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Effects of Exercise and Pharmacological Intervention on Electromechanical Function of The Heart in the Goto-Kakizaki Type 2 Diabetic Rat.

Khawla Abdulla Al-Ali

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EFFECTS OF EXERCISE AND PHARMACOLOGICAL INTERVENTION ON ELECTROMECHANICAL FUNCTION OF THE HEART IN THE GOTO-KAKIZAKI TYPE 2 DIABETIC RAT

Khawla Abdulla Al-Ali

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Chris Howarth

June 2015
Declaration of Original Work

I, Khawla Abdulla Al-Ali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "Effect of exercise and Pharmacological Intervention on Electromechanical Function of the Heart in the Goto-Kakizaki Type 2 Diabetic Rat", hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me under the supervision of Professor Chris Howarth, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

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Abstract

Type 2 diabetes mellitus accounts for more than 90% of cases of diabetes mellitus. Cardiovascular diseases are the major causes of morbidity and mortality in diabetic patients. A variety of diastolic and systolic dysfunctions have been reported. The severity of the abnormalities depends on the patients’ age and diabetes duration. The aim of the study was to investigate the age-dependent, exercise and pharmacological intervention on electromechanical function of the heart in the Goto-Kakizaki (GK) type 2 diabetic rat. mRNA expression was assessed in ventricular muscle with real-time RT-PCR. Ventricular myocytes shortening, intracellular Ca$^{2+}$ transport and L-type Ca$^{2+}$ current were measured with video edge detection, fluorescence photometry and whole cell patch clamp techniques, respectively. In vivo biotelemetry was used to measure the electrocardiogram. In young GK (8-10 weeks) rat, mRNA expression of Atp1a3, Cacna1h, Scn1b and Hcn2 were upregulated and Slc9a1, Hcn4, Kcnq2/4 and Kcnj2 were downregulated. Amplitude of ventricular myocyte shortening and intracellular Ca$^{2+}$ transient were unaltered, time to peak shortening was prolonged and time to half decay of the Ca$^{2+}$ transient was shortened in GK myocytes. Physical exercise is well established as a valuable form of non-pharmacological therapy. Experiments were performed in GK and control (10-11 months) following 2-3 months of treadmill exercise training. Expression of mRNA encoding Tpm2, Gja4, Atplb1, Cacna1g, Cavb2, Hcn2, Kcnq3 and Kcnel were upregulated and Gja1, Kcnj2 and Kcnk3 were downregulated in hearts of sedentary GK rats compared to sedentary controls. Gja1, Cav3 and Kcnk3 were upregulated and Hcn2 was down-regulated in hearts of exercise
trained GK compared to sedentary GK controls. Amplitude of ventricular myocyte shortening, Ca\(^{2+}\) transients and L-type Ca\(^{2+}\) current were not significantly altered.

The effects of the anti-diabetic drug Pioglitazone on ventricular myocyte shortening and Ca\(^{2+}\) transport in addition to electrocardiogram were also investigated. Pioglitazone (0.1-10) \(\mu\)M reduced the amplitude of shortening in ventricular myocytes from GK and control rats. Pioglitazone reduced the amplitude of the Ca\(^{2+}\) transient and modest reductions in L-type Ca\(^{2+}\) current in GK and control myocytes. Heart rate in GK rats was reduced. Although Pioglitazone reduced blood glucose in GK rats it had little effect on heart electrocardiogram.

**Keywords:** Type 2 diabetes, Exercise, Pioglitazone, Heart, Ventricular myocytes.
Title and Abstract (in Arabic)

دراسة أثر التمارين الرياضية والتدخل الدوائي على وظائف القلب الكهروميكانيكية في الجردنان المصابة بداء السكري من النوع الثاني (Goto-Kakizaki)

المختص

يعتبر النوع الثاني من مرض السكري هو الأكثر شيوعًا حيث يحتل 90% من حالات مرضى السكري. وتعد أمراض القلب والأوعية الدموية أحد أهم أسباب الحالات المرضية والوفيات لدى مرضى السكري، حيث وجد من خلال عدة دراسات بحثية اختلافات في مراحل الانتقاص والانسياس الوظيفي للقلب. وتعتبر هذه الاختلافات على عمر المرضى ومدة المرض.

لقد قمنا في هذا البحث بدراسة أثر العمر، والتمارين الرياضية والتدخل الدوائي على وظائف القلب الكهروميكانيكية للجردنان المصابة بداء السكري من النوع الثاني ومقارنةها بالجردنان السليمة. وباستخدام تقنية تفاعل البوليميراز السلسلي قمنا بدراسة الحمض النووي في خلايا العضلة القلبية. كما قمنا بقياس انقباض الخلايا القلبية، والنقل الداخلي للكالسيوم وحجم قنوات الكالسيوم وذلك بالإضافة لقياس الوظائف الحيوية في الجردنان كالتحفيز الكهربائي للقلب.

لاحظنا من خلال الدراسة على الجردنان الصغيرة في العمر (8-10 أسابيع) للجينات المسؤولة عن بروتينات العضلات والخلايا القلبية، والنقل الداخلي للكالسيوم، والنقل الخارجي وعينات قنوات الكالسيوم والبوتاسيوم والكالسيوم اختلافات مستقرة سواء كانت زيادة أو نقص في تمثيل الجينات مقارنة بالجردنان السليمة. حيث لم يتعكس اختلاف الجينات بشكل ملحوظ على قدرة الخلايا القلبية على الانقباض وطريقة تداول الكالسيوم داخل الخلية القلبية للجردنان المصابين بالسكري.

من المعروف أن التمارين الرياضية تعتبر من المعالجات غير الدوائية الفعالة لمرضى السكري. ولذا قمنا بإجراء أثر التمارين الرياضية للجردنان البالغة (10-11 شهر) لمدة شهرين إلى ثلاثة أشهر، ومن ثم درسنا تمثيل الجينات للخلايا القلبية ووجدنا أيضا اختلافات متعددة سواء كانت زيادة أو نقص في تمثيل الجينات في الجردنان المصابين بالسكري مقارنة بالجردنان السليمة، وكذلك اختلافات في التي تتعثر للتمارين الرياضية مقارنة بالجردنان التي لم تتعرض للتمارين الرياضية. وبينت الدراسة كذلك أن قدرة الخلايا القلبية على الانقباض وطريقة تداول الكالسيوم داخل الخلية القلبية لم تتاثر في الجردنان المصابين بالسكري أو الجردنان التي تعرضت للتمارين الرياضية.
ومدَّى تأثيره على قدرة الخلايا القلبية على الانقباض وأيضاً النقل الحلوي للكلسيوم وجه ردود الفعل الكالسيومية، وقد أثبتت الدراسة تأثيره المثبت على كل العوامل في كل من خلايا العضلة القلبية للجرذان المصابة بالسكري والسليمه. وعندما استُخلِص البُحث على الدراسة الحيوية (Biotelemetry) والتي بثبت أن معدل ضربات القلب للحيوانات المصابة بالسكري أقل من الحيوانات السليمة وان البطانيات تساعد في خفض مستوى السكر في الدم بدون تأثير سلبي على قراءات التخطيط الكهربائي للقلب.

مفاتيح البحث الرئيسيه: النوع الثاني لمرض السكري، الرياضة، بيوغلينزون، القلب، خلايا القلب البطينية.
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I have been most fortunate to have such wonderful friends and colleagues such as Dr. Lina Al-Kury, Dr. Zakeya Alrašbi, Ms. Fadwa, Ms. Abrar Ashoor and Dr. Amal Al Haddad for making this journey a memorable experience.

All my gratitude goes to my beloved mother and wonderful brothers and sisters for their prayers and for always believing in me. I could not have done this without them.
Dedication

To my father who is always in my heart, my beloved mother, brothers and sisters
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<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AFI</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV node</td>
<td>Aterioventricular node</td>
</tr>
<tr>
<td>Ba⁺</td>
<td>Barium</td>
</tr>
<tr>
<td>BMP</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BS</td>
<td>Brugada syndrome</td>
</tr>
<tr>
<td>Cdna</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CXE</td>
<td>Control + exercise</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induce calcium release</td>
</tr>
<tr>
<td>CMHS</td>
<td>College of Medicine and Health sciences</td>
</tr>
<tr>
<td>CS</td>
<td>Control sedentary</td>
</tr>
<tr>
<td>CX37</td>
<td>Connexin 37</td>
</tr>
<tr>
<td>CX43</td>
<td>Connexin 43</td>
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<tr>
<td>CXs</td>
<td>Connexins</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DCM</td>
<td>Diabetic cardiomyopathy</td>
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<tr>
<td>DKA</td>
<td>Diabetic ketoacidosis</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidylpeptidase 4</td>
</tr>
<tr>
<td>E-C coupling</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra acetic acid</td>
</tr>
<tr>
<td>Eₖ</td>
<td>K⁺ equilibrium potential</td>
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<tr>
<td>Eₘ</td>
<td>Membrane potential</td>
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<tr>
<td>Eₙa</td>
<td>Na⁺ equilibrium potential</td>
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<tr>
<td>FAT</td>
<td>fatty acid translocase</td>
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<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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</table>
GDM  Gestational diabetes mellitus
GK   Goto-kakizaki
GKEX  GK + exercise
GKS  GK sedentary
GLP-1  Glucagon like peptide
GLUT4  Glucose transporter 4
HbA1C  Hemoglobin A1C
       Hyperpolarization-activated cyclic nucleotide-gated channels
HCN  HCN
HDL  High density lip-protein
hERG  The human ether-ago-go-related gene
HR  Heart rate
HRV  Heart rate variability
i.p  Intraperitoneal
ICD  Intercalated disc
IDF  International Diabetes Federation
If  Pacemaker current
IGT  Impaired glucose tolerance
IK  K+ currents
IK1  Inward rectifying K+
IKr  Rapid delayed rectifier K+ channels
IKS  Slow delayed rectifier K+ channel
IKur  Ultrarapid delayed rectifier K+ channels
IL-6  Interleukin-6
Ito  Transient outward K+
KACH  Acetylcholine sensitive K channel
KATP  ATP sensitive K channel
KIR  Inward rectifying K+
LBD  Ligand binding domain
LDL  Low density lipoprotein
LV  Left ventricular
Mody diabetes  Maturity-onset diabetes of the young
mRNA  Messenger RNA
NA  Nano Ampere
NDDG  National Diabetes Data Group
OGTT  Oral glucose tolerance test
PAI-1  Plasminogen activator inhibitor-1
PF  Peco farad
PIO  Pioglitazone
PPARγ  Peroxisome proliferator-activated receptor γ
PPARα  Peroxisome proliferator-activated receptor α
PPARβ/δ  Peroxisome proliferator-activated receptor β/δ
PPRE  PPAR response element
PROactive study  Prospective pioglitazone clinical trial in macrovascular events
QTL  Quantitative trait locus
RCL  Resting cell length
Real time RT-PCR  Real time reverse transcription polymerase chain reaction
RECORD  Rosiglitazone Evaluated for Cardiovascular Outcome and regulation of Glycemia in diabetes
RYR  Ryanodin receptor
SA node  Sinoatrial node
SDS  Sodium Dodecylsulphate
SERCA  SR Ca²⁺-ATPase
SR  Sarcoplasmic reticulum
T1DM  Type 1 Diabetes Mellitus
T2DM  Type 2 diabetes mellitus
TBS  Tris-buffered saline
TBST  Tris-buffered saline containing 0.05% Tween 20
TEA  Tetraethylammonium
THALF  Time to half
TnC  Troponin C
TNFα  Tumor necrosis factor-α
TnI  Troponin I
TnT  Troponin T
TPK  Time to peak
TS  Timothy syndrome
T-tubules  Transverse tubules
TZD  Thiazolidinedione
UAEU  United Arab Emirates University
VGCCs  Voltage-gated Ca²⁺ channels
VGPCs  Voltage-gated K⁺ channels
VGSCs  Voltage-gated Na⁺ channels
WHO  World Health Organization
WS  Wolfram's syndrome
Chapter 1: Introduction

1.1 The heart

The heart is a muscular organ containing four chambers. The upper two chambers, left and right atria are separated by the interatrial septum. The lower two chambers, left and right ventricles are separated by the interventricular septum. Heart valves are located between each atrium and ventricle. The tricuspid valve controls blood flow between the left atrium and left ventricle and the mitral valve controls blood flow between the right atrium and the right ventricle. The aortic valve is located between the left ventricle and the aorta, and the pulmonary valve is located between the right ventricle and the pulmonary artery. Blood flow in and out of the heart is regulated by two circuits, namely, the pulmonary circuit which carries deoxygenated blood from the right ventricle to the lung via the pulmonary arteries and carries oxygenated blood to the left atrium via pulmonary veins and the systemic circuit which carries oxygenated blood from the left ventricle to the rest of the body via the aorta and returns deoxygenated blood in to heart via superior and inferior vena cava (Figure 1.1).

The heart wall consists of three layers, the outer layer called the epicardium, contains squamous cells and a network of fibro-elastic connective tissue. The middle layer called the myocardium is the muscular layer and it makes up the vast majority of the heart's thickness and consists of cardiac muscle cells (cardiac myocytes), connective tissue and blood vessels. The inner layer is the endocardium which is a thin layer which lines the heart chamber and is made up of squamous cells, collagen and elastic
fiber and a layer of smooth muscle. The heart tissue itself is supplied with blood through the coronary arteries that arise from the aortic sinus. Parallel to the coronary arteries there are coronary veins. The veins are responsible for collecting deoxygenated blood from heart tissue into a large vessel called the coronary sinus which empties blood into the right atrium. The heart is an energetic organ that requires large amounts of ATP to support its muscular activity. Under physiological conditions, optimal cardiac function depends on the efficient matching of energy production to energy expenditure. Oxidation of long chain fatty acid and glucose in the mitochondria are the main sources for ATP production.

The heart is under extrinsic control by the autonomic nervous system. It is richly innervated by branches of sympathetic and parasympathetic fibers which mainly terminate on the sinoatrial (SA) node and atrioventricular (AV) node. Stimulation of sympathetic nerves causes the release of norepinephrine at nerve endings which increases membrane permeability to calcium ions (Ca^{2+}) by opening voltage-gated L-type Ca^{2+} channels and enhances pacemaker current (I_{p}), which increase the rate of sinus rhythm and rate of conduction of impulses and result in cardiac acceleration. On the other hand, activation of parasympathetic nerves liberates acetylcholine (ACh) at nerve endings and causes an increase of cell membrane permeability to potassium ions (K^+) and produces hyperpolarization by suppressing I_{p}. These effects slow the firing rate in the SA node and decrease excitation of the conduction system and result in reduction in the rate and force of contraction of the heart [1]. Cardiac muscle contraction is also regulated by intrinsic mechanisms. According to Frank and Starling law of the heart, as the heart fills with blood the walls of the heart is
stretched. The greater the filling volume and hence, the greater the stretch, the greater will be the force of cardiac muscle contraction [2, 3].

Figure 1.1: Structure of the heart. Adapted from http://www.articleweb.org/blog/wp-content/gallery/draw-biology-diagram/draw-biology-diagram-13.jpg
1.1.1 Cardiac myocyte electrophysiology

The structural unit of the myocardium is the cardiac myocyte. Cardiac myocytes are the most physically energetic cells in the body contracting constantly throughout their lifespan. Unlike skeletal muscle cells, typical human ventricular myocytes have variable branching morphology, they are approximately 100-150 μm in length and about 20-35 μm in diameter. Cardiac myocytes are surrounded by a unique plasma membrane known as the sarcolemma. The sarcolemma is composed of a hydrophobic phospholipid bilayer and hydrophilic head groups. A variety of ion channels, pumps and ion exchangers are embedded in the sarcolemma, all of which regulate the movement of ions in and out of the myocyte. The transverse tubules (T-tubules) are finger-like invaginations which extend from the sarcolemma to the interior of the cell. Each T-tubule contains a large number of voltage-gated L-type Ca\textsuperscript{2+} channels.

Cardiac myocytes are highly packed with mitochondria to provide a steady supply of adenosine triphosphate (ATP) which is required as a source of energy for cardiac muscle contraction. Cardiac myocytes contain contractile proteins called myofilaments which include actin, myosin and associated regulatory proteins, arranged in a regular array of thick (myosin) and thin (actin) filaments. The sarcomere is the fundamental unit of the myofilament and is bordered by the Z lines. The interaction between actin and myosin generates muscle contraction [4, 5]. The regulatory proteins associated with actin and myosin include tropomyosin and the troponin complex. The troponin complex is made up of three subunits, troponin T (TnT, or tropomyosin binding subunit), troponin C (TnC or the Ca\textsuperscript{2+} binding subunits) and troponin I (TnI, inhibitory subunit which also binds actin).
The sarcoplasmic reticulum (SR) is an intracellular network which surrounds the contractile proteins. It stores and releases Ca\(^{2+}\) that is important in the regulation of cardiac muscle contraction. At multiple sites within this network the membrane flattens and is tightly pressed against T-tubules to form terminal cisternae (SR cisternae). Ca\(^{2+}\) is released from the SR via the Ca\(^{2+}\) channel called the ryanodine receptor (RYR). Three different RYRs have been identified in mammalian tissue including RYR1, RYR2 and RYR3. RYR2 is the predominant form in cardiac muscle. The relationship between T-tubules and RYR2 receptor is critical to the process of excitation-contraction coupling (E-C coupling) [6].

Adjacent myocytes are held together by specialized cell to cell junctions called intercalated discs (ICD). The ICDs provide mechanical and electrical connections between cardiac myocytes. Along the longitudinal parts of the ICDs, the sarcolemma of adjacent myocytes come into close proximity and fuse at various places to form gap junctions, which provide low-resistance pathways to allow the spread of cardiac impulses between myocytes [7]. Gap junctions in the heart are composed of connexin proteins. There are three major connexin isotypes in cardiac muscle; connexin 43 (CX43), connexin 45 and connexin 40. CX43 is the most prominent in mammalian cardiac muscle [8-11].

The contractile capabilities of myocytes have to be generated in a highly synchronous fashion to produce an efficient heartbeat. This requires both the initiation and orderly spread of the wave of electrical activation from one myocyte to the next, throughout the heart. The electrical activation of the myocyte is known as the action potential (AP). The AP is mediated by sequences of opening and closing ion channels and
result in changes in the membrane potential ($E_m$). In the resting state the membrane potential ranges from -60 to -90 mV. Compared to the extracellular compartment, the intracellular compartment is negatively charged. This is an essential physiologic condition that facilitate the diffusion of ions across the membrane down their concentration gradient and generates a potential difference that counteracts the diffusion and determines the electrochemical equilibrium according to the Nernst equation [12].

APs differ from one region of the heart to another depending on the cell types. In general APs can be classified into two types: pacemaker and non-pacemaker APs. Pacemaker cells are primarily located in the SA and AV nodes of the heart and have pacemaker potentials that depolarize spontaneously towards threshold. Non-pacemaker APs found throughout the heart except in the nodal regions and are characterized by fast responses with fast depolarization rate.

A typical ventricular myocyte AP lasts for 200-300 ms (species dependent) and has 5 sequential phases 0-4 (Figure 1.2). Phase 4 is where the resting membrane potential is approximately -90 mV. This is attributed mainly to the selective permeability of the cell membrane to K\(^+\), especially inward rectifying K\(^+\) (K\(_{IR}\)), and the concentration gradient for K\(^+\) that exists across the cell membrane close to the Nernst equilibrium potential for K\(^+\) ($E_K$) [3, 13, 14]. Upon arrival of suprathreshold electrical stimuli, the membrane permeability to K\(^+\) decreases and voltage-gated Na\(^+\) channels (VGSCs) open allowing a large influx of Na\(^+\) current ($I_{Na}$) into the cell, driving the $E_m$ towards the Na\(^+\) equilibrium potential ($E_{Na}$) and producing rapid depolarization from -90 mV to +10 mV (Phase 0) [15]. Phase 1 is a brief repolarization during which transient
outward $K^+$ ($I_{oa}$) channels are opened and there is an outward flow of $K^+$ current ($I_K$), producing a transient shift of the $E_m$ to approximately 0 mV [16]. Phase 2 lasts for 100-200 ms and is initiated by opening of slow L-type $Ca^{2+}$ channels to produce the plateau phase of the AP. During Phase 3 $Ca^{2+}$ channels are inactivated, while the delayed rectifier $K^+$ channel activates and conducts $I_K$ in the outward direction. Finally, $K_{IR}$ channels re-open during Phase 4 of the AP [15].

There is a strong association between the AP and cardiac muscle contraction. Rapid depolarization precedes the development of force while peak force occurs towards the end of repolarization. Relaxation of the muscles usually occurs during Phase 4. The events of muscle contraction proceed in parallel to the events of the AP.
Figure 1.2: Ventricular action potential. The ventricular action potential is generated mainly by inward (Na\(^+\) and Ca\(^{2+}\)) and outward (K\(^+\)) transmembrane ion currents. Numbers (0-4) denote the different phases of the ventricular action potential. Phase 0 is depolarization. Phase 1 is fast repolarization. Phase 2 plateau. Phase 3 is repolarization and Phase 4 is the resting phase of the action potential. The individual ionic membrane currents and their time course are illustrated. Modified from Hoekstra et al. 2012 [17].
1.1.2. Ionic basis of the action potential

1.1.2.1 Voltage-gated Na\(^+\) channels

Hodgkin and Huxley were the first to characterize the VGSCs by recording \(I_{Na}\) using voltage clamp techniques. They demonstrated three key features of VGSCs: voltage-dependent activation, rapid inactivation and selective ion conductance [18]. In the past 60 years, the measurement of ion flux, channel affinity binding and protein purification of VGSC have been progressively analyzed and studied. The VGSCs are responsible for the rapid depolarization (Phase 0) of AP of the excitable cells, including nerve, muscle and neuroendocrine cells. It plays a crucial role in the propagation of APs and also helps determine the duration of the AP. For example in cardiac myocytes, some Na\(_{v1.5}\) channels (subtype of VGCS which is highly expressed in the heart) may reopen during the plateau phase generating late inward current [19].

The VGSC structure (Figure 1.3) consists of a pore forming subunit \(\alpha\) (260 kDa) associated with an auxiliary \(\beta\) subunit (30-35 kDa). The \(\alpha\) subunit is required for channel function (current conductance) while the \(\beta\) subunit is responsible for kinetics and voltage dependence of channel gating, and is involved in channel localization and interaction with various extracellular and intracellular components. Four \(\beta\) subunits have been found encoded in the human genome. VGSC in adult central nervous system and heart contain \(\beta_1\) through \(\beta_4\) subunits, where as only \(\beta_1\) subunit is expressed in adult skeletal muscle [19, 20]. The \(\alpha\) subunit consists of four homologous domains (I-IV), each consists of six transmembrane \(\alpha\) helices segments (S1-S6). The S4 segments in each domain have positively charged amino acid residues at every third
position. These residues serve as gating charges to initiate channel activation during membrane depolarization. The intracellular loop connecting homologous domain III and IV serves as the inactivation gate folding into the channel structure and blocking the pore from the inside. The β subunit is one transmembrane segment protein with a large N-terminal extracellular domain and a short C-terminal intracellular segment [19].

Nine mammalian VGSCs have been identified (Na\textsubscript{1.1}-Na\textsubscript{1.9}). These are functionally expressed with > 50% homology in amino acid sequence in the transmembrane and extracellular domains [19, 21]. Na\textsubscript{1.5} subtype is found in cardiac myocytes [22, 23].

VGSCs are a site of action for different pharmacological agents. For example, antiarrhythmic drugs and local anesthetics act as VGSC blockers, while veratridin, batrachotoxin and aconitine act as VGSC activators [21]. Mutation in SCN5A, the gene that encodes cardiac Na\textsubscript{1.5} has been shown to be involved in many different cardiac electrical disorders including, Brugada syndrome (BS), long QT syndrome, idiopathic ventricular fibrillation and complex phenotypes of ventricular arrhythmias and dilative cardiomyopathy [24-26].
Figure 1.3: Structure of voltage-gated Na⁺ channel. The α and β subunits of the Na⁺ channel are illustrated. Roman numerals indicate the α subunit domains; segments 5 and 6 are the pore-lining segments and the S4 helices make up the voltage sensors. Adapted from Abriel 2007 [27].
1.1.2.2 Voltage-gated Ca\textsuperscript{2+} channels

Voltage-gated Ca\textsuperscript{2+} channels (VGCCs) were first identified by Fatt and Katz in 1953 [28]. VGCCs are the key signal transducers of membrane potential. They have the ability to convert the electrical signal of an AP into an intracellular Ca\textsuperscript{2+} transient and thus initiate and regulate many physiological processes. In many different cell types, VGCCs mediate entry of Ca\textsuperscript{2+} current (I\textsubscript{Ca}) which in turn can initiate hormone secretion or synaptic transmission and also regulate enzyme activity, gene expression, and other biochemical activities [29-32]. Activation of VGCCs in cardiac myocytes initiate and regulate contraction through the process of E-C coupling [33].

VGCCs are classified according to their pharmacological and biophysical properties (activation thresholds, conductance, and inactivation time) into several functional channel types including T-type, L-type, N-type, P/Q-type, and R-type. T-type open at low membrane potentials and inactivate very rapidly. R-type opens with intermediate membrane potential while L-type open at high membrane potentials with slow inactivation [34, 35]. These subtypes are classified as follows, Cav1.1-Cav1.4, Cav2.1-Cav2.3 and Cav3.1-Cav3.3 [36-38].

In cardiac muscle, both L-type Ca\textsuperscript{2+} channels (long lasting/ subtypes Cav1.1, Cav1.2, Cav1.3 and Cav1.4) and T-type Ca\textsuperscript{2+} channels (transient/ subtypes Cav3.1, Cav3.2 and Cav3.3) play an important role in cardiac myocyte contraction and automaticity [15]. In the heart, L-type Ca\textsuperscript{2+} currents and T-type Ca\textsuperscript{2+} currents are mediated by Cav1.2 and Cav3.2 types of the \( \alpha 1 \) subunit, respectively [39, 40].
VGCCs are a complex of five subunits, named α1, α2, β, γ, and δ (Figure 1.4). The α1 subunit consists of four repeated domains (I-IV) with each containing six transmembrane segments. The α1 subunit is a voltage sensor which is responsible for the Ca\(^{2+}\) current conductance [35, 41].

Unlike VGSCs, the β subunit of VGCCs is intracellular α helices [42]. The α2 and δ subunits form an extracellular disulfide-linked α2δ dimer. Co-expression of the β subunit and α2δ dimer enhances the expression and modulates the voltage dependence and kinetics of gating [38, 43, 44]. The γ subunit is a glycoprotein with four transmembrane segments [45]. Purification of different VGCCs showed that γ subunits were not expressed in some tissues depending on the VGCC types. L-type Ca\(^{2+}\) channels in cardiac myocytes are associated with α1, β, and α2δ but not γ subunits [46]. Three different α1 subunits have been identified by cDNA cloning and functional expression in mammalian cells or *Xenopus* oocytes [47, 48]. In addition, individual α1 subunit types are associated with multiple types of β subunits or α2δ dimers producing different shifts in the kinetics, voltage dependence of gating and/or level of functional expression of different α1 subunits.

In the clinical setting, numerous drugs that are used in the therapy of cardiovascular diseases act by mediating the function of L-type Ca\(^{2+}\) channels. These drugs are thought to act at three separate but allosterically coupled receptor sites. Phenylalkylamines (such as verapamil) block the L type Ca\(^{2+}\) channels by binding to the receptors that are found in the S6 segments in domains III and IV [49, 50]. Dihydropyridines act as L-type Ca\(^{2+}\) channel activators or inhibitors by binding to the receptors in S5 segment of the domain III and in S6 segments in domains III and IV.
A third receptor site which is targeted by the benzothiazepines is thought to overlap with the phenylalkylamines receptors [46].

L-type Ca\textsuperscript{2+} channelopathies are involved in the pathogenesis of various cardiac diseases. Timothy syndrome (TS) is an inherited arrhythmogenic disorder that is linked to mutations of the cardiac L-type Ca\textsuperscript{2+} channel [52]. Down regulation of L-type Ca\textsuperscript{2+} channels are found to be a contributory factor for atrial fibrillation [53]. Mutations of the L-type Ca\textsuperscript{2+} channel and their β subunit have been reported in BS, a disease which is characterized by a shorter QT-interval [54].
Figure 1.4: Structure of the voltage-gated Ca\(^{2+}\) channel. The \(\alpha, \beta, \gamma\) and \(\alpha_2\delta\) subunits of the voltage gated Ca\(^{2+}\) channel is illustrated. Roman numerals (I-IV) indicate the domains of \(\alpha\) subunit; segments 5 and 6 are the pore-lining segments and the S4 helices make up the voltage sensors. Adapted from Lehmann-Horn & Jurkat-Rott 1999 [55].
1.1.2.3 L-type Ca\(^{2+}\) channel and excitation contraction coupling

The L-type Ca\(^{2+}\) channel and I\(_{Ca}\) have a fundamental role in initiating cardiac myocyte E-C coupling (Figure 1.5). This can be defined as the process by which an electrical stimulus is converted into a mechanical response.

Arrival of an AP causes conformational changes that lead to the opening of the \(\alpha_1\) subunit pore of the L-type Ca\(^{2+}\) channel and Ca\(^{2+}\) current enters the cytosol down its electrochemical gradient. This small inward flow of Ca\(^{2+}\) current binds to RYR2 and triggers a large release of Ca\(^{2+}\) from the SR into the cytosol by a process known as calcium-induced calcium release (CICR) [33, 56]. The concentration of intracellular free Ca\(^{2+}\) increases 10-fold from 100 nM at rest to about 1 \(\mu\)M [33, 46]. This increase of intracellular Ca\(^{2+}\) is known as the Ca\(^{2+}\) transient. The Ca\(^{2+}\) binds to TnC which causes conformational changes which in turn releases TnI from actin and facilitates myosin and actin cross-bridge interaction to produce cardiac muscle contraction. For relaxation to occur, Ca\(^{2+}\) must be removed from the cytosol. Ca\(^{2+}\) is taken up into the SR via the SR Ca\(^{2+}\)-ATPase (SERCA) [33, 57] and effluxed from the cell primarily on the \(Na^+/Ca^{2+}\) exchange (removes approximately the same amount of Ca\(^{2+}\) that entered the cell through L-type Ca\(^{2+}\) channel) [14, 58-60]. To a smaller extent Ca\(^{2+}\) may also be transported out of the cell by the sarcolemmal Ca\(^{2+}\)-ATPase and taken up into the mitochondria by the Ca\(^{2+}\) uniporter [14, 33, 58].

The amplitude and duration of the Ca\(^{2+}\) transient and the sensitivity of the myofilaments to Ca\(^{2+}\) are known to be important regulators of muscle contraction. The most important determinants of the amplitude of the Ca\(^{2+}\) transient are the Ca\(^{2+}\)
content of the SR and the amount of Ca\(^{2+}\) that enters the cytosol via the L-type Ca\(^{2+}\) channel.

The Ca\(^{2+}\) content of the SR is dependent on the amount of Ca\(^{2+}\) that is pumped from the cytosol into the SR during relaxation. Therefore, decreased uptake of Ca\(^{2+}\) by the SR decreases the amplitude of Ca\(^{2+}\) transient and muscle contraction [61, 62].
Figure 1.5: Excitation-contraction coupling. Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels causes the release of Ca\(^{2+}\) from the SR via ryanodine receptor (RyR) resulting in the generation of a Ca\(^{2+}\) transient and muscle contraction. Relaxation occurs when the Ca\(^{2+}\) is pumped back into the SR by the SERCA pump and effluxed from the myocyte by the NCX (Na\(^{+}/Ca^{2+}\) exchanger). Adapted and modified from https://www.studyblue.com/notes/note/n/chapter-14-cardiovascular-physiology/deck/9845939
1.1.2.4 Potassium channels

A variety of $K^+$ channels function either to maintain the resting potential near the $E_K$ or to repolarize the AP [63]. $K^+$ channels are broadly classified on the basis of their function, molecular structure, and electrical properties. Within the heart, the major classes of voltage-gated $K^+$ channels (VGPCs) are $K_{IR}$ and leak channels. The varied expression of $K^+$ channels contributes to the variability of AP morphology in different regions of the heart [64].

The VGPCs include the rapidly activating and inactivating $I_{to}$ and the delayed outward rectifiers including the ultrarapid ($I_{kur}$), rapid ($I_{kr}$) and slow ($I_{ks}$) $K^+$ channels. Structurally VGPCs (Figure 1.6) are very similar to the VGSCs, however, the channel pore subunit is a heteromultimeric complex of four $\alpha$-subunits (each $\alpha$-subunit is composed of four domains (I-IV), each domain containing six transmembrane segments (S1-S6) instead of one single $\alpha$-subunit as is found in VGSCs. These channel pore subunits are covalently attached to a regulatory $\beta$-subunit.

$I_{to}$ is a brief transient current which is activated during the early phase of AP repolarization [65]. It was first described as an early outward current in sheep purkinje fibers [66]. Subsequent work identified the presence of $I_{to}$ in most heart tissue including ventricles, atria, and in SA and AV nodes. [67-70].

Several studies have classified $I_{to}$ according to their recovery kinetics into fast $I_{to}$ ($I_{to,f}$) and slow $I_{to}$ ($I_{to,s}$) [71]. Molecular studies on cardiac tissue have revealed distinct $I_{to}$ subfamilies. Kv4.2 and Kv4.3 encode $I_{to,f}$ and Kv4.1 encode $I_{to,s}$ channels. [72-74]
I_{Kur}, I_{Kr} and I_{Ks} are involved in late phase of repolarization (Phase 3). The channels open sequentially at membrane potentials near to +10 to +20 mV. They differ from each other based on their activation/inactivation kinetics, pharmacological sensitivity and conductance [64, 75].

I_{Kur} has a very rapid activation and is encoded by KCNA5, which is expressed in the atrium more than in the ventricle. It is more selective to the K^+ blockers 4-aminopyridine (4-AP) than either I_{Kr} or I_{Ks}. However, it is relatively insensitive to tetraethylammonium (TEA), barium (Ba^+) and class III antiarrhythmics of the methanesulfonanilide group [76-80].

A subunit of I_{Kr} encoded by KCNH2 is activated and inactivated rapidly [81], has a large single channel conductance. It is selectively blocked by class III antiarrhythmics of the methanesulfonanilide group. I_{Ks} is the dominant repolarizing current. It has a smaller channel conductance with slow activation and inactivation kinetics. A subunit of I_{Ks} is encoded by KCNQ1 [82].

Collectively, the level of expression of the three subtypes of the delayed rectifier K^+ channel is greater in atrium than the ventricle and this is explained by the shorter AP in atria compared to ventricles.

In physiological and pathological conditions, consideration should be taken that the role of VGPCs on cardiac function must be integrated in the context of the other ion channels. For example, in some heart diseases there is a reduction in I_{in} density and consequently a delay in the rate of early repolarization, which may lead to a reduction
in L-type Ca\textsuperscript{2+} current and decrease in release of Ca\textsuperscript{2+} from the SR which in turn exacerbates impaired Ca\textsuperscript{2+} homeostasis in the heart [65, 83].

The K\textsubscript{IR} channels including K\textsuperscript{+} inward rectifier (K\textsubscript{i}), acetylcholine sensitive K\textsuperscript{+} channel (K\textsubscript{ACh}) and ATP sensitive K\textsuperscript{+} channel (K\textsubscript{ATP}) contribute to the late stage of repolarization and play an important role in setting the resting potential close to E\textsubscript{K}. The structure of K\textsubscript{IR} contains two transmembrane domains (M1 and M2) connected by a pore and intracellular N- and C-termini [63, 64, 75, 84], and K\textsubscript{i} is expressed more in ventricle than in atrium [85].

The I\textsubscript{K,ACh} channel regulates heart rate (HR) and conduction in response to ACh which is released from the cardiac parasympathetic nerves. Following the binding of ACh to the muscarinic M2 receptor, the G\beta\delta subunit of G-protein receptor interacts directly with the cytoplasmic N- and C-termini of K\textsubscript{ACh} channel and activates the channel. When these channels are activated, the membrane of the myocyte is hyperpolarized, and this is a result of the slow rate of spontaneous depolarization. The K\textsubscript{ACh} are distributed primarily in SA and AV nodes and atrial myocytes [64].

K\textsubscript{ATP} channels respond to changes in the metabolic state of the myocyte and intracellular ATP concentration. Based on their molecular properties, two distinct subtypes have been described in the heart. One is localized in the sarcolemmal membrane, while the second is localized in the mitochondrial membrane. These channels are activated during cell stress when the ATP/ADP ratio is altered and provide large conductance of I\textsubscript{K}. Blockers of these channels, for example sulfonylurea agents are used in treatment of type two diabetes mellitus (T2DM).
The K⁺ selective channels are important in the genesis of several atrial and ventricular arrhythmias. Dysregulation of $I_{no}$ has also been postulated to play a pivotal role in BS. LQTS type 1 and type 2 syndromes have been reported with abnormalities in genes encoding $I_{Ks}$ ($KCNQ1$) and $I_{Kr}$ ($KCNH2$) respectively.
Figure 1.6: Structure of potassium channels. (a) Voltage-gated $\text{K}^+$ channel. The $\alpha$ subunit is a six-transmembrane domain one-pore-region of a tetrameric complex four comprising $\alpha$ subunits. (b) Inward-rectifying $\text{K}^+$ channel ($\text{K}_{\text{IR}}$). The $\alpha$ subunit is one transmembrane domain one-pore-region of tetrameric complex of four $\alpha$ subunits. Modified from Tamargo et al. 2004 [86].
1.1.3 Conduction system of the heart

The heart has a specialized conduction system which facilitates electrical excitation in an orderly and sequential manner during each cardiac cycle. APs generated in the SA node, spread into the atria and the AV node, via the AV bundle (Bundle of His), to the Bundle branches (interventricular septum), to the Purkinje fibers and the ventricles. The spread of conduction varies in different regions of the heart. The conduction is very slow in the AV node and this plays an important role in timing atrial and ventricular systole. Conduction is very rapid in the AV bundle, Bundle branches and Purkinje fibers, the rapid conduction in these fibers coordinates electrical activation of the ventricles and therefore the synchrony needed for efficient ventricular function [87, 88].

The conduction of electrical activity in the heart also depends on current flow between adjacent myocytes (longitudinal conduction). Gap junction proteins (mainly CXs) provide low resistance electrical pathways between adjacent myocytes that are essential for propagation of electrical impulses throughout the heart [87]. Electrical activity of the heart is commonly recorded by an array of electrodes placed on the body surface and the resulting voltage trace is called an electrocardiogram (ECG). The normal ECG represents a series of waves including P, QRS and T and typically each ECG record lasts for 800 ms (Figure 1.7). These waves initially have been identified by the father of the ECG, Einthoven Willem [89]. The P-wave is the first upward deflection and indicates atrial depolarization. The QRS complex normally begins with a downward deflection (Q) which represents depolarization of the basal portion of interventricular septum. The large upward deflection (R) represents
depolarization of the apical portion of the interventricular septum and ventricles and
the downward (S) represents depolarization of the basal portion of the ventricles. The
QRS complex collectively represents ventricular depolarization. The T wave is
normally a small upward deflection and represents ventricular repolarization.

The P-R interval is the duration between the onset of P wave and the onset of the Q
wave, which signifies atrial depolarization and conduction of impulses through the
AV node. The Q-T interval is the duration between the onset of the Q-wave and the
end of the T-wave. This interval represents a full ventricle cycle including ventricular
depolarization and repolarization.

The ECG can also provide various secondary measurements of heart function. For
example heart rate variability (HRV), which is the variation of beat to beat interval,
can be measured from the ECG. Analysis of HRV can be used to assess
sympathovagal function.

The clinical significance of the ECG recording is in its use to diagnose a variety of
cardiac pathologies [90]. For example, when the amplitude of the P-wave is elevated
this may represent right atrial hypertrophy. If this is accompanied by a broad based P-
wave this may indicate left atrial hypertrophy or dilatation. When the P-wave is
absent or inverted this may suggest atrial fibrillation. In Bundle branch block QRS is
prolonged or deformed. If the T-wave is tall and broad based with slight asymmetry,
this may predict acute myocardial ischemia. The P-R interval is prolonged in
bradycardia and first degree heart block, and shortened in tachycardia. Prolonged Q-
T interval is associated with myocardial infarction and atrial fibrillation [91].
Abnormalities of HRV have been shown to be associated with an increased risk of cardiac dysfunction in diabetic patients.

Figure 1.7: Normal electrocardiogram. The P-wave represents atrial depolarization, P-R interval represents atrial repolarization, QRS complex represents ventricular depolarization. The period between S and T wave (S-T segment) represents ventricular repolarization. QT interval represents the time between ventricular depolarization and repolarization and the R-R interval represents heart rate (time between beats). Modified from Ashley & Niebauer 2004 [92]
1.2 Diabetes mellitus

1.2.1 Historical background of diabetes mellitus

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders resulting from a failing in insulin secretion, insulin action, or both. Insulin deficiency leads to chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism [93].

3500 years ago an Egyptian physician described a disease that caused patients to lose weight rapidly and urinate frequently. Later the ancient Greeks described the disease as “a melting down of the flesh and limbs into urine”. By 1798 John Rollo recognized excess sugar in the blood as well as the urine. Diabetes comes from the Greek word for “pipe-like or siphon” and describes patients who pass excessive amounts of urine. Mellitus is Latin for “honey-sweet” and is a description of the sweetness of the patient’s urine. In the middle of the nineteenth century the link between the pancreas and DM was identified. In 1889 Mikowski and Von Mering showed that pancreatectomy in dogs caused a state of polyuria. As soon as the link between the pancreas and diabetes was recognized, researchers focused on treating diabetes with pancreatic extracts. Within a year of the first successful pancreatic extraction, insulin was sufficiently purified for therapeutic administration to patients with DM. Chemical identification of insulin was reported by Abel in 1926. At present, more than 300 insulin analogues have been identified, including about 70 animal, 80 chemically modified, and 150 biosynthetic insulin preparations. As drug treatments were being developed it was also increasingly recognized that diet had an important role to play in DM. Oral hypoglycemic agents, developed over the last 50 years, have been the primary mode of treatment for type 2 diabetes mellitus (T2DM).
1.2.2 Epidemiology of diabetes mellitus

DM is a serious global health problem. Currently there are more than 300 million people suffering from DM worldwide. A further increase of almost 50% of diabetic cases is anticipated by the year 2025 [94-96]. Worldwide rates of DM are similar in men and women, although they are slightly higher in men < 60 years of age and in women > 65 years of age. DM is now recognized as one of the top ten leading causes of death in adults. In 2011 DM caused 4.6 million deaths worldwide according to the International Diabetes Federation (IDF). In the United Arab Emirates (UAE) epidemiological studies have shown that DM is a serious and worsening medical problem. The prevalence of DM in the UAE is among the highest in the world. The highest prevalence is found in the population of the Pacific island of Nauru [95]. In 2005 the reported prevalence of DM in UAE citizens was 25% rising to 40% after the age of 55 [97]. Limited UAE regional data suggest that the prevalence of DM in 1995 was only 6% [98].

1.2.3 Diagnostic criteria for diabetes mellitus

The diagnostic criteria and classification of diabetes was first proposed by the World Health Organization (WHO) in 1965. Thereafter, frequent modifications were applied by different national and international organizations and expert groups including the National Diabetes Data Group (NDDG), IDF and the American Diabetes Association (ADA). The current WHO guidelines are based on one of three criteria: fasting blood glucose, abnormal oral glucose tolerance test (OGTT) and hemoglobin A1C (HbA1C) (Table1.1). The classic features of DM include polyuria, polyphagia, polydipsia and unexplained weight loss however, other features may also exist including fatigue,
blurred vision, frequent infections, poor wound healing, dry itchy skin and numbness and tingling in hands, legs and feet. In 2003, the ADA expert committee on the diagnosis and classification of DM recommended lowering the threshold for fasting blood glucose from 6.1 mM (110 mg/dl) to 5.6 mM (100 mg/dl).

Table 1.1: Diabetes diagnostic criteria

<table>
<thead>
<tr>
<th>Condition</th>
<th>2 hours glucose mM (mg/dl)</th>
<th>Fasting glucose mM (mg/dl)</th>
<th>HbA1c %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>≤ 7.8 (≤ 140)</td>
<td>≤ 6.1 (≤ 110)</td>
<td>≤ 6.0</td>
</tr>
<tr>
<td>Impaired fasting glycaemia</td>
<td>≤ 7.8 (≤ 140)</td>
<td>≥ 6.1 (≥ 110) &amp; ≤ 7.0 (≤ 126)</td>
<td>6.0-6.4</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td>≥ 7.8 (≥ 140) &amp; ≤ 11.1 (≤ 200)</td>
<td>≤ 7.0 (≤ 126)</td>
<td>6.0-6.4</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>≥ 11.1 (≥ 200)</td>
<td>≥ 7.0 (≥ 126)</td>
<td>≥ 6.5</td>
</tr>
</tbody>
</table>

1.2.4 Pre-diabetes stage (intermediate hyperglycaemia)

The WHO defines impaired fasting glucose as a fasting plasma glucose level of \( \geq 6.1 \) mM and \( \leq 7 \) mM and impaired glucose tolerance as 2 hour plasma glucose, post glucose challenge of \( \geq 7.8 \) to \( \leq 11.1 \) mM. If the glucose levels do not meet the criteria for diabetes, yet are higher than those considered normal, those people would be classified as pre-diabetic [99, 100]. This state indicates the relatively high risk for future development of T2DM. According to the ADA expert panel, 70% of individuals with pre-diabetes will progress to diabetes within 10 years of onset [101, 102]. Pre-diabetes is associated with the simultaneous presence of insulin resistance and pancreatic beta cell dysfunction that starts before glucose level changes are detectable. Pre-diabetes begins with an accumulation of excess free fatty acids in adipose tissue, as well as non-adipose tissue such as liver, muscle, and pancreas (visceral fat accumulation). The deposition of visceral fat induces the release of pro-inflammatory adipocytokines such as tumor necrosis factor-\( \alpha \) (TNF\( \alpha \)), interleukin-6 (IL-6), leptin and macrophage migration inhibitor factor, which in turn leads to decreased insulin sensitivity [103]. As indicated from large randomized clinical trials, appropriate lifestyle modifications and pharmacologic interventions in individuals with pre-diabetes have the potential to delay or even prevent development of T2DM.

1.2.5 Classification of diabetes mellitus

1.2.5.1 Type 1 Diabetes mellitus

Type 1 Diabetes Mellitus (T1DM) accounts for approximately 5% to 10% of all cases of diabetes. The incidence rate increases with having a first degree relative with T1DM [104]. Earlier the disease was known as insulin dependent diabetes mellitus which is an
autoimmune disease causing destruction of pancreatic beta cells and an absolute insulin deficiency [105]. The beta cell mediated autoimmune destruction is thought to be triggered by an environmental event, such as a viral infection [106] and genetically determined susceptibility factors increase the risk of such autoimmune phenomena. The peak incidence of T1DM occurs in childhood and adolescence. The onset generally occurs before the age of 30 years, but may be diagnosed at any age [107]. Epidemiological studies have reported no significant gender differences in incidence among individuals diagnosed before age 15 years, however, after the age of 25 years, there is a male excess in incidence [108]. Type 1 diabetic individuals are characteristically of normal weight or thin in stature. T1DM patients are absolutely dependent on exogenously administered insulin for survival. In the absence of insulin treatment people with T1DM are highly prone to diabetic ketoacidosis (DKA) [109].

1.2.5.2 Type 2 diabetes mellitus

T2DM, previously called non-insulin dependent diabetes mellitus accounts for roughly 90% of all affected individuals. The pathogenesis of this form of diabetes is characterized by the following three pathologies: (1) peripheral resistance to insulin; (2) increased production of glucose by the liver and (3) altered pancreatic insulin secretion [110, 111]. T2DM is often associated with a strong familial, probably genetic predisposition. The major risks for developing T2DM are increasing age, sedentary lifestyle and obesity [95, 109, 112-114] and of great concern is the recent increase in T2DM in children [115]. 80% to 90% of cases are obese [116] and obesity itself adds additional insulin resistance which might be alleviated by weight reduction. Furthermore, people with an increased percentage of body fat distributed predominantly
in the abdominal region also have an increased risk for development of T2DM [112, 113]. Hypertension and high blood cholesterol are pathological conditions which also can potentially increase the incidence of T2DM [117]. Unlike T1DM, type 2 diabetic patients do not require exogenous insulin for survival since they still produce insulin. A variety of oral pharmacological agents are used to maintain the blood glucose level. The mechanisms of actions of these pharmacological agents include reduction of insulin resistance in the periphery, decreased hepatic glucose production, decreased gut carbohydrate absorption and promotion of pancreatic beta cell insulin production.

1.2.5.3 Other specific types of diabetes mellitus

Gestational diabetes mellitus (GDM) accounts for about 3% to 10% of diabetes which resembles T2DM in manifestation and etiology. As its name suggests, GDM first appears during pregnancy, commonly between 24 and 28 weeks of gestation. It is proposed that, hormones secreted by the placenta including estrogen, progesterone, growth hormone, corticotrophin-releasing hormone and prolactin decrease the function of insulin, resulting in high blood glucose level. Treatment with dietary changes and/or drugs (usually insulin) can reduce the potential of perinatal complications. Although blood glucose level usually normalizes after birth, 70% of women with GDM eventually develop T2DM [118].

Many genetic abnormalities are associated with an increased incidence of DM. These include Wolfram’s Syndrome (WS) which is an autosomal recessive disorder. Polymorphism in WS associated with T2DM is characterized by insulin deficiency and absence of insulin secreting cells [119, 120]. Maturity-onset diabetes of the young (MODY) refers to a monogenetic disorder of beta cell dysfunction leading to
hyperglycemia which occurs before the age of 25 years. It is characterized by a slow-onset of symptoms including ketosis and impaired insulin secretion [121-123]. Leprechaunism and the Rabson-Mendenhall syndrome are two early onset diseases that have mutations in the insulin receptor gene causing alteration in insulin receptor function and eventually disturbances in blood glucose homeostasis [124, 125]. In addition, DM could be secondary to other pathological conditions such as pancreatitis or trauma. Viruses also have a role in causing diabetes by directly infecting and destroying pancreatic beta cells. Congenital rubella, cytomegalovirus or adenoviruses are most likely to be involved in the pathogenesis of diabetes [126-128].

1.2.6 Pathophysiological mechanism of type 2 diabetes mellitus

The pathogenesis of T2DM as mentioned earlier (section 1.2.5.2) is characterized by the following three disorders: (1) peripheral resistance to insulin, especially in muscle cells; (2) increased production of glucose by the liver and (3) altered pancreatic insulin secretion. Physiologically, insulin elicits its action by binding and activating a specific plasma membrane receptor with tyrosine kinase activity which leads to translocation of glucose transporters to the cell surface and uptake of glucose.

Obesity, physical inactivity and high circulating levels of a number of inflammatory elements such as cytokines strongly modulate insulin action in various body organs including adipose tissue, skeletal muscles and liver and may adversely affect the response of insulin receptors to circulated insulin. This may alter the insulin signaling which in turn may lead to chronic hyperglycemia. Furthermore, the beta cell abnormalities include both reduced beta cell mass, disruptions of beta cell function and reduced ability to secrete sufficient insulin to maintain glucose homeostasis.
Hyperglycemia disrupts organ function by increasing oxidative stress which can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA and tissue injury [129].

1.2.7 Genetic factors influencing type 2 diabetes mellitus

There is strong evidence that T2DM is an inherited disease. This inherent susceptibility has been attributed to a complex genetic origin. The risk of developing T2DM is approximately 3-4 times higher among first degree relatives of diabetic subjects compared to subjects without a family history of diabetes [130]. In 2007 genome studies demonstrated the presence of different T2DM susceptibility loci in different ethnic groups. About 11 predisposition genes/loci have been identified [131]. Interestingly, most of the identified T2DM susceptibility genes exert their effect by affecting beta cell function. The genetic predispositions of T2DM are classified as either monogenic or polygenic. The monogenic forms of diabetes include MODY, neonatal diabetes, mitochondrial diabetes and others. MODY diabetes results from mutation in any of at least eight different genes (Table 1.2).
Table 1.2: Candidate genes involved in MODY

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene loci</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODY 1</td>
<td>20q12-q13.1</td>
<td>Hepatocyte nuclear factor-4α</td>
</tr>
<tr>
<td>MODY 2</td>
<td>7p15-p13</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>MODY 3</td>
<td>12q24.2</td>
<td>Hepatocyte nuclear factor-1α</td>
</tr>
<tr>
<td>MODY 4</td>
<td>13q21.1</td>
<td>Insulin promoter factor-1</td>
</tr>
<tr>
<td>MODY 5</td>
<td>17cen-q21.3</td>
<td>Hepatocyte nuclear factor-1β</td>
</tr>
<tr>
<td>MODY 6</td>
<td>2q32</td>
<td>NEUROD-1</td>
</tr>
</tbody>
</table>
Genetic studies (summarized in Table 1.3) have identified several adipose-expressed genes likely to contribute to the risk of T2DM [132]. The gene variant with the greatest effect on the risk of T2DM is peroxisome proliferator-activated receptor γ (PPARγ). The pro12Ala polymorphism found in PPARγ is strongly associated with the risk of insulin resistance producing T2DM. KCNJ11 encodes the islet KATP- Kir6.2, is located on 11p15.1 and mediates glucose-stimulated insulin secretion. It is also involved in T2DM, where glutamate (E) is substituted for lysine (K) at position 23 (E23K) [133]. More recently, studies reported variants within a novel gene, TCF7L2, as a putative susceptibility gene for type 2 diabetes. TCF7L2 encodes transcription factor 7-like 2 and regulates pro-glucagon gene expression and thus influences the production of glucagon-like peptide-1 [134, 135]. An additional candidate linked to T2DM maps to the region 3q, APM1 gene, encoding the differentiated adipocyte-secreted protein ACRP30/adiponectin, an adipokine [136]. Mutation analyses have also identified the widespread SNB19, 43, 63 variant of the calpain-10 (CAPN10) which is located on chromosome 2q37 and increases the risk of both insulin resistance and insulin secretion [137-139].
<table>
<thead>
<tr>
<th>Gene</th>
<th>Loci</th>
<th>Variants implicated</th>
<th>Encoded proteins</th>
<th>Function of proteins</th>
<th>Putative mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF7L2</td>
<td>10.25</td>
<td>DG105478, SNP790314 in intron 3</td>
<td>Transcription-factor-7-like 2</td>
<td>Wnt-signalling, pancreas</td>
<td>Insulin, glucagon secretion</td>
</tr>
<tr>
<td>PPARγ</td>
<td>3p25</td>
<td>Pro12Al</td>
<td>Peroxisome-proliferator activated receptor γ</td>
<td>Nuclear receptor (transcription factor)</td>
<td>Adipose-tissue-related insulin resistance</td>
</tr>
<tr>
<td>FTO</td>
<td>16q12</td>
<td>Introns 2</td>
<td>Fat mass and obesity protein</td>
<td>Nucleic acid demethylase</td>
<td>Obesity (via insulin resistance)</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>11p15.1</td>
<td>E23K</td>
<td>Potassium-inward rectifier 6.2</td>
<td>Potassium channel, β cell</td>
<td>β-cell dysfunction</td>
</tr>
<tr>
<td>ABCC8</td>
<td>11</td>
<td>E23K</td>
<td>Sulfonyle receptor</td>
<td></td>
<td>Insulin secretion</td>
</tr>
<tr>
<td>IRS1</td>
<td>2q36</td>
<td>Gly972Arg</td>
<td>Insulin receptor substrate</td>
<td>Insulin receptor</td>
<td>Insulin signaling cascade</td>
</tr>
<tr>
<td>CDKAL1</td>
<td>6p22.3</td>
<td>Noncoding</td>
<td>CDK5-regulatory-subunit-associated-protein 1-like 1</td>
<td>---</td>
<td>β-cell development, regeneration</td>
</tr>
<tr>
<td>IGF2BP2</td>
<td>3q27</td>
<td></td>
<td>Insulin-like growth factor 2 mRNA binding protein 2</td>
<td>Transport IGF2 mRNA (translation)</td>
<td>Insulin sensitivity</td>
</tr>
<tr>
<td>SLC30A8</td>
<td>8q24.11</td>
<td>R.325W</td>
<td>Solute carrier family 30, member 8</td>
<td>Zinc transporter in β cell</td>
<td>β-cell dysfunction</td>
</tr>
<tr>
<td>HHEX</td>
<td>10</td>
<td></td>
<td>Haemopoietically expressed homoeobox or insulin-degrading enzyme</td>
<td>---</td>
<td>pancreas or liver development; IDE: insulin action, insulin-release disturbance</td>
</tr>
<tr>
<td>CAPN10</td>
<td>2q37</td>
<td>SAN 19,43,44 and 63</td>
<td>Calpain-10</td>
<td>Calcium regulated cysteine protease</td>
<td>Insulin secretion and resistance</td>
</tr>
</tbody>
</table>
1.2.8 Type 2 diabetes mellitus and associated complications

The major cause of the high morbidity and mortality rate associated with T2DM is development of a group of microvascular and macrovascular complications affecting multiple organ systems. The onset and progression of these complications is strongly linked to the presence of sustained hyperglycemia and may be accompanied by other disorders such as hypertension and dyslipidemia which are commonly seen in people with T2DM. Macrovascular complications primarily affect large blood vessels resulting in systemic diseases such as coronary artery disease, stroke and peripheral vascular disease. Microvascular complications are those affecting smaller blood vessels, primarily the kidneys (nephropathy) which can result in total kidney failure, eyes (retinopathy) characterized by damage to the retina of the eye which can lead to vision loss and neural abnormalities (neuropathy) which result in loss of feeling and ultimately may lead to ulceration and amputation of the toes, feet and lower limbs [109, 140].

1.2.8.1 Impact of type 2 diabetes mellitus on the cardiovascular system (diabetic cardiomyopathy)

Data from epidemiological and clinical trials have confirmed a higher incidence of cardiovascular diseases in the type 2 diabetic population, independent of other potential etiological factors such as hypertension or coronary artery diseases [141-143]. The risk of heart failure is about 4 fold in type 2 diabetic comparable to non-diabetic patients and is more likely in people over the age of 64 years [144, 145]. Diabetic cardiomyopathy (DCM) is defined as ventricular dysfunction secondary to DM associated with hypertrophy, fibrosis and significant cardiac dysfunction [146-149]. Although the pathogenesis of DCM is not completely understood, the suggested mechanisms of
development of DCM may include reduced energy production due to decreases in mitochondrial respiration and pyruvate dehydrogenase activity, cardiac contractile dysfunction and impaired intracellular \( \text{Ca}^{2+} \) homeostasis [150, 151]. Structurally diabetic hearts may have increased interventricular septum thickness, left atrial diameter and left ventricular (LV) mass index [152]. Myocardial fibrosis was initially described by Rubler et al in 1972 following anatomical dissection of hearts from 4 patients with diabetes and heart failure but without arterial hypertension or coronary artery disease. These hearts showed LV hypertrophy and fibrosis without evidence of coronary artery atheroma or other substrate pathology [146]. These findings were confirmed later by several histological and experimental studies. Most of the results showed an increase in myocardial density in diabetic subjects compared with control subjects [153]. Specific biomarkers for collagen synthesis or collagen degradation have been used in clinical practice to detect myocardial fibrosis and are found to be high in diabetic patients.

1.2.8.2 Impact of type 2 diabetes mellitus on mechanical function of the heart

Patients with T2DM show a higher incidence of cardiac arrhythmias, including ventricular fibrillation and sudden death. The ECG of type 2 diabetic patients may show several alterations from normal patterns, many of them related to the QT interval [143, 154]. Doppler imaging, echocardiography, radionuclide angiography and other techniques have demonstrated a variety of diastolic and systolic dysfunctions in type 2 diabetic patients and the severity of the abnormalities depend on the patients’ age and the duration of diabetes. Diastolic dysfunction is the most prominent mechanical defect and is thought to be the earliest detectable functional abnormality in DCM [155, 156]. Diastolic dysfunction has been associated with increases in LV mass, decreased
compliance and slower rates of myocardial relaxation [157, 158]. Hemodynamic abnormalities include reduced LV ejection fraction, impaired myocardial velocity at early diastole, abnormal relaxation during the early filling phase, prolonged isovolumetric relaxation, lower peak systolic and early diastolic velocity, impaired diastolic relaxation and filling and reduced peak filling rate [152, 159-165].

1.2.8.3 Molecular basis of diabetic cardiomyopathy

The development of DCM and the cellular and molecular perturbations associated with the pathology are complex and multifactorial. Several experimental studies have shown that genes encoding proteins that are involved in Ca\(^{2+}\) transport and cardiac muscle contraction are variously altered in DCM. Some alterations in the regulation of gene expression may be associated with cardiac muscle functional defects, whilst others may be associated with compensatory mechanisms. The \(\beta2\) subunit of the L-type Ca\(^{2+}\) channel has been reported to play important roles in many functional aspects of the Ca\(^{2+}\) channel and in particular it has a role in modifying inactivation kinetics [166]. In diabetic rat heart, the L-type Ca\(^{2+}\) channel \(\beta2\) subunit gene (Cacnb2) was found to be downregulated compared to control rat heart. Myosin light chain 2 (Myl2) and SERCA1 (Atp2a1) were also downregulated. Mutations in Myl2 have been shown to cause hypertrophic cardiomyopathy, which is characterized by ventricular hypertrophy associated with reduced compliance of the heart and myofibrillar disarray [167]. SR Ca\(^{2+}\) ATPases (SERCA\(s\)) play a major role in the control of Ca\(^{2+}\) signaling [168]. Previous experiments have demonstrated that overexpression of SERCA1 increased shortening amplitude and enhanced relaxation kinetics in failing human myocytes [169]. Interestingly, SERCA1 (Atp2a1) was downregulated in diabetic animal models.
Gating of Cav1.2 channels may regulate Ca\(^{2+}\) influx and excitability in neurons, cardiac and arterial smooth muscle. Upregulation of Caenalc, which encodes the α1 subunit of the Cav1.2 ion channel, may provide an early compensatory mechanism [170]. If upregulation of Caenalc translated into upregulation of the α1 subunit, then this might alter the voltage sensitivity and perhaps the activation and/or inactivation properties of the L-type Ca\(^{2+}\) channel, which in turn might provide a compensatory mechanism for the reduced density and prolonged inactivation of L-type Ca\(^{2+}\) current. T-type Ca\(^{2+}\) channels are implicated in cardiac automaticity and cardiovascular remodeling. Upregulation of Caenalγ and Caenalh, the two isoforms of T-type Ca\(^{2+}\) channel genes in the heart, may contribute to changes in channel activity [171, 172]. Previous studies have demonstrated altered Ca\(^{2+}\) sensitivity in the regulation of the cardiac actin-myosin filament in diabetic heart and this might be attributed in part to changes in expression of contractile proteins [173]. One factor that can affect myofilament sensitivity to Ca\(^{2+}\) is intracellular pH [174]. Decreased Na\(^{+}/H^{+}\) exchange activity which has been previously demonstrated in diabetic rat heart, leads to a decrease in pH and as a consequence, a decrease in myofilament sensitivity to Ca\(^{2+}\) [175]. Previous studies have demonstrated abnormal SR Ca\(^{2+}\) transport, including SR Ca\(^{2+}\) content and reduced rates of Ca\(^{2+}\) release and resequestration in diabetic heart and these defects have been associated with decreased SR Ca\(^{2+}\)-ATPase and ryanodine receptor proteins [176].

### 1.2.8.4 Diabetic heart and impaired calcium handling

Ca\(^{2+}\) is a critical regulator of cardiac function, because it links electrical depolarization of the cardiomyocyte with contraction [33]. Several experimental studies at the cellular level have linked the alteration of intracellular Ca\(^{2+}\) homeostasis and the incidence of
cardiac dysfunction in the diabetic heart [177, 178]. Cardiac Ca\textsuperscript{2+} signaling in diabetes has been most extensively studied in myocytes isolated from the ventricles of various diabetic rodent models. It was found that during diabetes there was an impairment in SR Ca\textsuperscript{2+} transport [179]. Decreased SR Ca\textsuperscript{2+} content and decreased spontaneous SR Ca\textsuperscript{2+} release and/or decreased SERCA function will result in a reduction in the amplitude of the Ca\textsuperscript{2+} transient and prolonged Ca\textsuperscript{2+} transient decay during relaxation phase [180]. The molecular mechanism for SERCA depression may be partly attributed to changes in mRNA level (gene expression) of the SERCA protein which has been reported in diabetic heart. Moreover, the sarcolemma Ca\textsuperscript{2+} pump and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity together with efflux of Ca\textsuperscript{2+} through the plasma membrane are depressed in the diabetic heart [181]. Additionally, alteration in mitochondrial Ca\textsuperscript{2+} transport might have a role in diabetic rat heart [178]. Cardiac β-adrenoceptor function is also altered in diabetic animals. These alterations include depressed β-receptor number, a reduced response to β-adrenergic agonists and a significant alteration in the response and sensitivity to perfusate Ca\textsuperscript{2+} stimulation [182-184]. These results collectively support the hypothesis that DM leads to an alteration in Ca\textsuperscript{2+} transport in the heart.

1.2.9 Experimental models of diabetes mellitus

In 1890, when Von Merhring and Minkowski began studying the digestive functions of the pancreas they discovered that the animals on which they had performed a pancreatectomy developed acute DM. Since then a large number of experimental models have been developed to study diabetes and its complications. These include chemical-induced models which utilize diabetogenic agents such as epinephrine, glucagon, growth hormones, streptozotocin and alloxan [185-187], diet-induced models
which involve feeding animals with modified diets which may be enriched with fructose or sucrose [178] and various genetic models of diabetes including the Zucker and Goto-Kakizaki (GK) rats [188-190]. Diabetes has been studied in different species including mouse, rat, rabbit, cat and dog. The general characteristics of DM found in different experimental models may include hyperglycemia, hyper- or normo- or hypoinsulinemia, polyuria, polydypsia, polyphagia, glucosuria and hyperlipidemia [191]. To varying degrees these experimental models exhibit diabetic complications including neuropathy, nephropathy, cardiomyopathy, atherosclerosis and hypertension.

1.2.9.1 The Goto-Kakizaki rat, an animal model of type 2 diabetes mellitus

The GK rat, a polygenic model of T2DM, is one of the best characterized animal models used for studying T2DM and its related complications. The GK diabetic rat was produced by Goto and his collaborators through selective inbreeding of Wistar rats with abnormal glucose tolerance repeated over several generations in Japan in 1973 [190]. It is characterized by moderate but stable fasting hyperglycaemia, hypoinsulinaemia, normolipidaemia, impaired glucose tolerance which appears at 2 weeks of age and an early onset of diabetic complications [192-194]. Impaired insulin sensitivity in the liver, skeletal muscle and adipose tissues has also been reported. Impaired insulin secretion and hepatic glucose overproduction (hepatic insulin resistance) are early events in diabetic GK rats which contribute to the development of hyperglycaemia. In adult GK rats the total pancreatic beta cell mass is decreased by 60% and this is accompanied by a decrease in pancreatic insulin stores and insulin secretion, similar characteristics that are seen in human T2DM [195]. Table 1.4 shows the comparison of pancreatic beta cell functional characteristics in GK rat and type 2 diabetic human.
Table 1.4: Comparison of pancreatic beta cells in GK rat and human type 2 diabetic

<table>
<thead>
<tr>
<th>Features</th>
<th>GK Rat</th>
<th>Humans T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin response to glucose</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Glucose oxidation</td>
<td>Decreased/ not</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>changed</td>
<td></td>
</tr>
<tr>
<td>Insulin response to GLP-1</td>
<td>Not changed</td>
<td>Not changed</td>
</tr>
<tr>
<td>m- GPDH activity</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>ATP/ADP ratio</td>
<td>Decreased</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

1.2.9.2 Genetic basis of diabetes mellitus in the Goto-Kakizaki rat

The use of an animal model can facilitate the search for genetic components involved in the onset and progression of T2DM. The GK rat is an excellent polygenic model for studying genetic susceptibility to T2DM and exhibits mild fasting glucose intolerance as early as 2 weeks of age (high basal plasma insulin levels) and impaired glucose tolerance by 4 weeks of age. Genetic studies in human populations and GK rat have identified regions of the human chromosome and rat chromosome showing evidence of significant linkage to diabetes-related phenotypes. The search for identification of the morbid genes in GK rat using a quantitative trait locus (QTL) approach has led to identification of loci that regulate fasting plasma glucose and insulin levels, glucose tolerance, insulin secretion and adiposity (Table 1.5) [196, 197]. *Niddml*, recently identified as a short region locus of *Niddml*, contributes to defective insulin secretion and is homologous to a region on human chromosome 10 that is associated with T2DM and includes *TCF7L2* [198, 199]. *IDE* and *SHIP2* genes localized in chromosome 1 in GK rat, encoding insulin degrading enzyme and phospholipid phosphatase respectively have also been implicated in T2DM [200, 201].
Table 1.5: Candidate genes involved in pathogenesis of diabetes mellitus in the GK rat

<table>
<thead>
<tr>
<th>Loci /gene</th>
<th>Linkage phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niddm1</td>
<td>IGT + impaired insulin secretion + insulin resistance with age associated with obesity and dyslipidemia</td>
</tr>
<tr>
<td>Sub loci</td>
<td></td>
</tr>
<tr>
<td>Niddm1b</td>
<td></td>
</tr>
<tr>
<td>Niddm1i</td>
<td></td>
</tr>
<tr>
<td>Nidd/gk1</td>
<td></td>
</tr>
<tr>
<td>Ide</td>
<td>IGT + reduce lipogenesis in fat + blunted insulin stimulated glucose transmembrane uptake</td>
</tr>
<tr>
<td>hpp1l or</td>
<td>Negative regulator of insulin signaling</td>
</tr>
<tr>
<td>Ship2</td>
<td></td>
</tr>
<tr>
<td>Niddm2</td>
<td>Fasting glucose + IGT</td>
</tr>
<tr>
<td>Weight 1</td>
<td>Body weight and fasting insulin level</td>
</tr>
<tr>
<td>Nidd/gk5</td>
<td>Insulin secretion and plasma triglycerides, phospholipids and HDL, LDL and total cholesterol</td>
</tr>
<tr>
<td>Niddm3</td>
<td>Fasting glucose + IGT</td>
</tr>
</tbody>
</table>

 1.2.9.3 Goto-Kakizaki heart function

The GK model provides an opportunity to study the effects of T2DM on the heart without other complications such as obesity. It was previously demonstrated that the heart of GK rats is insulin-resistant with a decreased insulin-stimulated glucose uptake and an impaired insulin signaling pathway [202]. Heart function in GK rats is frequently compromised. Several studies have revealed that the GK heart is bradycardic.
Biotelemetry data have demonstrated that HR and HRV were significantly reduced in GK rats [203]. In addition the GK heart action potential is modestly prolonged [192]. Magnetic resonance imaging has demonstrated reduced myocardial blood flow and reduced ejection fraction, mainly as a result of a reduction in LV longitudinal contraction [204]. The results obtained from ventricular myocytes isolated from GK rats are controversial. Some studies have shown that the amplitude of shortening and corresponding amplitude of the Ca$^{2+}$ transient were unaltered [205] whilst others have shown increased amplitude of the intracellular Ca$^{2+}$ transient in ventricular myocytes from 18-month old GK rats [192].

1.2.10 Type 2 diabetes mellitus management and consequences for heart function

1.2.10.1 Exercise and heart function

An abundance of evidence exists for prescribing exercise therapy in the treatment of a variety of diseases including metabolic syndrome-related disorders (T2DM, dyslipidemia, hypertension and obesity), heart, pulmonary and muscle diseases, depression and asthma. Epidemiological studies have shown that regular physical activity promotes cardiorespiratory fitness and that diabetic individuals who maintain a physically active lifestyle are much less likely to develop cardiovascular diseases related to diabetes. The positive physiological adaptation of the normal heart during exercise training (EXT) is manifested as increased delivery of blood to the peripheral vasculature to meet the enhanced demands of the musculoskeletal and pulmonary systems. This is usually achieved with an increased cardiac output (CO) through a combined increase of HR, stroke volume (SV), and/or myocardial contractility [206, 207]. The most significant benefits of physical EXT on the diabetic heart are due to
improving glucose and lipid metabolism and reduced insulin resistance however, the mechanisms underlying these beneficial effects are still unclear. In skeletal muscles, it was proposed that the beneficial effects of EXT on glucose homeostasis could be attributed to an increase in cellular glucose transporter 4 (GLUT4) in response to insulin [208-210].

An increase in abdominal fat accumulation and loss of muscle mass are associated with the development of insulin resistance. EXT results in preferential loss of fat from central regions of the body leading to a reduction of insulin resistance. EXT can prevent muscle atrophy and stimulate muscle development [211]. Several experimental studies in diabetic animals have shown that EXT has positive effects on beta 1 adrenoceptor density and these beneficial effects are due in part to normalization of the sympathetic outflow and improvement in the responsiveness of the myocardium to autonomic stimulation [212, 213]. Moreover, exercise reduces circulating levels of ANG II, aldosterone, vasopressin, neuropeptide Y, ANP and pro-inflammatory mediators [214]. EXT significantly improves cardiac function by enhancing both fractional shortening and ejection fraction.

1.2.10.2 Pharmacological therapy

A number of oral pharmacological agents developed during the last 60 years have been successfully used in the treatment of T2DM. The mechanisms of action of these agents to lower blood glucose are distinct. Currently, there are seven classes of oral hypoglycemic agents approved by the FDA. Sulfonylureas stimulate insulin secretion upon binding to their receptors which are located in the beta cells of the pancreas. They block K_{ATP} channels, which induces depolarization of the membrane, Ca^{2+} influx
1.2.10.3 Peroxisome proliferator-activated receptors

The PPARs belong to a family of nuclear receptors, classified as transcription factors, which have the ability to bind to DNA and regulate the expression of various genes. Some of these genes play diverse roles in cell differentiation, development and metabolism [222-224]. In mammals, three PPARs encoded by different genes have been identified thus far, including PPARα, PPARγ and PPARβ/δ. PPARs control the expression of genes involved in numerous physiological responses such as adipogenesis, lipid metabolism, inflammation and metabolic homeostasis [225].

Each PPAR isoform has unique actions which are attributable to distinct tissue expressions and differential responses to different ligands [223, 226]. PPARα is expressed in liver, kidney, heart and brown adipose tissue [227] and has a major role in β oxidation of fatty acid. Activation of PPARα induces expression of the fatty acid transport protein (FATP) and fatty acid translocase (FAT) both of which are responsible for transporting fatty acids across the cell membrane [228, 229]. They also enhance the expression of enzymes involved in fatty oxidation such as long chain fatty acid acetyl CoA synthase and others [228]. Fibrate drugs are the molecular target of PPARα and have been proven effective in the treatment of dyslipidemia. PPARβ/δ shares similar function with PPARα and is expressed in a wide range of brain and adipose tissue, heart and skeletal muscles [225, 230]. PPARγ is widely expressed in white adipose tissue and other tissues and is a regulator of adipogenesis and a modulator of whole body lipid metabolism and insulin sensitivity [231].
1.2.10.4 The role of Peroxisome proliferator-activated γ receptor

PAPAR γ1, PAPAR γ2 and PAPAR γ3 isoforms have been identified [232, 233]. In structure PPARγ is similar to other PPARs and comprises four functional domains including a highly conserved DNA binding domain (DBD) composed of two zinc fingers, an N-terminal transactivation domain (AF1), a C-terminal ligand binding domain (LBD) and a ligand-dependent transactivation function domain (AF2) [226].

After ligand binding PPARγ undergoes conformational changes and forms a heterodimer with the retinoid X receptor (nuclear receptor), this heterodimer recognizes a specific DNA response element called PPAR response element (PPRE) in the promoter region of target genes, which recruits cofactor proteins including coactivators or corepressors.

Several endogenous ligands for PPARγ have been described including fatty acid and their derivatives, eicosanoids, lysophosphatidic acid and nitrolionic acid [224, 234-236]. TZDs are potent exogenous agonists of PPARγ, and have been used for the treatment of hyperglycemia in T2DM since 1997 [237, 238] (Figure 1.8).

The actions mediated by PPARγ modulate the transcription of a number of genes responsible for improved insulin sensitivity. These genes mainly regulate factors which are known to play an important role in the development of insulin resistance in T2DM such as fatty acid, adiponectin and inflammatory markers.

Activation of PPARγ by TZDs promote fatty acid uptake into adipose tissue, promote adipogenesis and induce adipocyte differentiation to enhance storage of fat, thus reduce circulating fatty acid concentration. Activation of PPARγ also enhances adiponectin
production in adipose cells and increases adiponectin plasma concentration. TZDs binding to PPARγ are thought to reduce plasma levels of inflammatory mediators such as TNFα and plasminogen activator inhibitor-1 (PAI-1) and IL-6 [232, 239-242].

The role of PPARγ in glucose homeostasis was confirmed in human and animal genetic studies. Obese mice with a mutation on serine 112 of PPARγ showed a decrease in PPARγ phosphorylation and an increase in PPARγ activity, thus protecting mice from development of obesity and associated insulin resistance [243]. On the other hand, mice lacking PPARγ are predisposed to developing insulin resistance [244]. Similar results were found in humans. Dominant negative mutation in human PPARγ cause severe insulin resistance, while carriers of Pro12Ala polymorphism in the PPARγ gene reduces the risk of T2DM and improves glucose homeostasis [245, 246].
Figure 1.8: Structure and molecular mechanism of action of peroxisome proliferator-activated receptor γ (PPARγ). (a) PPARγ structure has four functional domains: the transactivation domain (AF-1), DNA binding domain, ligand binding domain (LBD) and ligand dependent transactivation AF-2 domain. (b) Thiazolidinediones (TZDs) binds PPARγ, the PPARγ and retinoid X receptor (RXR) heterodimer binds to PPARγ to PPAR response elements (PPRE) recruit diverse cofactors that modulate the transcriptional activity of the target gene.
1.2.10.5 Thiazolidinedione: Pioglitazone

TZDs showed great promise as PPARγ receptor-mediated oral therapy for the treatment of T2DM. Clinical studies have revealed their effectiveness at lowering fasting and postprandial blood glucose and improving insulin sensitivity. The potency of these agents in lowering glucose is strongly correlated with receptor binding and agonism [247]. Troglitazone, the first TZD marketed in 1997 for the treatment of T2DM, showed beneficial effects on insulin sensitivity, blood glucose level and circulating free fatty acids. In 2000, troglitazone was withdrawn from the market due to serious hepatotoxicity [248]. Rosiglitazone was approved in 2000 for treatment of T2DM. However, large meta analysis of randomized clinical trials that evaluated the glycemic control of rosiglitazone revealed that rosiglitazone was associated with a significant increase in the risk of myocardial infarction and death from cardiovascular diseases [249]. In 2010, the European Medicines Agency recommended suspension of rosiglitazone, while the FDA severely restricted its use [250, 251]. In 2013 the FDA removed this restriction on the basis of the results of Rosiglitazone Evaluated for Cardiovascular Outcome and regulation of Glycemia in diabetes (RECORD) clinical trial which indicated no elevation of the risk of heart attack or death in patients treated with rosiglitazone [252].

Currently, pioglitazone (PIO) is the only TZD approved for second or third line treatment of T2DM [253]. It can be administered as a monotherapy or in combination with metformin, sulfonylureas, exenatide, dipeptidyl peptidase 4 inhibitors or insulin [254]. The clinical pharmacology data following oral dose of 30 mg PIO showed a peak plasma concentration achieved 2 hrs after administration, while steady state is achieved
after 4-7 days of dosing. PIO undergoes hepatic metabolism via cytochrome P450 2C8 into six metabolites (M-I - M-VI). Among them, M-II, M-III and M-IV are active metabolites. The efficacy of metabolite M-III shows similar to unchanged PIO efficacy, while metabolite M-IV shows three fold higher activity than unchanged PIO and M-II has a minimal efficacy. The mean plasma elimination half-life of unchanged PIO in man is 5-6 hrs and for total active metabolites 16-23 hrs.

Besides its insulin sensitizing effects, PIO has been reported to lower blood pressure in human and experimental animals [255-258]. In animal studies PIO appears to improve vascular tone and decrease endothelial dysfunction by stimulating of nitric oxide and reducing endothelin-1 production. PIO compared with rosiglitazone has beneficial effects on triglycerides, and total cholesterol [259]. PIO may also exert an anti-inflammatory effect as well as decreasing hepatic inflammation in overweight rats on high cholesterol and fructose diet [260]. Clinical and experimental studies have also shown that PIO has atheroprotective effects in humans and animals with atherosclerosis [261, 262].

Data from clinical studies has revealed that PIO has other TZD side effects. A major side effect of PIO is weight gain which is due to expansion of the subcutaneous fat depot and this may vary greatly depending on the individual and on the treatment regimen employed. Another important side effect is sodium and water retention, which can provoke edema, anemia and even aggravate or reveal heart failure [263, 264]. Clinical trials have shown that PIO treatment is associated with reductions in bone formation and density and an increased risk of fracture and/or osteoporosis particularly in women [265]. Furthermore, the use of PIO has been associated with an increased risk
of bladder cancer [255]. In 2011, and based on the risk of bladder cancer the FDA
issued a warning, while France suspended and Germany restricted the use of PIO.

1.2.10.6 Pioglitazone: Heart benefit or risk?

Following concerns about the cardiovascular safety of rosiglitazone, greater caution in
the use of TZDs particularly in patients with a history of heart failure was urged.
Several meta analysis and clinical and experimental studies have been conducted to
evaluate the effects of PIO on cardiovascular outcomes. Although the side effects of
PIO include sodium and water retention, which can provoke edema and aggravate or
reveal heart failure, several clinical studies have shown that PIO exerts cardiovascular
benefits. A randomized prospective PIO clinical trial in macrovascular events
(PROactive study) demonstrated that PIO was associated with a reduction in mortality,
non fatal MI and stroke in patients with type 2 diabetes compared with placebo [255].
The results of a meta analysis conducted in 2007 which included 19 trials and 16,390
patients with drug duration ranging from 4 months to 3.5 years showed a significant
lower risk of death, MI or stroke among diabetic patients receiving PIO. However, PIO
increased severe heart failure with no increase in mortality [266]. Another clinical study
has shown that PIO does not affect heart function in diabetic patients receiving PIO
treatment [267].

In asymptomatic T2DM patients with LV diastolic dysfunction, adding PIO does not
induce any adverse or favorable changes in heart function, despite improvement in
glycemic control, insulin sensitivity, lipid profile and blood pressure [268]. A study
conducted in T2DM patients having systolic dysfunction demonstrated an excess of
overnight hospitalization for revisable heart failure for patients treated with PIO,
without an excess of cardiovascular mortality or aggravated cardiac function [269]. Other studies have shown PIO attenuates congestive heart failure-induced atrial structural remodeling and atrial fibrillation in rabbit heart [270] and improves LV remodeling and function in mice with post-myocardial infarction heart failure [271]. It also reduces angiotensin II induced cardiac hypertrophy in rats in vitro and in vivo [272]. In another study, PIO ameliorated LV hypertrophy and fibrosis in the heart of hypertensive rats [273] and attenuated cardiac hypertrophy and fibrosis in angiotensin II-induced cardiac hypertrophy in mice [274]. In addition, PIO has been shown to protect against ischemia/reperfusion injury when administered prior to ischemia in isolated perfused rat heart [275].
Hypothesis

The progression of electromechanical dysfunction in diabetic heart might be delayed or normalized by exercise training or pharmacological anti-diabetic treatment.

The main objectives of the project were to investigate the effects of:

1. DM on ventricular myocyte contraction and intracellular Ca\(^{2+}\) in young GK type 2 diabetic rat.

2. DM on expression of genes encoding various cardiac muscle proteins in young GK type 2 diabetic rat.

3. Exercise training on ventricular myocyte contraction, intracellular Ca\(^{2+}\) transport in adult GK type 2 diabetic rat.

4. Exercise training on expression of genes encoding various cardiac muscle proteins in adult GK type 2 diabetic rat.

5. Exercise training on expression of various cardiac proteins in adult GK type 2 diabetic rats.


7. PIO on ventricular myocyte contraction, intracellular Ca\(^{2+}\) transport in adult GK type 2 diabetic rat.

8. PIO on cardiac electrical conduction in adult GK type 2 diabetic rat.
Chapter 2: Materials and Methods

2.1 Ethical approval

All experimental procedures were performed with approval of the Animal Research Ethics Committee of the College of Medicine and Health sciences (CMHS), United Arab Emirates University (UAEU), Al Ain, UAE.

2.2 Experimental animals

Experiments were conducted in male GK type 2 diabetic rats and age-matched male Wistar controls. GK rats were obtained from Taconic (Germantown, New York, USA) and age-matched Wistar control rats were bred at our animal facility, CMHS, UAEU. The animals were accommodated in the animal facility, in polypropylene cages (43 x 22.5 x 20.5 cm; five rats/cage). The temperature was maintained between 22-24 °C, 50% humidity and animals were exposed to 12 hrs/12 hrs day/night cycle. The animals were fed a standard maintenance diet (Emirates Feed Factory, Abu Dhabi, UAE) ad libitum with free access to water.

Body weight, fasting blood glucose and blood glucose at 120 min after an interaperitoneal (i.p) injection of glucose (2 g glucose/kg body weight) were measured periodically. GK rats with normal fasting blood glucose and IGT were excluded from the experiments. For in vitro studies, body weight, heart weight and non-fasting blood glucose were measured immediately prior to experiments.

2.3 Experimental design

In one set of experiments, young GK rats at 8 weeks of age (early onset diabetes) were used. Ventricular myocyte contractility and intracellular Ca²⁺ concentration were
measured and expression profiles of genes encoding a variety of cardiac muscle proteins were evaluated. The effects of chronic diabetes and the effect of different diabetes treatment intervention including exercise training as non-pharmacological and PIO as pharmacological treatment were evaluated using animals at 8-10 months of age. This was done by evaluating ventricular myocyte shortening, intracellular Ca\(^{2+}\) concentration, SR Ca\(^{2+}\) transport, myofilament sensitivity to Ca\(^{2+}\) and L-type Ca\(^{2+}\) current. Furthermore, ventricular muscle morphology and expression of genes encoding a variety of cardiac muscle proteins were evaluated in sedentary and exercise trained GK and control animals. The effects of PIO on shortening and Ca\(^{2+}\) transport in ventricular myocytes from GK and control animals were investigated. Finally, an *in vivo* biotelemetry study was conducted to investigate the effects of PIO on the ECG in GK and control animals.

2.4 Methods

2.4.1 Ventricular myocyte isolation

LV myocytes were isolated using enzymatic and mechanical dispersal techniques according to previously described methods [276]. Animals were euthanized by decapitation using a guillotine. The abdomen was opened horizontally and the ribs and sternum were removed to access the chest cavity. The heart was exposed and elevated by cradling it gently (to avoid injury) in the finger tips. Hearts were removed rapidly and immersed in a cold perfusate (in mM: 130.0 NaCl, 5.4 KCl, 1.4 MgCl\(_2\), 0.75 CaCl\(_2\), 0.4 NaH\(_2\)PO\(_4\), 5.0 HEPES, 10.0 glucose, 20.0 taurine and 10.0 creatine; pH 7.3) to limit any ischemic injury during the period between excision and the restoration of vascular perfusion. The heart was cleared of extraneous tissue and debris and with the aid of
forceps, the aorta was slipped over the tip of a cannula and mounted for retrograde perfusion according to the Langendorff method. Published reviews by Skrzypiec-Spring in 2007 and more recently by Bell in 2011 described the general principles of this technique [277, 278]. The Langendorff apparatus (Figure 2.1) consists of a cannula attached to the outflow of a heat exchanger. A peristaltic pump (Minipuls3, Gilson, France) was set to deliver isolation solution at a constant flow rate of 8 ml/g heart/min. All perfusion procedures were performed with tissue being maintained at 37 °C and perfusion solutions continuously aerated with 95% O2 and 5% CO2. Hearts were perfused with cell isolation solution containing in mM: 130.0 NaCl, 5.4 KCl, 1.4 MgCl2, 0.75 CaCl2, 0.4 NaH2PO4, 5.0 HEPES, 10.0 glucose, 20.0 taurine and 10.0 creatine; pH 7.3). When the heart contraction had stabilized perfusion was switched for 4 min to Ca2+-free cell isolation solution containing 0.1 mM ethylene glycol tetra acetic acid (EGTA). The purpose of the low Ca2+ solution is to loosen junctions between cardiac myocytes. Solution was then switched to cell isolation solution containing 0.05 mM Ca2+, 0.60 mg/ml type I collagenase (Worthington Biochemical Corp, Lakewood, NJ, USA) and 0.075 mg/ml type XIV protease (Sigma, Taufkirchen, Germany) for 6 min. The enzyme cocktail loosens collagen and other linkages between cardiac myocytes. The heart was then removed from the Langendorff system, the ventricle tissue excised from the heart, minced and gently shaken in collagenase-containing isolation solution supplemented with 1% bovine serum albumin (BSA) for 4 min at 37 °C. Cells were filtered from this solution through a 300 μm nylon mesh. The filtrate containing ventricular myocytes was centrifuged at 400 rpm for 1 min. Subsequently, the supernatant was removed and the cell pellet was re-suspended in cell isolation
solution containing 0.75 mM Ca^{2+}. This process was repeated four times using tissue remaining in the nylon mesh. Ventricular myocytes were collected from the second, third and fourth shakes and stored at 4 °C and used over a period of 1-8 hrs. Ventricular myocytes were freshly isolated on a daily basis. Experiments were performed in ventricular myocytes that displayed normal rod-shaped morphology with clear striations (Figure 2.2).

Body weight, heart weight and non-fasting blood glucose were measured immediately prior to experiments. Hearts were weighed before canulation. Immediately, following sacrifice of the animal, blood was collected in tubes containing ethylene-diaminetetraacetic-acid (EDTA) and then centrifuged at 3,000 rpm for 15 min. The supernatant (plasma) was transferred to eppendorf tubes and stored at -80 °C.
Figure 2.1: Langendorff apparatus. Arrangement of system (upper panel). (a) Solution reservoirs, (b) Heart exchanger, (c) Peristaltic pump and (d) Thermo circulator. Magnified picture of Heat exchanger and heart warming jacket (lower left panel) and Magnified picture of cannulated heart (lower right panel).
Figure 2.2: Micrographs of isolated myocytes. A collection of ventricular myocytes (upper panel), (a) Viable myocytes and (b) Non-viable myocytes. Single ventricular myocyte (lower panel).
2.4.2 Exercise training protocol

Thirty male GK and thirty male Wistar control rats aged 8 months were divided into 4 subgroups, each containing 15 rats. 2 subgroups of GK and control rats received exercise training whilst the other 2 subgroups of GK and control rats continued a sedentary lifestyle. Exercise training was performed on a treadmill (EXER-4, Columbus Instruments, Columbus, OH, USA) (Figure 2.3) according to previously described protocols with minor modifications [279, 280]. Daily 1 hr exercise training sessions were repeated 5 days per week for a period of 2-3 months. Each exercise training period began with a 10 min warm-up during which time the belt speed was gradually increased from zero to training speed. During week 1, the training belt speed was 10 m/min, during weeks 2-3 belt speed was increased to 15 m/min and during week 4 belt speed was maintained at 15 m/min and the belt gradient was increased from 0° to 10°. Thereafter, the exercise training belt speed was increased to 20 m/min. If rats stopped running they would move backwards on to an electrical grid where they would receive a small electrical shock (200 ms pulse of 2 mA electric current).
Figure 2.3: Treadmill used for exercise training. (a) Control unit, (b) Electric grids, and (c) Exercise belt.
2.4.3 Expression of mRNA

Expression of genes encoding a range of cardiac muscle proteins was assessed using real-time reverse transcription polymerase chain reaction (Real time RT-PCR) modified from previously described methods [281].

2.4.3.1 Sample collection

Samples of left ventricle tissue were acquired from the apex of the heart in vivo prior to removal of the heart for myocyte isolation (Section 2.4.1). Samples of left ventricle tissue were collected and placed immediately in RNAlater (AM7021, Life Technologies, Carlsbad, CA, USA) and kept overnight at room temperature to allow thorough penetration of the tissue. Tissue samples were then frozen at -80 °C pending further processing.

2.4.3.2 RNA extraction and quantification

Isolation of total RNA from the tissue was performed using the SV Total RNA Isolation System (Promega, Madison, USA) according to the manufacturer’s instructions. In brief, frozen LV samples were cut into small sections with a sterile razor blade. The tissue (< 30 mg) was homogenized in RNA lysis buffer containing β-mercaptoethanol (BMP) to disrupt cells, precipitate cellular proteins and release the RNA into solution. Phase separation was done with the addition of RNA dilution buffer to the homogenized samples. The lysates were incubated for 3 min at 70 °C and cell debris was collected by centrifugation (10 min; 12,000 rpm; 25 °C). The supernatant (containing RNA) was mixed in a fresh Eppendorf tube with 95% ethanol and transferred onto a spin column. After centrifugation (1 min; 12,000 rpm; 25 °C), the elute was discarded and the column was washed with wash buffer. After an additional centrifugation step (1
min; 12,000 rpm; 25 °C). DNase I mix was applied to the membrane of the columns and samples were incubated for 15 min at 25 °C. Digestion was stopped by the addition of DNase I stop mix and the columns were centrifuged (1 min; 12,000 rpm; 25 °C). Following two wash steps with wash buffer (1 min and 2 min; 12,000 rpm; 25 °C), the column was transferred to a sterile Eppendorf tube and RNase-free water was added. After incubation for 1 min at 25 °C, the RNA was eluted by centrifugation (2 min; 12,000 rpm; 25 °C). The concentration and purity of the RNA was determined by measuring the absorbance at 260 nm (A260) and the ratio of the absorbance at 260 and 280 nm (ND-1000, NanoDrop). Samples were aliquoted and stored at -80 °C, pending further processing.

2.4.3.3 Reverse transcription to cDNA

A two-step RT-PCR procedure was used to generate complementary DNA (cDNA). Total RNA (500 ng) was converted into cDNA in a 25 μl PCR reaction with 10 x RT buffer 2.0 μl, 25 x dNTP Mix (100 mM) 0.8 μl, 10 x RT random primers 2.0 μl, MultiScribe™ Reverse Transcriptase 1.0 μl, RNase inhibitor 1.0 μl, and Nuclease free H2O (High Capacity cDNA Reverse Transcription Kit (4374966, Applied Biosystems, USA)). Reverse transcription was carried out using the following parameter values: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min on the Veriti thermal cycler (Applied Biosystems, USA).

2.4.3.4 Real time reverse transcription polymerase chain reaction

Gene expression was performed using custom Taqman Low Density Arrays (Format 32, 4346799, Applied Biosystems, USA). The TaqMan assays were pre-loaded in each reaction well of the array in triplicate for each RNA sample. The target genes for real-
Table 2.1. Rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an endogenous control. 100 ng of cDNA (RNA-equivalent) was loaded together with 2 x TaqMan Gene Expression Master Mix (No AmpErase UNG, Applied Biosystems, USA) made up to 100 μl per port. Real-time RT-PCR was performed on a 7900HT Fast ABI Prism 7900HT Sequence Detection System (Applied Biosystems, USA). The PCR thermal cycling parameters were run in standard mode as follows: 50 °C for 2 min, 94.5 °C for 10 min, followed by 40 cycles of 97 °C for 30 sec and 59.7 for 1 min. Results were initially analyzed using ABI Prism 7900HT SDS, v2.4. All remaining calculations and statistical analysis were performed by the SDS RQ Manager 1.1.4 software using the 2-ΔΔCt method with a relative quantification RQmin/RQmax confidence set at 95%.
Table 2.1: Target genes, alternative names and roles of genes selected for real-time RT-PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative name</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiac muscle proteins and associated regulatory proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Myh6</strong></td>
<td>Myosin, heavy polypeptide 6, cardiac muscle, alpha</td>
<td>Muscle contraction</td>
</tr>
<tr>
<td><strong>Myh7</strong></td>
<td>Myosin, heavy polypeptide 7, cardiac muscle, beta</td>
<td>Muscle contraction</td>
</tr>
<tr>
<td><strong>Myhpc3</strong></td>
<td>Myosin binding protein C</td>
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</tr>
<tr>
<td><strong>Myl1</strong></td>
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<td>Muscle contraction</td>
</tr>
<tr>
<td><strong>Myl3</strong></td>
<td>Myosin, light chain 3</td>
<td>Muscle contraction</td>
</tr>
<tr>
<td><strong>Actc1</strong></td>
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<td>Troponin 1 type 3, cardiac</td>
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</tr>
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<td>Troponin T2, cardiac</td>
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</tr>
<tr>
<td><strong>Tpm1</strong></td>
<td>Tropomyosin 1, alpha</td>
<td>Regulation of muscle contraction</td>
</tr>
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<td>Tropomyosin 2, beta</td>
<td>Regulation of muscle contraction</td>
</tr>
<tr>
<td><strong>Tpm4</strong></td>
<td>Tropomyosin 4</td>
<td>Regulation of muscle contraction</td>
</tr>
<tr>
<td><strong>Dbi</strong></td>
<td>Titin protein homolog</td>
<td>Scaffolding for sarcomere assembly</td>
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<tr>
<td><strong>Intercellular proteins</strong></td>
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<td></td>
</tr>
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<td>Intercellular communication</td>
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</tr>
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<td>Dsp</td>
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<td>Caveolae membranes</td>
</tr>
<tr>
<td>Cav3</td>
<td>Caveolin 3</td>
<td>Caveolae membranes</td>
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</tr>
<tr>
<td>Casq2</td>
<td>Calsequestrin 2</td>
<td>SR Ca^{2+} binding protein</td>
</tr>
<tr>
<td>Ptn</td>
<td>Phospholamban</td>
<td>SR Ca^{2+} pump regulation</td>
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<td>Calm2</td>
<td>Calmodulin 2</td>
<td>Regulation of ion channels</td>
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<td>Calcium/calmodulin</td>
<td>Regulation of Ca^{2+} homeostasis</td>
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<td>Prkaa2</td>
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<td>Regulation of cellular energy metabolism</td>
</tr>
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<td><strong>Cell membrane transport</strong></td>
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<td>L-type Ca²⁺ channel</td>
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<td>Calcium channel, alpha 1G subunit</td>
<td>T-type Ca²⁺ channel</td>
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<td>Calcium channel, alpha 1H subunit</td>
<td>T-type Ca²⁺ channel</td>
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<td>Ca²⁺ channel modulation</td>
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<td>Ca²⁺ channel modulation</td>
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<td>Pacemaker current (Iₕ)</td>
</tr>
<tr>
<td>Genotype</td>
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<td>-------------</td>
<td>--------------</td>
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<tr>
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<td>Pacemaker current (Iᵢ)</td>
</tr>
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<td><em>Hcn4</em></td>
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<td>Pacemaker current (Iᵢ)</td>
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<td>Delayed rectifier K⁺ channel (Iₚ)</td>
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<td>Delayed rectifier K⁺ channel (Iₚ)</td>
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<td>Potassium channel, shaker-related subfamily, member 4</td>
<td>Slow transient outward K⁺ channel (Iₒ⁺)</td>
</tr>
<tr>
<td><em>Kcn5</em></td>
<td>Potassium channel, shaker-related subfamily, member 5</td>
<td>Ultra-rapidly activating delayed rectifier (Iₜᵢᵣ)</td>
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<td><em>Kcn7</em></td>
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<td>Ultra-rapidly activating delayed rectifier (Iₜᵢᵣ)</td>
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<tr>
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<td>A-type potassium channel, protein 2</td>
<td>Transient outward K⁺ channel (Iₒ⁺)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
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<td>Delayed rectifier $K^+$ channel ($I_{K,1}$)</td>
</tr>
<tr>
<td>KCaM1</td>
<td>Potassium voltage-gated channel, Shab-related subfamily, member 1</td>
<td>Delayed rectifier $K^+$ channel ($I_{K,1}$)</td>
</tr>
<tr>
<td>KCaM1</td>
<td>Potassium voltage-gated channel, Shal-related family, member 1</td>
<td>Fast transient outward $K^+$ channel ($I_{o,f}$)</td>
</tr>
<tr>
<td>KCaM1</td>
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<td>Fast transient outward $K^+$ channel ($I_{o,f}$)</td>
</tr>
<tr>
<td>KCaM1</td>
<td>Potassium voltage-gated channel, Isk-related subfamily, member 1</td>
<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
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<td>KCaM1</td>
<td>Potassium voltage-gated channel, Isk-related subfamily, member 2</td>
<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
</tr>
<tr>
<td>KCaM1</td>
<td>Potassium voltage-gated channel, Isk-related subfamily, member 3</td>
<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
</tr>
<tr>
<td>KCaM1</td>
<td>Potassium voltage-gated channel, Isk-related subfamily, member 4</td>
<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
</tr>
<tr>
<td>KCaM1</td>
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<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
</tr>
<tr>
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<td>Slow delayed rectifier $K^+$ channel ($I_{K,s}$)</td>
</tr>
<tr>
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<td>Rapid delayed rectifier $K^+$ channel ($I_{K,r}$)</td>
</tr>
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<td>Potassium channel, subfamily K, member 3</td>
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</tr>
<tr>
<td>KCaM1</td>
<td>Potassium intermediate/small conductance calcium activated channel, subfamily N, member 2</td>
<td>n/a</td>
</tr>
</tbody>
</table>
2.4.4 Measurement of ventricular myocyte shortening

The apparatus used to measure ventricular myocyte shortening is illustrated in Figure 2.4 and measurements were performed using previously described techniques [282]. In brief, freshly isolated 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, Gottingen, Germany) and viewed with a 20X objective lens and with a high speed video camera (Myotrac, Crystal Biotech, UT, USA). Myocytes were superfused (3-5 ml/min) with normal Tyrode (NT) containing the following in mM: 140.0 NaCl, 5.0 KCl, 1.0 MgCl₂, 10.0 glucose, 5.0 HEPES, 1.8 CaCl₂ (pH 7.4). Inflow of NT solution to the chamber was controlled by two micro pumps (P07002-39/P07002-33, Cole-Parmer, USA). Outflow and the level of fluid in the chamber was controlled by a glass tube dipping into the chamber and connected to a waste bottle which was connected to a vacuum line. The temperature of the chamber solution was maintained at 35-37 °C by a temperature controller (TC-20, NPI, Germany), which comprised a heating coil wound around the inflow line and a thermostart dipping into the chamber solution. Ventricular myocytes were stimulated at 1 Hz (SD-5 Stimulator, Grass Instrument, USA) using 2 platinum electrodes positioned at either side of the chamber. Myocyte shortening was recorded with a video edge detection system (VED-114, Crystal Biotech, Northborough, MA, USA).

Myocytes with normal rod-shaped morphology and stable shortening during electrical stimulation were selected for experiments. Cursors of the video edge system were positioned over the edges. Resting cell length (RCL), time to peak (TPK) shortening, time to half (THALF) relaxation and amplitude of shortening (expressed as a % of
resting cell length) were measured. Data were acquired and analyzed with Signal Average software (v 6.37, Cambridge Electronic Design, Cambridge, UK).

2.4.5 Measurement of intracellular Ca^{2+} concentration

The fluorescence photometry system shown in Figure 2.4 was used to measure intracellular Ca^{2+} concentration in myocytes loaded with the fluorescent indicator fura-2 AM (F-1221, Molecular Probes, Eugene, OR, USA).

Myocytes were loaded with the fluorescent indicator fura-2 AM according to previously described techniques [282]. In brief, 6.25 µl of a 1.0 mM stock solution of fura-2 AM, dissolved in dimethylsulphoxide (DMSO) 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. After loading with fura-2 AM, myocytes were centrifuged at 400 rpm, washed with NT to remove extracellular fura-2 and then left for 30 min to ensure complete hydrolysis of the intracellular ester. To measure intracellular Ca^{2+} concentration, myocytes were alternately illuminated by 340 and 380 nm light using a monochromator (Cairn Research, Favergham, UK) which changed the excitation light every 2 ms. The resulting fluorescence emitted at 510 nm was recorded by a photomultiplier tube and the ratio of the emitted fluorescence at the two excitation wavelengths (340/380 ratio) was calculated to provide an index of intracellular Ca^{2+} concentration. Resting fura-2 ratio, TPK Ca^{2+} transient, THALF decay of the Ca^{2+} transient and the amplitude of the Ca^{2+} transient were measured in electrically stimulated (1 Hz) myocytes maintained at 35-36 °C. Data were acquired and analyzed with Signal Averager software v 6.37 (Cambridge Electronic Design, Cambridge, UK.)
Figure 2.4: Electrophysiology system. (a) Anti-vibration table, (b) Farady cage, (c) Inverted microscope, (d) Cell perfusion system, (e) Temperature control system, (f) Video edge detection system, (g) Florescence photometry system and (h) Electrical stimulator.
2.4.6 Measurement of sarcoplasmic reticulum Ca\(^{2+}\) transport

SR Ca\(^{2+}\) was assessed using previously described techniques [61, 282]. After establishing steady state Ca\(^{2+}\) transients in electrically stimulated (1 Hz) myocytes maintained at 35-36 °C and loaded with fura-2, stimulation was paused for a period of 5 sec. Caffeine (20 mM) was then applied for 10 sec using a solution switching device customized for rapid solution exchange [283]. Electrical stimulation was then resumed and the Ca\(^{2+}\) transients were allowed to recover to steady state. SR-releasable Ca\(^{2+}\) was assessed by measuring the amplitude of the caffeine-evoked Ca\(^{2+}\) transient. Fractional release of SR Ca\(^{2+}\) was assessed by comparing the amplitude of the electrically-evoked steady state Ca\(^{2+}\) transients with that of the caffeine-evoked Ca\(^{2+}\) transient. Ca\(^{2+}\) refilling of the SR was assessed by measuring the rate of recovery of electrically-evoked Ca\(^{2+}\) transients following application of caffeine.

2.4.7 Assessment of myofilament sensitivity to Ca\(^{2+}\)

Myofilament sensitivity to Ca\(^{2+}\) was assessed using previously described techniques [284]. In some cells shortening and fura-2 ratio were recorded simultaneously. Myofilament sensitivity to Ca\(^{2+}\) was assessed from phase-plane diagrams of fura-2 ratio vs. 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\(^{2+}\) [285, 286].
2.4.8 Measurement of L-type Ca\textsuperscript{2+} current

L-type Ca\textsuperscript{2+} current was measured using whole-cell patch-clamp according to previously described techniques [281]. Figure 2.5 shows the arrangement of the patch clamp system. Cells were mounted on the stage of an inverted microscope (Nikon ECLIPSE Ti, Nikon Instruments Inc., USA). L-type Ca\textsuperscript{2+} current was recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) linked to an A/D interface (Digidata 1200, Molecular Device, USA) connected to a computer. The analog signal was filtered using an eight-pole Bessel filter with a bandwidth of 5 kHz and digitized at a sampling rate of 10 kHz under software control (pClamp 8.2, Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were fabricated from filamented BF150-86-10 borosilicate glass (OD = 1.5 mm, Sutter Instrument, CA, USA) using a horizontal puller (Sutter Instrument Co., CA, USA). Pipette tips were fire-polished using a microforge (Zeiss ID03, Germany). The pipette was carefully moved to the cell surface by using a three-axis micromanipulator (PatchStar, Scientifica, UK). A tight seal (Gigaseal) between the membrane and the tip of the pipette was obtained by the application of suction through the pipette. Further suction was applied in order to disrupt the membrane and achieve whole cell configuration. Cells that had stable currents within the third to fifth min after establishing whole cell mode were used in the majority of our experiments. The whole cell bath solution contained the following in mM: 145 NaCl, 2 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 HEPES and 10 glucose, pH was adjusted to 7.35. The rate of solution flow to the recording chamber was controlled by two micropumps (Micropump, Inc., WA, USA).
The pipette solution contained the following in mM: 140 CsCl, 2 MgCl_2, 10 TEACl, 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 -60 mV to +70 mV in 10 mV steps from a holding potential of -50 mV. The amplitudes of currents were normalized to cell membrane capacitance (nA/PF). The steady-state inactivation of Ca^{2+} current was measured as the relationship of amplitude of the peak current at a test pulse of 0 mV to amplitude of the peak current at 1000 ms long pre-pulses to various voltages between -50 and +30 mV. Normalized peak currents measured after these pre-pulses were plotted against the respective pre-pulse potential. Time course of recovery from inactivation was investigated using a two-pulse protocol. Two 100 ms depolarizing pulses to +10 mV were separated by inter-pulse intervals with variable duration. Peak Ca^{2+} current amplitude measured by the second pulse was normalized to that measured by the first pulse and their ratio was plotted against the inter-pulse interval. Data were acquired and analyzed with pClamp software v 8.2.
Figure 2.5: Patch clamp system. (a) Patch pipette, (b) Micromanipulator, (c) Inverted light microscope, (d) Anti-vibration table, (e) Faraday cage to shield the system from ambient electrical noise, (f) Amplifier, (g) Analogue to digital converter and (h) Micromanipulator control.
2.4.9 Western blot

2.4.9.1 Sample collection, protein extraction and quantification

Samples of left ventricle tissue were acquired from the apex of the heart, frozen immediately in liquid nitrogen and stored at -80 °C pending further processing. For processing, frozen left ventricle tissues were allowed to thaw and were then weighed, washed with ice-cold 0.9% normal saline, minced and homogenized (25% w/v) in isotonic 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl florid (PMSF-protease inhibitor, Sigma Aldrich). The homogenate was centrifuged for 10 min at 6000 rpm at 4 °C and the supernatant was collected. The protein content was measured by Bradford assay according to previously described techniques [287]. Samples were aliquoted and stored at -80 °C. All the procedures were performed on ice to prevent proteolysis of proteins. Protein content was quantified using spectrophotometry (Beckman Du-70, ALT, EL, USA).

2.4.9.2 Preparation of Sodium dodecyl-sulphate polyacrylamide gel electrophoresis

The apparatus used for preparation of the sodium dodecyle-sulphate (SDS) Polyacrylamide gel electrophoresis included a vertical clamp stand, cassette plates, casting stand, separator and comb. 10% SDS poly acrylamide gel was prepared using two different layers of acrylamide gel. The separating gel (10 ml gel containing, 2.5 ml of 1.5 M Tris-base pH 8.8, 100 µl of 10% SDS, 3.33 ml of 30% acrylamide/bis, 50 µl of 10% ammonium persulfate (APS), 5 µl of TEMED and 4.02 ml distilled water) was prepared and loaded into the apparatus. This layer is responsible for separating the proteins by size.
Distilled water was added onto the top of the separating gel to prevent the gel from drying. When the gel polymerized, water was removed, and the stacking gel (5 ml gel containing, 1.75 ml of 0.5 M Tris-HCL pH 6.8, 50 μl of 10% SDS, 650 μl of 30% acrylamide/bis, 25 μl of 10% APS and 10 μl of TEMED) was loaded onto the top of the separating gel. A comb was inserted into the stacking gel in order to form wells. Once the gel was set, the comb was removed and the SDS-PAGE was ready for loading.

2.4.9.3 Protein expression

Proteins equivalent to 25-70 μg (Table 2.2) from sedentary control, exercise trained control, sedentary GK and exercise trained GK rats were mixed with a sample buffer (12.5% of 0.5 M Tris-base pH 6.8, 10% glycerol, 20% of 10% (w/v) SDS, 5% 2-mercaptoethanol and 2.5% of 0.05% (w/v) bromophenol blue) to give a final volume of 30-40 μl. Samples were heated for 10 min at 95 °C followed by a brief spin for 10 sec.

About 15 μl of sample mixture and 5 μl of pre-stained standard protein-molecular weight marker (Prestained SDS-PAGE Standards, broad range Bio-Rad Laboratories, Hercules, CA, USA) were loaded onto the prepared 10% SDS gel and electrophoresed at 100 V in running buffer (25 mM Tris-base, 192 mM glycine and 0.1% SDS, pH 8.3). After the run, the gel was detached from the cassette plate and washed for 20 min with transfer buffer at room temperature. The separated proteins were then transferred electrically at 80 V on to a nitrocellulose membrane (Schleicher & Schuell, Protran) for 2 hrs at 4 °C in transfer buffer (119 mM Tris-base, 192 mM glycine and 20% methanol) as previously described [288, 289]. A successful protein transfer was indicated by the complete transfer of pre-stained molecular weight markers onto the nitrocellulose membrane. Non-specific binding sites were then blocked by incubating the membrane
overnight in 5% fat free milk at 4 °C, washed twice with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) for 10 min each, and then probed with primary antibodies as listed in Table 2.2 for 2 hrs at room temperature. Primary antibodies were diluted in 5% fat free milk blocking agent. The membrane was washed in several changes of TBST and TBS buffers, and then incubated with secondary antibody for 1 hr at room temperature. Later, the membranes were washed twice for 10 min with TBST and twice with TBS buffer (TBS buffer was prepared as a 10 x stock solution and diluted 1:10 to make 1 x working solution. 1 x TBS buffer contained 20 mM Tris-HCl and 150 mM NaCl, pH 7.4).

2.4.9.4 Blot development and protein detection

The blot was developed using Thermo Scientific Pierce ECL 2 Western Blotting Substrate. Each blot was incubated with 1.23 ml working solution for 5 min at room temperature. The substrate working solution was prepared by mixing substrate A and substrate B in a 40:1 ratio. The blot was removed and dried using absorbent tissue to remove excess liquid. The blot was placed on a Typhoon Imager (Typhoon FLA 9500) with the protein side facing down, and scanned using the 473 nm excitation and LPB (510LP) emission filter in fluorescence mode to visualize the protein bands. The protein bands were then quantified using ImageQuant TL 7.0 software. To check for equal loading, each of the blots were also incubated with stripping buffer containing 62.5 mM Tris-HCl; pH 6.7, 100 mM β-mercaptoethanol, 2% SDS, for 1 hr at 56 °C. This step was essential to remove primary and secondary antibodies from the blot. The blot was then blocked overnight with 5% fat free milk blocking agent at 4 °C and incubated with internal control antibody. β-actin was used as an internal control. The density of each
band was then normalized to the internal control. The intensity of the bands was calculated and expressed as a relative intensity compared to the control sedentary proteins, which was arbitrarily taken as 100%. The analyses were representative data of three independent experiments.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein size (kDa)</th>
<th>Protein (μg)</th>
<th>Primary antibody</th>
<th>Secondary antibody-dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connexin 37</td>
<td>45-55</td>
<td>70</td>
<td>Connexin 37 antibody (M-19).</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution 1:100</td>
<td></td>
</tr>
<tr>
<td>Connexin 43</td>
<td>43</td>
<td>50</td>
<td>Connexin 43 antibody (D-7).</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution 1:200</td>
<td></td>
</tr>
<tr>
<td>L-type Ca(^{2+})</td>
<td>50-70</td>
<td>50</td>
<td>L-type Ca(^{2+}) CP 2 antibody (F-29).</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution 1:200</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>34</td>
<td>25</td>
<td>Tropomyosin antibody (C-3).</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution 1:200</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>42</td>
<td>50</td>
<td>β-Actin antibody (C-4).</td>
<td>1:3000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution 1:100</td>
<td></td>
</tr>
</tbody>
</table>

Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, CA, USA.
2.4.10 Drug preparation for ventricular myocyte experiments

PIO (E6910, Sigma-Aldrich) was dissolved in 100% DMSO to produce 0.5, 5 and 50 mM stock solutions which were stored at -20 °C. PIO solutions were prepared by addition of stock solutions to NT to achieve 0.1, 1 and 10 mM bath solution. Final DMSO concentration was 0.02% in all test solutions. Experimental solutions were prepared from stock solution immediately prior to each experiment. Therefore, the cells were perfused with the freshly made bath solutions containing the desired concentrations of the drugs.

2.4.11 In vivo Biotelemetry

Figure 2.6a-e shows the procedures and apparatus used in the biotelemetry study. Heart biopotential was monitored with a biotelemetry system (Data Sciences Int., St Paul, MN, USA) using previously described techniques [203, 290]. The system comprised the transmitter devices (TA11CTA-F40, Data Sciences Int.), the receivers (RPC-1, Data Sciences Int.) a data exchange matrix (20CH, Data Sciences Int) and a personnel computer for system configuration, control, acquisition, storage and analysis.

2.4.11.1 Surgery

The transmitter devices were surgically implanted in 7 male GK and 7 male Wistar control rats, 6 months of age, under general anesthesia (sodium pentoobarbitone, 45 mg/kg, i.p). Transmitter devices were inserted into the peritoneal cavity with the electrodes protruding caudally (towards the tail) and arranged in Einthoven bipolar lead II configuration (right foreleg and left side of chest at the level of the last rib). Transmitters were activated by use of a permanent magnet switch.
2.4.11.2 Experimental protocol

Before surgical implantation of the transmitter devices, non-fasting blood glucose and body weight were measured. After an overnight fast a glucose tolerance test was administered. Blood glucose was measured at time zero (fasting blood glucose) and 120 min following administration of glucose (2 g/kg body weight, i.p). Surgical implantation of transmitters was then performed. Data acquisition commenced one week after surgery and continued throughout the study. Body weight and non-fasting blood glucose was measured at the end of each PIO dose period and at the end of the experiment. Animals received standard rat chow ad libitum throughout the experiment.

2.4.11.3 Treatment protocol

During the first few weeks animals received normal drinking water. During week 4 animals received Water/DMSO (0.19% v/v), week 5-6 Water/DMSO/PIO 2.5 mg, week 7-8 Water/DMSO/PIO 5 mg, week 9-10 Water/DMSO/PIO 10 mg, week 11-12 Water/DMSO/PIO 20 mg and weeks 13-14 back to Water/DMSO.

2.4.11.4 Drug preparation for biotelemetry study

PIO tablets (Actos - Takeda Pharmaceutical, Tokyo, Japan) were used in this experiment. A tablet was crushed into a fine powder, dissolved in DMSO and diluted with water to form a suspension. The suspension was used to prepare the required concentration of PIO (according to the treatment protocol in Section 2.4.11.3) and dissolved in a small volume of water. When the animals had consumed the drug containing water, the bottles were refilled with water. Drug suspension was prepared fresh on a daily basis. Final DMSO concentration was 0.19 v/v throughout the study.
2.4.11.5 Data collection

ECG data was collected for 5 min/hour/animal/24 h. From the collected ECG data, secondary physiological measurements were determined, including the average 5 min HR and HRV.
Figure 2.6: Biotelemetry study. (a) Surgical tools, (b) Preparing animal for surgery, (c) Implanting the transmitter in intraperitoneal cavity and (d) administration of antibiotic and (e) Animal cages sitting on biotelemetry receivers.
2.4.12 Transmission electron microscopy

2.4.12.1 Sample processing

Freshly isolated left ventricle samples from 4 groups of animals were cut into 4 mm x 4 mm pieces and transferred into Karnovsky fixative (2.5% paraformaldehyde, 25% EM grade glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.2) in glass vials. Samples were washed in 0.1 M phosphate buffer 3 times for 15 min each and stored at 4 °C until further processing. Samples were then treated with 1% osmium tetroxide buffered in 0.1 M phosphate buffer for one hour at room temperature on a rotamixer, followed by washing with distilled water and dehydration in graded ethanol as follows: 15 min each in 30%, 50%, 70%, 80%, 90% ethanol, and 2 changes in absolute ethanol and propylene oxide. Tissues were then treated with agar resin-propylene oxide mixture as 1:1, 1:2 and 1:3 ratios for 1 hour each, and transferred into pure resin at 4 °C overnight.

Resin was composed of 23 g Agar 100 epoxy resin, 13 g Dodecenylysuccinic anhydride, 14 g Methyl nadic anhydride, and 1.1 Tri Dimethylaminomethyl phenol.

Tissues were then embedded in fresh resin using flat molds. Polymerization was performed at 65 °C for 24 hrs. Ultrathin sections (90-100 nm) were cut with a Diatone knife (Agar Scientific, Essex, England) and mounted on 3.0 mm 200 mesh copper grids. The sections were stained with saturated uranyl acetate for 50 min and lead citrate solution for 25 min. Ultrathin sections were scanned and photographed using a Phillips CM10 electron microscope (Eindhoven, The Netherlands).
2.4.12.2 Morphometric analysis

A number of electron micrograph sections were taken per rat in each group. The number of mitochondria in a given area was counted manually. The sarcomere length and width were measured using Image J software. The average number of mitochondria number and the average sarcomere length and width were calculated. Intercalated disc features were evaluated visually and membrane convolution/length unit was measured.

2.4.13 Statistical analysis

Results were expressed as the mean ± SEM of ‘n’ observations. ‘n’ refers to number of animals or number of ventricular myocytes. Data were analyzed by the Independent samples t-test, Paired samples t-test or analysis of variance (ANOVA) with Bonferroni post hoc as appropriate, using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY). P value < 0.05 were considered significant and P < 0.01 were considered highly significant.
Chapter 3: Results and Discussion

3.1 Shortening and intracellular Ca$^{2+}$ in ventricular myocytes and expression of genes encoding cardiac muscle proteins in early onset type 2 diabetic Goto-Kakizaki rats

3.1.1 Introduction

The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [95]. The association between type 2 diabetes and obesity is very strong and cardiovascular disease is the major cause of morbidity and mortality in diabetic patients [96, 291]. The GK rat is one of the best characterized genetic animal models of type 2 diabetes. This strain was created by selective breeding of an outbred colony of Wistar rats, with selection for high glucose levels in an oral glucose tolerance test [190]. Type 2 diabetes in the GK rat appears to be polygenic with at least three different loci involved in the disease [197]. General characteristics of the GK rat include fasting hyperglycaemia, impaired secretion of insulin in response to glucose both in vivo and in isolated pancreas, raised HbA$_1c$ hepatic and peripheral insulin resistance and a variety of late complications including nephropathy and neuropathy [190]. The etiology of diabetes in GK rats includes a decrease of pancreatic insulin stores and reduced beta-cell mass and proliferative capacity, reduced beta cell sensitivity to glucose, impaired insulin release, under expression of GLUT2 in beta cells and reduction in GLUT4 protein in GK hearts. Previous studies, which have been generally performed in adult animals, have demonstrated a variety of contractile dysfunctions in GK rat heart including decreased heart rate, decreased ejection fraction, mainly as a result of a loss in LV longitudinal contraction, and prolonged time-course of shortening.
and/or relaxation in ventricular myocytes. These contractile dysfunctions have been partly attributed to defects in Ca\(^{2+}\) transport and myofilament sensitivity to Ca\(^{2+}\) [192, 204, 285]. Defective contraction and Ca\(^{2+}\) transport may in turn be attributed to changes in the expression of genes and remodeling of proteins. Altered expression of genes that encode various L-type Ca\(^{2+}\) channel proteins (\textit{Cacna1c, Caenb2, Caenad1}) and cardiac muscle proteins (\textit{Myh6, Myh7, Myl2, TnnI}) has been reported in GK rats fed high fat diets and in Zucker type 2 diabetic fatty rat [281, 284].

The aim of the current study was to characterize the expression pattern of genes encoding cardiac muscle proteins and myocyte shortening and intracellular Ca\(^{2+}\) concentration in young (8-10 weeks of age) type 2 diabetic GK rat heart.

### 3.1.2 Results

#### 3.1.2.1 General characteristics of the animals

In order to characterize the diabetic state in the early stage of diabetes of the GK and age matched control rats, blood glucose and body weight were measured at a young age (7 weeks). Fasting blood glucose was significantly ($P < 0.01$) elevated in GK rats (124.40 ± 9.86 mg/dl) compared with control animals (73.10 ± 2.48 mg/dl). After an overnight fast and at 120 min following a glucose challenge (2 g glucose/kg body weight, i.p), blood glucose was significantly ($P < 0.01$) elevated in GK rats (218.50 ± 23.93 mg/dl) compared with control animals (95.60 ± 3.65 mg/dl). These results confirmed the diabetic state of the GK rats.

At 8–10 weeks of age, the experiments were started. The general characteristics of GK and control rats obtained immediately before the experiment are shown in Table 3.1.
Body weight was not significantly ($P > 0.05$) altered, non-fasting blood glucose and heart weight were significantly ($P < 0.01$) elevated and heart weight to body weight ratio was not significantly ($P > 0.05$) altered in GK rats compared with age-matched control animals.
Table 3.1: General characteristics of GK compared to control rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>218.50 ± 8.65</td>
<td>246.40 ± 11.97</td>
</tr>
<tr>
<td>Non-fasting blood glucose (mg/dl)</td>
<td>118.40 ± 6.53</td>
<td>166.40 ± 14.33**</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.96 ± 0.03</td>
<td>1.05 ± 0.02**</td>
</tr>
<tr>
<td>Heart weight to body weight ratio (mg/g)</td>
<td>4.43 ± 0.11</td>
<td>4.33 ± 0.15</td>
</tr>
</tbody>
</table>

Animals were 8-10 weeks of age. Data are mean ± S.E.M. Statistical comparisons were performed using independent samples t-test, n = 10 animals. ** P < 0.01
3.1.2.2 Expression of cardiac muscle mRNA

Expression of cardiac muscle messenger RNA (mRNA) was assessed in young 8-10 week GK and age-matched control rats using real time RT-PCR assay as described in section 2.4.3. Levels of mRNA reflecting the expression of genes encoding a variety of cardiac muscle and ion channel proteins are shown in Figure 3.1-3.7. Expression of genes encoding cardiac muscle proteins (Myh6/7, Myhpc3, Myl1/3, Actc1, Tnni3, Tnn2, Tpm1/2/4 and Dbi) and intercellular proteins (Gja1/4/5/7, Dsp and Cav1/3) were not significantly (P > 0.05) altered in GK compared with control ventricle (Figure 3.1 and Figure 3.2, respectively). Expression of genes encoding some membrane pump and exchange proteins (Atp1a1/2, Atp1b1 and Slc8a1) (Figure 3.3) were not significantly (P > 0.05) altered, whilst others were either significantly (P < 0.05) upregulated (Atp1a3) or significantly (P < 0.01) downregulated (Slc9a1). Relative expression of Atp1a3 was 2.61 ± 0.69 versus 0.84 ± 0.23, and Slc9a1 was 0.62 ± 0.07 versus 1.08 ± 0.08 in GK compared with control ventricle. Expression of genes encoding some calcium channel proteins (Caena1c1g, Caena2d1/2d2 and Caenb1/2), sodium channel proteins (Scn5a) and potassium channel proteins (Kcnab1, Kcnd1/2/3, Kcne1/4, Kcne3, Kcnj2, Kcnj4 and Kcnj2) were not significantly (P > 0.05) altered, whilst others were either significantly (P < 0.05) upregulated (Caena1h, Scn1b and Hcn2) or significantly (P < 0.05) downregulated (Hcn4, Kcn2a/b and Kcnj2) (Figure 3.4 and 3.6, respectively). Relative expression of Caena1h was 0.95 ± 0.16 versus 0.47 ± 0.09, Scn1b was 1.84 ± 0.16 versus 1.11 ± 0.11 and Hcn2 was 1.55 ± 0.15 versus 1.03 ± 0.08 in GK compared with control ventricle. Relative expression of Hcn4 was 0.16 ± 0.03 versus 0.37 ± 0.08, Kcna2 was 0.35 ± 0.03 versus 0.80 ± 0.11,
\( Kcn4 \) was 0.79 ± 0.25 versus 1.90 ± 0.26 and \( Kcnj2 \) was 0.52 ± 0.07 versus 0.78 ± 0.08 in GK compared with control ventricle. Expression of genes encoding \( \text{Ca}^{2+} \) transport (\( Atp2a1/2 \) and \( \text{Ryr}2 \)) and \( \text{Ca}^{2+} \) regulatory proteins (\( \text{Casq}2, \text{Pln}, \text{Calm}2, \text{Camk}2d \) and \( \text{Prkca}2 \)) were not significantly \( (P > 0.05) \) altered (Figure 3.7).
Figure 3.1: Expression of genes encoding various muscle proteins. The relative quantities versus Gapdh of genes encoding various muscle proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. Arb = Arbitrary.
Figure 3.2: Expression of genes encoding intercellular proteins. The relative quantities versus Gapdh of genes encoding various intercellular proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. Arb = Arbitrary.
Figure 3.3: Expression of genes encoding membrane pump and exchanger proteins. The relative quantities versus Gapdh of genes encoding various membrane pumps and exchanger proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. * P < 0.05, ** P < 0.01. Arb = Arbitrary.
Figure 3.4: Expression of genes encoding calcium channel proteins. The relative quantities versus Gapdh of genes encoding various calcium channel proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. * P < 0.05. Arb = Arbitrary.
Figure 3.5: Expression of genes encoding sodium channel proteins. The relative quantities versus Gapdh of genes encoding various sodium channel proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. ** P < 0.01. Arb = Arbitrary.
Figure 3.6: Expression of genes encoding potassium channel proteins. The relative quantities versus Gapdh of genes encoding various potassium channel proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. * P < 0.05, ** P < 0.01. Arb = Arbitrary.
Figure 3.7: Expression of genes encoding intracellular calcium regulatory proteins. The relative quantities versus *Gapdh* of genes encoding various intracellular transport proteins in ventricular muscle. Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. Tissue samples are from 9-10 hearts. Arb = Arbitrary.
3.1.2.3 Ventricular myocyte shortening and intracellular Ca\textsuperscript{2+}

Ventricular myocytes were isolated from young animals (8-10 weeks of age) using enzymatic and mechanical dispersal technique as described in section 2.4.1. Ventricular myocyte shortening and intracellular Ca\textsuperscript{2+} concentration were measured by video-edge detection and fluorescence photometry as described in sections 2.4.4 and 2.4.5, respectively. Resting cell length (Figure 3.8a), THALF relaxation (Figure 3.8c) and amplitude of shortening (Figure 3.8d) were not significantly (P > 0.05) altered; however, TPK shortening (Figure 3.8b) was significantly (P < 0.05) prolonged in GK (125.38 ± 3.38 ms) compared with control myocytes (115.03 ± 3.32 ms). Resting fura-2 ratio (Figure 3.9a), TPK Ca\textsuperscript{2+} transient (Figure 3.9b) and amplitude of the Ca\textsuperscript{2+} transient (Figure 3.9d) were unaltered, whereas THALF decay of the Ca\textsuperscript{2+} transient (Figure 3.9c) was significantly (P < 0.01) reduced in GK (148.32 ± 4.88 ms) compared with control myocytes (183.46 ± 5.76 ms).
Figure 3.8: Ventricular myocyte shortening. Recordings of ventricular myocyte shortening in electrically stimulated (1 Hz) cells. (a) Mean resting cell length. (b) Time to peak shortening. (c) Time to half relaxation of shortening and (d) Amplitude of shortening, expressed as a percentage of resting cell length (RCL). Data are mean ± S.E.M. Statistical comparisons were performed using independent samples t-test. n = 33-37 cells from 5 hearts. * P < 0.05
Figure 3.9: Ventricular myocyte intracellular Ca\textsuperscript{2+}. Recordings of intracellular Ca\textsuperscript{2+} in electrically stimulated (1 Hz) ventricular myocytes. (a) Mean resting fura-2 ratio, (b) Time to peak Ca\textsuperscript{2+} transient, (c) Time to half decay of the Ca\textsuperscript{2+} transient and (d) Amplitude of the Ca\textsuperscript{2+} transient. Data are mean ± S.E.M. Statistical comparisons were performed using unpaired student t test. n = 37-41 cells from 5 hearts. ** P < 0.01
3.1.3 Discussion

The major findings of the study were as follows: (i) upregulation of genes encoding Atp1a3, Cacna1h, Sen1b, and Hcn2 in GK compared with control left ventricle; (ii) downregulation of genes encoding Slec9a1, Hcn4, Kcna2, Kcna4 and Kcnj2 in GK compared with control left ventricle; and (iii) unaltered amplitude of shortening and intracellular Ca\(^{2+}\) transient and prolonged TPK shortening and shortened THALF decay of the Ca\(^{2+}\) transient in ventricular myocytes from young (8-10 weeks of age) GK compared with control rats.

Expressions of genes encoding nine of the 67 measured cardiac muscle proteins were significantly altered: Atp1a3, Cacna1h, Sen1b and Hcn2 were upregulated, whilst Slec9a1, Hcn4, Kcna2, Kcna4 and Kcnj2 were downregulated in GK compared with control ventricle.

The Na\(^+-\)K\(^+-\)ATPase is found in virtually all animal cells and has major regulatory functions, including the maintenance of intra- to extracellular Na\(^{+}\) and K\(^{+}\) concentration gradients, membrane excitability and contractility, as well as secondary transport processes. It comprises a catalytic \(\alpha\) subunit (100-112 kDa) and a glycosylated \(\beta\)-subunit (40-60 kDa). The expression of the \(\alpha3\) subunit in humans is confined to adult brain and heart [292-294]. Although the specific role of Atp1a3 in heart is still not understood, upregulation of Atp1a3 might have an effect on catalytic activity of the Na\(^+-\)K\(^+-\)ATPase, which might in turn alter the intracellular Na\(^{+}\)-K\(^{+}\) balance and have implications for excitation-contraction coupling. The \(\beta\) subunit plays an essential role in regulating cardiac contractility, and its loss is associated with significant
pathophysiology of the heart [295]. The gene \textit{Atp1b1}, which encodes the β1 subunit was unaltered [295].

In the myocardium, the \( \text{Na}^+\text{-H}^+ \) exchanger isoform1 (\textit{Slc9a1}) is a plasma membrane protein that regulates intracellular pH. Activation of the exchanger leads to the extrusion of one intracellular proton in exchange for one extracellular sodium [296]. \textit{Slc9a1} was downregulated in GK ventricle. A previous study in the Zucker diabetic fatty rat has also reported downregulation of \textit{Slc9a1} [284]. Downregulation of \textit{Slc9a1}, if accompanied by a reduction in exchange protein, would be expected to reduce activity of the exchanger, which perhaps in turn might lead to an accumulation of intracellular protons. Acidification of the intracellular environment would be expected to have a variety of effects on excitation-contraction coupling, including altered myofilament sensitivity to \( \text{Ca}^{2+} \); a feature that has been previously demonstrated in ventricular myocytes from ageing GK rats and in streptozotocin-induced diabetic rats [203, 280, 297].

The voltage-gated T-type \( \text{Ca}^{2+} \) channel \( \text{Cav3.2} \) (\( \alpha_{1H} \)) subunit, responsible for T-type \( \text{Ca}^{2+} \) current, is expressed in different tissues, including cardiac muscle, and participates in pacemaker activity [298]. The gene \textit{Caen1h} encodes the \( \alpha_{1H} \) subunit of the T-type \( \text{Ca}^{2+} \) channel, and upregulation might be expected to modify T-type \( \text{Ca}^{2+} \) current. Changing expression of \textit{Caen1h} might be a compensatory response to the reductions in HR that has been previously reported in young GK rats [203]. L-type \( \text{Ca}^{2+} \) channels are formed by heteromultimeric complexes of \( \alpha, \beta \) and \( \gamma \) subunits [299]. The major L-type voltage-gated calcium channels in the heart consist of an \( \alpha_{1C} \) (\( \text{Cav1.2} \)) subunit, usually associated with an auxiliary \( \beta \) subunit (\( \text{Cav1β2} \)) [300]. In young GK
ventricle. Cacna1c, Cacnb1 and Cacnb2, which encode α1C, β1 and β2 subunits and play an important role in the regulation of Ca\(^{2+}\) current, were unaltered [301].

Voltage-gated Na\(^+\) channels are heteromeric proteins that function in the generation and propagation of action potentials in muscle and neuronal cells and are composed of one α and two β subunits, where the α subunit provides channel activity. Type 5 α protein, encoded by Scn5a, is responsible for the initial upstroke of the action potential, and type 1 β protein, encoded by Scn1b, contributes to the modulation of the kinetics of channel inactivation. Expression of Scn5a was unaltered and Scn1b was upregulated in GK ventricle. Interestingly, previous studies have shown that mutations in Scn1b are associated with Brugada syndrome and defects in cardiac conduction [302].

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to the initiation and regulation of cardiac rhythm. The pacemaker current (I\(_{\text{f}}\)) is encoded by the HCN family. HCN isoforms, including Hcn2 and Hcn4, are present in ventricular tissue of the heart [303, 304]. Previous studies have demonstrated that Hcn2 and Hcn4 channel proteins can associate with each other in rat ventricle and that different ratios of Hcn2 and Hcn4 transcripts overlapping in different tissues might contribute to tissue-specific properties of I\(_{\text{f}}\) [305]. Upregulation of Hcn2 and downregulation of Hcn4 might be compensatory responses to the reductions in HR that have been previously reported in young GK rats [203].

In contrast to the classical I\(_{\text{Kr}}\) and I\(_{\text{Ks}}\), it has become evident that many cardiac tissues express delayed rectifier currents with faster kinetics, similar to those found in nerve and muscle, and these currents have been designated as I\(_{\text{kur}}\). A variety of I\(_{\text{kur}}\) channels comprising different shaker-related clones, including Kv1.2, Kv1.3 and Kv1.6, have
been identified in different species and to different extents in different regions of the heart [306, 307, 76]. Downregulation of *Kcna2*, if accompanied by a reduction in expression of *Kv1.2* channel protein, might alter the time course of repolarization and contribute to the reduced HR previously reported in the GK rat [203].

Rapidly activating and inactivating cardiac *I*_{to} are expressed in most mammalian cardiomyocytes and contribute to the early phase of action potential repolarization and to plateau potentials. The *I*_{to,d} and *I*_{to,s} are differentially expressed in the myocardium, and distinct pore-forming (α) subunits underlie the two *I*_{to} components. *Kcna4* encodes *I*_{to,s} channels, and downregulation of *Kcna4* protein might alter the time course of repolarization and contribute to the reduced HR previously reported in the GK rat [203]. Various K⁺ channels are involved in phase 3 repolarization of the cardiac action potential, and these channels include *Kv1.1* (*hERG1*), *Kv7.1* (*Kcnq1*) and *Kir2.1* (*Kcnj2*), which are the α subunits that conduct *IKr*, *IKs* and *IK1*, respectively [308]. Reduced *Kir2.1* would be likely to prolong the terminal phase of the cardiac action potential [309]. Down-regulation of *Kcna4*, if accompanied by a reduction in expression of *Kv1.4* channel protein, might alter the time course of repolarization and might also contribute to reduced HR [203].

Cardiac muscle function was assessed to investigate whether the changing pattern of genes might be associated with changes in the amplitude and/or time course of ventricular myocyte shortening. Whilst resting cell length, fura-2 ratio, THALF relaxation and amplitude of shortening and TPK and amplitude of the Ca²⁺ transient were unaltered, the TPK shortening was prolonged and THALF decay of the Ca²⁺ transient was shortened in GK myocytes compared with control myocytes. Alterations
in the kinetics of L-type Ca\(^{2+}\) current and/or SR Ca\(^{2+}\) release might contribute to the prolonged TPK shortening, whilst alterations in the kinetics of Ca\(^{2+}\) extrusion, primarily in the Na\(^+\)/Ca\(^{2+}\) exchange and/or SR Ca\(^{2+}\) uptake, might contribute to the shortened THALF decay of the Ca\(^{2+}\) transient in GK myocytes. Consistent with the present study, El Omar et al (2004) reported unaltered amplitude of shortening and Ca\(^{2+}\) transient in GK myocytes [205]. Unaltered amplitude of shortening accompanied by unaltered TPK and decreased THALF relaxation of shortening and increased amplitude of the Ca\(^{2+}\) transient accompanied by unaltered TPK and THALF decay of the Ca\(^{2+}\) transient have been previously reported in ventricular myocytes from elderly GK rats (18 months of age) [192]. Altered expression of certain genes, including those that encode Atp1a3 and Slc9a1, might have implications for intracellular Na\(^+\) and H\(^+\) balance, which in turn might have effects on the process of excitation-contraction coupling. Gene expression experiments were performed on ventricular muscle tissue, which contains not only muscle but also vascular and connective tissues. In the future, more extensive experiments might be conducted to investigate whether any of the reported changes in gene expression might be attributed partly to changes occurring in tissues other than cardiac muscle.

3.1.4 Conclusion

The pattern of 67 genes encoding cardiac muscle, intercellular, membrane pump and exchanger, calcium, sodium and potassium channel and intracellular Ca\(^{2+}\) and Ca\(^{2+}\) regulatory proteins have been characterized in the cardiac ventricle of young GK rats. Even at this early stage of diabetes, the expression of genes encoding some membrane pumps, exchangers and calcium, sodium and potassium channel proteins were altered.
Further understanding of the changes that occur in the heart during the early stages of diabetes may provide opportunities to develop interventions and treatment strategies that might prevent or delay the onset of serious functional disorders as the disease progresses.
3.2 Effects of exercise training on the excitation-contraction coupling, expression of genes encoding cardiac muscle proteins L-type Ca\(^{2+}\) current, cardiac muscle protein expression and ventricular morphology in the aging Goto-Kakizaki type 2 diabetic rat

3.2.1 Introduction

DM has reached pandemic proportions worldwide. In the UAE DM is a serious and worsening medical problem. In 2005 the reported prevalence of DM in UAE citizens was 25% rising to 40% after the age of 55 [97]. Cardiovascular disease is the major cause of morbidity and mortality in diabetic patients and hearts of diabetic patients are frequently in a compromised condition [291]. A variety of diastolic and systolic dysfunctions have been reported in T2DM patients and the severity of the abnormalities depend on the patients' age and the duration of diabetes [152, 155, 161, 163, 164, 310-312]. Although, several novel forms of intervention aiming at newly identified therapeutic targets are currently being developed for T2DM, it is well established that physical exercise continues to be one of the most valuable forms of non-pharmacological therapy. An abundance of evidence exists for prescribing exercise therapy in the treatment of a variety of diseases including metabolic syndrome-related disorders (insulin resistance, T2DM, dyslipidemia, hypertension, obesity), heart and pulmonary diseases, muscle, bone and joint diseases and cancer, depression and asthma [313]. Physical activity has been reported to influence several signs and symptoms of DM, including blood glucose concentration, insulin action and cardiovascular risk factors and long-term exercise has been repeatedly associated with lower occurrence rates of T2DM [314].
In this study, the effect of DM and exercise training as a form of non-pharmacological therapy for DM on the expression of genes encoding cardiac muscle proteins and on ventricular myocyte shortening and intracellular Ca$^{2+}$ in the aging (10-11 month) GK rat has been investigated.

3.2.2 Result

3.2.2.1 General characteristics of the animal

Thirty male GK and thirty male Wistar control rats aged 8 months were divided into 4 subgroups, each containing 15 animals. Two subgroups of GK and control rats received exercise training performed as described in Section 2.4.2, whilst the other 2 subgroups of GK and control rats continued a sedentary lifestyle. A glucose tolerance test was performed in the resting state 2 months into the exercise training program. After 2 months of exercise training, blood glucose concentrations 120 min following a glucose challenge in sedentary GK rats (236.27 ± 15.12 mg/dl) was significantly (P < 0.01) higher than blood glucose in sedentary controls (93.93 ± 2.32 mg/dl). Exercise training modestly reduced blood glucose in GK (207.36 ± 12.13 mg/dl) and in control (89.60 ± 2.78 mg/dl) rats however, the reductions in blood glucose with exercise did not reach significance (P > 0.05).

Body weight, heart weight and non-fasting blood glucose, measured immediately before experiments, were significantly (P < 0.01) increased in sedentary GK compared to sedentary control rats. Exercise training modestly reduced body weight, blood glucose and increased heart weight to body weight ratio in both GK and control rats although the differences did not reach significance (P > 0.05) (Table 3.2).
Table 3.2: General characteristic of the animals

<table>
<thead>
<tr>
<th></th>
<th>Control sedentary (CS)</th>
<th>Control + exercise (CEX)</th>
<th>GK sedentary (GKS)</th>
<th>GK + exercise (GKEX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>400.33 ± 12.17</td>
<td>371.73 ± 11.80</td>
<td>443.64 ± 7.94</td>
<td>418.93 ± 6.80</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.37 ± 0.05</td>
<td>1.34 ± 0.04</td>
<td>1.60 ± 0.04</td>
<td>1.64 ± 0.05</td>
</tr>
<tr>
<td>Heart weight / Body weight (mg/g)</td>
<td>3.43 ± 0.07</td>
<td>3.62 ± 0.09</td>
<td>3.61 ± 0.08</td>
<td>3.92 ± 0.13</td>
</tr>
<tr>
<td>Non-fasting blood glucose (mg/dl)</td>
<td>91.67 ± 1.74</td>
<td>89.67 ± 1.58</td>
<td>161.29 ± 12.77</td>
<td>133.07 ± 9.79</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. 15 animals in each group.

- Body weight: CS vs. GKS and CEX vs. GKEX P < 0.05; CEX vs. GKS P < 0.01
- Heart weight: CS vs. GKS, CS vs. GKEX, CEX vs. GKS and CEX vs. GKEX P < 0.01
- Heart weight/body weight: CS vs. GKE X P < 0.01
- Non-fasting blood glucose: CS vs. GKS, CS vs. GKE X. CEX vs. GKS and CEX vs. GKE X P < 0.01
3.2.2.2 Ventricle myocyte shortening

After 2 months of exercise training, ventricular myocytes from exercise trained animals and sedentary rats were isolated using enzymatic and mechanical dispersal techniques as described in Section 2.4.1. Ventricular myocyte shortening was measured by a video edge detection system as described in Section 2.4.4. Characteristics of ventricular myocyte shortening are shown in Figure 3.10. Myocyte resting cell length was significantly (P < 0.05) longer in sedentary GK compared to sedentary control myocytes and was not additionally altered by exercise training in GK or control myocytes (Figure 3.10a). TPK shortening was not significantly (P > 0.05) different in myocytes from sedentary GK compared to sedentary control rats. Exercise training significantly (P < 0.05) reduced TPK in myocytes from control rats compared to those from sedentary control rats (Figure 3.10b). THALF relaxation of shortening was not significantly (P > 0.05) different in myocytes from sedentary GK compared to sedentary control rats and was not additionally altered by exercise training (Figure 3.10c). Amplitude of shortening was not significantly (P > 0.05) altered in myocytes from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.10d).
Figure 3.10: Ventricular myocyte shortening. Recordings of ventricular myocyte shortening in electrically stimulated (1 Hz) cells. (a) Mean resting cell length, (b) Time to peak shortening, (c) Time to half relaxation relaxation of shortening and (d) Amplitude of shortening expressed as a percentage of resting cell length (RCL). Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. n = 44-67 cells from 5-6 hearts. Lines above bars indicate significance (P < 0.05).
3.2.2.3 Ventricular myocyte intracellular Ca$^{2+}$

Intracellular Ca$^{2+}$ concentration was measured by fluorescence photometry, as described in Section 2.4.5, using ventricular myocytes isolated from sedentary and exercise trained groups. Characteristics of ventricular myocyte intracellular Ca$^{2+}$ are shown in Figure 3.11. Resting fura Ca$^{2+}$ was not significantly (P > 0.05) different between sedentary GK and sedentary control myocytes, however, exercise training significantly (P < 0.05) increased resting fura Ca$^{2+}$ in exercise trained GK compared to sedentary GK myocyte (Figure 3.11a). TPK Ca$^{2+}$ transient was not significantly (P > 0.05) different in myocytes from sedentary GK compared to sedentary control rats and was not additionally altered by exercise training in myocytes from GK and control rats (Figure 3.11b). THALF decay of the Ca$^{2+}$ transient was not significantly (P > 0.05) altered in myocytes from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.11c). Amplitude of the Ca$^{2+}$ transient was not significantly (P > 0.05) altered in myocytes from either sedentary GK compared to sedentary control rats or by exercise training (Figure 3.11d).
Figure 3.11: Ventricular myocyte intracellular Ca$^{2+}$. Recordings of ventricular myocyte intracellular Ca$^{2+}$ in electrically stimulated (1 Hz) cells. (a) Mean resting fura-2 ratio (340/380 nm), (b) Time to peak Ca$^{2+}$ transient, (c) Time to half decay of Ca$^{2+}$ transient and (d) Amplitude of Ca$^{2+}$ transient. Data are means ± S.E.M., statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 40-46 cells from 5-6 hearts. Lines above bars indicate significance P < 0.05.
3.2.2.4 Sarcoplasmic reticulum Ca$^{2+}$ transport

SR Ca$^{2+}$ transport was measured in ventricular myocytes loaded with fluorescence fura-2 AM as described in section 2.4.6. Ventricular myocytes were obtained from the hearts of sedentary and exercise trained rat hearts.

A typical recording, in a control myocyte, illustrating the experimental protocol is shown in Figure 3.12a. The amplitude of electrically-evoked Ca$^{2+}$ transients were not significantly (P > 0.05) altered in myocytes from GK rats compared to controls. Interestingly, electrically-evoked Ca$^{2+}$ transients were significantly (P < 0.05) increased in myocytes from exercise trained GK compared to sedentary GK rats (Figure 3.12b). Caffeine-evoked Ca$^{2+}$ transients were not significantly (P > 0.05) altered in myocytes from GK rats compared to controls or by exercise training (Figure 3.12c). Fractional release of Ca$^{2+}$ (fraction of caffeine-evoked Ca$^{2+}$ transient that is released during electrically-evoked stimulation) was not significantly (P > 0.05) altered in myocytes from GK rats compared to controls, however, it was significantly (P < 0.05) increased in myocytes from exercise trained control compared to sedentary control rats (Figure 3.12d). Recovery of the Ca$^{2+}$ transient following application of caffeine and resumption of electrical stimulation was not significantly (P > 0.05) altered in myocytes from GK rats compared to controls or by exercise training (Figure 3.12e).
Figure 3.12: Sarcoplasmic reticulum Ca\textsuperscript{2+}. (a) Typical record illustrating the effects of 1Hz electrical stimulation (ES) and rapid application of caffeine on fura-2 ratio in a myocyte from a sedentary control rat. (b) Mean amplitude of electrically-evoked and (c)
Caffeine-evoked Ca\textsuperscript{2+} transients. (d) Mean amplitude of sarcoplasmic reticulum fractional Ca\textsuperscript{2+} release and (e) Recovery of the electrically-evoked intracellular Ca\textsuperscript{2+} after application of caffeine. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. n = 27-52 cells from 5-6 hearts. Lines above bars indicate significance (P < 0.05).
3.2.2.5 Myofilament sensitivity to $\text{Ca}^{2+}$

Myofilament sensitivity to $\text{Ca}^{2+}$ 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 the late relaxation of the twitch contraction as described in Section 2.4.7. The position of the trajectory reflects the relative myofilament response to $\text{Ca}^{2+}$ and hence, can be used as a measure of myofilament sensitivity to $\text{Ca}^{2+}$ (Spurgeon et al., 1992). A typical recording of shortening and $\text{Ca}^{2+}$ transient with time in a myocyte from a control rat is shown in Figure 3.13a. A phase-plane diagram of fura-2 ratio versus cell length is shown in Figure 3.13b. The mean gradients during the periods 500-800, 500-700 and 500-600 ms are shown in Figures 3.13c-e, respectively. Gradients were not significantly ($P > 0.05$) altered in myocytes from GK rats compared to controls or by exercise training. It was interesting to note a difference in gradient between myocytes from exercise trained GK compared to sedentary GK, however, the difference did not reach significance.
Figure 3.13: Myofilament sensitivity to Ca$^{2+}$. (a) Typical record of shortening and fura-2 ratio unit (RU) in a myocyte from a sedentary control rat. (b) Typical phase plane diagram of Fura-2 ratio unit (RU) versus Cell length in electrically stimulated (1 Hz) myocyte from a sedentary control rat. The solid arrow in (b) indicates where the gradient was measured. Mean gradient of the fura-2-cell length trajectory during late relaxation of the twitch contraction during the periods (c) 500-800 ms, (d) 500-700 ms and (e) 500-600 ms. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 6-24 cells from 5-6 hearts. Lines above bars indicate significance (P < 0.05).
3.2.2.6 L-type Ca\textsuperscript{2+} current

Ventricular myocytes from sedentary and exercise trained animals were isolated using enzymatic and mechanical dispersal techniques as described in Section 2.4.1. L-type \(\text{Ca}^{2+}\) current was measured using whole cell patch-clamp techniques as described in Section 2.4.8. Typical recordings of L-type \(\text{Ca}^{2+}\) current in sedentary control myocytes during activation, inactivation and restitution experiments are shown in Figure 2.14a-c, respectively. Amplitude of L-type \(\text{Ca}^{2+}\) current at test voltages ranging between -60 and +70 were not significantly (\(P > 0.05\)) altered in myocytes from either sedentary GK compared to sedentary control rats or with exercise training (Figure 3.14d). Although the currents were not significantly different, it was interesting to note that the largest amplitude of L-type \(\text{Ca}^{2+}\) current generated at a test potential of 0 mV was observed in exercise trained control, followed by sedentary control, exercise trained GK and smallest in sedentary GK myocytes. Inactivation of L-type \(\text{Ca}^{2+}\) current at pre-pulse test voltages ranging between -50 and +30 were not significantly (\(P > 0.05\)) altered in myocytes from either sedentary GK compared to sedentary control rats or by exercise training (Figure 3.14e). Restitution of L-type \(\text{Ca}^{2+}\) current with inter-pulse times ranging between 20 and 700 ms were not significantly (\(P > 0.05\)) altered in myocytes from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.14f).
Figure 3.14: L-type Ca\textsuperscript{2+} current. Typical recordings (a) Activation, (b) Inactivation and (c) Restitution of L-type Ca\textsuperscript{2+} current in a ventricular myocyte from a sedentary control rat. (d) Mean activation current at voltages ranging from -60 to +70 mV. (e) Mean inactivation curves at pre-pulse voltages ranging from -50 to +30 mV and (f) Mean restitution curves at inter-pulse timings ranging from 20 to 700 ms in. Data are mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 9-20 cells from 5 to 6 hearts. Sedentary GK (GKS), sedentary control (CS), exercised trained GK (GKEX) and exercise trained control (CEX) rats.
3.2.2.7 Expression of cardiac muscle mRNA

Expression of mRNA encoding a variety of cardiac muscle, intercellular, intracellular Ca\(^{2+}\) transport and regulatory, cell membrane transport, sodium, calcium and potassium channel proteins in ventricular muscle was assessed using real time RT-PCR as described in section 2.4.3. The results obtained from sedentary and exercise trained groups are shown in Figure 3.15-3.21. The results show that expression of mRNA encoding cardiac muscle proteins *Myh6/7, Myhpc3, Myl1/3, Actc1, Tnnt3, Tnnt2, Tpm1/4* and *Dbi* were not significantly (P > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.15). Expression of mRNA encoding *Tpm2* was significantly (P < 0.05) upregulated in ventricular muscle from sedentary GK compared to sedentary control rats (Figure 3.15). Expression of mRNA encoding intercellular proteins *Gja5/7* and *Cav1* were not significantly (P > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.16). *Gja1* was significantly (P < 0.05) downregulated and *Gja4* was significantly (P < 0.05) upregulated in ventricular muscle from sedentary GK compared to sedentary control rats (Figure 3.16). *Gja1* and *Cav3* were significantly (P < 0.05) upregulated in ventricular muscle from exercise trained GK compared to sedentary GK rats (Figure 3.16). Expression of mRNA encoding intracellular Ca\(^{2+}\) transport and regulatory proteins *Atp2a1, Atp2a2, Ryr2, Casq2, Pln* and *Calm2* were not significantly (P > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.17). Expression of mRNA encoding cell membrane transport proteins *Atp1a1/2/3, Slc8a1/3* and *Slc9a1* were not significantly (P > 0.05) altered in ventricular muscle from
sedentary GK compared to sedentary control rats or by exercise training (Figure 3.18). However, Atp1bl was significantly (P < 0.05) upregulated in ventricular muscle from sedentary GK rats compared to sedentary controls (Figure 3.18). Expression of mRNA encoding Ca\(^{2+}\) channel proteins Ca\(\text{calc}\), Ca\(\text{na}2d\)/2, Ca\(\text{cnb}1/3\) and Ca\(\text{nb}4\) were not significantly (P > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats or by exercise training (Figure 3.19). Ca\(\text{nal}\)g and Ca\(\text{nb}2\) were significantly (P < 0.05) upregulated in ventricular muscle from sedentary GK rats compared to sedentary controls (Figure 3.19). Expression of mRNA encoding Na\(^{+}\) channel proteins Scn5a, Scn1b and Scn3b were not significantly (P > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats (Figure 3.20). Expression of mRNA encoding Na\(^{+}\) channel protein Scn3b was significantly (P < 0.05) upregulated in exercise trained GK compared to exercise trained control rats (Figure 3.20). Figure 3.21a & b shows the expression of mRNA encoding K\(^{+}\) channel proteins Hcn4, Kcnq2/4/5/7, Kcnj3/5/8/11/12, Kcnd2, Kcnab1, Kcnb1, Kcnd1/2/3, Kcne2/3, Kcng2, Kcnh2 and Kcnj2 were not significantly (p > 0.05) altered in ventricular muscle from sedentary GK compared to sedentary control rats or by exercise training. Hcn2, Kcna3, Kcen1 were significantly (P < 0.05) upregulated and Kcnj2 and Kcnj3 were downregulated in ventricular muscle from sedentary GK rats compared to sedentary controls. Hcn2 was significantly (P < 0.05) downregulated (Figure 3.21a) and Kcnj3 was significantly (P < 0.05) upregulated (Figure 3.21b) in ventricular muscle from exercise trained GK compared to sedentary GK rats.
Figure 3.15: Expression of mRNA encoding various cardiac muscle proteins. The relative quantities versus Gapdh of genes encoding various cardiac muscle proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
Figure 3.16: Expression of mRNA encoding various intercellular proteins. The relative quantities versus Gapdh of genes encoding various intercellular proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
Figure 3.17: Expression of mRNA encoding various intracellular \( \text{Ca}^{2+} \) transport proteins. The relative quantities versus \textit{Gapdh} of genes encoding various intracellular \( \text{Ca}^{2+} \) transport proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, \( n = 7-10 \) hearts. Lines above bars indicate significance (\( P < 0.05 \)). Arb = Arbitrary.
Figure 3.18: Expression of mRNA encoding various cell membrane transport proteins. The relative quantities versus Gapdh of genes encoding cell membrane transport proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
Figure 3.19: Expression of mRNA encoding various Ca\(^{2+}\) channel proteins. The relative quantities versus Gapdh of genes encoding various Ca\(^{2+}\) channel proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
Figure 3.20: Expression of mRNA encoding various Na\(^+\) channel proteins. The relative quantities versus Gapdh of genes encoding various Na\(^+\) channel proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
Figure 3.21: Expression of mRNA encoding various K\(^+\) channel proteins. (a) & (b) The relative quantities versus Gapdh of genes encoding various K\(^+\) channel proteins in ventricular muscle. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7-10 hearts. Lines above bars indicate significance (P < 0.05). Arb = Arbitrary.
3.2.2.8 Expression of ventricular muscle proteins

The results from real time RT-PCR showed alteration in the expression of genes encoding a number of cardiac muscle proteins. To investigate, if these alterations of genes have been translated into expression of corresponding proteins, immunoblot experiments were conducted. Experiments were performed using tissues acquired from the same animals that were used in the real time RT-PCR experiments. The results of real time RT-PCR showed that, the expression of mRNA encoding Gaj1, Gaj4, Cacnb2 and Tpm2, were altered in sedentary GK compared to sedentary control therefore, the evaluation of the expression these proteins were investigated.

Expression of connexin 37 (CX37), connexin 43 (CX43), L-type Ca\(^{2+}\)-beta 1 subunit and tropomyosin 2 (beta) sedentary and exercise trained GK and control rats were evaluated by immunoblot assay as described in Section 2.4.9

Figure 3.22a shows immunoblots of the proteins and β-actin as an internal control. Quantitative densitometric data corrected to β-actin and normalized to control sedentary for CX 37, CX 43, L-type Ca\(^{2+}\) and tropomyosin are shown in Figure 3.22b to 3.22e, respectively. No significant (P > 0.05) alterations were observed in CX43, CX37, L-type Ca\(^{2+}\) and tropomyosin proteins in sedentary GK rats compared to sedentary control rat or following exercise training.
Figure 3.22: Expression of cardiac muscle protein. (a) Immunoblots of cardiac proteins. Quantitative densitometric corrected to β-actin and normalized to control rats for (b) Connexin 37, (c) Connexin 43, (d) L-type Ca^{2+}, and (e) Tropomyosin. The average data was obtained from three separate experiments. Results are expressed as mean ± S.E.M.
Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. Sedentary control (Sed-cont), exercise trained control (Ext-cont), sedentary GK (Sed-GK) and exercise trained GK (Ext-GK).
3.2.2.9 Morphological study

The ultrastructural morphology of left ventricle in sedentary and exercise trained animals was assessed using transmission electron microscopy as described in Section 2.4.12. At a magnification of X18000, no substantial changes were observed in ventricular tissue from sedentary GK compared to sedentary control rats or in exercise trained compared to sedentary rats. LV myocyte structure was intact and preserved in all groups of rats (Figure 3.23).

Mitochondria were densely packed between the myofibrils. The quantitative results are shown in Figure 3.24. The number of mitochondria was modestly but not significantly (P > 0.05) reduced in sedentary GK (8.0 ± 0.7) compared to sedentary control rats (9.0 ± 0.8) however, exercise training significantly (P < 0.05) increased the mitochondria number in exercise trained GK (11 ± 0.8) compared to sedentary GK rats, with no significant (P > 0.05) changes observed in controls. Sarcomere organization was regular in arrangement and clear striations were seen in all groups. Sarcomere length was measured as the distance between two Z-bands and results are shown in Figure 3.25a. No significant (P > 0.05) alteration was observed in sarcomere length between sedentary GK (3.07 ± 0.02) and sedentary control (2.95 ± 0.05). Exercise training significantly (P < 0.05) reduced sarcomere length in exercise trained GK (2.67 ± 0.09) and in exercise trained control (2.73 ± 0.02) rats compared to sedentary GK and control rats, respectively. Sarcomere width was also measured and results are shown in Figure 3.25b. Sarcomere width in sedentary GK (1.88 ± 0.09) was significantly (P < 0.01) wider compared to sedentary control (1.47 ± 0.07) rats. Exercise training significantly
(P < 0.01) increased sarcomere width in exercise trained GK (2.73 ± 0.14) and exercise trained control (2.31 ± 0.08) rats.

The ICDs were evaluated from electron micrographs under a magnification of X7000 (Figure 3.26a). Random fields of many longitudinal sections showed preserved structure of ICDs in all experimental groups. Visually, more ICD were observed in GK sedentary compared to the other groups. The convolutions per given length of ICD membrane were measured (Figure 3.26b). There was no significant (P > 0.05) difference in the average number of convolution between sedentary GK and sedentary control rats. Exercise training significantly (P < 0.01) increased the number of convolutions in exercise trained GK compared to sedentary GK rats.
Figure 3.23: Electron micrographs of longitudinal sections of rat left ventricular myocyte. Sedentary control (Sed-cont), exercise trained control (Ext-cont), sedentary GK (Sed-GK) and exercise trained GK (Ext-GK). Sarcomere Length (SL), Sarcomere width (SW), Mitochondria (M) and Z-line (Z). Magnification X18000.
Figure 3.24: Average numbers of mitochondria. Results are expressed as mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. 21-24 electron micrographs were analyzed from 4 rats. Line above bar indicates significance (P < 0.05).
Figure 3.25: Cardiac myocyte sarcomere. (a) Sarcomere length and (b) Sarcomere width. Results are expressed as mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. 36-54 electron micrographs were analyzed from 4 rats. Line above bar indicate significance (P < 0.05).
Figure 3.26: Intercalated disc. (a) Electron micrograph of intercalated disc of left ventricular tissue and (b) Relative convolution number of intercalated disc membrane. Results are expressed as mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. 11-22 electron micrographs were analyzed from 3 rats. Line above bar indicate significance (p < 0.05). Sedentary Control (Sed-cont), Exercise trained control (Ext-cont), Sedentary GK (Sed-GK) and Exercise trained GK (Ext-GK). Intercalated disc (ICD). Magnification X7000.
3.2.3 Discussion

This study investigated the effects of exercise training on excitation-contraction coupling and expression of mRNA encoding cardiac muscle proteins in the adult GK type 2 diabetic compared to age-matched Wistar control rats. The major findings were: (i) amplitude of shortening was not altered in ventricular myocytes from sedentary GK compared to sedentary control rats or by exercise training, (ii) amplitude of Ca\(^{2+}\) transients was not altered in ventricular myocytes from sedentary GK compared to sedentary control rats or by exercise training, (iii) SR Ca\(^{2+}\) content and myofilament sensitivity to Ca\(^{2+}\) were not altered, (iv) L-type Ca\(^{2+}\) current (activation, inactivation and restitution) was not altered in ventricular myocytes from sedentary GK compared to sedentary control rats or by exercise training, (v) expression of mRNA encoding Tpm2, Gja4, Atp1b1, Caclang, Caenb2, Hcn2, Kcna3 and Kcnel were upregulated and Gjal, Kcnj2 and Kcnk3 were downregulated in sedentary GK compared to sedentary control rats and Gjal, Cav3 and Kcnk3 were upregulated and Hcn2 was downregulated in exercise trained GK compared to sedentary GK, (vi) expression of various cardiac protein including CX 37, CX 43, L-type Ca\(^{2+}\) channel and tropomyosin were not altered in sedentary GK compared to sedentary control or by exercise training and (vii) morphologically, mitochondria number was increased in exercise trained GK compared to sedentary GK. The sarcomere length was shorter in exercise trained GK and control compared to sedentary GK and control rats, respectively. In contrast, the sarcomere width was longer in exercise trained GK and control compared to sedentary GK and control rats, respectively. ICD membrane convolutions were increased in exercised trained GK compared to sedentary GK ventricles.
After 2 months of exercise training a glucose tolerance test was applied following an overnight fast in all four groups of rats. The results showed that 120 min following an intraperitoneal injection of glucose (2 g/kg body weight), blood glucose levels were significantly (P < 0.05) elevated in sedentary GK rats compared to sedentary controls demonstrating impaired glucose uptake in GK rats. Exercise training reduced blood glucose to a small extent in control and to a larger extent in GK rats, however, the difference did not reach statistical significance (P > 0.05). Non-fasting blood glucose measured immediately before experiment was significantly (P < 0.05) elevated in GK sedentary compared to control sedentary rats. Exercise training reduced blood glucose to a small extent in control and to a larger extent in GK rats however, the difference did not reach statistical significance (P > 0.05). Previous studies have shown that long term (2, 8 and 14 months of age) non-fasting serum insulin is reduced in the GK rat compared to age-matched controls whilst fasting-insulin is reduced at 2 months and increased at 8 and 14 months of age in GK rats compared to age-matched controls [315]. It has also been shown that exercise training for 9 weeks reduced the levels of fasting insulin in exercise trained GK rats compared to age-matched controls and calculation of the insulin sensitivity index determined that training significantly increased insulin sensitivity [316].

Body weight and heart weight were significantly increased in sedentary GK rats compared to sedentary controls. Collectively, these characteristics are typical of features widely displayed in T2DM [203].

Ventricular myocyte shortening and Ca²⁺ transport were generally well preserved in sedentary GK rats compared to sedentary controls and after exercise training. The time
course of shortening and Ca\(^{2+}\) transient were altered by exercise training. The TPK shortening was unaltered by exercise training in GK compared to sedentary GK rats and reduced by exercise training in control compared to sedentary control rats.

Despite generally well preserved myocyte shortening there were a variety of changes in expression of ventricle mRNA in sedentary diabetic compared to control heart and as a result of exercise training.

Expression of mRNA encoding cardiac muscle proteins in young (8–10 weeks) [317] and in adult (10-11 months) GK rats [318] are compared in Table 3.3. It is interesting to note the changing profile of mRNA expression as the GK rats aged. Expressions of Tpm2, Gja4, Atplb1, Cavnalg, Cavnb2, Kena3 and Knel1 were upregulated whereas Gja1 and Kenk3 were downregulated with age. Downregulation of Sle9al, Hcn4, Kena2, and Kena4 in young animals normalized with age. Upregulation of Caen1h normalized with age. Hcn2 was upregulated and Kcnj2 was downregulated in sedentary GK rats from an early age.

The effects of exercise training on expression of mRNA encoding cardiac muscle proteins in adult GK and control rats are summarized in Table 3.4. Interestingly mRNA expression of Gja1, Cav3 and Kenk3 were upregulated and Hcn2 was downregulated by exercise training in GK but was unaltered by exercise training in control rats. Expression of mRNA encoding Tpm2 was upregulated in aging sedentary GK rat. Tropomyosin controls muscle contraction by inhibiting actin-myosin interaction in a calcium-sensitive manner and mutations in the Tpm2 gene may lead to changes in the affinity for actin resulting in either muscle weakness or defective Ca\(^{2+}\) activation of contractility [319].
Expression of mRNA encoding Gja1 was downregulated and Gja4 was upregulated in aging sedentary GK compared to sedentary control rat. In contrast Gja1 was upregulated in GK ventricle by exercise training. Connexins are the pore forming subunits of gap junction channels and these channels regulate membrane permeability in individual cells or couple between adjacent cells to form gap junctions and thereby providing a pathway for regulated intercellular communication. CX43 which is encoded by Gja1, is a major protein of gap junctions in the heart and is vital to the synchronicity of contraction in the heart. Mutations in various connexin proteins including CX43 are known to be associated with cardiac arrhythmias [320]. Altered expression of the gene encoding Gja1, if accompanied by a reduction in CX43 protein, might partly underlie the low heart rates which have been previously reported in GK rat heart [203]. Interestingly, the present results show that exercise training appeared to upregulate the expression of Gja1 in exercise trained GK rat ventricle. Multiple epidemiological studies have associated a single nucleotide polymorphism in the Gja4 gene, coding for CX37, with increased risk for atherosclerosis and myocardial infarction and atherosclerosis is frequently involved in the pathophysiology of DM [321].

Atp1b1 was upregulated in aging sedentary GK compared to sedentary control rat ventricle. The Na\(^+\)/K\(^+\)-ATPase is responsible for maintaining the electrochemical gradient of Na\(^+\) and K\(^+\) ions across the plasma membrane and is composed of two essential α- and β subunits, both of which have multiple isoforms. The β subunit regulates the assembly of α/β heterodimers and translocation of sodium pump protein to the plasma membrane. Upregulation of Atp1b1, if accompanied by an increase in β subunit protein, might be expected to increase activity of the pump. Loss of Na\(^+\)/K\(^+\)- β1
is associated with significant pathophysiology of the heart [295]. The present results show that Caclalg and Caclh2 were upregulated in ventricle of aging sedentary GK compared to sedentary controls. T-type Ca\(^{2+}\) channel current is involved in the generation of the pacemaker potential in SA nodal cells. Previous studies have shown that disruption of the gene encoding Caclalg abolishes T-type Ca\(^{2+}\) current in isolated cells from the SA node and the AV node without affecting L-type Ca\(^{2+}\) current [322]. Caclh2 encodes the β2 subunit of the L-type Ca\(^{2+}\) channel. Previous studies have demonstrated that in embryonic cardiomyocytes altered expression of the β2 subunit has a large effect on L-type Ca\(^{2+}\) current however, in adult cardiomyocytes the effects are well tolerated [300]. The results have shown that Caclh2 was upregulated in sedentary GK ventricle however, Ca\(^{2+}\) signaling including amplitude of the Ca\(^{2+}\) transient and L-type Ca\(^{2+}\) current, the primary trigger for SR Ca\(^{2+}\) release, were not significantly altered in myocytes from aging sedentary GK rat.

The present study has shown that Hcn2, Kena3, Kcnel were upregulated and Kcnj2 and Kcnk3 were downregulated in aging sedentary GK compared to sedentary control rat ventricle. Hcn2 was downregulated and Kcnk3 was upregulated in GK ventricle after exercise training. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to the initiation and regulation of cardiac rhythm. The pacemaker current (I\(_{f}\)) is encoded by the HCN family. HCN isoforms including Hcn2 and Hcn4 have been identified in ventricle tissue [303, 304]. Hcn2 was downregulated by exercise training in GK ventricle bringing Hcn2 towards sedentary control level. Previously, it was reported that Kena3 encodes the voltage-gated Kv1.3 channel which is expressed in different regions of guinea pig and ferret heart [306, 307]. In the heart Kv1.3 channels contribute
to repolarization and membrane potential. The physiological effect of upregulation of
Kcnq3 in ventricle tissue remains to be clarified. Cardiac repolarization is controlled by
the I_Kr and I_Ks. The human ether-ago-go-related gene (hERG) encodes I_Kr, whereas
KCNQ1 and KCNE1 together encode I_Ks. Decreases in I_Kr or I_Ks can cause long QT
syndrome, a cardiac disorder with a high risk of sudden death [323, 324]. Upregulation
of KCNE1 might be associated with changed kinetics of cardiac repolarization. Various
K+ channels are involved in phase 3 repolarization of the cardiac action potential and
these channels include Kv11.1 (hERG1), Kv7.1 (Kcnq1) and Kir2.1 (Kcnj2) which are
the α-subunits that conduct I_Kr, I_Ks, and I_K1 [308]. Reduced Kir2.1 might prolong the
terminal phase of the cardiac action potential which in turn may lead to a reduction in
HR [309]. Two pore domain K+ channels mediate background K+ currents, stabilizing
resting membrane potential and expediting action potential repolarization. In the heart,
two pore domain K+ Kcnk3 (Task-1) channels are implicated in the cardiac plateau
current [325]. Task-1 carries a background or “leak” current and its inhibition is
sufficient to delay repolarization, causing prolongation of the action potential duration,
and in some cases, early after depolarizations [326]. Interestingly, exercise training
normalized GK ventricle Kcnk3 towards sedentary control level.

Immunoblot assay was done to evaluate whether the alteration of mRNA encoding
cardiac proteins was translated into alterations of protein expression. Despite the real-
time RT-PCR results showing downregulation in the expression of Gjal and
upregulation of Gja4, Cacnb2 and Tpm2 in sedentary GK compared to sedentary control and upregulation of Gjal in exercise trained GK compared to sedentary GK rats,
the expressions of corresponding proteins encoded by these genes were not significantly
(P > 0.05) altered in sedentary GK compared to sedentary control or by exercise training. Previous studies comparing the mRNA and protein levels concluded that the correlation is poor [327, 328]. A study by Schwanhausser has shown that mRNA levels explain only around 40% of the variability in protein levels [329].

The lack of changes of proteins, despite changes in mRNA, may partly explain the preserved function in myocyte shortening and Ca$^{2+}$ transport in GK compared to control rats.

This study has also shown the effects of diabetes and exercise training on myocardial ultrastructure. The ultrastructure was generally well preserved in all groups. The mitochondria are important determinants of myocyte function and survival. Classically, approximately 40% of cardiomyocyte volume is mitochondria. The mitochondria are essential for supplying the heart with ATP to sustain heart function. Impairment of mitochondrial function and energy metabolism in diabetic heart has been reported in numerous clinical [330, 331] and experimental studies [332, 333]. Results from the present study showed mitochondrial numbers were not significantly altered in sedentary GK compared to sedentary control rats. Exercise training significantly increased mitochondrial number in exercise trained GK compared to sedentary GK rats and to a similar extent in exercise trained control compared to sedentary control rats. Whilst the number of mitochondria was not significantly altered, the biochemical activities might change. A previous study has reported that mitochondrial function of chronically diabetic rats and in particular ATP and phosphocreatine levels were altered and endurance training was able to reverse this alteration [334]. Exercise training increased cardiac output to meet the enhanced demands of the musculoskeletal and pulmonary
Enhanced mitochondrial number and generation of ATP can greatly benefit cardiac function during exercise training [207, 335, 336].

It is well known that cardiac hypertrophy is an adaptive response to stress on the heart [337-339]. Cardiac hypertrophy is broadly classified into pathologic hypertrophy as observed in diabetes, and physiologic hypertrophy as a consequence for example of regular exercise. In exercise training, the myocardial mass is increased in order to increase performance of the heart and the hypertrophy is reversible [340]. In contrast, in diabetes increased myocardial mass is irreversibly associated with increased interstitial fibrosis, cell death and cardiac dysfunction. At the cellular level significant morphological changes contribute to the hypertrophic process including increase of myocyte size, enhanced protein synthesis and remodeling of sarcomere organization.

The present study revealed that sedentary GK hearts were heavier compared to sedentary control rats, while the impact of exercise training on heart weight was not significant. Cardiac hypertrophy facilitates the increased workload by increasing contractile capacity. The contractile function of the heart relies on the sarcomeres. Sarcomere length was reduced in exercise trained GK and exercise trained control compared to sedentary GK and sedentary control rats, respectively. However, hypertrophic remodeling produced by exercise training was accompanied by an increase in sarcomere width in exercise trained GK and exercise trained control rats.

Classically, the ICDs are described as irregularly-spaced dark bands between myocytes, and contain different structures including desmosomes that are responsible for mechanical coupling and gap junctions that are responsible for electrical coupling of the myocytes. In the current study, ICD morphology was evaluated, to investigate cardiac
systems. Enhanced mitochondrial number and generation of ATP can greatly benefit cardiac function during exercise training [207, 335, 336].

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cell-to-cell junction remodeling in diabetes and exercise training. ICD membrane convolution was measured per length unit. A significant increase in membrane convolutions was observed in exercise trained GK compared to sedentary GK and to a lesser extent in exercise trained control compared to sedentary control rats. Generally, a flattened convolution leads to loosening of attachments and may explain poor conduction in the myocardium. On the other hand a high degree of membrane convolution may be associated with greater electrical and mechanical attachments and enhanced myocardial conduction.

3.2.4 Conclusion

In the previous section (3.1.2) employing hearts from 8 to 10 week old GK and age-matched control rats 67 genes encoding cardiac muscle proteins and ventricular myocyte shortening and intracellular Ca\(^{2+}\) concentrations were investigated. The results showed that even in these young animals there were alterations in the pattern of mRNA. In adult GK rats a continuation in the changing pattern of mRNA which in some cases was modified by exercise training was observed. However, these changes were not associated with significant alterations in the expression of some cardiac muscle proteins or in either ventricular myocyte shortening or Ca\(^{2+}\) transport.

In T2DM a derangement in cardiac muscle contraction and cellular Ca\(^{2+}\) transport usually occurs later in life and it is possible that some of the changes in mRNA are taking place to protect cardiac muscle function and exercise training may have a beneficial therapeutic role as the animals develop chronic diabetes later in life.
Table 3.3: Expression of genes encoding cardiac muscle proteins in ventricle from young and aging GK rat

<table>
<thead>
<tr>
<th>Genes</th>
<th>Young (8-10 weeks) GK vs. Control</th>
<th>Aging (10-11 months) GK vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiac muscle proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tpm2</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><strong>Intercellular proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gja1</em></td>
<td>No change</td>
<td>Downregulation</td>
</tr>
<tr>
<td><em>Gja4</em></td>
<td>No change</td>
<td>Upregulated</td>
</tr>
<tr>
<td><strong>Cell membrane transport</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Slc9a1</em></td>
<td>Downregulation</td>
<td>No change</td>
</tr>
<tr>
<td><em>Atp1b1</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><strong>Calcium channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cacna1g</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><em>Cacna1h</em></td>
<td>Upregulation</td>
<td>No change</td>
</tr>
<tr>
<td><em>Cacnb2</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><strong>Sodium channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scn1b</em></td>
<td>Upregulation</td>
<td>No change</td>
</tr>
<tr>
<td><strong>Potassium channel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hcn2</em></td>
<td>Upregulation</td>
<td>Upregulation</td>
</tr>
<tr>
<td><em>Hcn4</em></td>
<td>Downregulation</td>
<td>No change</td>
</tr>
<tr>
<td><em>Kcnb2</em></td>
<td>Downregulation</td>
<td>No change</td>
</tr>
<tr>
<td><em>Kcnb3</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><em>Kcnb4</em></td>
<td>Downregulation</td>
<td>No change</td>
</tr>
<tr>
<td><em>Kcnf2</em></td>
<td>Downregulation</td>
<td>Downregulation</td>
</tr>
<tr>
<td><em>Kcn1</em></td>
<td>No change</td>
<td>Upregulation</td>
</tr>
<tr>
<td><em>Kcnk3</em></td>
<td>No change</td>
<td>Downregulation</td>
</tr>
</tbody>
</table>
Table 3.4: Effects of exercise training on expression of genes encoding cardiac muscle proteins in ventricle from aging GK and control rats

<table>
<thead>
<tr>
<th>Genes</th>
<th>Aging (10-11 months)</th>
<th>Aging (10-11 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GK Exercise vs. GK</td>
<td>Control Exercise vs. Control Sedentary</td>
</tr>
<tr>
<td></td>
<td>Sedentary</td>
<td>Sedentary</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercellular proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Gja1$</td>
<td>Upregulation</td>
<td>No change</td>
</tr>
<tr>
<td>$Cav3$</td>
<td>Upregulation</td>
<td>No change</td>
</tr>
<tr>
<td>Potassium channel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Kcnk3$</td>
<td>Downregulation</td>
<td>No change</td>
</tr>
<tr>
<td>$Kcn2$</td>
<td>Upregulation</td>
<td>No change</td>
</tr>
</tbody>
</table>
3.3 Effects of pioglitazone on excitation-contraction coupling, L-type Ca\(^{2+}\) current and electrical conduction in the Goto-Kakizaki type 2 diabetic rat heart

3.3.1 Introduction

Pioglitazone (PIO) belongs to the thiazolidinedione group of oral antidiabetic agents and is an agonist of peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) [253, 341]. It can be administered as a monotherapy or in combination with metformin, sulfonylureas, exenatide, dipeptidyl peptidase 4 inhibitors or insulin [254] for the treatment of T2DM. PIO in combination with any insulin containing regimen slightly increases the risk of hypoglycaemic episodes, causes weight gain and oedema, due to fluid retention, and increases the risk for the development and/or exacerbation of heart failure [342, 343]. PIO improves fasting and postprandial levels of insulin sensitivity, plasma glucose and lipid levels and also decreases systolic and mean arterial pressure in spontaneously obese, insulin-resistant rhesus monkey [257]. High oral doses of PIO (500–1000 mg/kg) administered to mice produce hypertrophy of the heart and mild congestion of the liver and kidneys [344]. PIO attenuates congestive heart failure-induced atrial structural remodeling and atrial fibrillation in rabbit heart [270]. Major side effects of PIO include sodium and water retention, which can provoke oedema, anemia and even aggravate or reveal heart failure, hepatotoxicity and increases in edema and weight gain [263, 264]. In addition to an increased risk of heart attack and stroke, cardiac electrical conduction abnormalities are frequently observed in diabetic patients [345-347]. These include various arrhythmias [345-347], atrioventricular block [348], prolonged QT interval and sudden death [346, 347, 349]. Hyperglycaemia has been linked to a prolonged QT interval, and the presence of QT abnormalities are
commonly used as indicators of mortality in diabetic patients [349, 350]. It is also notable that the incidence of Bundle branch block, bradyarrhythmias and atrioventricular block are significantly higher in diabetic patients than in the general population [348]. PIO has been shown to improve LV remodeling and function in mice with post-myocardial infarction heart failure and reduce infarct size by 51% in PPAR-γ knockout mice [271, 351]. Preconditioning with nidcorandil and PIO reduced lactate accumulation, counteracted oxidative stress, improved energy production and reduced ventricular arrhythmias in myocardial ischemia/reperfusion injury in rats [352]. PIO reduced angiotensin II-induced cardiac hypertrophy in vitro and in vivo in rats [272]. Myocardial hypertrophy induced by glucose and insulin was inhibited directly by PIO in primary cultured rat cardiac myocytes [353]. PIO ameliorated left ventricular hypertrophy and fibrosis in the heart of hypertensive rats and attenuated cardiac hypertrophy and fibrosis in angiotensin II-induced cardiac hypertrophy in mice [273, 274]. LV/body weight ratio was increased and myocyte size and atrial natriuretic factor were increased in PIO treated aortic banded rats [354]. PIO ameliorated the histological (reduced area of cardiac fibrosis) and functional cardiac damage (improved systolic and diastolic cardiac performance) induced by angiotensin II hypertensive rats by a mechanism that might be related to its antioxidative action [355]. PIO also protected against ischemia/reperfusion injury when administered prior to ischemia in isolated perfused rat heart [275] and inhibited cardiomyocyte apoptosis and reduced mitochondrial ultrastructure injury and membrane potential loss in ischemic/reperfused rat heart [356]. Infarct size induced in rat heart was smaller in PIO treated animals and the limiting effects were dependent on activation of mitochondrial I_{KATP} [357]. In
canine ventricular myocytes action potential duration was shortened by PIO at concentration ≥ 10 μM and this was accompanied by inhibition of several transmembrane ion currents including L-type Ca^{2+} current, the rapid and slow component of delayed rectifier K^{+} current and the transient outward K^{+} current [358]. PIO attenuated angiotensin II-induced connective tissue growth factor expression and proliferation in atrial fibroblast and also suppressed AngII- induced L-type Ca^{2+} current remodeling in HL-1 cells [359]. The aims of the current study were to investigate the acute effects of PIO on ventricular myocyte shortening and Ca^{2+} transport, and the effects of PIO on cardiac electrical conduction in type 2 diabetic GK rat.

3.3.2 Results

3.3.2.1 Acute effects of pioglitazone on ventricular myocyte shortening and Ca^{2+} transport

3.3.2.1.1 General characteristics of the animal

Experiments were performed in cardiac myocytes isolated from GK rats, aged 8-10 months, and age-matched controls. Body weight, heart weight and non-fasting blood glucose were measured immediately before experiments. The results are shown in Table 3.5. Body weight and heart weight were significantly (P < 0.01) higher in GK compared to control rats. Non-fasting blood glucose was significantly (P < 0.01) elevated in GK compared to control rats. Heart weight to body weight ratio was not significantly (P > 0.05) altered in GK compared to control rats.
Table 3.5: General characteristics of Goto-Kakizaki rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GK</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight(g)</td>
<td>352.5 ± 11.6</td>
<td>417.83 ± 5.1</td>
<td>0.00</td>
</tr>
<tr>
<td>Heart weight(g)</td>
<td>1.2 ± 0.03</td>
<td>1.4 ± 0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>3.4 ± 0.07</td>
<td>3.2 ± 0.03</td>
<td>0.057</td>
</tr>
<tr>
<td>Blood glucose(mg/dl)</td>
<td>96.9 ± 2.1</td>
<td>131.5 ± 3.6</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M. Statistical comparisons were performed using independent samples t-test. n = 11-12.
3.3.2.1.2 Effects of acute pioglitazone on ventricular myocyte shortening

Experiments were conducted on ventricular myocytes isolated from 8-10 month GK and age-matched control rats. Myocytes were isolated using enzymatic-mechanical dispersal technique as described in Section 2.4.1. Myocyte shortening was measured using a video edge detection system (Section 2.4.4). Myocytes were exposed to 10 min NT containing different concentration of PIO dissolved in DMSO. The final concentration of DMSO in all PIO and NT solutions was 0.02%, as mentioned in the drug preparation (Section 2.4.10).

Figure 3.27 shows the effects of 10 min PIO (0.1-10 μM) on the amplitude of myocyte shortening. Amplitude of shortening was significantly (P < 0.05) and progressively reduced in GK myocytes following treatment with 1 and 10 μM PIO and in control myocytes following treatment with 0.1, 1 and 10 μM PIO.

Resting cell length and the kinetics of myocyte shortening were measured in myocytes exposed to 1 μm PIO. A typical recording of shortening in a control myocyte superfused with NT or NT + 1 μM PIO for 10 min is shown in Figure 3.28a. Resting cell length in GK and control myocytes was unaltered by PIO (Figure 3.28b). TPK shortening was significantly (P < 0.05) prolonged in GK and control myocytes and THALF relaxation of shortening was significantly (P < 0.05) prolonged only in GK myocytes with 1μM PIO (Figure 3.28c-d). Amplitude of shortening was unaltered in GK (7.37 ± 0.44) compared to control (8.5 ± 0.38) myocytes. PIO significantly (P < 0.05) reduced the amplitude of shortening in GK (5.9 ± 0.39) and control (6.94 ± 0.34) myocytes (Figure 3.28e).
Figure 3.27: Effects of different concentrations of pioglitazone (PIO) 0.1-10 μM on ventricular myocyte shortening. Recordings of ventricular myocyte shortening were performed in electrically stimulated (1 Hz) myocytes. Data are mean ± S.E.M. Statistical comparisons were performed using paired sample t-test (within the same myocyte analysis) and independent samples t-test (for analysis of data from different groups), n = 14-19 cells from 9-10 hearts. Horizontal lines above graph bars indicate significance (P < 0.05).
Figure 3.28: Effects of pioglitazone (PIO) on shortening. Recordings were performed in electrically stimulated (1 Hz) myocytes. (a) Typical records of shortening in a control myocyte superfused with either normal Tyrode (NT) or NT + 1 μM PIO, (b) Resting cell length (RCL), (c) Time to peak shortening, (d) Time to half relaxation of shortening, and (e) Amplitude of shortening. Data are mean ± S.E.M. Statistical comparisons were performed using paired sample t-test (within the same cell analysis) and independent samples t-test (for analysis of data from different groups), n = 22-24 cells from 8 hearts. Horizontal lines above graph bars illustrate significant difference (P < 0.05).
3.3.2.1.3 Effects of acute pioglitazone on ventricular myocyte intracellular Ca\(^{2+}\) concentration

In these experiments, the effect of 10 min bath application of PIO (1 \(\mu\)M) on the resting intracellular Ca\(^{2+}\) levels and on the amplitudes and kinetics of Ca\(^{2+}\) transients were investigated using fluorescence photometry as described in Section 2.4.5. The effects of 10 min bath application of 1 \(\mu\)M PIO on intracellular Ca\(^{2+}\) is shown in Figure 3.29. A typical recording of Ca\(^{2+}\) transient in a control myocyte superfused with NT or 10 min NT + 1 \(\mu\)M PIO is shown in Figure 3.29a. Resting fura-2 ratio was not significantly (P > 0.05) altered in GK or control myocytes or by PIO (Figure 3.29b). TPK Ca\(^{2+}\) transient was not altered in GK compared to control myocytes and was significantly prolonged by PIO in GK and control myocytes (Figure 3.29c). THALF decay of the Ca\(^{2+}\) transient was significantly (P < 0.05) shortened in GK compared to control myocytes (Figure 3.29d). Amplitude of the Ca\(^{2+}\) transient was significantly (P < 0.05) reduced in GK and control myocytes by PIO (Figure 3.29e).
Figure 3.29: Effects of pioglitazone (PIO) on intracellular Ca\textsuperscript{2+} concentration. Recordings in electrically stimulated (1 Hz) myocytes. (a) Typical records of Ca\textsuperscript{2+} transient in a control myocyte superfused with either normal Tyrode (NT) or NT + 1 μM PIO, (b) Resting fura-2 ratio, (c) Time to peak Ca\textsuperscript{2+} transient, (d) Time to half relaxation of Ca\textsuperscript{2+} transient, and (e) Amplitude of Ca\textsuperscript{2+} transient. Data are mean ± SEM. Statistical comparisons were performed using paired sample t-test (within the same cell analysis) and independent samples t-test (for analysis of data from different groups). \( n = 20-24 \) cells from 8 hearts. Horizontal lines above graph bars indicate significance (\( P < 0.05 \)).
3.3.2.1.4 Effects of pioglitazone on myofilament sensitivity to Ca\(^{2+}\)

In some experiments myocyte shortening and intracellular Ca\(^{2+}\) transients were recorded simultaneously (Section 2.4.7) before and after 10 min application of 1 μm PIO. Typical records of myocyte shortening and fura-2 ratio in NT and a typical phase plane diagram of fura-2 ratio vs. cell length in NT are shown in Figures 3.30a & 3.30b, respectively. The gradient of the fura-2-cell length trajectory during late relaxation of the twitch contraction was not significantly (P > 0.05) altered in GK compared to control myocytes or by PIO (1 μm) during the periods 500-800 ms (Figure 3.30c), 500-700 ms (Figure 3.30d) and 500-600 ms (Figure 3.30e).
Figure 3.30: Effects of pioglitazone (PIO) on myofilament sensitivity to Ca^{2+}. (a) Typical records of shortening and Ca^{2+} transient recorded simultaneously in a control myocyte superfused with either normal Tyrode (NT) or NT + 1 \mu M PIO. (b) Typical phase plane diagram of fura-2 ratio unit (RU) vs. cell length in a control myocyte. The solid arrow and bar in (b) indicates where the gradient was measured. Gradient of the fura-2-cell length trajectory during late relaxation of twitch contraction during the
Figure 3.30: Effects of pioglitazone (PIO) on myofilament sensitivity to Ca\(^{2+}\).
(a) Typical records of shortening and Ca\(^{2+}\) transient recorded simultaneously in a control myocyte superfused with either normal Tyrode (NT) or NT + 1 \(\mu\)M PIO. (b) Typical phase plane diagram of fura-2 ratio unit (RU) vs. cell length in a control myocyte. The solid arrow and bar in (b) indicates where the gradient was measured. Gradient of the fura-2-cell length trajectory during late relaxation of twitch contraction during the
period (c) 500-800 ms, (d) 500-700 ms and (e) 500-600 ms. Data are mean ± SEM. Statistical comparisons were performed using paired sample t-test (within the same cell analysis) and independent samples t-test (for analysis of data from different groups), n = 21-26 cells from 8 hearts.
3.3.2.1.5 Effects of pioglitazone on sarcoplasmic reticulum Ca\(^{2+}\) transport

The effects of 10 min application of PIO (1 µm) on SR Ca\(^{2+}\) were assessed in fura-2 loaded myocytes from GK and control rat (Section 2.4.6). A typical record of the experimental protocol in a control myocyte is shown in Figure 3.31a. Amplitude of the caffeine evoked Ca\(^{2+}\) transient was not significantly (P > 0.05) altered in GK compared to control myocytes or by PIO (Figure 3.31b). SR fractional release of Ca\(^{2+}\), measured as the relationship between electrically evoked and caffeine evoked Ca\(^{2+}\) transient, was significantly (P < 0.05) increased in GK compared to control myocytes and was further increased by PIO (Figure 3.31c). Recovery of the Ca\(^{2+}\) transient following application of caffeine was not significantly (P > 0.05) altered in GK compared to control myocytes or by PIO (Figure 3.31d).
Figure 3.31: Effects of pioglitazone (PIO) on sarcoplasmic reticulum (SR) Ca$^{2+}$ transport. (a) Typical record of experimental protocol in control myocyte, (b) Amplitude of caffeine-evoked Ca$^{2+}$ transient, (c) Fractional release of Ca$^{2+}$, and (d) Recovery of the Ca$^{2+}$ transient following rapid application of caffeine. Data are mean ± S.E.M. Statistical comparisons were performed using paired sample t-test (within the same cell analysis) and independent samples t-test (for analysis of data from different groups), n = 25-36 cells from 10 hearts. Horizontal lines above graph bars indicate statistical significance (P < 0.05).
3.3.2.1.6 Effects of pioglitazone on L-type Ca\textsuperscript{2+} current

The effects of 10 min PIO (1 \textmu m) on myocyte L-type Ca\textsuperscript{2+} current was also investigated, using whole cell patch clamp as described in Section 2.4.8.

The electrophysiological protocols (upper panel) that were used and typical current records of Ca\textsuperscript{2+} current in a control myocyte (lower panel) during activation, inactivation and restitution experiments are shown in Figures 3.32a, b and c, respectively. Over a range of test potentials the amplitude of L-type Ca\textsuperscript{2+} current was modestly reduced in GK compared to control myocytes and was further reduced by PIO (Figure 3.32d). At a test potential of 0 mV the amplitude of L-type Ca\textsuperscript{2+} current was largest in control myocytes (7.4 ± 1.3 pA/pF), then control myocytes treated with 1 \textmu m PIO (6.1 ± 1.4 pA/pF), then GK myocytes (5.8 ± 0.9 pA/pF) and smallest in GK myocyte treated with 1 \textmu m PIO (5.0 ± 0.7 pA/pF) (Figure 3.32e). Inactivation of L-type Ca\textsuperscript{2+} current, was not significantly (P > 0.05) altered in GK compared to control myocytes or by 10 min application of 1 \textmu m PIO (Figure 3.32f). Similarly, the time course of recovery from inactivation was not significantly (P > 0.05) altered in GK compared to control myocytes or by PIO (Figure 3.32g).
Figure 3.32: Effects of pioglitazone (PIO) on amplitude of L-type Ca^{2+} current. Voltage protocols (upper panel) and typical records (lower panel) of (a) Activation, (b) Inactivation, and (c) Restitution of L-type Ca^{2+} current in a control myocyte. (c) Activation current at voltages ranging from -60 to +70 mV, (e) Mean L-type Ca^{2+} current evoked by test potentials to 0 mV, (f) Inactivation curve at pre-pulse voltage ranging from -60 to +30 mV. (g) Restitution curve at inter-pulse timing ranging from 25 to 70 ms. Data are mean ± SEM. Statistical comparisons were performed using paired sample t-test (within the same cell analysis) and independent samples t-test (for analysis of data from different groups). n = 9-11 cells from 4-6 hearts.
3.3.2.2 The effects of pioglitazone on cardiac electrical conduction

The effect of oral PIO on electrical conduction of the heart in vivo was also investigated in GK and control rats. Biotelemetry techniques were used (Section 2.4.11) to monitor the ECG in GK and control rats. The surgical procedures were performed in 7 GK and 7 controls at 7 months of age (Section 2.4.11.1). HR, HRV, QRS complex, QT, cQT and PQ were evaluated after PIO treatment. Animals received 2.5 to 20 mg/kg body weight of PIO according to the protocol described in Section 2.4.11.3. PIO was prepared as described in Section 2.4.11.4.

3.3.2.2.1 General characteristics of the animal

Prior to implantation of the transmitter devices, the fasting blood glucose was significantly (P < 0.05) higher in GK rats (101.3 ± 5.6 mg/dl) compared to age matched controls (73.9 ± 5.6 mg/dl). Blood glucose at 120 min after glucose injection (2 g/kg body weight, i.p) was 283.7 ± 24.5 mg/dl in GK rats compared to 91.9 ± 4.0 mg/dl in controls. Average body weight, fasting blood glucose and blood glucose at 120 min following glucose administration were all significantly (P < 0.05) higher in GK rats compared to controls (Table 3.6).
Table 3.6: General characteristics of experimental animals prior to implantation of transmitters

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>GK (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>345.3 ± 10.5</td>
<td>407.7 ± 7.4*</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>73.9 ± 5.6</td>
<td>101.3 ± 5.6*</td>
</tr>
<tr>
<td>Glucose tolerance (mg/dl) at 120 min</td>
<td>91.9 ± 4</td>
<td>283.7 ± 24.5*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Statistical comparisons were performed using independent samples t-test. *P < 0.01
3.3.2.2 Effects of pioglitazone on body weight and blood glucose

The effects of PIO on body weight and blood glucose of GK rats and controls were measured periodically at different stages of the study. The results are shown in Figure 3.33. Body weights of GK rats were significantly (P < 0.05) higher than controls at all stages of the experiment. PIO had no significant (P > 0.05) effects on body weight in either GK rats or controls. Non-fasting blood glucose in GK rats and controls measured at different stages of the study are shown in Figure 3.34. At the start of the experiment blood glucose was significantly (P < 0.05) higher in GK rats compared to controls. With increasing concentrations of PIO blood glucose in GK rats was reduced towards control values. PIO appeared to have little effect in controls.
Figure 3.33: Effects of pioglitazone (PIO) on body weight. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day. Lines above bars indicate significance (P < 0.05).
Figure 3.34: Effects of pioglitazone (PIO) on non-fasting blood glucose. Data are mean ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day. Lines above bars indicate significance (P < 0.05).
3.3.2.2.3 Effects of pioglitazone on heart rate and heart rate variability

Biotelemetry data was collected for 5 min/hr/animal/24 hr/day and analyzed using one-way ANOVA followed by Bonferroni. HR was modestly lower in GK rats compared to controls (Figure 3.35). PIO had no significant ($P > 0.05$) effect on HR in either GK or control rats. HRV was modestly elevated in GK compared to control rats (Figure 3.36). PIO had no significant ($P > 0.05$) effect on HRV in either GK or control rats.
Figure 3.35: Effects of pioglitazone (PIO) on heart rate. (a) Typical records of heart rate, and (b) Mean heart rate during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day. Lines above bars indicate significance (P < 0.05).
Figure 3.36: Effects of pioglitazone (PIO) on heart rate variability. (a) Typical recording of heart rate variability, and (b) Mean heart rate variability during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day.
3.3.2.2.4 Effects of pioglitazone on the electrocardiogram

The QRS complex, QT, cQT and PQ intervals were analyzed during the study. QRS complex duration was modestly but not significantly ($P > 0.05$) reduced in GK compared to control rats and was not altered by PIO (Figure 3.37). QT interval (Figure 3.48) was modestly but not significantly ($P > 0.05$) prolonged and QT interval corrected for HR (Figure 3.49) was not different in GK compared to control rats and were not altered by PIO. Interestingly, the PQ interval was significantly ($P < 0.05$) prolonged in GK compared to controls rats at the start of the study (Figure 3.40). PIO had no significant ($P > 0.05$) effect on the PQ interval in either GK or control rats.
Figure 3.37: Effects of Pioglitazone (PIO) on QRS complex duration. (a) Typical records of QRS interval, and (b) Mean QRS interval during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day.
Figure 3.38: Effects of pioglitazone (PIO) on QT interval. (a) Typical recording of QT interval, and (b) Mean QT interval during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day.
Figure 3.39: Effects of pioglitazone (PIO) on cQT interval. (a) Typical recording of corrected QT interval, and (b) Mean corrected QT interval during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni, n = 7. Numbers on the X-axis represent the dose in mg/kg/day.
Figure 3.40: Effects of pioglitazone (PIO) on PQ interval. (a) Typical recording of PQ interval, and (b) Mean PQ interval during administration of different concentrations of PIO. Data are means ± S.E.M. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni. n = 7. Numbers on the X-axis represent the dose in mg/kg/day. Line above bar indicate significance (P < 0.05).
3.3.3 Discussion

In recent years much of the research has focused on the general effects of PIO on cardiovascular function. In this study the effects of PIO on ventricular myocyte shortening and Ca\(^{2+}\) transport and on the electrical conduction system of the diabetic heart were investigated.

Experiments were performed in GK rats, 8-10 months of age, and age-matched controls. Diabetes status was confirmed by elevated blood glucose.

The major findings of this study were as follows: (i) PIO in the range 0.1-10 \(\mu\)M reduced the amplitude of shortening to similar extents in GK and control myocytes, (ii) PIO (1 \(\mu\)M) reduced the amplitude of the Ca\(^{2+}\) transient to similar extents in GK and control myocytes, (iii) Myofilament sensitivity to Ca\(^{2+}\) was not altered in GK compared to control myocytes or by PIO, (iv) SR Ca\(^{2+}\) fractional release was increased in GK compared to control myocytes and was additionally increased by PIO, (v) L-type Ca\(^{2+}\) current was modestly decreased in GK compared to control myocytes and further decreased by PIO.

It has been reported that blood glucose is elevated in GK rats as young as 1 month old [360]. Body weight was higher in GK compared to control rat. A previous study reported no significant difference at 2 months and significantly lower gain in weight at 7 months in GK compared to control rats [203]. PIO belongs to the TZD group of oral antidiabetic agents, and is an agonist of PPAR\(\gamma\) [361] which is associated with altered glucose homeostasis [362-364]. Numerous experimental and clinical studies have demonstrated the favorable effects of PIO to modulate cardiovascular risk factors.
related to T2DM [255, 266, 365-367]. Furthermore, previous studies have shown that
the effects of PIO on the cardiovascular system are initiated at the pre-diabetes stage in
type 2 diabetic rat [368]. In this study PIO (1 μM) significantly reduced the amplitude
of shortening and prolonged the TPK shortening in GK and control myocytes to similar
extents. Resting cell length was unaltered in GK compared to control rat or by PIO. In a
previous study PIO treatment reduced cardiac cell length in myocytes from transgenic
rats over expressing renin compared to non-transgenic littermates [369]. To investigate
the possible mechanism underlying the effects of PIO on myocyte shortening, further
experiments were conducted. PIO decreased the amplitude and prolonged the TPK of
the Ca\(^{2+}\) transient in GK and control myocytes, suggesting that the negative inotropic
effects of PIO might be partly attributed to altered mechanisms of Ca\(^{2+}\) transport. The
effect of PIO on myofilament sensitivity to Ca\(^{2+}\) was also investigated. Myofilament
sensitivity to Ca\(^{2+}\) was not altered by PIO suggesting that the negative inotropic effects
of PIO could not be attributed to altered myofilament sensitivity to Ca\(^{2+}\). Release of
Ca\(^{2+}\) from the SR during the process of E-C coupling contributes greatly to the
generation of the Ca\(^{2+}\) transient in cardiac myocytes [33]. Therefore, SR Ca\(^{2+}\) transport
was assessed before and after PIO treatment. Caffeine-evoked SR Ca\(^{2+}\) release was not
altered in GK compared to control myocytes or by PIO. However, the fractional release
of Ca\(^{2+}\) was increased in GK compared to control myocytes and was additionally
increased by PIO. These results may suggest that the SR Ca\(^{2+}\) release channel in
diabetic heart and during PIO treatment is more sensitive to electrical stimulation. The
recovery of the Ca\(^{2+}\) transient was not altered in GK compared to control or PIO treated
myocytes. During the recovery process Ca\(^{2+}\) is taken back into the SR via the SERCA
pump and extruded from the cell primarily via the Na⁺/Ca²⁺ exchanger [33]. Further studies will be required to investigate the effects of PIO on these mechanisms. L-type Ca²⁺ current is the primary trigger for SR Ca²⁺ release. Amplitude of Ca²⁺ current was modestly reduced in GK compared to control myocytes and was further modestly reduced by PIO. Inactivation and the time course of recovery from inactivation were not altered in either GK or control myocytes or PIO treated myocytes. Previous studies in canine ventricular myocytes showed that several trans-membrane ion currents including L-type Ca²⁺ current were inhibited by ≥ 10 μM PIO [358].

The main findings of the biotelemetry experiments a follows: (i) Body weight was higher in GK compared to control rats and PIO treatment in the range 2.5 to 20 mg/kg did not significantly alter body weight in either GK or control rats. (ii) Non-fasting blood glucose was significantly elevated in GK compared to control rats and with increasing concentrations of PIO treatment blood glucose in GK was progressively normalized towards control rat values. (iii) HR was modestly lower in GK compared to control rats and was not altered by PIO. (iv) HRV was modestly higher in GK compared to control rats and was not altered by PIO. (v) QRS complex duration and QT interval were not significantly different in GK compared to control rats and were not altered by PIO and (vi) PQ interval was prolonged in GK compared to control rats and was not altered by PIO.

Body weight was higher in GK rats compared to controls a finding that is consistent with some previous studies [192, 203, 317, 318]. TZDs are typically associated with weight gain and increased risk of oedema [370] so it was interesting to note that PIO, in the range of concentrations used in this study, had little effect on body weight in either
GK rats or controls. Consistent with previous studies, the non-fasting blood glucose was elevated in GK rats compared to controls [203, 317, 318] and treatment with increasing concentrations of PIO gradually improved blood glucose in GK towards control rat values.

HR was modestly reduced in GK compared to control rats and was not additionally altered by PIO. Previous in vivo biotelemetry studies have also reported reductions in HR in GK aged between 2 and 15 months compared to control rats [203]. Reductions in HR might be attributed to altered autonomic nerve control and/or altered intrinsic control of the heart. Action potential experiments in spontaneously beating isolated heart have shown reductions in HR suggesting that intrinsic mechanisms at least partly underlie the reduced HR in GK rats [192]. HRV was modestly increased in GK compared to control rats and was not additionally altered by PIO. Alterations in HRV, albeit modest, might suggest changes in sympathovagal control of the heart [371, 372]. The ECG provides valuable measures of electrical conduction in the heart. The QRS complex, which represents ventricular depolarization, was modestly but not significantly reduced in GK compared to control rats and was not additionally altered by PIO. Interestingly, previous studies have demonstrated prolonged QRS complex duration in GK compared to control rats, however, the differences were only significant in young 2 month old rats and, consistent with the current study, were no longer significant in older animals aged 7 and 15 months [203]. Prolonged QT interval, which represents the time from ventricular depolarization to repolarization, is a frequently reported finding in diabetic patients [373]. In the current study QT interval was modestly but not significantly prolonged and QT when corrected for HR was not altered.
in GK compared to control rats and was not additionally altered by PIO. This finding is consistent with previous studies where small prolongations of QT interval were reported in GK rats aged 2, 7 and 15 months [203]. The PQ interval, time between atrial depolarization and the start of ventricular depolarization, was prolonged in GK compared to control rats and appeared to be modestly reduced in GK towards control rats with increasing concentration of PIO. This finding may suggest prolonged conduction time between the atria and ventricles which might be attributed to atrioventricular node dysfunction. Reduced HR and prolonged sinoatrial node conduction time have been previously demonstrated in the streptozotocin-induced diabetic rat heart [373, 374].

3.3.4 Conclusion

Collectively, the results have shown that PIO has negative inotropic effects on GK and control myocytes which might be partly attributed to altered mechanisms of Ca\(^{2+}\) transport. The biotelemetry study has shown that PIO in the range 2.5-20 mg/kg administered orally during a period of 14 weeks had favorable effects on blood glucose and little or no effect on HR, HRV and QRS complex duration, QT and PQ intervals.
Chapter 4: Summary

The expression pattern of 67 genes encoding cardiac muscle, intercellular, membrane pump and exchanger, calcium, sodium and potassium channel and intracellular Ca\(^{2+}\) and Ca\(^{2+}\) regulatory proteins were characterized in cardiac ventricle of young GK rats. Even at this early stage of diabetes, the expression of mRNA encoding some membrane pump and exchange proteins were either upregulated (Atp1a3) or downregulated (Slc9a1). Expression of mRNA encoding some calcium and potassium channel proteins were upregulated (Cacn1h, Scn1b, Hcn2) or downregulated (Hcn4, Kcn2/4, Kcnj2). Although amplitude of ventricular myocyte shortening and intracellular Ca\(^{2+}\) transient were unaltered, TPK shortening was prolonged and THALF decay of the Ca\(^{2+}\) transient was shortened in GK myocytes compared to controls. Further understanding of the changes that occur in the heart during the early stages of diabetes may provide opportunities to develop interventions and treatment strategies that might prevent or delay the onset of severe functional disorders as the disease progresses.

In T2DM a derangement in cardiac muscle contraction and cellular Ca\(^{2+}\) transport usually occurs later in life and it is possible that some of the changes in mRNA are taking place to protect cardiac muscle function.

It is well established that physical exercise continues to be one of the most valuable forms of non-pharmacological therapy. Experiments were performed in GK and control rats aged 10-11 months following 2-3 months of treadmill exercise training.

Expression of mRNA encoding Tpm2, Gja4, Atp1b1, Cacn1g, Caenb2, Hcn2, Kcn2a3 and Kcnj1 were upregulated and Gja1, Kcnj2 and Kcnk3 were downregulated in hearts
of sedentary GK rats compared to sedentary controls. *Gjal*, *Cav3* and *Kcnk3* were upregulated and *Hcn2* was downregulated in hearts of exercise trained GK compared to sedentary GK controls. However, amplitude of shortening, Ca$^{2+}$ transients and L-type Ca$^{2+}$ current were not significantly altered in ventricular myocytes from sedentary GK rats compared to sedentary controls or by exercise training. Although, the expression of mRNA encoding *Gjal*, *Gja4*, *Cacnb2* and *Tpm2* were altered in sedentary GK compared to sedentary control and *Gjal* were upregulated in exercise trained GK compared to sedentary GK rats, the expressions of corresponding proteins encoded by these genes were not altered in sedentary GK compared to sedentary control or by exercise training.

The ultrastructuture of ventricular myocytes was generally well preserved in all groups. The number of mitochondria was not significantly altered in sedentary GK compared to sedentary control rats. Exercise training significantly increased mitochondrial number in exercise trained GK compared to sedentary GK rats and to a similar extent in exercise trained control compared to sedentary control rats. Sarcomere organization was regular in arrangement and clear striations were seen in all groups. Sarcomere length was measured, no alteration was observed in sarcomere length between sedentary GK and sedentary control. Exercise training reduced sarcomere length in exercise trained GK and in exercise trained control rats compared to sedentary GK and control rats, respectively. Sarcomere width in sedentary GK was wider compared to sedentary control rats. Exercise training increased sacromere width in exercise trained GK and exercise trained control rats.
Despite widespread therapeutic uses of PIO for the treatment of T2DM, the effects of PIO on the heart remain unclear. It's well known that diabetic patients suffer from an increased risk of heart attack and stroke and cardiac electrical conduction abnormalities. Therefore the effect of PIO was incorporated into the current study to investigate the benefits and risks of PIO treatment.

Experiments were performed in ventricular myocytes from GK and control rats aged 8-10 months. PIO (1 μM) reduced the amplitude and prolonged time to peak shortening and reduced the amplitude and prolonged the TPK Ca$^{2+}$ transient to similar extents in GK and control myocytes. Sarcoplasmic reticulum fractional release of Ca$^{2+}$ was increased in GK compared to control myocytes and was additionally increased by PIO. Activation of L-type Ca$^{2+}$ current was modestly reduced in myocytes from GK compared to control rats and was further reduced by PIO treatment. Inactivation and restitution of L-type Ca$^{2+}$ current were unaltered in GK compared to control myocytes or by PIO treatment.

Collectively, PIO had negative inotropic effects in GK and control myocytes and the effects may partly be attributed to altered mechanisms of Ca$^{2+}$ transport.

The biotlemetry study has shown that PIO in the range 2.5-20 mg/kg administered orally during a period of 14 weeks had favorable effects on blood glucose and little or no effects on HR, HRV and QRS complex duration, QT and PQ intervals.
Chapter 5: Limitation of the study and future direction

- mRNA expression was performed in ventricle tissue which, in addition to ventricular myocytes, also contains other types of cells. Although technically difficult it might be interesting to repeat the mRNA studies in individual ventricular myocytes and to investigate whether changes in mRNA expression translate into changes in protein expression.

- Ventricular myocyte experiments were performed at 1 Hz. It might be interesting to investigate the effects of different stimulation frequencies on E-C coupling.

- Heart function of GK rat was only slightly compromised. It might be interesting to investigate the effects of PIO in severely compromised diabetic heart.

- It might be interesting to investigate the effects of higher doses of PIO (> 20 mg/kg body weight) and longer duration of treatment on the ECG.

- APs recorded in isolated perfused heart (not included in this study) showed prolongation at 50% and 70% repolarization and reduced HR in GK compared to control rats. It might be interesting to investigate the effects of PIO on the ion channel currents that are responsible for AP repolarization.

- PIO undergoes hepatic metabolism into active and inactive metabolites. It might be interesting to investigate the effects of the various metabolites of PIO in myocytes from diabetic heart.
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355. Sakamoto A, Hongo M, Furuta K, Saito K, Nagai R, Ishizaka N. Pioglitazone ameliorates systolic and diastolic cardiac dysfunction in rat model of angiotensin II-


List of Publications

At the time of writing this thesis, the majority of the content has been published or submitted for publication.


- **Salem KA**, Sydorenko V, Qureshi MA, Oz M, Howarth FC. Acute effects of pioglitazone on ventricular myocyte shortening and L-type calcium current in the type 2 diabetic Goto-Kakizaki rat (submitted).