge cycling rate which in turn may be related to the alteration of expression of different myofilament myosin isoforms.

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