Myofilament sensitivity to Ca^{2+} in ventricular myocytes from the Goto–Kakizaki diabetic rat

Frank Christopher Howarth · M. A. Qureshi

Abstract The chronic effects of type 2 diabetes mellitus on myofilament sensitivity to Ca^{2+} in ventricular myocytes from the Goto–Kakizaki (GK) rat have been investigated. Experiments were performed in ventricular myocytes isolated from 17-month GK rats and age-matched Wistar controls. Myocytes were loaded with fura-2 (an indicator for intracellular Ca^{2+} concentration) and the fura-2 ratio (340/380 nm), and shortening were measured simultaneously in electrically stimulated myocytes. Myofilament sensitivity to Ca^{2+} was assessed from phase-plane diagrams of fura-2 versus cell length by measuring the gradient of the fura-2–cell length trajectory during late relaxation of the twitch contraction. Non-fasting and fasting blood glucose were elevated in GK rats compared to controls. Fasting blood glucose was 151.5 ± 15.3 mg/dl ($n = 8$) in GK rats compared to 72.1 ± 3.6 mg/dl ($n = 9$) in controls. At 120 min after intraperitoneal injection of glucose (2 g/kg body weight), blood glucose was 570.8 ± 36.8 mg/dl in GK rats compared to 148 ± 8.6 mg/dl in controls. Amplitude of shortening was significantly increased in myocytes from GK rats ($6.56 \pm 0.54\%$, $n = 31$) compared to controls ($5.05 \pm 0.43\%$, $n = 36$), and the amplitude of the Ca^{2+} transient was decreased in myocytes from GK rats (0.23 ± 0.02 RU, $n = 31$) compared to controls (0.30 ± 0.02 RU, $n = 36$). The fura-2–cell length trajectory during the late stages of relaxation of the twitch contraction was steeper in myocytes from GK rats (89.2 ± 16.6 μm/RU, $n = 27$) compared to controls (31.9 ± 5.9 μm/RU, $n = 35$).

Increased amplitude of shortening, accompanied by a decrease in amplitude of the Ca^{2+} transient, might be explained by an increased myofilament sensitivity to Ca^{2+}.

Keywords Type 2 diabetes mellitus · Goto–Kakizaki rat · Heart function

Introduction

The effects of type 2 diabetes mellitus (DM) on heart function are poorly understood, and in general, manifestations of cardiac dysfunction in the experimental setting are only evident several months following the onset of DM. The Goto–Kakizaki (GK) rat is a genetic animal model of type 2 DM which was created by selective breeding of an outbred colony of Wistar rats, with selection for high glucose levels in an oral glucose tolerance test [1]. Several studies have investigated the effects of DM on heart function in the GK rat. In isolated perfused hearts, El Omar et al. reported unaltered left ventricular diastolic pressure and left ventricular pressure rise (LV dP/dt_{max}) and fall (LV dP/dt_{min}) and in ventricular myocytes, unaltered amplitude of twitch shortening and amplitude of the Ca^{2+} transient [2]. A magnetic resonance imaging study reported reduced myocardial blood flow, reduced ejection fraction mainly as a result of loss in left ventricular longitudinal contraction [3]. A recent study in ventricular myocytes reported unaltered amplitude of shortening and an increase in amplitude of the Ca^{2+} transient which might be attributed to an altered relationship between intracellular Ca^{2+} and the myofilaments [4]. The aim of this study was to investigate the chronic effects of DM on myofilament sensitivity to Ca^{2+} in ventricular myocytes from GK rats aged 17 months.

F. C. Howarth (✉) · M. A. Qureshi
Department of Physiology, Faculty of Medicine & Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, UAE
e-mail: chris.howarth@uaeu.ac.ae

Methods

Experimental model

Groups of young male GK and age-matched control Wistar rats were maintained on the same diet and water ad libitum. Experiments were conducted at 17 months of age. Glucose tolerance tests were applied after an overnight fast [5]. Blood glucose was measured (One Touch II Blood Glucose Meter, LifeScan Inc., USA) at time 0 and 30, 60, 120 and 180 min after an intraperitoneal injection of glucose (2 g/kg body weight). Principles of laboratory animal care were followed throughout. Approval for this project was obtained from the Faculty of Medicine & Health Sciences Ethics Committee, United Arab Emirates University.

Ventricular myocyte isolation

Single ventricular myocytes were isolated according to previously described techniques [6] with minor modifications. Animals were killed humanely with a guillotine, and hearts were then removed rapidly and mounted in Langendorff mode. Hearts were perfused at a constant flow of 8 ml g heart^{-1} min^{-1} and at physiological temperature (36–37°C) with 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 animals. When the heart had stabilized, perfusion was continued for 4 min with Ca^{2+}-free isolation solution containing 0.1 mM EGTA and then for 6 min with isolation solution containing 0.05 mmol/l Ca^{2+}, 0.75 mg/ml collagenase (type 1; Worthington Biochemical Corp., USA) and 0.075 mg/ml protease (type X1V; Sigma, Germany). 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+}. Ventricular myocytes were isolated from five control and five GK rats.

Simultaneous measurement of ventricular myocyte shortening and intracellular Ca^{2+} concentration

Myocytes were loaded with the fluorescent indicator fura-2 AM (F-1221, Molecular Probes, USA) as described previously [6]. 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 normal Tyrode solution (see below) to remove extracellular fura-2 and then left for 30 min to ensure complete hydrolysis of the intracellular ester. 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 containing 1.8 mmol/l Ca^{2+} at room temperature 25–27°C. The animals used in these experiments were 17 months of age. At this elderly age, ventricular myocytes are less robust compared to cells isolated from younger animals. In order to facilitate the simultaneous measurement of myocyte shortening and intracellular Ca^{2+} experiments were performed at room temperature.

Unloaded shortening was used as an index of contractility [7]. 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)], the time to peak (TPK) shortening and time from peak shortening to half (THALF) relaxation were recorded. To measure intracellular Ca^{2+} concentration, myocytes were alternately illuminated by 340 and 380 nm light using a monochromator (Cairn Research, UK) 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, the TPK Ca^{2+} transient and the THALF relaxation of the Ca^{2+} transient were recorded. Data were analysed using Signal Averager (v 6.16, Cambridge Electronic Design, UK).

Assessment of myofilament sensitivity to Ca^{2+}

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+} [8].

Solutions

The Ca^{2+}-free isolation solution contained (in mmol/l): 130 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 normal Tyrode solution contained (in mmol/l): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 5 HEPES, 1.8 $CaCl_2$ (pH 7.4).

Statistics

Results were expressed as the mean ± SEM of 'n' observations. 'n' Refers to the number of animals or cells. Statistical comparisons were performed using the

independent sample t-test. $P < 0.05$ was considered to indicate a significant difference.

Results

General characteristics

Experiments were performed in GK animals and age-matched controls at 17 months of age. Neither body weight or heart weight was significantly ($P > 0.05$) different in GK rats compared to controls. Mean body weight was 399 ± 22.1 g ($n = 5$) in GK rats compared to 436.0 ± 43.2 g ($n = 5$) in controls. Heart weight was 1.50 ± 0.05 g ($n = 5$) in GK rats compared to 1.52 ± 0.06 g ($n = 5$) in controls. Non-fasting and fasting blood glucose were significantly ($P < 0.05$) elevated in GK rats compared to controls. Non-fasting blood glucose was 188.8 ± 10.1 mg/dl ($n = 5$) in GK rats compared to 101.4 ± 6.1 mg/dl ($n = 5$) in controls. Fasting blood glucose was 151.5 ± 15.3 mg/dl ($n = 8$) in GK rats compared to 72.1 ± 3.6 mg/dl ($n = 9$) in controls. The results of the glucose tolerance test are shown in Fig. 1. Animals were fasted overnight and after a glucose challenge (2 g/kg bodyweight, i.p.), blood glucose was measured at 30, 60, 120 and 180 min. At 17 months blood glucose was significantly elevated at 30, 60, 120 and 180 min in GK rats compared to controls. At 120 min blood glucose was 570.8 ± 36.8 mg/dl ($n = 8$) compared to 148.1 ± 8.6 mg/dl ($n = 9$) in controls.

Ventricular myocyte shortening and intracellular Ca^{2+}

Typical records of shortening and Ca^{2+} transients in electrically stimulated (1 Hz) myocytes from GK and control rats are shown in Fig. 2a. Resting cell length was not significantly altered in ventricular myocytes from GK rats (109.3 ± 2.6 μm, $n = 31$) compared to controls (109.7 ± 2.4 μm, $n = 36$). TPK shortening was significantly prolonged in ventricular myocytes from GK rats (337.5 ± 8.0 ms, $n = 31$) compared to controls (302.7 ± 7.8 ms, $n = 36$) (Fig. 2b). THALF relaxation was also significantly prolonged in ventricular myocytes from GK rats (275.4 ± 10.7 ms, $n = 31$) compared to controls (231.3 ± 11.1 ms, $n = 36$) (Fig. 2c). Amplitude of shortening was significantly increased in myocytes from GK rats ($6.56 \pm 0.54\%$, $n = 31$) compared to controls ($5.05 \pm 0.43\%$, $n = 36$) (Fig. 2d).

Resting fura-2 ratio (340/380) was not significantly altered in ventricular myocytes from GK rats (1.23 ± 0.04 RU, $n = 31$) compared to controls (1.32 ± 0.04 RU, $n = 36$). Neither the TPK nor THALF relaxation of the Ca^{2+} transient was significantly altered in ventricular myocytes from GK rats compared to controls. TPK Ca^{2+} transient and THALF relaxation of the Ca^{2+} transient were, respectively, 104.3 ± 5.9 and 199.0 ± 6.9 ms ($n = 31$) in ventricular myocytes from GK rats compared to 91.7 ± 4.0 and 199.1 ± 6.3 ms ($n = 36$) in controls. The amplitude of the Ca^{2+} transient was significantly reduced in myocytes from GK rats (0.23 ± 0.02 RU, $n = 31$) compared to controls (0.30 ± 0.02 RU, $n = 36$).

Myofilament sensitivity to Ca^{2+}

Typical phase-plane diagrams of length versus fura-2 ratio in myocytes from GK rats and controls are shown in Fig. 3a. Myofilament sensitivity to Ca^{2+} was assessed by measuring the gradient of the fura-2–cell length trajectory during late relaxation of the twitch contraction. Measurements were carried out at 600–700 ms (Fig. 3b), 700–800 ms (Fig. 3c), 800–900 ms (Fig. 3d) and 600–900 ms (Fig. 3e). 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+} [8]. The gradients of the trajectory for GK myocytes were significantly greater at 600–700 and at 600–900 ms compared to controls. Gradients for GK myocytes compared to controls were, respectively: 49.3 ± 11.4 ($n = 27$) and 23.4 ± 6.1 ($n = 35$) μm/RU at 600–700 ms; 22.8 ± 6.8 ($n = 27$) and 12.5 ± 2.4 ($n = 35$) μm/RU at 700–800 ms; 12.2 ± 4.4 ($n = 27$) and 5.0 ± 1.3 ($n = 35$) μm/RU at 800–900 ms; 89.2 ± 16.6 ($n = 27$) and 31.9 ± 5.9 ($n = 35$) μm/RU at 600–900 ms.
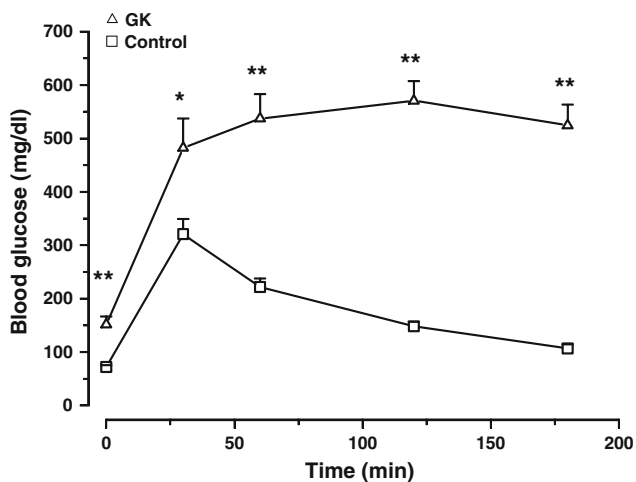

Fig. 1 Uptake of glucose from blood following a glucose challenge at 17 months of age. After an overnight fast animals received an intraperitoneal injection of glucose (2 g glucose/kg body weight). Blood glucose was measured at time 0 and at 30, 60, 120 and 180 min after administration of glucose. Data are mean $\pm$ SEM, $n = 8$–9 animals. * $P < 0.05$, ** $P < 0.01$

Fig. 2 Ventricular myocyte shortening and intracellular Ca^{2+}. (a) Typical records of shortening and Ca^{2+} transient in myocytes from control (left panel) and GK (right panel) rat hearts. (b) TPK shortening, (c) THALF relaxation, (d) amplitude of shortening, (e) TPK Ca^{2+} transient, (f) THALF relaxation of the Ca^{2+} transient and (g) amplitude of the Ca^{2+} transient in ventricular myocytes from GK and control rat hearts. Data are mean ± SEM, $n = 31$–36 cells from five hearts
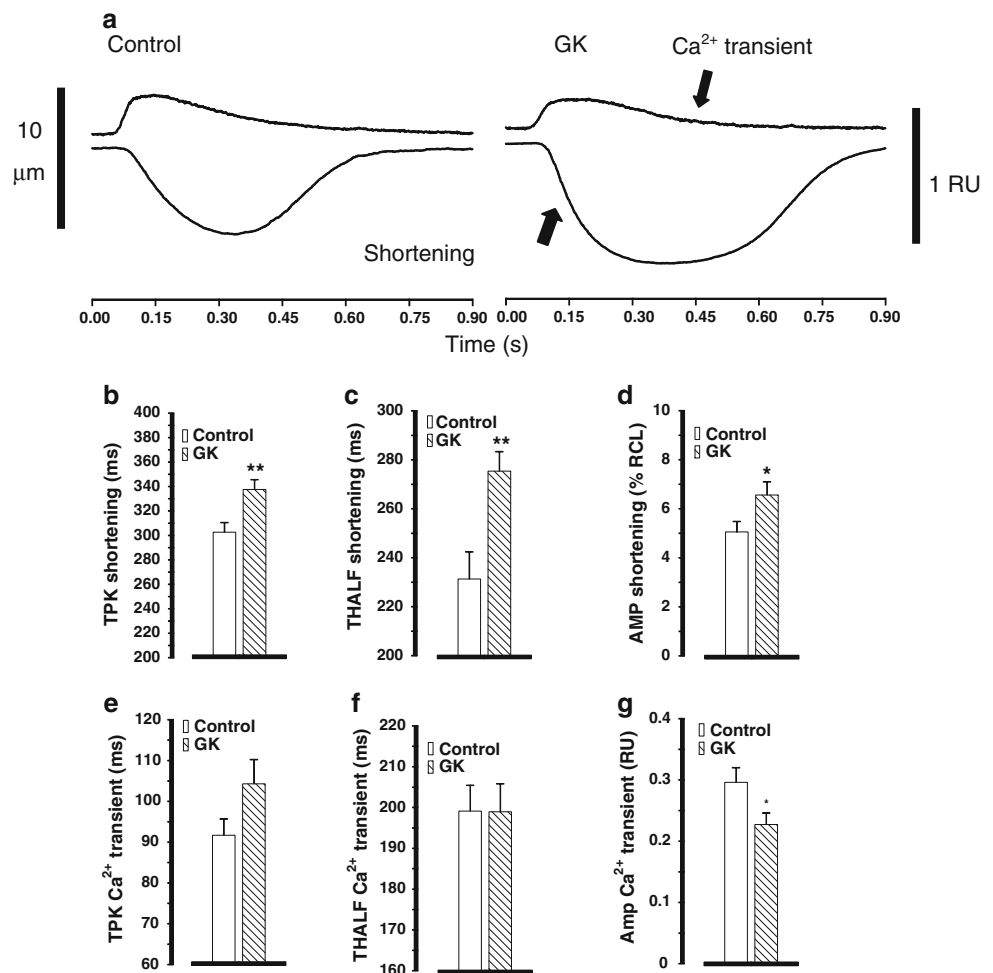
Discussion

Experiments were performed in GK rats and Wistar controls aged 17 months. The major findings of this study were: (i) TPK and THALF relaxation of shortening were prolonged and amplitude of shortening was increased in GK myocytes compared to controls; (ii) amplitude of the Ca^{2+} transient was reduced in GK myocytes compared to controls; (iii) the gradient of the trajectory during the relaxation phase of the twitch contraction was significantly greater for GK myocytes compared to controls.

The GK rats displayed increased non-fasting and fasting blood glucose, characteristics which are typical of type 2 DM. Previous studies have demonstrated elevations in blood plasma glucose in GK rats from as young as 1 month after weaning [9]. The GK rats also displayed glucose intolerance which is consistent with impaired secretion of insulin and peripheral insulin resistance [1, 9], which may contribute to cardiac hypertrophy [10].

The time course of myocyte shortening, including the TPK and THALF relaxation, was prolonged, and the amplitude of shortening was increased in electrically stimulated (1 Hz) ventricular myocytes from GK rats compared to age-matched controls. Previous studies in isolated perfused heart preparations from younger animals have reported unaltered left ventricular diastolic pressure and left ventricular pressure rise (LV dP/dt_{max}) and fall (LV dP/dt_{min}) and unaltered amplitude of myocyte twitch shortening [2]. Another study which employed magnetic resonance imaging in female GK rats demonstrated reduced myocardial blood flow, reduced ejection fraction mainly as a result of loss in left ventricular longitudinal contraction [3]. Interestingly, there is exaggerated diastolic dysfunction during hypoxia [2], and ageing female GK rat heart has an increased susceptibility to ischaemic injury compared to male GK rat heart [11]. A more recent study also demonstrated unaltered amplitude of shortening in ventricular myocytes from GK rats compared to controls [4].

Although the time course of the Ca^{2+} transient was not significantly altered, the amplitude of the Ca^{2+} transient was reduced in myocytes from GK rats compared to controls. Previous studies have reported unaltered or increased intracellular Ca^{2+} levels in GK animals [2, 4, 10]. Increased, or unaltered, amplitude of myocyte shortening accompanied
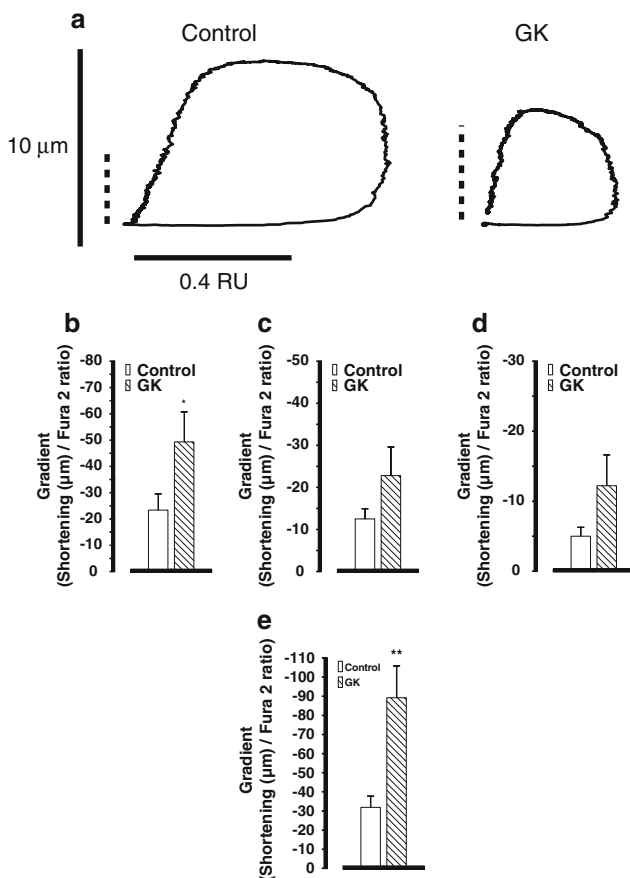
Fig. 3 Myofilament sensitivity to Ca^{2+}. (**a**) Typical phase-plane diagrams of length versus fluorescence for twitch contractions in myocytes from control (left panel) and GK (right panel) rat hearts. Dotted lines indicate the time (600–900 ms) during which trajectory measurements were calculated. Mean trajectories at (**b**) 600–700, (**c**) 700–800, (**d**) 800–900, (**e**) 600–900 ms during the relaxation phase of contraction. Data are mean $\pm$ SEM, $n = 25$–35 cells from five hearts. * $P < 0.05$, ** $P < 0.01$

by decreased amplitude of the Ca^{2+} transient may be explained by altered myofilament sensitivity to Ca^{2+}. Previous studies in the STZ-induced diabetic rat have demonstrated diminished sensitivity of myofilaments to Ca^{2+} and alterations in myosin isoenzyme pattern, for example from V1 to V3 isoenzyme, which in turn may partly underlie the contractile defects which have been frequently reported in the diabetic heart [12–14]. An increase in systolic Ca^{2+} might provide a mechanism to compensate for defective myofilament sensitivity to Ca^{2+}.

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 during the relaxation phase reflects the relative myofilament response to Ca^{2+} and, hence, can be used as a measure of myofilament sensitivity to Ca^{2+} [8].

The gradient of the trajectory during the relaxation phase of the twitch contraction was significantly greater for GK myocytes compared to controls suggesting that myofilaments sensitivity to Ca^{2+} is increased in GK myocytes compared to controls. Previous studies in STZ-induced diabetic rats, which is a widely used experimental model of type 1 DM, have also demonstrated an increase in myofilament sensitivity to Ca^{2+} [15–17]. In the STZ-induced diabetic rat heart decreased expression of SR Ca^{2+}-ATPase and ryandodine receptor protein partly underlies the decreased uptake and release of Ca^{2+} which in turn contributes to the prolonged time course and decreased amplitude of the Ca^{2+} transient [18]. Interestingly, there was a decrease in the amplitude of the Ca^{2+} transient, whilst there was a small increase in the amplitude of myocyte shortening. Alterations in myofilament sensitivity to Ca^{2+} might provide a compensatory mechanism to preserve mechanical function of the diabetic heart.

It is concluded that altered myofilament sensitivity to Ca^{2+} partly underlies contractile dysfunction in ventricular myocytes from the GK diabetic rat.

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