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ORIGINAL ARTICLE

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Effects of hyperosmotic shrinking on ventricular myocyte shortening and intracellular Ca^{2+} in streptozotocin-induced diabetic rats

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Abstract Evidence exists for a specific diabetic cardiomyopathy independent of concurrent vascular disease. We tested the hypothesis that chronic hyperglycaemia found in streptozotocin- (STZ) induced diabetic rats leads to an altered response to and contractile effects of hyperosmotic shrinkage in ventricular myocytes. Analysis confirmed significant hyperglycaemia and revealed significant blood hyperosmolarity in STZ-treated rats. Myocyte volume changes, shortening and intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients were measured in cells superfused with normal Tyrode (NT, 300 mmol/kg) and then hyperosmotic Tyrode (HT, 440 mmol/kg) at 35–36°C. Shrinking significantly reduced the amplitude of shortening, whilst the $[Ca^{2+}]_i$ transient was significantly increased. The time course of both shortening and the $[Ca^{2+}]_i$ transient were prolonged in myocytes from STZ-treated compared to control rats. Time to peak shortening was 130.3 ms in STZ compared to 100.2 ms in control myocytes. Time to peak $[Ca^{2+}]_i$ transient was 70.8 ms in STZ compared to 44.6 ms in control myocytes and the time from peak to half recovery was 191.0 ms in STZ compared to 169.1 ms in control myocytes. Fractional SR Ca^{2+} release, assessed by the application of caffeine, was increased by shrinking. However, the effects of raised extracellular osmolarity on volume changes, contractility and $[Ca^{2+}]_i$ were not altered by the chronic hyperglycaemia found in STZ-treated rats.

Keywords Diabetes · Heart · Hyperosmosis · Shrinkage · Streptozotocin · Ventricular myocytes

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Introduction

Diabetes can be associated with cardiovascular dysfunction [1, 2, 3] and in experimental models of type 1 diabetes, a variety of alterations in the amplitude and time course of contraction and the intracellular Ca^{2+} ($[Ca^{2+}]_i$) transient have been reported to occur in single ventricular myocytes [4, 5, 6, 7]. Changes in contractile proteins [8], intracellular Ca^{2+} homeostasis [6] and cardiac electrophysiology [9] have been proposed to account for the alterations in contractile function.

It is well known that changes in extracellular osmolarity have profound effects on excitation–contraction coupling and the contractility of cardiac muscle [10, 11, 12, 13, 14]. Exposure to hyposmolar solutions decreases contractility [11, 12, 15]. Moderately hyperosmolar solutions (up to ≅420 mosmol/l) cause an increase in both resting and developed tension, whereas further increases in osmolarity (to ≅600 mosmol/l) have a negative inotropic effect [11, 16]. In addition, shrinking is thought to modulate signalling pathways that influence myocyte function [17].

In the streptozotocin (STZ) rat model of diabetes, considerable hyperglycaemia [4, 5, 6, 7] and hyperlipidaemia [18] may result in hyperosmotic loads being placed upon myocytes. However, the contractile response of diabetic myocytes to hyperosmotic solutions has not previously been reported. The aim of this study therefore was to test the hypothesis that the effects of raised extracellular osmolarity on volume changes, contractility and Ca^{2+} transport in ventricular myocytes isolated from STZ-treated rats are altered when compared to control animals.

Materials and methods

Induction of diabetes

Diabetes was induced by a single i.p. injection of STZ (60 mg/kg; Sigma) administered to young male Wistar rats (200–250 g; bred

in house). The STZ was dissolved in a citrate buffer solution (0.1 mol/l citric acid, 0.1 mol/l sodium citrate; pH 4.5).

Age-matched controls received an equivalent volume of the citrate buffer solution alone. Both groups of animals were maintained on the same diet and water ad libitum until they were used 8–12 weeks later. Principles of laboratory animal care were followed throughout. Approval for this project was obtained from the Faculty of Medicine and Health Sciences ethics committee.

Ventricular myocyte isolation

Single ventricular myocytes were isolated according to previously described techniques [19] with minor modifications. In brief, rats were killed humanely by stunning followed by cervical dislocation and their hearts were removed quickly and mounted on a Langendorff perfusion apparatus. Hearts were perfused retrogradely at a constant flow of 8 ml/g heart per minute with a HEPES-based salt solution (isolation solution – see below) containing 0.75 mmol/l Ca^{2+}. Perfusion flow rate was adjusted to allow for differences in heart weight between STZ-treated and control animals. When the coronary circulation had cleared of blood, perfusion was continued for 4 min with Ca^{2+}-free isolation solution containing 0.1 mM EGTA, and then for 6 min with solution containing 0.05 mmol/l Ca^{2+}, 0.75 mg/ml collagenase (type 1; Worthington, N.J., USA) and 0.075 mg/ml protease (type X1V; Sigma). After this time, the ventricles were excised from the heart, 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 isolation solution containing 0.75 mmol/l Ca^{2+}.

Measurement of shortening

Ventricular myocytes were allowed to settle on the glass bottom of a Perspex chamber mounted on the stage of an inverted microscope (Axiovert 35, Zeiss, Germany). Myocytes stimulated at 1 Hz were superfused (3–5 ml/min) with a HEPES-based normal Tyrode solution (NT solution – see below) containing 1 mmol/l Ca^{2+} at 35–36°C. Unloaded shortening was used as an index of contractility [20]. Shortening was followed using a video edge detection system (VED-114, Crystal Biotech, USA). The degree of shortening [expressed as a % of resting cell length (RCL)] and the kinetics of shortening, time to peak shortening (t_{pk}) and time from peak to half relaxation ($t_{1/2}$) were recorded. To assess the effect of hyperosmotic stress on cells, a rapid solution-changing device was used [21] to apply either NT or hypertonic Tyrode (HT) for a period of 5 min.

Measurement of myocyte volume

The video image of a myocyte, observed with a ×40 oil-immersion microscope objective was used to calculate cell volume based on the formula Volume (pl)=(πwdl)/4, where *wdl* represents cell width×depth×length and the cell is assumed to be an elliptical cylinder with a cell depth of 1/3 cell width [15, 22]. Cell volume was calculated in NT solution and volumes in the presence of HT were expressed as volumes relative to this control value for each myocyte.

Measurement of the Ca^{2+} transient

Myocytes were loaded with the fluorescent indicator fura-2/AM (F-1221, Molecular Probes, USA) as described previously [23]. In brief, 6.25 µl of a 1.0 mmol/l stock solution of fura-2/AM (dissolved in dimethylsulphoxide) was added to 2.5 ml of cells to give a final fura-2 concentration of 2.5 µmol/l. Myocytes were shaken gently for 10 min at 24°C (room temperature). After loading, myocytes were centrifuged, washed with NT to remove extracellular fura-2 and then left for 30 min to ensure complete hydrolysis of the intracellular ester.

To measure $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$.

Sarcoplasmic reticulum (SR) Ca^{2+} release was assessed using previously described techniques [23]. After establishing steady-state Ca^{2+} transients in electrically stimulated (1 Hz) myocytes loaded with fura-2, stimulation was stopped for a period of 5 s. Caffeine (20 mmol/l) was then rapidly applied for 10 s using a solution-switcher device [21]. Electrical stimulation was then restarted and the Ca^{2+} transients were allowed to recover to steady-state. SR Ca^{2+} was assessed by comparing the amplitude of the steady-state Ca^{2+} transients with that of the caffeine evoked Ca^{2+} transient.

Solutions

The Ca^{2+}-free isolation solution contained (in mmol/l): 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 NT solution contained (in mmol/l): NaCl 140, KCl 5, $MgCl_2$ 1, glucose 10, HEPES 5, $CaCl_2$ 1 (pH 7.4). Sucrose (150 mmol/l) was added to NT to prepare the HT solution. The osmolarities, measured with a vapour pressure osmometer (Wescor, USA, 5500), in NT and HT solutions were 300 and 440 mmol/kg, respectively.

Statistical analysis

Results are expressed as the mean ±SEM of *n* observations. Statistical comparisons of the effects of diabetes and time-dependent effects of exposure to and recovery from hyperosmotic solution were performed using 2-way repeated-measures analysis of variance (2-RMANOVA) followed by Tukey corrected *t*-tests for multiple comparisons. Other tests were made with Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

Results

General characteristics of the STZ-treated rat

The general characteristics of STZ-treated rats compared with their age-matched controls are shown in Table 1. Diabetes was confirmed in STZ-treated rats by a significant, 4.5-fold, elevation of blood glucose. STZ-treated rats had significantly lower body weights and heart weights and elevated blood osmolarity compared with controls.

Table 1 General characteristics of STZ-treated and control rats. Data are mean ±SEM. Numbers of animals shown in *parentheses*

	Control	STZ
Animal weight (g)	307.1±6.3 (16)	232.8±10.5 (17)**
Heart weight (g)	1.21±0.02 (16)	1.04±0.02 (17)**
Blood glucose (mg/dl)	78.3±2.5 (16)	366.7±23.8 (17)**
Blood osmolarity (mosmol/kg)	302.4±1.2 (16)	324.3±3.1 (16)**

**$P<0.01$, independent samples *t*-test

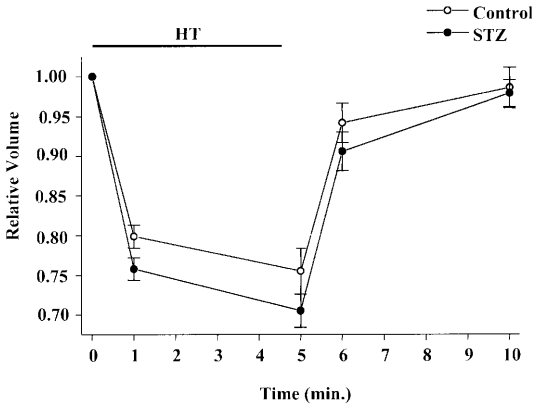

Fig. 1 Effect of exposure to and recovery from hyperosmotic solution (*HT*) on the relative volume of myocytes from control and streptozotocin- (STZ-) treated rats. Shrinkage significantly reduced cell volume but its effects on myocytes from STZ-treated rats were not different to those from control rats. Values are mean ±SEM of 12–14 observations. [P<0.001 for effect of HT, and P>0.05 for effect of STZ treatment, 2-RMANOVA, P<0.05, 1 min and 5 min of HT versus normal Tyrode (*NT*) and 1 min and 5 min of recovery, Tukey corrected *t*-test]

General characteristics of ventricular myocytes from STZ-treated rats

There were no clear visual differences between rod-shaped myocytes from control and STZ-treated animals. Measurements of a population of cells (n=83 cells), superfused with NT, found that resting cell length and width were not significantly (P>0.05) altered by STZ treatment. The cell volume (calculated from a smaller number of cells: control, 22.2±3.5 pl, n=12 cells; STZ, 18.7±1.9 pl, n=14 cells) was not significantly different either (P>0.05). Data for these experiments were acquired from four STZ-treated and four control rats.

Effect of hyperosmotic solution on cell volume

Exposure to HT solution caused a significant fall in cell volume (P<0.001, 2-RMANOVA, P<0.05 Tukey, HT versus NT) that was apparent after 1 min of exposure (Fig. 1). Recovery of relative volume was also seen 1 min after return to NT solution. The changes in relative volume of myocytes from STZ-treated rats were not significantly different to those of control cells (P>0.05, 2-RMANOVA, STZ versus control).

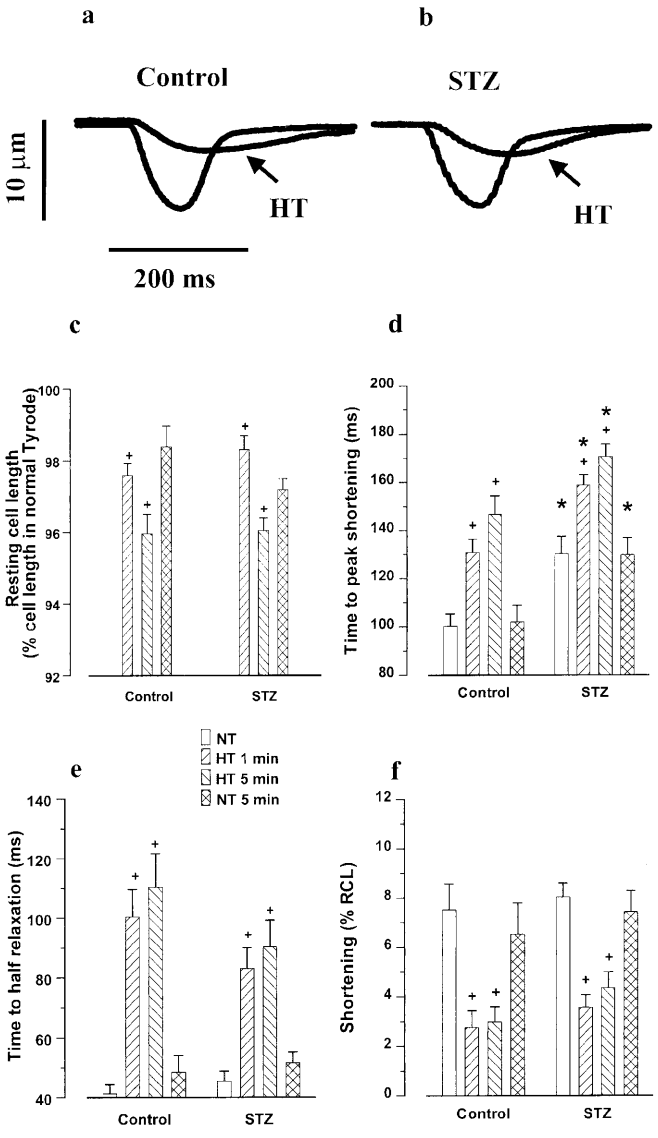

Fig. 2 Representative fast time-base recordings of unloaded shortening in ventricular myocytes isolated from: **a** control or **b** STZ-treated rats superfused with either normal (*NT*) or hyperosmotic solution (*HT*) for 5 min. Mean data showing resting cell length (**c**), time to peak shortening (**d**), time to half relaxation of shortening (**e**) and amplitude of shortening (*RCL* resting cell length) (**f**) in ventricular myocytes, superfused with normal (*NT*), followed by 1 and 5 min of exposure to hyperosmotic solution (*HT*) and 5 min of wash with NT, from control or STZ-treated rats. Values are mean ±SEM of 8–16 observations. (P<0.001 for effect of HT in all cases, and P=0.01 for effect of STZ treatment; **d** only, 2-RMANOVA.) ^{+}P<0.05, HT versus NT; $^{*}P$<0.05, STZ versus control, Tukey corrected *t*-test

Characteristics of myocyte shortening in NT and HT solution

Superfusion with HT changed both the amplitude and the time course of shortening in myocytes from STZ-treated and control rats Fig. 2a, b). Resting cell length was significantly decreased on exposure to HT and returned towards normal upon wash (Fig. 2c). The amplitude of shortening was significantly reduced in myocytes from STZ-treated and control rats after 1 or 5 min of HT. However, the differences in basal contractility and in the response to HT were not significantly different between myocytes from STZ-treated and control rats Fig. 2f). Mean t_{pk} shortening was significantly increased in myocytes from STZ-treated rats compared to controls in NT and was significantly increased in myocytes from STZ-

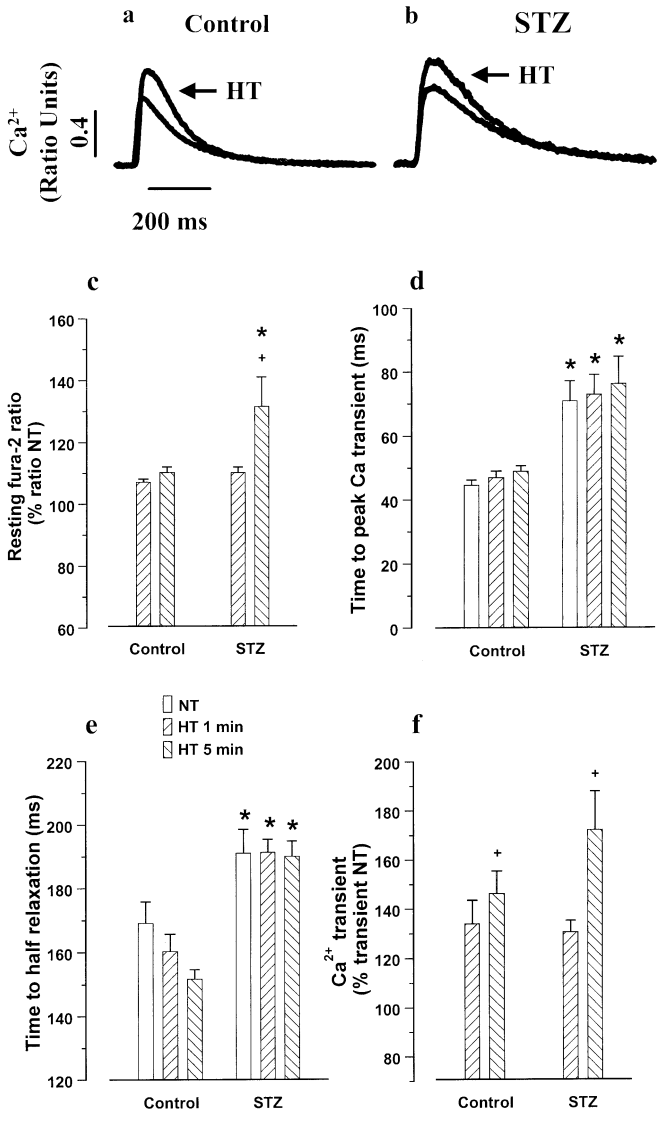

Fig. 3a–f Representative fast time-base recordings of Ca^{2+} transients in ventricular myocytes isolated from: **a** control or **b** STZ-treated rats, superfused with either normal (*NT*) or hyperosmotic solution (*HT*) for 5 min. Mean data showing resting fura-2 ratio (**c**), time to peak Ca^{2+} transient (**d**), time to half relaxation of Ca^{2+} transient (**e**) and amplitude of Ca^{2+} transient in ventricular myocytes (**f**), superfused with normal (*NT*), followed by 1 and 5 min of exposure to hyperosmotic solution (*HT*) and 5 min of wash with NT, from control or STZ-treated rats. Values are mean ±SEM of 8–16 observations. ($P<0.001$ for effect of HT in **c** and **f** and for diabetes in **d** and **e**, and $P=0.05$ for effect of diabetes **c**, 2-RMANOVA.) $^{+}P<0.05$, HT versus NT; $^{*}P<0.05$, STZ versus control, Tukey corrected *t*-test

treated and control rats after 1 or 5 min HT (Fig. 2d). Mean $t_{1/2}$ was significantly increased in myocytes from STZ-treated and control rats after 1 or 5 min of HT (Fig. 2e). The magnitude of the increases in t_{pk} and $t_{1/2}$ were not significantly different between myocytes from STZ-treated and control rats.

Characteristics of myocyte Ca^{2+} transient in NT and HT solution

The amplitude and time course of the Ca^{2+} transients were altered in myocytes from STZ-treated and control rats in response to HT (Fig. 3a, b). The resting fura-2 ratio in NT was not significantly ($P>0.05$) different in myocytes from STZ-treated compared to control rats.

Resting fura-2 ratio (expressed as a percentage of the fura-2 ratio in NT) was increased in myocytes from control and STZ-treated rats after 5 min of HT (Fig. 3c). The magnitude of increase in myocytes from STZ-treated rats was significantly greater than that in control cells at this time point. The amplitude of the Ca^{2+} transient was significantly increased in STZ-treated and control myocytes after 5 min of HT (Fig. 3f). The difference in the magnitude of response between myocytes from STZ-treated and control rats in HT was not significant.

Mean t_{pk} Ca^{2+} transient was significantly increased in myocytes from STZ-treated rats compared to controls in NT (Fig. 3d). The t_{pk} was not significantly altered in myocytes from STZ-treated or control rats after exposure to HT compared to NT (Fig. 3d). Mean $t_{1/2}$ Ca^{2+} transient was significantly increased in myocytes from STZ-treated rats compared to controls in NT but was not significantly altered in myocytes from STZ-treated or control rats after exposure to HT (Fig. 3e).

Characteristics of SR Ca^{2+} release in NT and HT solution

When electrical stimulation was stopped for a period of 5 s and 20 mmol/l caffeine rapidly applied, there was release of Ca^{2+} from the SR (Fig. 4a). In NT solution Ca^{2+} transients in response to caffeine were significantly greater than electrically evoked transients and (in agreement with data presented in Fig. 3f) the amplitude of the electrically stimulated Ca^{2+} transient was significantly increased in STZ-treated and control myocytes by exposure to 5 min of HT (Fig. 4b). However, in HT solution the amplitude of the caffeine-evoked Ca^{2+} transients in myocytes from STZ-treated and control rats was not significantly greater than that of the electrically evoked transients (Fig. 4b). SR fractional release (electrically evoked transients expressed as a percentage of caffeine-evoked transients) was not different between myocytes from STZ-treated and control rats but was significantly increased following exposure to HT (Fig. 4c). The repriming of the SR stores was tested by measuring the rate of Ca^{2+} transient recovery upon electrical stimulation following wash of caffeine. The rate of recovery was significantly increased by HT (Fig. 4d) but was not altered in diabetes. This effect reflects the larger $[Ca^{2+}]_i$ transients seen in HT, because the times taken for the transients to recovery to half their respective steady-state amplitude were not significantly different ($P>0.05$) between the groups (6.8±1.5 s for control cells in NT, $n=8$).

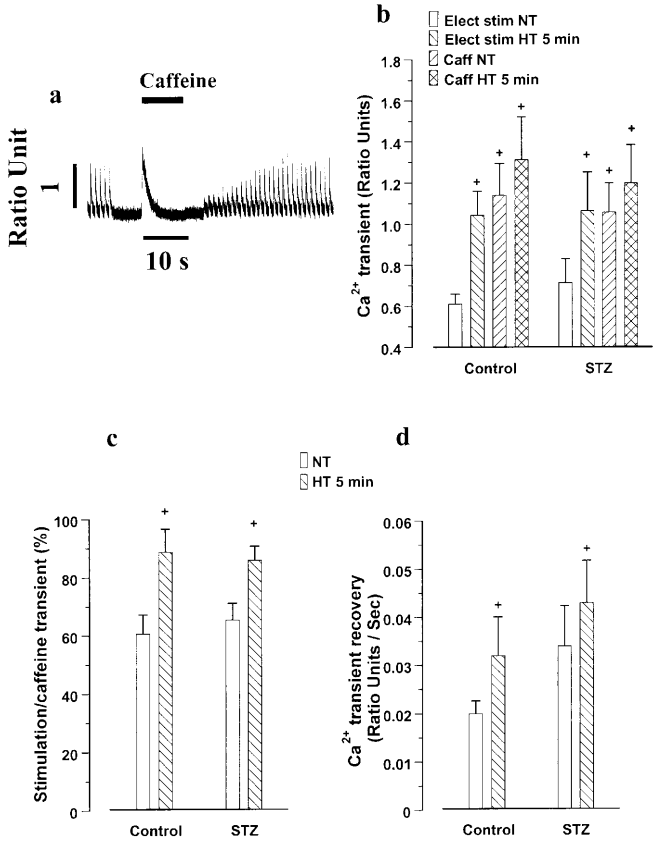

Fig. 4 **a** Typical chart recording showing the experimental protocol used to investigate SR Ca^{2+} release. Cells were electrically stimulated at 1 Hz, stimulation was then stopped for 5 s and 20 mmol/l caffeine was applied for 10 s. Electrical stimulation was restarted following the wash of caffeine. Mean data showing amplitudes of Ca^{2+} transient (fura-2 ratio) during electrical stimulation or exposure to caffeine (**b**), amplitude of Ca^{2+} transient during electrical stimulation expressed as a percentage of the Ca^{2+} transient during application of caffeine (**c**), recovery of the Ca^{2+} transient following application of caffeine after restarting electrical stimulation (**d**) in myocytes from control or STZ-treated rats superfused with either normal (*NT*) or hyperosmotic (*HT*) solution. Values are mean ±SEM of between 10 and 14 observations. ($P<0.001$ for effect of HT in all cases, 2-RMANOVA.) $^{+}P<0.05$, versus electrical stimulation in NT (**b**) or versus NT (**c**) and (**d**), Tukey corrected *t*-test

Discussion

In the present study, we have investigated for the first time the effects of increased extracellular osmolarity on the contractility of ventricular myocytes in the STZ-treated rat. The STZ-induced model of diabetes is well characterized. This model does not produce atherosclerosis and therefore is an excellent choice for the study of diabetic cardiomyopathy [24]. In the present study, at 2 months following the induction of diabetes, we observed a marked reduction in body and heart weight consistent with the diabetic state. Consistent with our initial hypothesis, in STZ-treated rats we observed an elevation of blood glucose and hypertonicity of blood, compared to controls.

When measuring $[Ca^{2+}]_i$ it should be acknowledged that ionic strength will influence the K_d of fura-2 and thus the relationship between fluorescence and $[Ca^{2+}]_i$.The effect of shrinking is such that the affinity for Ca^{2+} is decreased, giving a reduced 340/380 ratio for a given $[Ca^{2+}]_i$ [15, 25]. However, as we report an increase in 340/380 signal with shrinkage, we can be confident this qualitative effect is not artefactual. The literature have yet to agree on the effect of STZ treatment on levels of diastolic $[Ca^{2+}]_i$. One study reported a threefold elevation of $[Ca^{2+}]_i$ measured using Ca^{2+}-sensitive microelectrodes at 8 weeks after injection of STZ in the rat [26]. Using Ca^{2+}-sensitive dyes, others have reported no effect [27], or reductions [28] in diastolic $[Ca^{2+}]_i$ in this model of diabetes. In our study resting fura-2 ratios were not altered by STZ treatment.

Our observations on the effect of shrinking on the amplitude and time course of contraction and $[Ca^{2+}]_i$ are consistent with previous reports, e.g. [12, 16]. However, it is worth noting that we did not see biphasic effects on contractility as recently reported upon hypo-osmotic swelling of single rat myocytes [15]. Given the recent interest in the role of myofilament lattice spacing upon myofilament Ca^{2+} sensitivity (see [29, 30]), where osmotic compression is used as a tool to increase sensitivity in skinned preparations, it is interesting to note that in intact preparations (such as this study) the overall effect of hypertonicity is to decrease contraction and increase $[Ca^{2+}]_i$ transients, i.e. a decrease in sensitivity. It is thought that in intact preparations any increase in myofilament Ca^{2+} sensitivity, resulting from lattice compression, is outweighed by effects such as intracellular acidosis [31], decreased maximum force and decreased Ca^{2+} sensitivity due to increased $[K^+]_i$ [32].

We observed an increase in fractional SR Ca^{2+} release [33] upon shrinking. An opposite effect was seen in response to hypo-osmotic swelling [15]. It is possible that the increased Ca^{2+} transient, seen in response to shrinking, is due to increased SR load and it is known that fractional release is dependent upon SR load [33]. We do not believe that saturation of fura-2 was responsible for this finding as we could evoke 340/380 ratios significantly greater than those seen in experiments by rupturing myocyte membranes at the end of experiments.

Despite the increase in blood osmolarity demonstrated in STZ-treated rats we observed no differences in the time course or amplitude of the change in myocyte volume of STZ-treated and control cells upon exposure to and recovery from HT (Fig. 1). In addition the changes in contraction and $[Ca^{2+}]_i$ upon exposure to HT were not altered by diabetes (Figs. 2, 3, 4). Our observations are not consistent with the proposed hypothesis and we therefore conclude that STZ-induced diabetes does not alter the response to hyperosmotic shrinking.

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