Research article

Effects of prolactin on ventricular myocyte shortening and calcium transport in the streptozotocin-induced diabetic rat

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ABSTRACT

The physiological role of prolactin (PRL) in the heart, and in particular the diabetic heart, are largely unknown. The effects of PRL on ventricular myocyte shortening and Ca^{2+} transport in the streptozotocin (STZ) – induced diabetic and in age-matched control rats were investigated. PRL receptor protein, myocyte shortening, intracellular [Ca^{2+}], L-type Ca^{2+} current were measured by Western blot, cell imaging, fluorescence photometry and whole-cell patch-clamp techniques, respectively. Compared to normal Tyrode solution (NT), PRL (50 ng/ml) significantly ($p < 0.05$) increased the amplitude of shortening in myocytes from control (7.43 ± 0.38 vs. 9.68 ± 0.46 %) and diabetic (6.57 ± 0.24 vs. 8.91 ± 0.44 %) heart (n = 44–49 cells). Compared to NT, PRL (50 ng/ml) significantly increased the amplitude of Ca^{2+} transients in myocytes from control (0.084 ± 0.004 vs. 0.115 ± 0.007 Fura-2 ratio units) and diabetic (0.087 ± 0.007 vs. 0.112 ± 0.006 Fura-2 ratio units) heart (n = 36–50 cells). PRL did not significantly alter the amplitude of caffeine-evoked Ca^{2+} transients however, PRL significantly increased the fractional release of Ca^{2+} in myocytes from control (21 %) and diabetic (14 %) and heart. The rate of Ca^{2+} transient recovery following PRL treatment was significantly increased in myocytes from diabetic and control heart. Amplitude of L-type Ca^{2+} current was not significantly altered by diabetes or by PRL. PRL increased the amplitude of shortening and Ca^{2+} transients in myocytes from control and diabetic heart. Increased fractional release of sarcoplasmic reticulum Ca^{2+} may partly underlie the positive inotropic effects of PRL in ventricular myocytes from control and STZ-induced diabetic rat.

1. Introduction

Prolactin (PRL), also known as luteotropic hormone or luteotropin, is a peptide hormone that is classically associated with milk production. Expression of genes that encode various isoforms of PRL receptor have been reported in a variety of tissues including the brain, kidney, liver, skeletal muscle and heart [1, 2]. PRL acts in a cytokine-like manner and is an important regulator of the immune system. It has important cell cycle-related growth, differentiating and anti-apoptotic functions. As a growth factor, binding to cytokine-like receptors, it influences hematopoiesis, angiogenesis and is involved in the regulation of blood clotting through several pathways. The hormone acts in endocrine, autocrine and paracrine manner through the prolactin receptor and a large number of cytokine receptors [3].

Diabetes mellitus (DM) is a chronic metabolic disease that has reached pandemic proportions, affecting millions of individuals worldwide. The global prevalence of DM is expected to rise from 387 million in 2015 to 635 million in 2040 (https://www.idf.org/) and the prevalence of DM in the United Arab Emirates (UAE) is among the highest in the world [4]. DM is associated with substantial mortality, morbidity, and healthcare expenditure. Cardiovascular complications are the major cause of mortality and morbidity in individuals with DM and the largest contributor to the overall healthcare costs associated with DM [5]. DM is an independent risk factor for the development of heart failure [6, 7]. Diabetic cardiomyopathy (DCM) is a common but underestimated cause

of heart failure in DM. The development of DCM is multi-factorial and the proposed mechanisms underlying DCM include metabolic disturbances, myocardial fibrosis, small vessel disease, cardiac autonomic neuropathy and insulin resistance [8]. Disturbances in Ca^{2+} transport and the metabolic shift toward a greater use and storage of fatty acids are thought to play key roles in the development of DCM. At an early stage DCM is manifested by diastolic heart failure with preserved ejection fraction and in some patients this may progress to heart failure with reduced ejection fraction and overt systolic heart failure [6, 7, 8].

The streptozotocin (STZ) - induced diabetic rat is a widely used experimental model of DM. The general characteristics of the STZ-induced diabetic rat include hypoinsulinemia, hyperglycemia, dyslipidemia, polyuria, reduced body weight gain, polydipsia and polyphagia [9]. Cardiac abnormalities may include disturbances in heart rate, stroke volume, cardiac output, ejection fraction and rates of pressure development and relaxation [10, 11]. In animal models of DM there is a wealth of evidence demonstrating altered expression, activity and function of many of the transporters involved in the process of excitation-contraction coupling in cardiac muscle. These alterations include reduced release of Ca^{2+} from the sarcoplasmic reticulum (SR), reduced SR Ca^{2+} pump activity, and reduced myofilament Ca^{2+}-sensitivity which are all involved in DCM [10, 12].

Little is known about the physiological role of PRL in the heart and in particular the diabetic heart. Previous studies have reported that the PRL receptor gene is expressed in rat heart [1, 2, 13]. Sub-clinical impairment of left ventricular function has been reported in patients with prolactinomas [14]. Seasonal and sex-specific variations in the responsiveness of rabbit hearts to PRL have been reported [15]. In the isolated rat heart PRL causes disturbances in heart rate and heart rhythm [16]. PRL also produces a positive inotropic effect in isolated rat heart [17, 18]. PRL may protect cardiomyocytes against intermittent hypoxia-induced cell damage [23].

Previous studies have variously reported that PRL may be decreased or not significantly altered in the STZ-diabetic rat compared to control [19, 20, 21, 22, 23] therefore, it is of considerable interest to investigate the effects of PRL in diabetic heart. The aim of this study was to investigate the effects of PRL on ventricular myocyte shortening and Ca^{2+} transport in the STZ-induced diabetic rat.

2. Methods

2.1. Experimental model

Male Wistar rats were obtained from the Animal House Facility, College of Medicine & Health Sciences, UAE University. Rats were housed at 22 ± 1 °C on a 12 h light-dark cycle and provided with standard rat feed and water *ad libitum*. Ethical approval for this project was obtained from the UAE University Animal Research Ethics Committee and experiments were performed in accordance with institutional guidelines. Experiments were performed in ventricular myocytes isolated from STZ-induced diabetic rat, a well characterized animal model of DM, and age-matched controls [24, 25]. DM 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. Blood glucose was measured the day after STZ treatment to confirm diabetic status of the rats and rats with blood glucose levels of >200 mg/dl were considered to be diabetic. 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 prior to experiments. Experiments were performed 8–12 weeks after STZ treatment.

2.2. Expression of prolactin receptor protein

The expression of PRL receptor protein in rat left ventricle was assessed using Western immunoblotting techniques. Samples of left ventricle were collected from STZ and control rat hearts and rinsed with ice-cold saline. As a positive control, samples were also collected from the mammary glands of female rats that had delivered 5 days earlier. These samples were homogenized at a speed of 7 M/S for 1 min for 5 runs with 1 min gaps using a Bead Blaster homogenizer (D2400, Benchmark Scientific, USA), using RIPA lysis buffer (20–188, EMD Millipore, Billerica, MA, USA) containing protease and phosphatase inhibitors (A32955 and A32957, Thermo Scientific, USA) followed by brief sonication and centrifugation at 12000 rpm, 15 min, (5147 R, Eppendorf, Germany). The protein concentration was calculated using a Pierce BCA protein assay kit. The lysate was used for SDS-PAGE and Western blotting. Briefly, 20 μg of protein was electrophoretically separated using 12 % polyacrylamide gel and transferred onto PVDF transfer membrane (88518, Thermo Fisher Scientific, Rockford, IL, USA). Membranes were incubated overnight with primary antibody for PRL receptor protein (ab2772, Abcam) and GAPDH (SC-32233, Santacruz Biotechnology, USA). This was followed by incubation with HRP-conjugated goat anti-mouse secondary antibody (ab205719, Abcam) for 1 h at 1:10000 concentration. The blots were developed using the Super Signal West Pico Plus chemiluminescent substrate (34577, Thermo Scientific, Rockford, IL, USA). The blot images were acquired using a Sapphire Biomolecular Imager (Azure Biosystems, Dublin, California, USA) using chemiluminescent detection of HRP, coupled with color image acquisition of the protein ladder (26634, Spectra™ Multicolor Broad Range Protein Ladder, Thermo-Scientific, USA). Densitometric analysis of the protein bands was performed and quantified using Image J software. The ratio of specific protein optical density to that of GAPDH (loading control) was calculated.

2.3. Isolation of ventricular myocytes

Ventricular myocytes were isolated by enzymatic and mechanical dispersal techniques, according to the methods of Smail *et al.* 2016 and Hamouda *et al.* 2015, with small modifications [26, 27]. In brief, rats were euthanized using a guillotine. Hearts were removed rapidly and mounted for retrograde perfusion on a Langendorff system. Hearts were perfused at a flow rate of 8 ml/g heart^{-1}.min^{-1} and at 36–37 °C with cell isolation solution containing in mmol/l: 130.0 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 0.75 $CaCl_2$, 0.4 NaH_2PO_4, 5.0 HEPES, 10.0 glucose, 20.0 taurine and 10.0 creatine (pH adjusted to 7.3 with NaOH). When heart contraction had stabilized, 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 Langendorff perfusion system and the left ventricle was carefully dissected. Ventricle tissue was minced and gently shaken in collagenase-containing isolation solution supplemented with 1 % bovine serum albumin. Cells were filtered from this solution at 4 min intervals and re-suspended in cell isolation solution containing 0.75 mmol/l Ca^{2+}. The shaking and filtration process was repeated 4 times.

2.4. Measurement of ventricular myocyte shortening

Experiments were performed to investigate the effects of different concentrations of PRL (20–500 ng/ml) on shortening (contraction) in ventricular myocytes from control rat. Ventricular myocyte shortening was investigated using a video imaging technique according to the methods of Smail *et al.* 2016 and Hamouda *et al.* 2015, with small modifications [26, 27]. Ventricular myocytes were incubated at room temperature for 30 min with either normal Tyrode (NT) containing the following in mmol/l: 140.0 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 5.0 HEPES, 1.8 $CaCl_2$ – adjusted to pH 7.4 or NT containing PRL at various concentrations ranging from 20-500 ng/ml. After the 30 min incubation period shortening was measured in electrically stimulated (1 Hz)

Table 1. General characteristics of STZ-induced diabetic and age-matched control rats.

	Control rats	Streptozotocin-induced diabetic rats
Body weight (g)	422.76 ± 6.01	256.81 ± 7.85**
Heart Weight (mg)	1250.00 ± 10.00	990.00 ± 20.00**
Heart weight/Body weight (mg/g)	2.96 ± 0.033	3.92 ± 0.092**
Non-fasting blood glucose (mg/dl)	91.28 ± 1.39	514.88 ± 14.92**

Data are mean $\pm$ SEM, statistical comparisons performed using Independent samples t-test, n = 25–26 rats; ** = p < 0.01.

ventricular myocytes maintained at 35–36 °C with an IonOptix MyoCam imaging system (IonOptix Corporation, Milton, MA, USA). Resting cell length (RCL), time to peak (TPK) shortening, time to half (THALF) relaxation and amplitude (AMP) of shortening were measured. During

experiments the myocytes were continuously superfused with either NT or NT containing different concentrations of PRL. The concentration of PRL that produced the largest inotropic effect (50 ng/ml) was selected for subsequent experiments. Data were acquired and analyzed with IonOptix software (IonOptix Corporation, Milton, MA, USA).

2.5. Measurement of intracellular Ca^{2+} and sarcoplasmic reticulum Ca^{2+} transport

Intracellular Ca^{2+} and SR Ca^{2+} release were investigated using a fluorescence photometry technique, according to the methods of Smail *et al.* 2016 and Hamouda *et al.* 2015, with small modifications [26, 27]. In brief, after establishing steady state Ca^{2+} transients in electrically stimulated (1 Hz) myocytes maintained at 35–36 °C and loaded with fura-2 AM, stimulation was paused for a period of 5 sec. Caffeine (20 mM) was then applied for 10 sec using a solution switching device customized
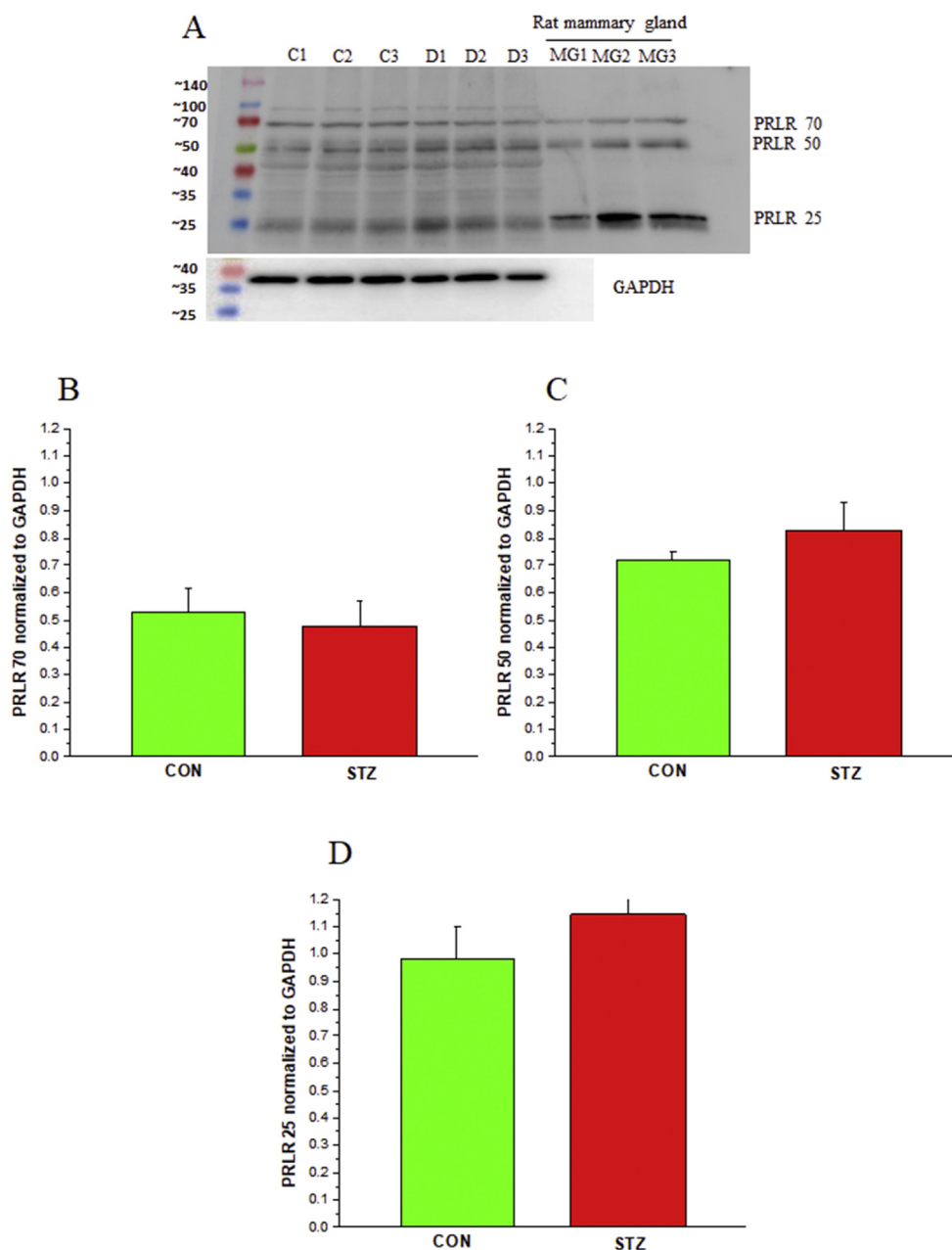

Figure 1. Expression of PRL receptor (PRLR) protein in ventricle tissue from STZ-induced diabetic and control hearts. Typical Western blots showing expression of PRLR protein in three control (C1 – C3) and 3 diabetic (D1-D3), GAPDH loading control and PRLR protein in tissue from 3 rat mammary glands (MG1-MG3) from 3 female rats, 5 days following delivery (A). Mean expression of PRLR at 70 KD (B) and PRLR at 50 KD (C) and PRLR protein at 25 KD (D). Non-adjusted images of the Western blots can be found in Supplementary File 1. Data are mean $\pm$ SEM, statistical comparisons performed using Independent samples t-test, n = 6 samples from diabetic and control hearts.

for rapid solution exchange [28]. Electrical stimulation was then resumed and the Ca^{2+} transients allowed to recover to steady state. Resting fura-2 ratio, TPK Ca^{2+} transient, THALF decay of the Ca^{2+} transient and AMP of the Ca^{2+} transient were measured. AMP of the caffeine-evoked Ca^{2+} transient and recovery of the electrically-evoked Ca^{2+} transient, following application of caffeine and resumption of electrical stimulation, were also measured. Fractional release of SR Ca^{2+} was calculated by comparing the AMP of the electrically-evoked steady state Ca^{2+} transients with that of the caffeine-evoked Ca^{2+} transient. Data were acquired and analyzed with IonOptix software (IonOptix Corporation, Milton, MA, USA).

2.6. Measurement of L-type Ca^{2+} current

Voltage-dependent L-type Ca^{2+} current was investigated according to modifications of previously described whole-cell patch-clamp techniques [26]. In brief $I_{Ca,L}$ was recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). The analog signal was filtered using a four-pole Bessel filter with a bandwidth of 5 kHz and digitized at a sampling rate of 10 kHz under software control (PClamp 10.6.2.2, Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were fabricated from filamented BF150-86-10 borosilicate glass (Sutter Instrument, CA, USA). The whole cell bath solution contained the following in mmol/l: 145 NaCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.35). The pipette solution contained the following in mmol/l: 140 CsCl, 2 $MgCl_2$, 10 TEA Cl, 10 EGTA, 10 HEPES, 1 MgATP (pH 7.25). Electrode resistances ranged from 3 to 5 MΩ, and seal resistances were 1–5 GΩ. Series resistances were compensated to >75 % of the uncompensated value. Experiments were performed at 34–36 °C. The current-voltage relationship was obtained by applying 300 ms test pulses in the range -40 mV to +70mV in 10 mV steps from a holding potential of -50 mV. Data were acquired and analyzed with pClamp software v 10.6.2.2 (Molecular Devices, Sunnyvale, CA, USA).

2.7. Statistics

The results were expressed as the mean ± SEM of 'n' observations. Statistical comparisons were performed using Independent samples t-test or one-way or two-way ANOVA and Bonferroni post hoc for multiple comparisons, as appropriate. P values of less than 0.05 were considered significant.

3. Results

3.1. General characteristics of diabetic rats

The general characteristics of diabetic and age-matched control rats are shown in Table 1. Body weight and heart weight were significantly (p < 0.01) lower, heart weight/body weight ratio and non-fasting blood glucose were significantly (p < 0.01) higher in diabetic rats compared to controls.

3.2. Expression of prolactin receptor protein in ventricle tissue from diabetic and control rat

The PRL receptor (PRLR) protein antibody (ab2772, Abcam) was able to pick up 3 isoforms of PRLR protein in diabetic and control ventricle tissue. A typical Western blot showing the expression of PRLR protein at 70, 50 and 25 KD, GAPDH and positive control Western blots in rat mammary gland are shown in Figure 1A. The records from the three mammary glands are from 3 female rats, 5 days following delivery. PRLR at 70, 50 and 25 KD were also identified in mammary gland tissue. Mean expression of PRLR 70 (Figure 1B), PRLR 50 (Figure 1C) and PRLR 25

(Figure 1D) protein were not significantly (p > 0.05) altered in ventricular tissue from diabetic compared to control heart.

3.3. Effects of prolactin on shortening in ventricular myocytes from diabetic and control rat

The effects of different concentrations of PRL (20–500 ng/ml) on control myocyte shortening are shown in Figure 2. Compared to normal Tyrode (NT) the AMP of shortening started to increase at 35 ng/ml and was significantly (p = 0.001) increased at 50 ng/ml and began to decrease at 75 ng/ml. Therefore, a concentration of 50 ng/ml PRL was selected for subsequent experiments.

The effects of PRL (50 ng/ml) on myocyte shortening in control and diabetic heart are shown in Figure 3. Typical records showing the effects of PRL on myocyte shortening in a control heart are shown in Figure 3A. RCL was not significantly (p > 0.05) altered in myocytes from diabetic compared to control heart in the presence or absence of PRL (Figure 3B). TPK shortening was significantly (p = 0.001) prolonged in myocytes from diabetic compared to control heart and was not additionally altered by PRL (Figure 4C). THALF relaxation of shortening was not significantly (p > 0.05) prolonged in myocytes from diabetic compared to control heart or by PRL (Figure 4D). AMP of shortening was not significantly (p > 0.05) altered in myocytes from diabetic compared to control heart. However, compared to NT the AMP of shortening was significantly (p = 0.001) increased by PRL in myocytes from control (7.43 ± 0.38 vs. 9.68 ± 0.46 % = 30%) and from diabetic (6.57 ± 0.24 vs. 8.91 ± 0.44 % = 36 %) hearts (Figure 4E). TWO-way ANOVA showed that the PRL had a statistically significant (p < 0.001) effect on the AMP of shortening whilst controlling for the presence or absence of diabetes.

Experiments were conducted to investigate if the effects of PRL (50 ng/ml) could be reversed following a 5 min washout with NT. The positive inotropic effects of PRL could be reversed in myocytes from control and diabetic heart. The AMP of shortening in myocytes from control heart treated with PRL (50 ng/ml) was 10.66 ± 0.54 (n = 7 cells) and following washout with NT was 7.91 ± 0.95 (n = 7 cells). The AMP of shortening in myocytes from diabetic heart treated with PRL (50 ng/ml)
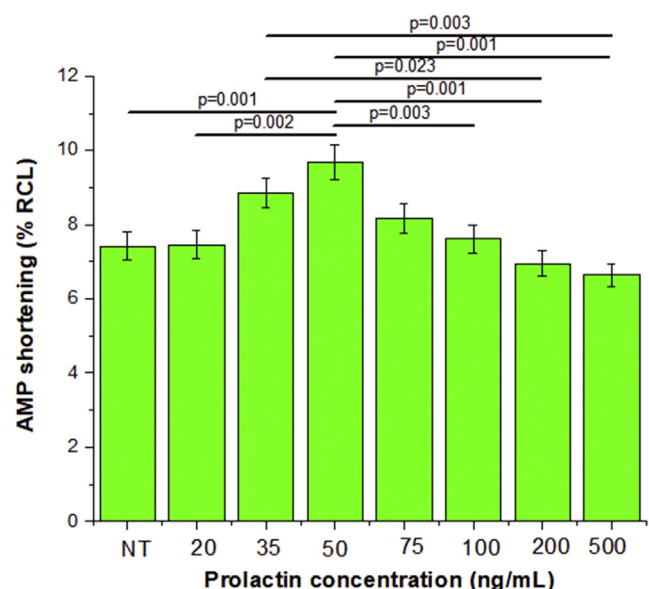

Figure 2. Effects of different concentrations of PRL (20–500 ng/ml) on shortening in ventricular myocytes from control heart. Data are mean ± SEM, statistical comparisons performed using ANOVA and Bonferroni post hoc for multiple comparisons, n = 38–49 cells for each concentration of PRL, from 8-10 hearts. Horizontal lines above bars indicate p < 0.05.
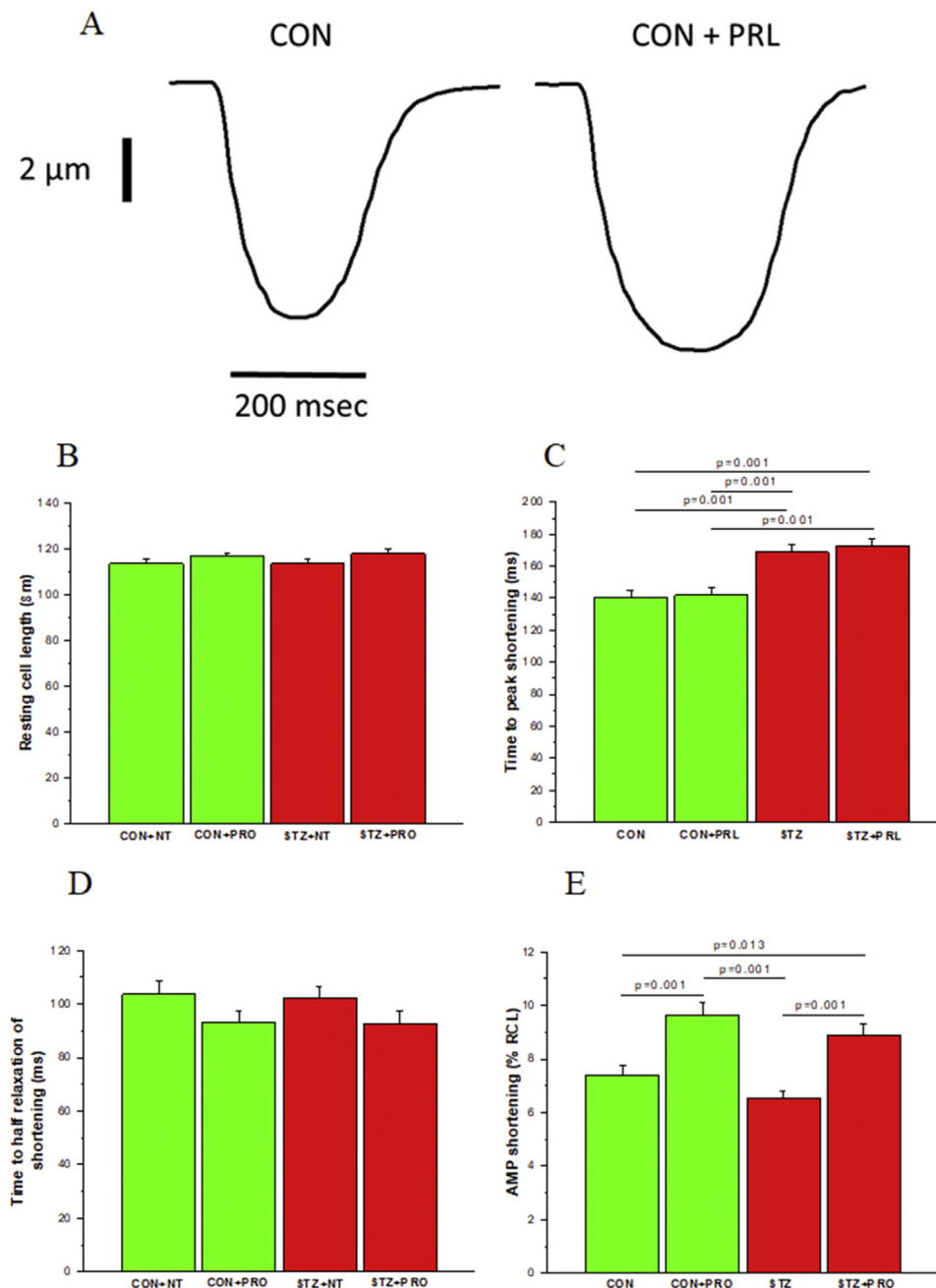
Figure 3. Effect of PRL (50 ng/ml) on shortening in ventricular myocytes from STZ-induced and control hearts. Typical records of shortening and the effects of PRL on shortening in a myocyte from a control heart (A). Graphs showing the effects of PRL on resting cell length (B), time to peak shortening (C), time to half relaxation of shortening (D) and amplitude (AMP) of shortening (E). Data are mean ± SEM, statistical comparisons performed using ANOVA and Bonferroni post hoc for multiple comparisons, n = 44–49 cells, from 9-10 hearts. Horizontal lines above bars indicate p < 0.05.

was 10.56 ± 0.87 (n = 10 cells) and following washout with NT was 7.14 ± 0.74 (n = 10 cells).

3.4. *Effects of prolactin on intracellular Ca^{2+} and sarcoplasmic reticulum Ca^{2+} in ventricular myocytes from diabetic and control rat*

The effects of PRL (50 ng/ml) on intracellular Ca^{2+} and SR Ca^{2+} in myocytes from diabetic and control heart are shown in Figure 4. Typical records showing the effects of PRL on electrically-evoked and caffeine-evoked Ca^{2+} transients in a myocyte from a control heart are shown in Figure 4A. Resting fura-2 ratio was significantly (p = 0.010) increased in myocytes from diabetic compared to control heart and was not additionally altered by PRL (Figure 4B). TPK Ca^{2+} transient was not significantly (p > 0.05) altered in myocytes from diabetic compared to control hearts in the presence or absence of PRL (Figure 4C). THALF decay of the

Ca^{2+} transient was significantly (p = 0.001) prolonged in myocytes from diabetic compared to control hearts and was not additionally altered by PRL (Figure 4D). AMP of electrically-evoked Ca^{2+} transients were not significantly (p > 0.05) altered in myocytes from diabetic compared to control hearts. Compared to superfusion with NT solution, PRL (50 ng/ml) significantly (p < 0.05) increased the amplitude of Ca^{2+} transients in myocytes from control (0.084 ± 0.004 vs. 0.115 ± 0.007 Fura-2 ratio units = 37 %, p = 0.001) and from diabetic (0.087 ± 0.007 vs. 0.112 ± 0.006, p = 0.009 Fura-2 ratio units = 29 %) heart (Figure 4E). Two-way ANOVA showed that the PRL had a statistically significant (p < 0.001) effect on the AMP of the Ca^{2+} transient whilst controlling for the presence or absence of diabetes. However, the analysis also showed that diabetes did not significantly (p = 0.945) influence the Ca^{2+} transient.

The amplitude of the caffeine-evoked Ca^{2+} transient was not significantly (p > 0.05) altered in myocytes from diabetic compared to control
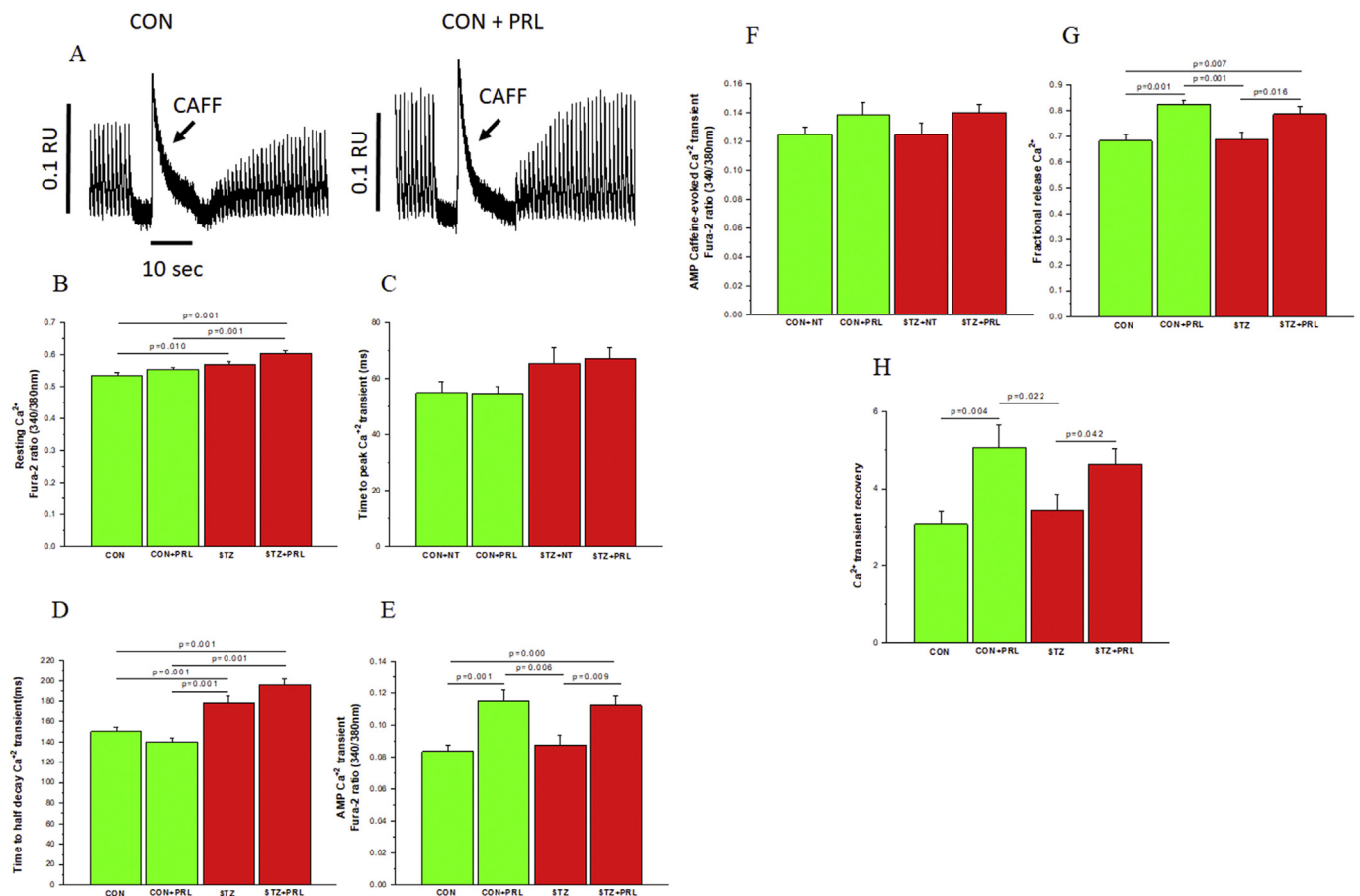
Figure 4. Effects of PRL on intracellular Ca^{2+} and sarcoplasmic reticulum Ca^{2+} transport in ventricular myocytes from STZ-induced diabetic and control hearts. Typical records of electrically-evoked and caffeine-evoked Ca^{2+} transients and the effects of PRL in a myocyte from a control heart (A). Graphs showing the effects of PRL on resting fura-2 ratio (B), time to peak Ca^{2+} transient (C), time to half decay of the Ca^{2+} transient (D), amplitude (AMP) of the electrically-evoked Ca^{2+} transient (E), AMP of the caffeine-evoked Ca^{2+} transient (F), fractional release of Ca^{2+} (G) and Ca^{2+} transient recovery following application of caffeine and resumption of electrical stimulation (H). Data are mean $\pm$ SEM, statistical comparisons performed using ANOVA and Bonferroni post hoc for multiple comparisons, n = 36–50 cells, from 5-6 hearts. Horizontal lines above bars indicate p < 0.05.

hearts in the presence or absence of PRL (Figure 4F). Fractional release of Ca^{2+} was significantly increased by PRL in myocytes from diabetic (14 %, p = 0.016) and control (21 %, p = 0.001) hearts (Figure 4G). The rate of recovery of the Ca^{2+} transient, following application of caffeine and resumption of NT was not significantly (p > 0.05) altered in myocytes from diabetic compared to control heart however, it was significantly increased by PRL in myocytes from control (p = 0.004) and diabetic (p = 0.042) hearts (Figure 4H).

3.5. Effects of prolactin on L-type Ca^{2+} current in ventricular myocytes from diabetic and control rat

The effects of PRL (50 ng/ml) on L-type Ca^{2+} current in myocytes from diabetic and control hearts are shown in Figure 5. The voltage clamp protocol (left panel) and a typical recording of L-type Ca^{2+} current in a myocyte from a control heart (right panel) are shown in Figure 5A. The amplitude of L-type Ca^{2+} current was not significantly (p > 0.05) altered in myocytes from diabetic compared to control hearts in the presence or absence of PRL over a range of test voltages between -40 to +60 mV (Figure 5B). For ease of comparison, the amplitude of L-type Ca^{2+} current evoked by a test pulse to 0 mV is shown in Figure 5C.

4. Discussion

The major findings of this study were as follows: (i) Three isoforms of prolactin (70, 50 and 25 KD) were identified in ventricle tissue from

control and diabetic rats, (ii) PRL increased the amplitude of shortening in ventricular myocytes from control (30 %) and diabetic (36 %) hearts and the effects of PRL could be reversed following washout, (iii) PRL increased the amplitude of the Ca^{2+} transient in ventricular myocytes from control (37 %) and diabetic (29 %) hearts, (iv) PRL increased the fractional release of Ca^{2+} in ventricular myocytes from control (21 %) and diabetic (14 %) hearts and (v) L-type Ca^{2+} current was not altered in myocytes from diabetic compared to control hearts or by PRL.

Using the Anti-Prolactin Receptor/PRL-R antibody ab2772 our experiments have demonstrated PRLR proteins at 70, 50 and 25 KD. Western blots performed in mammary gland provided positive controls. Previous studies have demonstrated that PRLR mRNA is expressed to varying extents in different tissues in rat, including heart [1, 2]. PRL and PRLRs are found in hearts of both genders [2] however, male rats were employed in this study as they have more stable endocrine profiles. The PRLRs were expressed to similar extents in control and diabetic ventricular tissue.

Having verified the presence of PRLR protein in control and diabetic heart, the effects of different concentrations of PRL on ventricular myocyte shortening were evaluated. It was interesting to observe that over the range of concentrations of PRL (20–500 ng/ml) only PRL at a concentration of 50 ng/ml had a significant positive inotropic effect in control myocytes. The range 20–500 ng/ml was selected as it reflects the physiological and pathophysiological (e.g., hyperprolactinemia) range of PRL in humans and in experimental animals [29, 30]. The AMP of shortening was not altered in myocytes from diabetic compared to
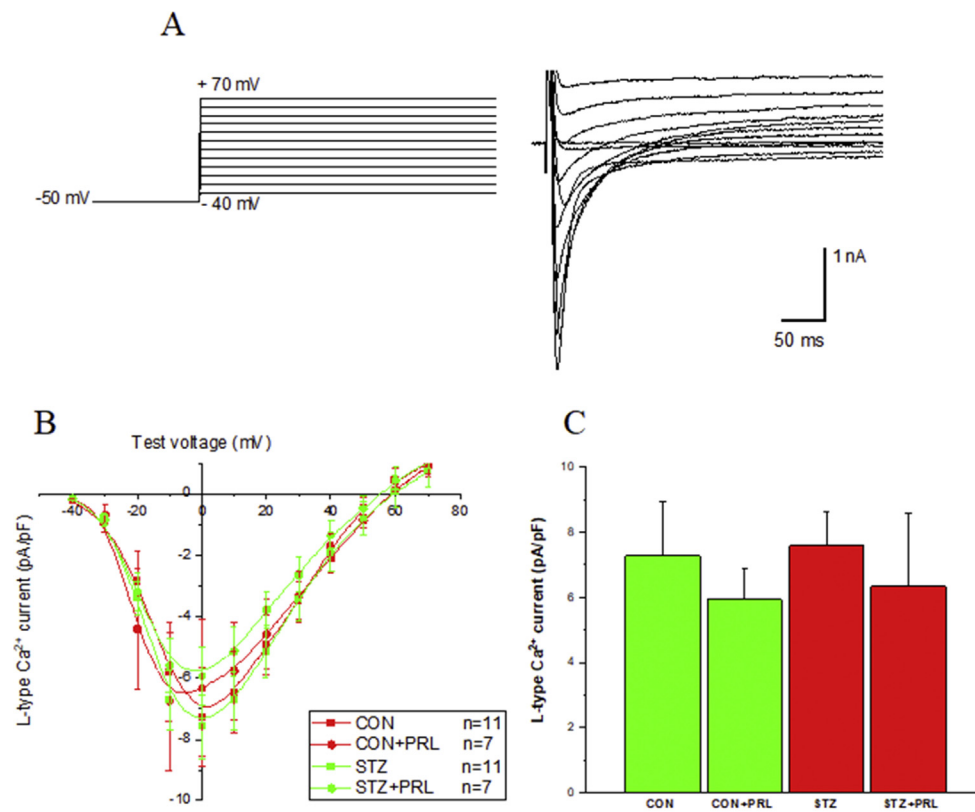
Figure 5. Effects of PRL on L-type Ca^{2+} current. Typical recordings of L-type Ca^{2+} current at different test potentials in a myocyte from a control heart (A). Current-voltage relationship (B) and amplitude of L-type Ca^{2+} current at a test potential of 0 mV (C). Data are mean ± SEM, statistical comparisons performed using ANOVA and Bonferroni post hoc for multiple comparisons, n = 7–11 cells, from 6-7 hearts.

control hearts a finding that is consistent with many previous studies in the STZ-induced diabetic rat [31, 32, 33]. Interestingly, the AMP of shortening was increased by PRL to similar extents in myocytes from diabetic and control hearts and could be reversed following washout. To our knowledge no other studies have investigated the effects of PRL in ventricular myocytes. A previous study in isolated perfused rat heart reported that PRL at 50 ng/ml produced a larger effect on contractile force than PRL at 200 ng/ml and the effects of PRL on contractile force reached a peak after 30 min and thereafter, remained stable [17]. This was the reason why a PRL incubation period of 30 min was selected prior to performing experiments. In myocytes from control heart PRL at 50 ng/ml produced an increase in AMP of shortening whilst concentrations of PRL in excess of 50 ng/ml did not further increase the AMP of shortening and this finding is consistent with a previous study performed in isolated heart [17] and might be explained by PRL receptor dimerization [34]. A previous study reported PRL produced concentration dependent alterations in heart rate and heart rhythm in the isolated perfused heart [16]. It has also been previously reported that indomethacin, an inhibitor of cAMP-dependent protein kinase, and propranolol reduced the inotropic effects of PRL [17]. These findings suggest that PRL may have an action via the beta-adrenergic signaling pathway.

The amplitude of the Ca^{2+} transient was unaltered in myocytes from diabetic compared to control hearts a finding that is consistent with several previous studies [33, 35]. However, the amplitude of the Ca^{2+} transient was increased by PRL in myocytes from diabetic and control hearts. In an attempt to identify the mechanism(s) underlying this increase in AMP of the Ca^{2+} transient the effects of PRL on SR Ca^{2+} was investigated. The amplitude of caffeine-evoked Ca^{2+} transient was not significantly altered in myocytes from diabetic compared to control hearts or by PRL suggesting that neither STZ-induced diabetes nor PRL altered the amount of Ca^{2+} that could be released from the SR by caffeine. The fractional release of Ca^{2+} was not altered in myocytes from

diabetic compared to control hearts. However, PRL increased fractional release of Ca^{2+} in myocytes from diabetic (14 %) and control (21 %) hearts, respectively. These results suggest that during electrical stimulation PRL is able to increase SR releasable Ca^{2+}. In an attempt to identify the mechanism(s) underlying the increased fractional release of Ca^{2+} caused by PRL the L-type Ca^{2+} current was measured. L-type Ca^{2+} current was not significantly altered either by STZ-induced diabetes or by PRL. Several previous studies have also reported unaltered L-type Ca^{2+} current in myocytes from STZ-induced diabetic rat [26, 36, 37]. Another possible mechanism underlying increased fractional release of Ca^{2+} might be altered function of the Na$^+$/Ca^{2+} exchange, the major mechanism for Ca^{2+} extrusion in myocytes. Previous studies have reported a reduction in Na$^+$/Ca^{2+} exchange current in myocytes from STZ-induced diabetic rat [38, 39]. Further studies will be required to investigate the effects of PRL on Na$^+$/Ca^{2+} exchange current.

The physiological function of PRL and the PRLR in the heart remain to be fully clarified. In the clinical setting typical male PRL values range from 2-8 ng/ml, typical female values range from 2-29 ng/ml, pregnant female values range from 6-210 ng/ml and values greater than 150–200 ng/ml are suggestive of a prolactin secreting pituitary adenoma. Several studies, including the current study, have demonstrated the presence of genes encoding PRLR protein and PRLR protein in many tissues including heart [1, 2]. The current study has demonstrated that PRL increases the AMP of contraction and that this positive inotropic effect is regulated, at least in part, by changes in intracellular Ca^{2+} concentration. It is tempting to speculate that PRL may increase cardiac output to meet the increased metabolic requirements, for example during pregnancy [40] and lactation [41]. Changes in heart function during pregnancy coincide with elevation of PRL and placenta lactogen. Increased body growth is another physiological situation that also affects the heart. This situation is dependent on growth hormone (GH) and it is possible that PRLR protein in heart might be activated by GH. Disease related

overproduction of GH/PRL can directly or indirectly affect the heart functions as can conditions related to altered tissue sensitivity to these hormones. In future studies it might be interesting to investigate the effects of PRL on the heart in young and old, male and female and during pregnancy and lactation.

In conclusion, diabetes was not associated with major changes in PRLR protein levels in ventricle. PRL (50 ng/ml) increased AMP of shortening and Ca^{2+} transient in myocytes from diabetic and control hearts. Increased fractional release of SR Ca^{2+} may partly underlie the positive inotropic effects of PRL in myocytes from diabetic and control hearts. Although a relationship exists between diabetes and PRL, it appears that the effects of PRL on electrophysiological parameters in the heart are similar between STZ-induced diabetic and control rats.

Declarations

Author contribution statement

F. Howarth: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

G. Norstedt: Conceived and designed the experiments; Analyzed and interpreted the data.

O. Boldyriev, M. Qureshi, O. Mohamed, K. Parekh and B. Venkataraman: Performed the experiments; Analyzed and interpreted the data.

S. Subramanya: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Shmygol and L.Al Kury: Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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