Dissertation

Effects of Modern Antidiabetic Concepts on Myocardial Function

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. The acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

Graz, 28th July 2019

Ewald Kolesnik

Disclosures

This thesis served a basis for two publications of original data and one review. I by myself acted either as first, shared first, or corresponding author. I did not include results or figures of these publications that were derived from other research groups without any contribution by myself; but I included these results in the discussion. Since I wrote large parts of these three manuscripts, I included these parts in this thesis. Corresponding chapters are marked with an asterisk (*) and information about the publication is provided in the footer. In order to give this thesis a clear structure, these chapters were revised. I informed all co-authors about the publication of this thesis. All co-authors have agreed to the inclusion of their published data in the dissertation and permission to reproduce illustrations and figures from own publications has been granted.

List of Publications and Affiliations

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At this point, some personal words: thanks to all, who feel addressed, especially to those, who supported me in any thinkable way during the exhausting period of this doctoral thesis and the triple burden consisting of daily routine in clinics, scientific work, and teaching duties.

I won’t forget it.

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Abbreviations and Definitions

AC	*adenylyl cyclase*
ACE	*angiotensin converting enzyme*
ADA	*American Diabetes Association*
ADP	*adenosine diphosphate*
AF	*atrial fibrillation*
AGEs	*advanced glycation end-products*
AMPK	*adenosine monophosphate-activated protein kinase*
AP	*action potential*
APD	*action potential duration*
ARB	*angiotensin receptor blocker*
ATP	*adenosine triphosphate*
AVR	*aortic valve replacement*
BDM	*butanedione monoxime*
BL	*baseline*
BMI	*body mass index*
Ca^{2+}	*ionized calcium*
$[Ca^{2+}]m$	*mitochondrial calcium*
CABG	*coronary artery bypass graft*
CAM	*calmodulin*
CAMKII	*calmodulin kinase II*
cAMP	*cyclic adenosine monophosphate*
cGMP	*cyclic guanosine monophosphate*
CO_2	*carbon dioxide*
cTnC	*cardiac troponin C*
DM	*diabetes mellitus*
DPP	*dipeptidyl peptidase*
EASD	*European Association for the Study of Diabetes*
EMA	*European Medicines Agency*
eNOS	*endothelial nitric oxide synthase*
Epac	*exchange protein directly activated by cyclic adenosine monophosphate*

FDA	*Food and Drug Administration*
GIP	*gastric inhibitory polypeptide*
GLUT	*glucose transporter*
GLP-1	*glucagon-like peptide*
GPCR	*G-protein coupled receptor*
GP	*guinea pig*
GPV	*guinea pig ventricle*
HFpEF	*heart failure with preserved ejection fraction*
Hz	*hertz*
I_K	*potassium (outward) current*
I_{SS}	*steady state current*
IR	*insulin receptor*
IU	*international units*
K^+	*ionized potassium*
K_{ATP}	*adenosine triphosphate – sensitive potassium channel*
L_{max}	*optimal length of a muscle strip*
LVEF	*left ventricular ejection fraction*
MACE	*major cardiovascular events*
mL	*milliliter*
mM	*millimolar*
mm²	*square millimeter*
mN	*millinewton*
MVR	*mitral valve replacement*
µM	*micromolar*
mV	*millivolt*
Na^+	*ionized sodium*
NCX	Na^+*- *Ca^{2+}*-exchanger*
NHE1	Na^+/H^+ *exchanger 1*
NKA	Na^+/K^+*-ATPase*
nM	*nanomolar*

NO	*nitric oxide*
nS	*nanosiemens*
n.s.	*not significant*
O_2	*oxygen*
PDE5	*phosphodiesterase 5*
PKA	*protein kinase A*
PKC	*protein kinase C*
PKG	*protein kinase G*
PLB	*phospholamban*
RAAS	*renin-angiotensin-aldosterone-system*
RT	*relaxation time*
SEM	*standard error of the mean*
SERCA2a	*sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2a*
SGLT	*sodium dependent glucose transporter*
SR	*sarcoendoplasmic reticulum*
WHO	*World Health Organization*

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Zusammenfassung

Hintergrund und Ziel: Dipeptidylpeptidase (DPP) 4 Inhibitoren, Glucagon-like Peptide (GLP) 1 Rezeptor Agonisten und Sodium dependent glucose transporter 2 (SGLT2) Inhibitoren stellen Vertreter moderner antidiabetischer Arzneistoffe dar. Verpflichtende kardiovaskuläre Outcome-Studien zeigten divergierende Ergebnisse hinsichtlich vorteilhafter Effekte auf kardiovaskuläre Endpunkte. Auch Hinweise auf mögliche schädliche Effekte konnten identifiziert werden. Bis heute sind direkte Interaktionen dieser Substanzen mit dem Myokard nicht bekannt. Ziel der Arbeit liegt in der Erforschung solcher Interaktionen.

Material und Methoden: Isolierte humane atriale (n = 146) und ventrikuläre (n = 30) Trabekel wurden in Experimenten mit 1 Hz bei 37°C elektrisch stimuliert und steigende Konzentrationen der DPP4 Inhibitoren Saxagliptin, Alogliptin und Sitagliptin sowie der SGLT2 Inhibitoren Empagliflozin, Dapagliflozin und T-1095 behandelt. In weiteren Experimenten wurden Einzeldosen von den GLP-1 Rezeptor Agonisten Exenatid und GLP-1 (7-36) Amid, vom GLP-1 Rezeptor Antagonisten GLP-1 (7-39) Amid und Isoproterenol sowie steigende Konzentrationen von Kalzium in Anwesenheit unterschiedlicher Energieträger (Glukose versus Pyruvat) untersucht. Dabei wurden funktionelle Effekte (entwickelte Kraft, diastolische Spannung und Relaxationszeiten) ermittelt. Zusätzlich wurden die akuten Auswirkungen von Saxagliptin auf die elektrophysiologische Funktion ventrikulärer Myozyten von Meerschweinchen (n = 16) mittels Patch-Clamp-Technik untersucht.

Ergebnisse: DPP4 Inhibitoren lösten in circa der Hälfte aller Trabekel Arrhythmien aus. Saxagliptin wirkte negativ inotrop und Alogliptin verlängerte signifikant die Relaxationszeit RT90. In den Patch-Clamp Experimenten führte Saxagliptin zu einer Verlängerung der Aktionspotenzialdauer sowie reduzierten Auswärtsströmen. Exenatid und GLP-1 (7-36) Amid führten zu einem transienten positiv inotropen Effekt, welcher in Anwesenheit von Glukose stärker ausgeprägt war als in Anwesenheit von Pyruvat. Isoproterenol und Kalzium führten zu ähnlicher positiver Inotropie unabhängig vom Energiesubstrat. SGLT2 Inhibitoren hatten keine funktionellen Effekte zur Folge, lediglich der kombinierte SGLT1/SGLT2 Inhibitor T-1095 bewirkte einen negativ inotropen Effekt.

Conclusio: Die Ergebnisse dieser Dissertation zeigen vielfältige direkte Interaktionen verschiedener moderner antidiabetischer Arzneistoffe mit dem Myokard auf. Insbesondere gilt dies für DPP4 Inhibitoren und GLP-1 Rezeptor Agonisten. Die hier gewonnen Daten stellen einerseits die Grundlage für weitere Untersuchungen dar, andererseits helfen diese bei der Interpretation divergierender Ergebnisse von klinischen Outcome Studien.

Abstract

Background and Aim: In type-2 diabetes mellitus dipeptidyl peptidase (DPP) 4 inhibitors, glucagon-like peptide 1 (GLP-1) receptor agonists and sodium dependent glucose transporter 2 (SGLT2) represent modern antidiabetic concepts. Mandatory cardiovascular outcome trials revealed diverging results ranging from beneficial to neutral with hints of possible harmful effects. Direct effects of these modern antidiabetic effects on myocardium have never been assessed, therefore this thesis aims on filling this gap of evidence.

Material and Methods: Isolated human atrial (n = 146) and ventricular (n = 30) muscle strips were electrically stimulated at 1 Hz and functional effects (developed force, diastolic tension, relaxation parameters) of modern antidiabetic concepts were assessed. Therefore, muscle strips were treated with increasing concentrations of the DPP4 inhibitors saxagliptin, alogliptin, or sitagliptin and the SGLT2 inhibitors empagliflozin, dapagliflozin, and T-1095. In another set of experiments, muscle strips were treated with a single dose of the GLP-1 receptor agonists exenatide and GLP-1(7–36) amide, the GLP-1 receptor antagonist GLP-1(9–36) amide, isoproterenol, and increasing concentrations of calcium in the presence of either glucose or pyruvate. Furthermore, the acute effect of saxagliptin on action potentials (APs, elicited by a stimulation frequency of 1 Hz) were measured in guinea pig ventricles (GPV; n = 16) using whole-cell patch-clamp technique.

Results: DPP4 inhibitors induced arrhythmic events in a same extend in almost half of the tested muscle strips. Here, saxagliptin induced a significant negative inotropic effect while alogliptin induced a significant prolongation of the relaxation time RT90. Furthermore, saxagliptin prolonged action potential duration and decreased the repolarizing potassium outward current density in GPV cells. Administration of exenatide and GLP-1(7–36) amide, but not GLP-1(9–36) amide, led to a transient positive inotropic effect in the presence of pyruvate and glucose. This effect tended to be more pronounced in glucose-treated muscle strips, while both isoproterenol and calcium exerted a strong positive inotropic effect with no difference regarding the energy substrate. The SGLT2 inhibitors empagliflozin and dapagliflozin exerted no functional effects on human atrial and ventricular muscle strips, while T-1095 induced a significant negative inotropic effect.

Conclusion: The results of this thesis provide an insight in direct effects of modern antidiabetic concepts on human myocardium and may serve as basis for further investigations. At least DPP4 inhibitors and GLP-1 receptor agonists interact strongly with myocardium. The presented data may help to explain discrepant outcomes of clinical trials.

1. Introduction

1.1 Glucose Metabolism and Diabetes Mellitus

The term diabetes mellitus (DM) includes various metabolic diseases that are defined as any form of dysregulation of glucose. The primary cause lies within a deficiency of insulin or a malfunction in its biosynthesis or action causing a disturbance in carbohydrate, fatty, and protein metabolism.

Complications of DM range from acute forms like hyperglycemic episodes, ketoacidosis with coma or drug-induced hypoglycemic episodes to chronic forms that mainly affect big and small blood vessels causing heart, kidney, brain, eye, nerve, and liver injuries. As the disease continues, affected organs may develop end-stage organ failure[1].

1.1.1 Scope of the Problem

In 2016, an estimated 1.6 million death were worldwide directly caused by DM and almost half of these deaths occur before the age of 70 years. Incidence and prevalence are worldwide increasing dramatically: in 1980 an estimated amount of 108 million people was affected. In 2014, this number rose to 425 million people. However, up to 630 million people suffering from DM are estimated within the year 2045[2].

In Austria, the prevalence of DM for the year 2017 is estimated with approximately 600.000 people (representing 7 % of the total population)[3]. This proportion of the total population is in line with other western countries. For example, in the United States of America 7.6 % of the total population is estimated to be affected by DM and the prevalence has risen since 2012 of remarkable 11 %[4].

The immense impact of DM is represented by its costs for treatment of the disease and its complications: these costs are estimated with approximately 5 % of the total healthcare expenses in Austria.

1.1.2 Biosynthesis of Insulin

Insulin is a protein consisting of 51 amino acids. The synthesis takes place in the beta cells in the pancreatic islets (islets of Langerhans). Here, the transcription of the protein proinsulin is promoted via the transcription factors pancreas duodenum homeobox 1 (PDX-1) and

hepatocyte nuclear factor-1 α (HNF-1α)[5]. Furthermore, proinsulin is processed several times to insulin and finally mature insulin is stored in granules within the beta cells. A depolarization of the beta cell leads to the secretion of the insulin granules in a calcium dependent manner.

1.1.3 Secretion of Insulin

This depolarization may be achieved by a surplus of any energy substrates as glucose, fatty acids, or amino acids. In this process, the most important energy substrate is glucose which enters the cell via the glucose transporter (GLUT) 2 and is metabolized via glycolysis and further the citrate acid cycle[6]. Here, adenosine diphosphate (ADP) gets converted to adenosine triphosphate (ATP). ATP inhibits the ATP-sensitive potassium channel (K_{ATP}) and causes a reduction of cellular potassium (K^+) outward current. This in turn leads to a buildup in the concentration of positively charged K^+ ions in the beta cell and further to a depolarization.

After depolarization, the voltage gated L-type calcium (Ca^{2+}) channel in the membrane of the beta cell opens and Ca^{2+} enters the cytoplasm. The latter part is a necessary step for the fusion of insulin granules with the cellular membrane and further the secretion of insulin.

Intestinal hormones as the gastric inhibitory polypeptide (GIP) or incretins as glucagon-like peptide 1 (GLP-1) can bind to their receptors on the surface of the beta cells and thereby amplify the insulin secretion by a protein kinase A (PKA) dependent phosphorylation of the L-type Ca^{2+} channel. This mechanism is called incretin-effect and increases the Ca^{2+} influx thus enhancing the insulin secretion[7]. Of note, this incretin-effect only amplifies the insulin secretion. However, the mandatory depolarization of the beta cell for insulin secretion is not affected and needs any form of energy substrate surplus, thus avoiding the risk of inducing hypoglycemia by this intrinsic mechanism.

Insulin gets secreted in a pulsatile way and induces a positive autocrine feedback on the beta cells[8]; however, this mechanism is not fully understood till date.

1.1.4 Effect of Insulin

Secreted insulin has a rather short half-life of approximately 6 minutes[9]. However, an allocation in the whole organism is achieved via the quick transportation in the blood. Insulin

binds to the insulin receptor (IR), a tyrosine kinase receptor with multiple existing subtypes that is expressed virtually by all mammalian cells[10].

Following the binding of insulin, a cascade of activations of second messengers is induced and causes different effects in different organ systems in order to reduce blood glucose levels[11]. This happens particularly in the liver, brain, adipose tissue, and all muscles. In detail, the GLUT4 is translocated into the cell membrane and glucose uptake gets promoted. In parallel, the synthesis of glycogen, fatty acids, triglycerides, amino acids, proteins, and glycolysis gets promoted while gluconeogenesis, lipolysis, proteolysis, and glycogenolysis is inhibited[12].

A schematic of the effects of insulin and the cellular and molecular mechanisms of the insulin secretion is shown in figure 1.

1.1.5 Pathophysiology, Diagnostic Criteria and Types of Diabetes Mellitus

The World Health Organization (WHO) published several reports on diabetes mellitus. The till-date valid classifications were published in the year 1999, since then cut-off values for the diagnosis of DM have been updated in 2006[13].

The European Association for the Study of Diabetes (EASD) and American Diabetes Association (ADA) followed the WHO-recommendations for the diagnosis of DM: eight hours fasting plasma glucose above 126 mg/dL (7.00 mmol/L), plasma glucose randomly measured or two hours after intake of 75 g glucose above 200 mg/dL (11.1 mmol/L), or hemoglobin A1C above 6.5 % (48 mmol/mol). If one criterion is fulfilled, the same test should be repeated at a different timepoint for the definite diagnosis of DM while no single test is regarded as superior[14,15]. Table 1 summarizes the definition of DM.

Type-2 DM represents the leading class of DM, while type-1 DM and other forms of DM are commonly rare. Irrespective of the etiology, the increased blood glucose concentrations favor glycations of proteins or lipids. These advanced glycation end-products (AGEs) cause a certain degree in loss-of-function in nearly every affected cell or protein. Moreover, inflammatory and oxidative pathways are promoted directly via a cellular receptor for AGEs or indirectly via glycation of low-density lipoprotein particles[16,17].

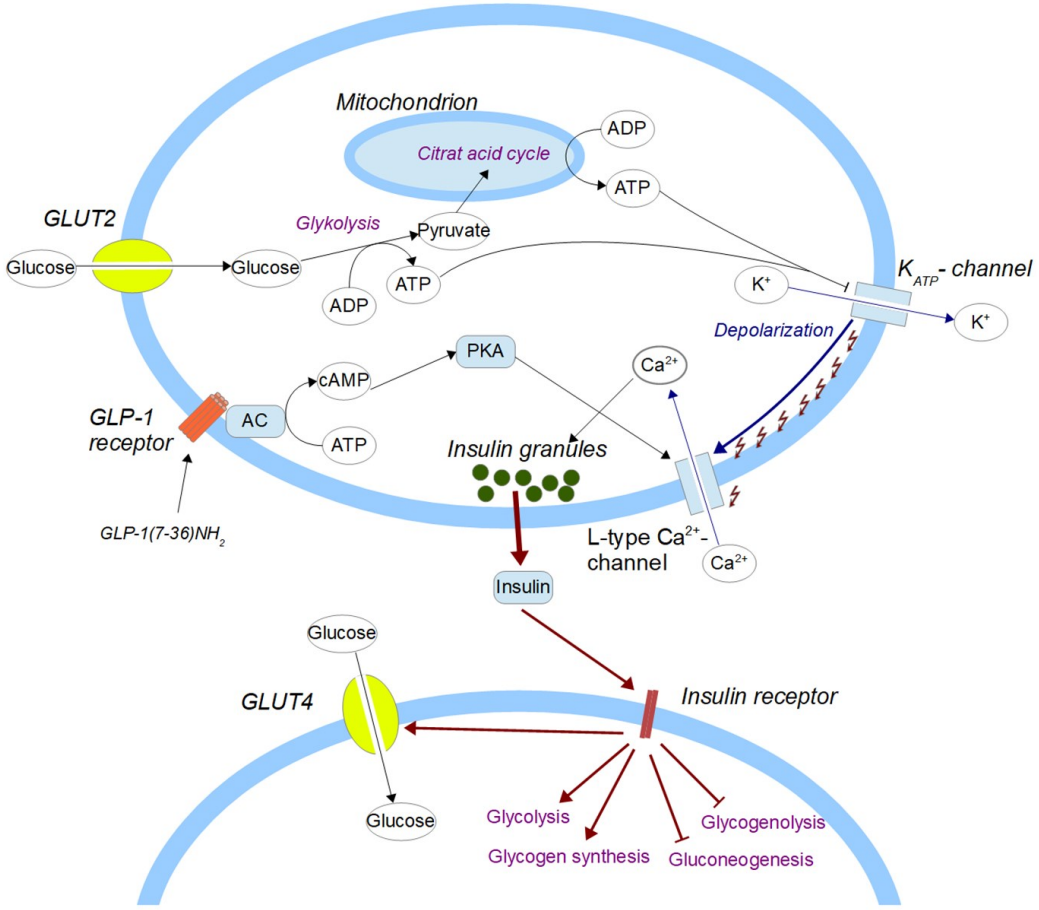

Figure 1: Schematic of the insulin secretion and the effects caused by insulin. The internalization of glucose inside a pancreatic beta cell is achieved via the GLUT2. Glucose is metabolized via glycolysis and further the citrate acid cycle. In this process, ADP gets converted to ATP. Increasing concentrations of the latter molecule may inhibit the K_{ATP} channel which is followed by an increase in the cellular K^+ concentration thereby depolarizing the cell membrane. This depolarization triggers the opening of voltage gated L-type Ca^{2+} channels and Ca^{2+} enters the cell. Ca^{2+} binds to the stored insulin granules and triggers the secretion of insulin. The insulin secretion is amplified by the incretin-effect: human GLP-1(7-36) amide may bind to its GLP-1 receptor. This GPCR is bound to an AC subunit which catalyzes the conversion of ATP to cAMP, a second messenger that activates the PKA. PKA itself phosphorylates the voltage gated L-type Ca^{2+} channel and thereby increases the Ca^{2+} influx following a depolarization. Of note, this incretin effect does not directly lead to insulin secretion, however, the energy substrate dependent insulin secretion gets significantly enhanced. Secreted insulin binds to its insulin receptor that is virtually expressed in the membranes of all mammalian cells. Insulin induces a (not shown) cascade of downstream signals with the aim to lower blood glucose levels: expression of GLUT4 and cellular glucose uptake, glycolysis and glycogen synthesis is promoted, while glycogenolysis and gluconeogenesis is inhibited. GPCR: G-protein coupled receptor; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate.

1.1.6 Type-1 and Type-3c Diabetes Mellitus

Chronic processes like the autoimmune pancreatitis may lead to the destruction of beta cells in the pancreatic islets (type-1 DM). A similar situation results from acute processes like a fulminant pancreatitis or from a pancreatectomy during a Whipple's procedure (both type-3c DM). These forms of DM lead likely to an absolute lack of insulin. In order to survive, patients must likely be treated with subcutaneously applicated insulin or insulin analogues, especially in the situation of type-1 DM[13].

1.1.7 Type-2 Diabetes Mellitus

A multitude of etiologies may cause this by far most common form of DM, type-2 DM. Beta cells in the pancreatic islets still produce and secret insulin, however, due to a decreased IR activity caused by a loss of sensitivity to insulin, the cellular glucose uptake is disturbed.

Method	Pathological measure
Randomly measured glucose	> 200 mg/dL > 11.1 mmol/L
2-hour oral glucose tolerance test (75 g of glucose)	> 200 mg/dL > 11.1 mmol/L
8-hour fasting plasma glucose	> 126 mg/dL > 7.00 mmol/L
A1C	> 6.5 % > 48 mmol/mol

Table 1: International standardized methods and pathological cut-off values for the diagnosis of DM. No single method is regarded as superior. To establish the diagnosis, a pathological measure must occur at two different timepoints.

In consequence, the plasma concentration of glucose increases. Initially, beta cells upregulate the insulin secretion in order to counteract the increased plasma glucose levels. As the disease progresses slowly, this compensatory mechanism will fail due to an increasing insulin resistance and subsequent relative lack of insulin.

Manifest type-2 DM is defined by chronically elevated plasma glucose levels. In the beginning of the disease, patients are normally asymptomatic, and the disease progresses slowly. The diagnosis of type-2 DM often follows the manifestation of microvascular complications like neuropathies, retinopathies, or nephropathies after years of persisting elevated plasma glucose levels.

The worldwide rapid increase of type-2 DM is a consequence of increasing wealth and a change in the lifestyle of people around the globe. Beside genetic risk factors, type-2 DM mainly results from a lack of physical activity and dietary patterns[13,18].

1.1.8 Other Forms of Diabetes Mellitus

Some rare conditions cause elevated plasma glucose levels without meeting the criteria for neither type-1 nor type-2 DM.

Genetic defects of the beta cells may cause a relative or absolute lack of insulin. Therein, a mutation in the chromosome 12 may cause a defect of the transcription factor HNF-1α and further impedes the biosynthesis of insulin[19].

Another known mutation in the chromosome 7 causes a malfunction of the enzyme glucokinase, which represents an essential enzyme of the glycolysis; in such conditions, beta cells are not able to metabolize glucose anymore which in turn inactivates the most important stimulus for insulin secretion[20].

Moreover, diseases of the exocrine pancreas like pancreatitis or cystic fibrosis may lead to an incomplete destruction of the insulin-producing beta cells. On the other hand, endocrine diseases like Cushing's syndrome or a pheochromocytoma lead to a pathological increase in endocrine hormones like cortisol, adrenalin, or glucagon which counteract insulin. Similarly, in the conditions of pregnancy concentrations of various endocrine hormones change and may lead to gestational diabetes. Normally, this form of DM disappears after completion of the pregnancy and a normalization of the hormone levels. Of note, a similar effect may occur drug induced[21-23].

1.1.9 Treatment of Diabetes Mellitus

In principle, the treatment of DM aims on the prevention of organ damage caused by high blood glucose levels or AGEs. Beside recommendations in physical activity and dietary patterns[24], multiple drugs with different pharmacological mechanisms have been developed and are currently in clinical use. The drug classes compose of the biguanide metformin, sulfonylureas, glinides, thiazolidinediones, alpha-glucosidase inhibitors, insulin, GLP-1 receptor agonists, DPP4 inhibitors, and SGLT2 inhibitors. The latter three drug classes have been developed in the late 2000s and considered as modern antidiabetic concepts[25]. Depending on the molecular structure, small molecules (metformin, thiazolidinediones, alpha-glucosidase inhibitors, sulfonylureas, DPP4 inhibitors, and SGLT2 inhibitors) can be administered orally while proteins (GLP-1 receptor agonists and insulin) need to be applicated subcutaneously in order to avoid a proteolysis by the intestine or liver. An overview of the drug classes and their antidiabetic mechanisms is given in table 2.

According to current guidelines of the ADA and EASD, the biguanide metformin should be initiated as first line therapy. Metformin does not interact with the insulin secretion; its antidiabetic effect is caused by inhibition of gluconeogenesis in the liver and an increase in the sensitivity of the insulin receptor. The latter mechanism is of particular interest in early stages of type-2 DM and may normalize blood glucose levels in these conditions[26].

Thiazolidinediones (rosiglitazone, pioglitazone) also do not influence the insulin secretion. This class of antidiabetics interact with the nucleus of cells and modify the process of transcription. Thereby, adipocytes synthesize less hyperglycemia-promoting molecules and myocytes improve their insulin sensitivity and metabolize glucose more efficiently[27].

Alpha-glucosidase inhibitors (acarbose, miglitol) inhibit the glucose uptake in the intestine without influencing the insulin secretion. This drug class counteracts especially hyperglycemic episodes after food intake[28].

Sulfonylureas (glibenclamide, glimepiride, carbutamide, tolbutamide, gliclazide) directly cause a secretion of insulin by the inhibition of the K_{ATP} channel and further depolarization of a pancreatic beta cell. This effect is independent of glucose; therefore, this drug class induces hypoglycemic events as a dangerous side effect. The newest guidelines on the treatment of DM do not recommend the use of sulfonylureas anymore, if any other drug class is available and financial issues do not matter[29].

Glinides (repaglinide, nateglinide) are closely related to sulfonylureas and act on the same mechanism with the same limitations[30,31].

Drug class	Application	Mechanism of antidiabetic action
Biguanides	*p.o.*	*Inhibition of gluconeogenesis in the liver and an increase in the sensitivity of the insulin receptor*
Thiazolidinediones	*p.o.*	*Inhibition of the synthesis of hyperglycemia-promoting molecules and improvement of insulin sensitivity*
Alpha-glucosidase inhibitors	*p.o.*	*Inhibition of the glucose uptake in the intestine*
Sulfonylureas Glinides	*p.o.*	*Promote the secretion of insulin by the inhibition of the K_{ATP} channel of beta cells*
DPP4 inhibitors	*p.o.*	*Increase of the incretin-effect*
SGLT2 inhibitors	*p.o.*	*Promote the excretion of glucose via urine*
GLP-1 receptor agonists	*s.c.*	*Amplification of the glucose-dependent insulin secretion of beta cells*
Insulin analogues	*s.c.*	*Direct activation of the insulin receptor*

Table 2: Overview of antidiabetic concepts, application forms, and important mechanisms of antidiabetic actions. p.o.: peroral administration; s.c.: subcutaneous application.

If other therapeutic concepts fail or in the presence of an absolute lack of insulin, insulin analogues need to be applicated as a replacement therapy strategy. This must be done subcutaneously due to their molecular structure of proteins. This antidiabetic therapy is long-established and at a certain point of the progression of DM inevitable. However, insulin itself causes metabolic side effects and represents a danger of hypoglycemic episodes, that in turn are discussed to represent a risk factor for mortality and heart failure[32,33].

1.1.10 Modern Antidiabetic Therapies

New antidiabetic drugs were constantly developed in order to achieve a better glycemic control compared to above mentioned concepts on the one hand and an easy – ideally peroral – application on the other hand. Finally, modern concepts aim also on the avoidance of hypoglycemic episodes[34]. These modern therapeutic agents consist of three classes, that are extensively described in the chapters 1.3.2 to 1.3.8 of this thesis.

Classical treatments on top of metformin like thiazolidinediones, alpha-glucosidase inhibitors, or sulfonylureas have serious disadvantages compared to the modern concepts: weaker antidiabetic effect or risk of hypoglycemic episodes due to direct secretion of insulin and undesirable side effects like increase in weight. Since DM consists a cardiovascular risk factor, cardiovascular diseases are common in patients suffering from DM. A convincing evidence for a beneficial effect on top of a cardiovascular safety profile has only been demonstrated for the modern antidiabetic concepts. Therefore, the classical approaches apart from metformin have dramatically lost their importance and are only recommended if cardiovascular diseases are excluded or in rare cases when an individualized antidiabetic therapy regimen is chosen for whatever reason[35].

*1.2 The Diabetic Heart & Heart Failure**

As mentioned in the previous section of this thesis, DM itself is considered as a cardiovascular risk factor. Additionally, DM aggravates potential risk factors of the cardiovascular system like arterial hypertension[36] and is associated with increased levels of blood lipids and obesity[37]. These conditions pave the way towards coronary artery disease, which indeed represents a common disease in diabetic patients[38]. In turn, chronic ischemia of the heart may lead to heart failure, which is the consequence due to any form of myocardial damage and is defined as “a clinical syndrome characterized by typical

* parts of this chapter have been published in BioMed Res. Int. (2017). DOI: 10.1155/2017/1253425.

symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral edema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress"[39]. The reduced cardiac output is followed by a compensatory homeostatic response, mainly driven by an uncontrolled upregulation of the renin-angiotensin-aldosterone-system (RAAS) resulting in vasoconstriction, increased cardiac preload, increased cardiac afterload, and increased sodium reabsorption. These factors promote each other in form of a vicious circle. Finally, this chronic hemodynamic stress leads to maladaptive remodeling processes in the heart (see figure 2).

Besides the well characterized macrovascular effects leading to coronary artery disease and corresponding clinical events, increasing data suggest that there are direct associations between diabetes and heart failure[40]. A 2-fold higher risk of heart failure in male diabetics and a 5-fold increase in risk in female patients with diabetes has already been demonstrated in the Framingham study[41] and this association is of particular importance in younger patients[42]. The underlying mechanisms include but are not limited to increased interstitial and perivascular fibrosis. This histological pattern was considered the basis for the term "diabetic cardiomyopathy" in the early 1970s[43]. This type of fibrosis is independent of coronary artery disease or hypertension[44]. Nonetheless, diabetic cardiomyopathy remains only moderately understood. AGEs and increased content crosslinking of collagen seem to play a significant role[45,46]. Besides histological findings, Ca^{2+} homeostasis is probably affected directly as indicated by lower activity levels of the sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) in diabetic hearts[47]. Moreover, SERCA2a is a major regulator of glucose transport in the healthy and diabetic heart via Ca^{2+} mediated GLUT 4 translocation[48].

There is robust evidence that metabolic abnormalities underlie the impaired myocardial function in heart failure. Metabolic parameters such as the ATP to phosphocreatine ratio (ATP/PCr) have been shown to predict outcome even better than left ventricular ejection fraction (LVEF) or the clinical NYHA class[49]. In addition, changes in myocardial metabolism show direct and acute effects on mechanical performance and this effect seems to be of particular importance in human myocardium. Insulin administration itself exerts positive inotropic effects in human ventricular myocardium via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Both mechanisms raise the load of the sarcoplasmic reticulum (SR) resulting in an increase of systolic Ca^{2+}-transients as well as an increase in myofilament sensitivity[50]. The metabolic changes upon insulin administration could be traced back to altered GLUT4

translocation and SGLT1 activation[51,52]. Additionally, insulin administration does not only result in acute functional effects, but also triggers various approaches modifying the energy substrate metabolism via an increased rate of pyruvate supply, as shown in vitro as well as in vivo[53,54].

Heart failure and diabetes interact bi-directionally. Besides an A1C dependent increased risk of developing heart failure in patients with diabetes mellitus, the prevalence of diabetes in heart failure patients is known to increase markedly over time (3.8% per year)[55]. Paradoxically, intensive blood glucose control alone does not improve the cardiovascular outcomes of diabetic patients and may increase mortality on contrary[56-58].

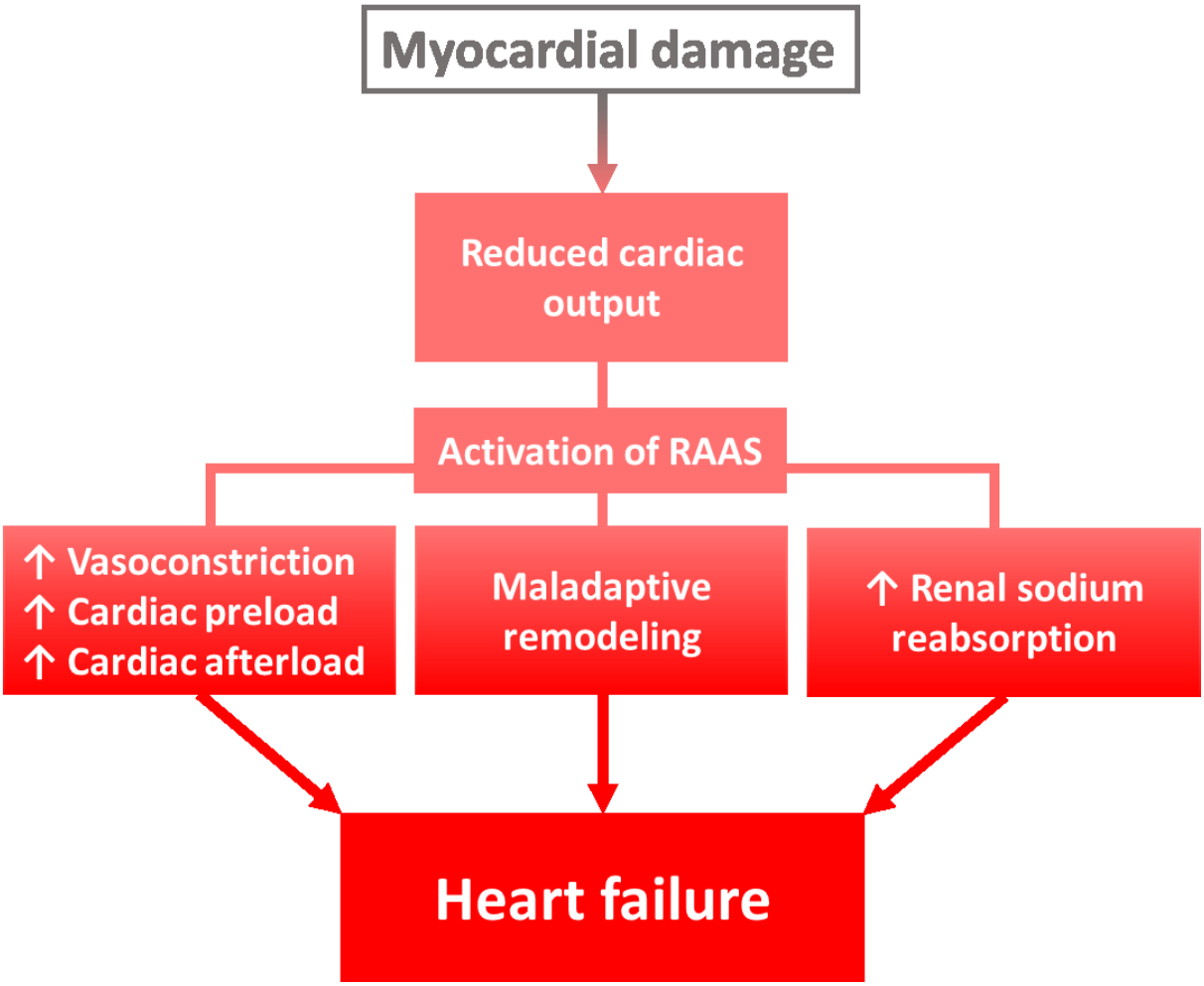

Figure 2: Schematic of the pathophysiology of heart failure

1.3 The Modern Antidiabetic Concepts*

In 2007, a meta-analysis by Nissen and Wolski found a significant increase in myocardial infarctions and cardiovascular death of diabetics under treatment with the thiazolidinedione rosiglitazone. The United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) reacted by mandating the demonstration of the cardiovascular safety profile of novel antihyperglycemic drugs, requiring a cardiovascular outcome trial[59].

This novel regulation has changed the landscape for clinical trials in the field of diabetes significantly and since 2008 more than 160,000 patients have been enrolled in cardiovascular outcome trials; an overview of these trials is given in figure 3[60]. Augmenting data on potential cardiovascular side effects of antidiabetic drugs is very valuable since millions of people are treated over many years. In most of these patients, multiple cardiovascular risk factors are commonly present; therefore, lowering the risk for macrovascular complications is one of the major tasks in current multifactorial diabetes management. Besides the classical primary ischemic endpoints, heart failure has emerged as an increasingly important endpoint in diabetes outcome trials over the last years[58].

1.3.1 Outcome Trials and Rationale of Modern Antidiabetic Strategies*

Since 2013, twelve of the FDA and EMA mandated trials have reported their results. There is no doubt that major cardiovascular events (MACE), death and heart failure are indeed robust clinical endpoints, however, some of the results such as the potential heart failure signal for the DPP4 inhibitor saxagliptin in SAVOR-TIMI 53 or the pronounced cardiovascular benefit of the SGLT2 inhibitors empagliflozin and canagliflozin were rather surprising. To sufficiently power these outcome trials while keeping the number of subjects and follow up duration within acceptable limits, patients with diabetes and high cardiovascular risk or previously diagnosed atherosclerotic disease are randomized in these trials.

However, the majority of patients with diabetes in routine care do not have a cardiovascular risk as high as represented by these trials[61]. This must be kept in mind, especially when findings from these outcome trials are extrapolated to patients with lower cardiovascular risk. Performing outcome trials in the primary prevention setting would be important to inform future diabetes treatment, although this is a challenging task: given a MACE rate of approximately one third as compared to subjects in the secondary prevention setting, trials in

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low cardiovascular risk patients would need to last longer, include more subjects or combine both approaches, leading to a significant increase in the costs for such trials[58].

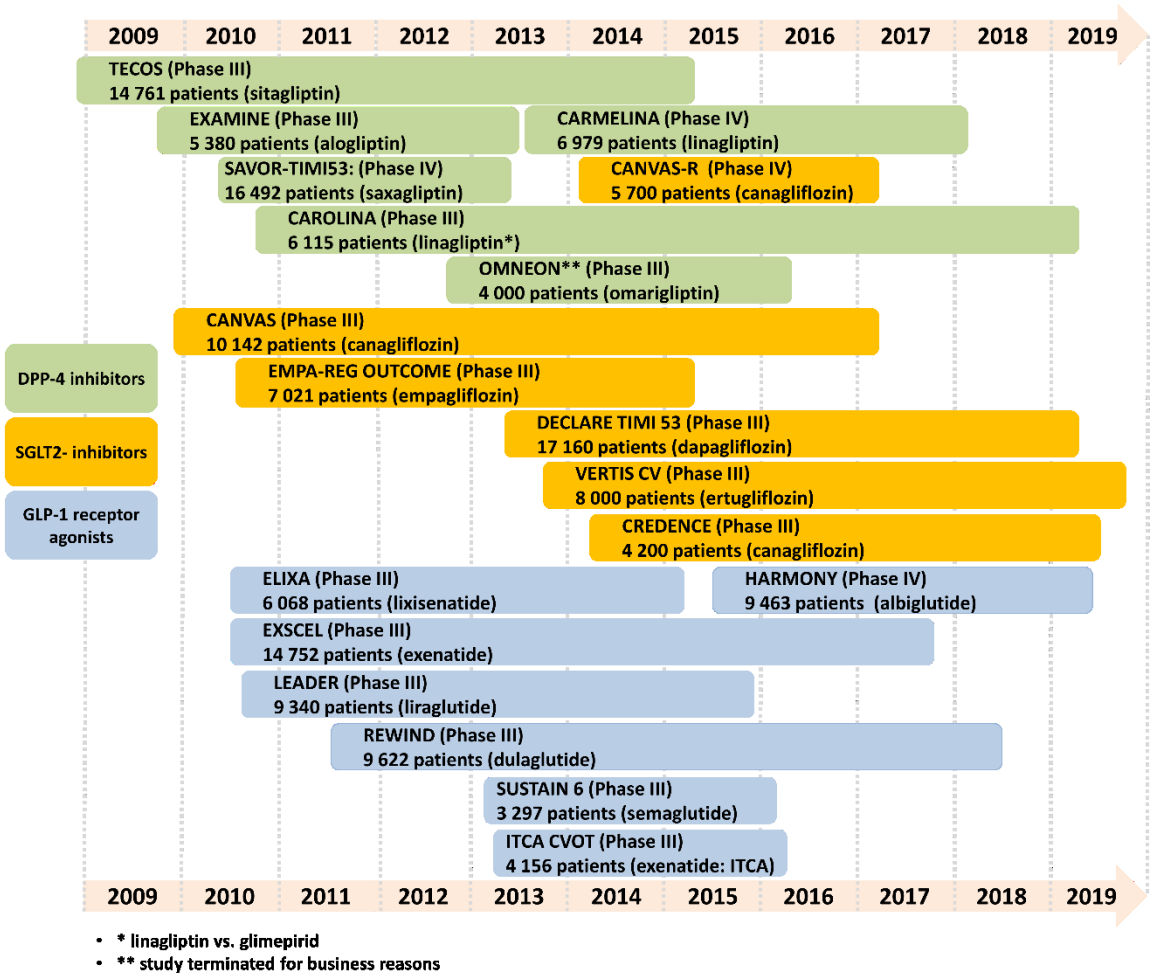

Figure 3: Overview of large cardiovascular outcome trials of DPP4 inhibitors (green), SGLT2 inhibitors (orange) and GLP-1 receptor agonists (blue). For DPP4 inhibitors, sitagliptin was investigated in the TECOS trial, alogliptin was investigated in the EXAMINE trial, saxagliptin was investigated in the SAVOR-TIMI 53 trial, and linagliptin was investigated in the CAROLINA and CARMELINA trials. For SGLT2 inhibitors, empagliflozin was investigated in the EMPA-REG OUTCOME trial, canagliflozin was investigated in the CANVAS trials and the CREDENCE trial, dapagliflozin was investigated in the DECLARE-TIMI 53 trial, and ertugliflozin was investigated in the VERTIS trial. For GLP-1 receptor agonists, liraglutide was investigated in the LEADER trial, lixisenatide was investigated in the ELIXA trial, exenatide was investigated in the EXSCEL trial, dulaglutide was investigated in the REWIND trial, semaglutide was investigated in the SUSTAIN 6 trial, and albiglutide was investigated in the HARMONY trial. This figure has been modified from the publication in the BioMed Research International journal; 2017. DOI: 10.1155/2017/1253425.

1.3.2 GLP-1 Receptor Agonists – Cardiovascular Outcome Trials*

The first cardiovascular outcome trial on glucagon-like peptide-1 receptor agonists was the ELIXA trial, which was designed to assess the effects of lixisenatide on the cardiovascular outcome in patients with type-2 diabetes mellitus who had an acute coronary event within 180 days of screening. For the primary composite endpoint (cardiovascular death, myocardial infarction, stroke), as well as for hospitalization for heart failure, no significant difference was observed between the treatment and placebo group[62]. The LEADER trial assessed the cardiovascular safety of liraglutide in patients with type-2 diabetes mellitus and a A1C ≥ 7%. Of the total enrolled subjects, 81.3% had pre-existing cardiovascular diseases. Liraglutide significantly reduced the rate of the first occurrence of the primary endpoint (cardiovascular death, non-fatal myocardial infarction, or non-fatal stroke) and all-cause mortality. The rates of non-fatal stroke, myocardial infarction and hospitalization for heart failure were non-significantly lower in the liraglutide group compared to the placebo group[63]. In the SUSTAIN-6 trial (semaglutide) patients with type-2 diabetes mellitus and established cardiovascular diseases, chronic heart failure, or chronic kidney disease, or ≥ 60 years with at least one cardiovascular risk factor, were enrolled. Semaglutide significantly reduced the risk for the primary endpoint (first occurrence of cardiovascular death, non-fatal myocardial infarction, or non-fatal stroke). The protective effect of semaglutide on composite end points seems to be mainly driven by the reduction of non-fatal stroke[64].

The results of LEADER and SUSTAIN-6 continue to hold promise that GLP-1 receptor agonists might improve cardiovascular morbidity in patients with type-2 diabetes mellitus. The HARMONY trial supports these findings where albiglutide showed a clear superiority against placebo in type-2 diabetics with established cardiovascular diseases[65]. However, the most recently published EXSCEL trial investigated 14.752 patients at cardiovascular risk and showed no inferiority of once weekly exenatide application compared to placebo on the one hand; but a superiority against placebo treatment was not reached on the other hand[66]. More insight will be gained by the immanent publication of the outcomes of the REWIND-trial for dulaglutide[67].

We do not yet fully understand the reasons for the diverging results in the currently published trials. Differences in the duration of action (short acting substances such as lixisenatide versus longer acting drugs like liraglutide or semaglutide) or differences within the amino acid sequences of the peptides are currently being discussed. The inclusion criteria and the duration of the follow-up or allowed other antidiabetic medication with known beneficial effects on the cardiovascular system may also serve as an explanation model[58]. For

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example, in the LEADER trial, the median follow-up duration was 3.8 years while in the EXSCEL trial the median follow-up duration was only 3.2 years. This half year difference does not seem to be that important at first glance. However, it must be kept in mind, that the median exposure towards the treatment showed a bigger difference between these trials, where liraglutide was applicated in median for 3.5 years, while exenatide was applicated only for 2.4 years.

1.3.3 GLP-1 Receptor Agonists – Structure & Antidiabetic Mechanism*

As described in the chapter 1.1.3 "Secretion of Insulin", GLP-1 receptor agonists enhance the glucose-dependent secretion of insulin thus avoiding the risk of hypoglycemic episodes. In Austria, GLP-1 receptor agonists are available as antidiabetic therapeutics since 2007. Currently, five substances are available: the short-acting exenatide and lixisenatide for postprandial use, and the long acting version of exenatide, liraglutide, dulaglutide, and semaglutide for basal therapy. All these mentioned drugs represent proteins, because of that their use is limited to a subcutaneous application.

As their name imply, GLP-1 receptor agonists can bind to the GLP-1 receptor; therefore, they induce similar effects like the intrinsic GLP-1 (7-36) amide that is mainly secreted by L-cells located in the ileum[68]. A trigger for this secretion is food intake. This mechanism, the so-called incretin-effect, leads to a postprandial amplification of the insulin secretion and is responsible for approximately 60 % of the total insulin secretion[69]. Moreover, by this mechanism unnecessary insulin secretion during fasting periods is avoided[58].

The chemical structure of GLP-1 receptor agonists is similar to the structure of the intrinsic GLP-1. This protein exists physiologically as two isoforms: the 30 amino acid containing GLP-1 (7-36) amide and the 31 amino acid containing GLP-1 (7-37) amide. Both isoforms are products of a breakdown of proglucagon[70]. Intrinsic GLP-1 is degraded rapidly within a few minutes by the enzyme DPP4 into its cleavage products GLP-1 (9-36) amide and GLP-1 (9-37) amide[71,72]. The latter two proteins can also bind to the GLP-1 receptor; however, they act as functional competitive antagonists. Therefore, GLP-1 (9-36) amide and GLP-1 (9-37) amide exert a regulatory function on GLP-1 (7-36) amide[73]. Clinically used GLP-1 receptor agonists represent proteins with a similar structure of intrinsic GLP-1. For example, exenatide shows a homology of approximately 53 % of the amino acid sequence of GLP-1 (7-36) amide[74], while liraglutide shows a homology of 97 %[75]. However, these GLP-1

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receptor agonists are resistant towards DPP4 breakdown, therefore a much stronger antidiabetic effect can be achieved.

1.3.4 The GLP-1 Receptor and Downstream Mechanisms*

GLP-1 receptor agonists bind specifically to their receptors. These receptors belong to the family of seven transmembrane G-protein coupled receptors (GPCR)[76]. After the binding of a ligand to the GPCR, a change of the conformation of the receptor is achieved via the breakdown of a molecule guanylyl triphosphate. This process activates the adenylyl cyclase (AC), which is positively coupled to GPCR and catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP).

Increased concentrations of cAMP activate the PKA which in turn increases the phosphorylation of the L-type Ca^{2+} channel. In pancreatic beta cells the Ca^{2+} influx increases through this mechanism further leading to an enhanced insulin secretion. Beside pancreatic beta cells, PKA induces relaxation in smooth muscle cells[77] and inhibits proliferation of cells after organ damage[78]. In endothelial cells, PKA inhibits processes that favor atherosclerosis[79].

A second target for cAMP is the exchange protein directly activated by cAMP (Epac). Epac itself represents a regulatory protein especially in cardiomyocytes and can activate other proteins like the calmodulin kinase II (CAMKII). Here, Epac finally regulates the ryanodine receptor (RYR) and is therefore involved in the Ca^{2+} homeostasis of cardiomyocytes. Of note, Epac is thereby also involved in the development of arrhythmias[80-82]

Besides increased insulin secretion, GLP-1 receptor activation leads to an inhibition of gastric and small bowel motility, reduces appetite and subsequently leads to weight loss[83]. In addition, human data suggests that this drug class improves cardiac function in patients with congestive heart failure, ameliorates endothelial dysfunction, and reduces the infarct size after ST-segment-elevation myocardial infarction[84-86].

Beneficial effects of GLP-1 receptor agonists have been attributed to direct action on myocardium, with the majority of these effects reported in ventricular cardiomyocytes. However, there are conflicting reports regarding GLP-1 receptor expression in cardiac tissue. Recent studies in mice and rats revealed that the GLP-1 receptor is exclusively localized in atrial cardiomyocytes[87-89]. Our research group already reported GLP-1 receptor expression in human right and left ventricular myocardium, although the expression levels were

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significantly lower compared to right atrial tissue[90]. This discrepancy between human and rodent tissue could be explained by species-related differences.

A recent study in normo- and hypertensive mice suggested that GLP-1 receptor activation in atrial cardiomyocytes increased cAMP levels, promoted Epac translocation to the membrane and increased atrial natriuretic peptide (ANP) secretion[87]. Epac functions in a PKA-independent manner and therefore, represents a novel mechanism for governing signaling specificity within the cAMP cascade[91]. A recent study by our lab reported significant Epac translocation from the cytosol to the cell membrane after GLP-1 receptor activation in human atrial myocardium[90]. Epac activation increases phosphorylation of cardiac troponin I (cTnI) in a protein kinase C (PKC) - dependent manner resulting in increased myofilament Ca^{2+} sensitivity and contractility[92]. GLP-1 receptor agonists significantly increased developed force in human atrial muscle strips, whereas GLP-1 (9-39) amide, a GLP-1 receptor antagonist, and H-89, a PKA inhibitor, blunted the inotropic effect of exenatide. In addition, exenatide increased the PKA-dependent phosphorylation of phospholamban (PLB) and GLUT1 translocation, but not GLUT4 translocation[90]. β-arrestin signaling downstream of GLP-1 receptor activation is another potential mechanism to increase cardiac contractility. β-arrestin, which is well-known for contributing to the termination of GPCR signaling[93], might regulate cardiac function and increase cardiac contractility via β-arrestin-mediated processes. Novel “biased ligands” that selectively recruit β-arrestin independent of G protein-mediated signaling have been described for the angiotensin II Type 1A receptor and the β1-adrenergic receptor[58,94-96].

1.3.5 DPP4 Inhibitors – Cardiovascular Outcome Trials*

While the drugs sitagliptin, alogliptin and saxagliptin were shown to be safe for the cardiovascular system in terms of the MACE, cardiovascular death, and heart failure endpoints, the SAVOR-TIMI53 trial showed a rather surprising signal for an increased risk for hospitalization of heart failure in the saxagliptin group of type-2 diabetics at cardiovascular risk[97], especially in the subgroups of impaired renal function and preexisting heart failure[98]. A similar trend – albeit not statistically significant – could be observed for alogliptin in the EXAMINE trial that investigated the cardiovascular outcomes of type-2 diabetics that were affected by an acute coronary syndrome or unstable angina[99]. In contrast, the TECOS trial did not show an increased rate for heart failure hospitalizations in type-2 diabetics with established cardiovascular diseases after sitagliptin administration[100], suggesting a potential difference between members of the DPP 4 inhibitor class. The CARMELINA trial reported a

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noninferior risk of a composite cardiovascular outcome for linagliptin against placebo in type-2 diabetics at high cardiovascular risk[101,102]. Another trial with linagliptin – the CAROLINA trial that compares the effect of linagliptin versus the sulfonylurea glimepiride – is still running and results are expected in the year 2020[103]. Recent meta analyses including the finished major and many smaller cardiovascular safety studies for DPP 4 inhibitors have different conclusions, ranging from no increased risk for the hospitalization of heart failure after DPP 4 inhibitor use[104] to an increased risk[58,105].

1.3.6 DPP4 Inhibitors – Antidiabetic and Pleiotropic Effects*

The enzyme DPP4 was first described more than 50 years ago[106]. It belongs to the prolyl oligopeptidase family, which contains a large number of biologically active enzymes like DPP6, DPP8, DPP9, and DPP10[107]. DPP4 has a complex three-dimensional structure consisting of several binding subsites and is expressed on the surface of various cells in different tissues[108]. Moreover, a soluble form exists[109].

DPP4 can breakdown proteins with a certain amino-acid sequence. Therefore, various targets for DPP4 – like GLP-1 – have been identified. Due to its manifold catalytic potential, DPP4 is also involved in the processes of inflammation, immune mediated diseases, tumor biology, and glycemic control[110,111].

Drugs that inhibit the DPP4 are authorized in Europe since 2008. These inhibitors are also called gliptins and show a different stereochemical structure[112]. Available substances are sitagliptin, linagliptin, alogliptin, saxagliptin, and vildagliptin.

Since DPP4 rapidly degrades the intrinsic GLP-1 receptor agonist GLP-1 (7-36) amide to its cleavage product GLP-1 (9-36) amide, inhibition of DPP4 theoretically expands the lifespan of GLP-1. Therefore, DPP4 inhibitors enhance the physiological glucose-depend incretin-effect. Moreover, direct antidiabetic effects of DPP4 inhibitors are discussed via till date not understood mechanisms[113]. Like GLP-1 receptor agonists, DPP4 inhibitors are relatively safe with respect to the induction of hypoglycemic events. All gliptins are small molecules and a peroral administration is possible.

Studies that examine the potential pleiotropic and non-glycemic effects of DPP4 inhibitors on various cells and tissues may help to understand and interpret the difference in the observed cardiovascular side effects in some of the clinical trials. Recently, many reviews have tried to clarify the effects caused by DPP 4 inhibitors. They interact strongly with the heart, vascular

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system, kidney, liver, neuro-endocrine system, immune system, and hematopoietic system affecting hormones or second messengers like brain natriuretic peptide (BNP), substance P, activation of chemokine and cytokine pathways, intracellular Ca^{2+} concentrations, and the release of nitric oxide (NO) shown in different animal models in vivo and ex vivo[114-118]. Interactions of DPP4 inhibitors with the cardiovascular system and cardiomyocytes were successfully revealed, yet, a direct link between DPP4 inhibitors and its effects on cardiac contractility is still unknown, and the corresponding downstream mechanisms have yet to be determined. Therefore, studies that explored effects of DPP4 inhibitors on the cardiovascular system are of particular interest.

For saxagliptin, overwhelming potential beneficial effects are reported in literature: it reduces the damage of blood vessels via the amelioration of the availability of NO and the reduction of cyclooxygenase-1-action derived vasoconstriction caused by induced type-2 diabetes mellitus in mice[119] and, similarly, leads to a restoration of damaged mitochondrial vascular function in diabetic rats[120]. Additionally, a reduction of blood pressure by increasing the bioavailability of NO in spontaneous hypertensive rats[121] and an improvement of cardiac function after myocardial infarction independent of glucose lowering could be demonstrated in diabetic rats[122]. One study clarified that saxagliptin alters the cyclic guanosyl-monophosphate (cGMP) – protein kinase G (PKG) – phosphodiesterase 5 (PDE5) axis in a swine model that mimicked heart failure with preserved ejection fraction (HFpEF) by aortic banding thus preventing left ventricular damages and improving left ventricular systolic and diastolic function[123].

Similar effects are reported for sitagliptin in diabetic rats. Sitagliptin improved endothelial function[124] and attenuated cardiac remodeling without affecting systolic function after myocardial infarction[125] while in normoglycemic rats with induced myocardial infarction, sitagliptin prevented fatal arrhythmias by attenuating GIP-dependent resistin signaling[126] and in a PKA dependent pathway[127]. Moreover, sitagliptin attenuated changes in the electrophysiological function in hypertensive rats[128] and counteracted induced HFpEF by improving the diastolic function, decreasing the generation of reactive oxygen species, and reducing pro-inflammatory biomarkers in the myocardium thus lowering mortality[129,130]. Similarly, one study proved the reduction of parameters of diastolic dysfunction and myocardial stiffness via the cGMP-PKG pathway after sitagliptin administration in obese diabetic mice[131].

Alogliptin could restore cardiac remodeling and prevent apoptosis via an Epac dependent and PKA independent mechanism in a model of ventricular pressure overload[132] and

inhibited inflammation in arteries that sustained damage by high low-density lipoproteins (LDL) concentrations[133] in mice. The reported potential beneficial effects might also be present in humans, one trial with a small number of participants showed increased coronary flow reserve and improved left ventricular ejection fraction in patients with type-2 DM and coronary artery disease within three month of alogliptin use[134].

For vildagliptin, conflicting results are reported: one study failed to show potential protective effects on cardiac function after myocardial infarction which thereby followed cardiac remodeling despite increased levels of GLP-1 in rats[135]. In contrast, other studies suggested that vildagliptin might reduce infarct size and preserve LVEF by reducing reactive oxygen species in a rat model of ischemia/reperfusion[136] and preventing hypertrophy of the left ventricle after continuous infusion of isoproterenol in rats by the inhibition of inflammatory markers[137]. Additionally, vildagliptin exerts effects via NO and the endothelial NO-synthase (eNOS) leading to an improved vascularization in a mouse model with surgical induced ischemia[138]. Focusing on the cardiovascular system, vildagliptin seems to exert similar effects as sitagliptin[139]. However, no large cardiovascular outcome trial for vildagliptin is being performed.

Finally, linagliptin improves diastolic function in a model of HFpEF in obese rats via an elevated expression of eNOS and improved SERCA2a activity[140]. The effect on eNOS availability could be demonstrated in non-obese mice as well[141]. Linagliptin also reduced angiotensin and glucose induced collagen formation in cardiac fibroblasts of mice by an anti-inflammatory mechanism (via NFkB)[58,142].

1.3.7 SGLT2 Inhibitors – Cardiovascular Outcome Trials*

In 2015, the EMPA-REG-OUTCOME trial demonstrated a significant reduction in MACE and all-cause mortality in subjects treated with the SGLT2 inhibitor empagliflozin[143]. Moreover, this landmark trial showed a striking 35% relative reduction in the rate of heart failure hospitalization in the empagliflozin group, an effect occurring very quickly after initiating treatment. These findings on MACE and heart failure hospitalization were confirmed in the recently published data from the CANVAS program with canagliflozin[144]. However, cardiovascular and all-cause mortality were not significantly reduced by canagliflozin, in contrast to empagliflozin. On the other hand, data derived from the DECLARE trial with dapagliflozin show a lower rate of cardiovascular death or hospitalization for heart failure but not a reduction of MACE[58,145].

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1.3.8 SGLT2 Inhibitors – Beyond Inhibition of SGLT2*

Glucose may enter a eukaryotic cell via two different membrane associated carrier proteins: the already presented GLUTs and SGLTs. The latter ones consist of two different glucose transporters: SGLT1 and SGLT2[146]. Since SGLT2 is mainly expressed in the proximal tubule of the kidneys and resorbs glucose and sodium, that is determined to be excreted via urine, inhibition of SGLT2 leads to an increased glucose and sodium excretion. Of note, this antidiabetic mechanism is independent of the effect of insulin and induces a direct elimination of glucose. However, preserved diuresis is a requirement for this mechanism of action. The first SGLT2 inhibitor approved by the EMA was dapagliflozin in the year 2012. The approval of empagliflozin followed in the year 2014.

Currently, several hypotheses are being discussed for the findings in the SGLT2 inhibitor trials. These include hemodynamic changes and increased hematocrit that are caused by a diuretic effect or changes in the cardiac fuel metabolism by an improved uptake of β-hydroxybutyrate under conditions of persistent hyperketonemia, all induced by SGLT2 inhibitors. Especially ischemic and therefore endangered myocardium may benefit from these effects[147,148]. For SGLT2 inhibitors, little data on cardiovascular side effects in animal models or in-vitro settings is available. This may be a consequence of the fact that the SGLT2 receptor is not expressed in myocardial tissue[52,143,149].

Mechanistically, cardiovascular side effects of SGLT2 inhibitors could either occur via unselective binding of compounds to SGLT1, which is not the case for most of the members of this drug class, or via receptor independent effects. Interestingly, the pattern of intracellular mechanisms seems to be different for various class members. Activation of the adenosine monophosphate-activated protein kinase (AMPK) for example has only been shown for canagliflozin but not for dapagliflozin or empagliflozin[150]. A pathway most likely influenced by all SGLT2 inhibitors in cardiomyocytes is the Na^+/H^+ exchanger 1 (NHE1) mediated decrease in intracellular Na^+ and Ca^{2+}, although this has only been reported for empagliflozin so far[58,151].

1.4 Gaps of Evidence

Despite the large population undergoing cardiovascular outcome trials, there is little mechanistic insight to derive from these data explaining cardiovascular harm or benefit. One would expect rather similar outcomes in the case of improved glycemic control. However, in

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the placebo-arm of the clinical trials this goal could be reached too in a lesser extent. Therefore, potential pleiotropic effects beyond glucose homeostasis could be responsible for the observed different outcomes of the clinical trials. As described in the previous section of this thesis, data derived from in vivo and in vitro experiments support this thesis. However, potential effects directly aiming on myocardium and their underlying molecular mechanisms of modern antidiabetic concepts remain mostly unknown.

1.5 Cardiac Signaling

There are only a few possible targets to alter myocardial function. As mentioned above, GLP-1 receptor agonists act on cardiomyocytes via the GLP-1 receptor. However, neither SGLT2 receptors are expressed on human cardiomyocytes nor DPP4 receptors are known to impact an influence on myocardial function. On the other hand, DPP4 inhibitors and SGLT2 inhibitors represent small molecules that could potentially pass the cell membrane and exert effects inside the cardiomyocytes.

The function of cardiomyocytes may be manipulated mainly by alteration of ion currents or the Ca^{2+} homeostasis, both reflected by the time course of the action potential. A detailed description of the cardiac signaling is shown in figure 4.

In principle, spontaneous action potentials are generated in pacemaker cells located in the sinus node in the right atrium by a tight interplay of transmembrane ion channels as the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and spontaneous local Ca^{2+} release from the SR via the RYRs Gap junctions enable the spread of excitation to neighboring cells. The action potential upstroke of myocytes of the working myocardium is generated by an influx of Na^{+} through rapidly opening voltage gated Na^{+} channels. A subsequent Ca^{2+} inward current via L-type Ca^{2+} channels triggers the Ca^{2+} induced Ca^{2+} release from the SR via the RYR[152]. Ca^{2+} then binds to cardiac troponin C (cTnC), a protein that blocks myosin from binding to actin. Binding of Ca^{2+} leads to a change in the conformation of cTnC and allows myosin binding with actin. Then, the ATP-dependent change in the conformation of myosin leads to a mechanical contraction of the myocyte. This whole process is called excitation-contraction-coupling[153]. Moreover, Ca^{2+} binds to calmodulin (CAM) and forms Ca^{2+}-CAM. Ca^{2+}-CAM inhibits the RYR in order to stop the Ca^{2+} flow on the one hand and activates the CAMKII on the other hand, which in turn phosphorylates the RYR, thus increasing the Ca^{2+} current of the next action potential[154,155].

In parallel, various K^+ channels open and repolarize the cell membrane to the resting level. Ca^{2+} is removed from the sarcoplasm by the SERCA2a and the Na^+- Ca^{2+}-exchanger (NCX). Na^+ and K^+ gradients are finally restored by the Na+/K+-ATPase (NKA). GPCR can enhance

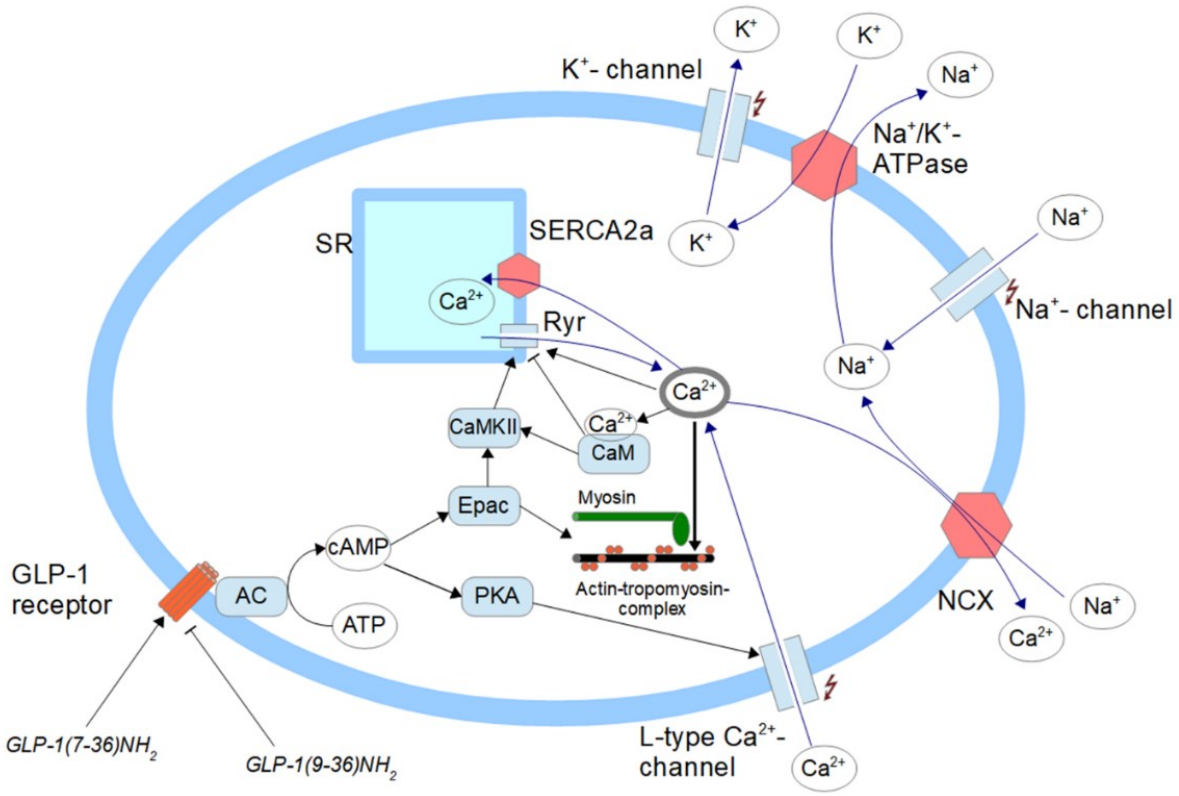

Figure 4: Overview of the cardiac signaling. An action potential is caused by the influx of Na^+ ions via HCN channels. The depolarization leads to the opening of Na^+ and L-type Ca^{2+} channels and causes an influx of Ca^{2+} ions. These Ca^{2+} ions open the RYR of the sarcoplasmic reticulum (SR; "calcium-induced calcium release"). Ca^{2+} interacts with tropomyosin, which further allows myosin binding on actin. This process is called excitation-contraction coupling. Repolarization is achieved by the elimination of Ca^{2+} from the sarcoplasm: Ca^{2+} gets pumped back into the sarcoplasmic reticulum via SERCA2a and out of the cell via NCX. In parallel, a K^+ efflux begins via different late-opening voltage gated K^+ channels. The Na^+/K^+-ATPase restores the Na^+ and K^+ gradients. Any GPCR is connected to an adenylyl cyclase (AC). Activation of GPCRs leads to a conversion of ATP to cAMP. This second messenger can activate the PKA which increases the Ca^{2+} influx via a phosphorylation of the L-type Ca^{2+} channel. Moreover, cAMP activates Epac, which regulates CAMKII and increases the activity of the RYR during the following depolarizations, thus increasing the Ca^{2+} transient. This figure has been modified from the publication in the BioMed Research International journal; 2017. DOI: 10.1155/2017/1253425.

the Ca2+ currents of a myocyte by the regulation of the enzyme AC. AC converts ATP to cAMP. The latter one activates the PKA, which represents an important regulator of myocardial inotropy. This enzyme can phosphorylate the L-type Ca^{2+} channel and therefore increases the sarcoplasmic Ca^{2+} concentrations[154].Another important regulator of myocardial inotropy is Epac. Like PKA, Epac is cAMP-dependent and activates the CAMKII, thus increasing the Ca^{2+} transients through the RYR in the following action potentials.

1.6 Aims of the Thesis

Given the importance and dimension of the topic, this thesis aims on identifying direct functional effects of GLP-1 receptor agonists, DPP4 inhibitors and SGLT2 inhibitors as well as the interaction with different energy substrates for GLP-1 receptor agonists. The results of this thesis consist of the muscle strip experiments performed with different experimental settings of modern antidiabetic concepts. Moreover, one series of patch-clamp experiments in guinea pig ventricle cells was performed in cooperation with the Institute of Biophysics of the Medical University of Graz.

The main goal is to elucidate functional effects on human myocardium. However, functional effects alone may be hard to interpret without additional information. Therefore, the DPP4 inhibitor saxagliptin was investigated in patch-clamp experiments. Further experiments finally revealed first mechanistic insights in the interaction of saxagliptin with myocytes; however, since these experiments were performed by different laboratories, these data are not part of the results of this thesis and therefore only being discussed in this thesis.

Another aim of this thesis is to assess the contribution of GLUT1 translocation after GLP-1 receptor activation. This effect could be demonstrated in previous experiments and may help to understand the positive inotropic effect of GLP-1 receptor agonists[88].

Basic science data revealing such direct interactions between modern antidiabetic concepts and myocardium or myocytes represent necessary fundamentals to positively understand and interpret their cardiovascular outcome trials. Further, the understanding of the functional effects and especially the underlying molecular mechanisms is essential for clear recommendations of antidiabetic drug treatment in patients with comorbidities beyond type 2 DM.

2. Materials and Methods

2.1 Overview and Ethics Approval for Human Muscle Strip Experiments

Functional effects of modern antidiabetic drugs that act on the GLP-1 / DPP4 and SGLT2 axis were mainly assessed by the measurement of the contractility of human muscle strips. Right atrial and left or right ventricular muscle strips were either exposed to a certain drug at single concentration or in a dose-response-relationship at increasing concentrations. Functional data was collected in real time in form of a beat-to-beat analysis. The experimental protocols and used concentrations are described in chapter 2.6 “Protocols for Human Muscle Strip Experiments” of this thesis.

The use of human tissue was approved by the ethics committee of the Medical University of Graz, Austria (19-109ex07/08) and all patients gave written informed consent according to the World Medical Association Declaration of Helsinki, 2013.

2.2 Buffer Solutions for Human Muscle Strip Experiments

All experiments with human muscle strips were performed with a modified Krebs–Henseleit solutions that mimic physiological circumstances and contain energy substrates to run experiments and inhibit autolytic processes. These solutions are hereafter named Tyrode’s solution. The composition of the buffer solutions is shown in table 3 and consists of 152 mM Na^+, 3.6 mM K^+, 0.6 mM Mg^{2+}, 0.2 mM Ca^{2+} (elevated during experiments to 2.5 mM), 129.5 mM Cl^-, 25 mM HCO_3^-, 1.3 mM $H_2PO_4^-$, and 0.6 mM SO_4^{2-}. In all but one experimental series 11.2 mM glucose served as energy substrate and is meant to be the energy substrate if not otherwise declared. Alternatively, 22.4 mM pyruvate served as energy substrate and is further labelled as pyruvate-Tyrode’s solution. Moreover, 5 IU insulin were added to the buffer solution used during experiments.

To achieve physiological pH values of approximately 7.400, all buffer solutions were constantly bubbled with carbogen gas (95 % O_2 and 5 % CO_2) whenever possible and during the whole experiments.

Krebs-Henseleit-Buffer			
Cations	**Concentrations**	**Anions**	**Concentrations**
Na^+	152 mmol/L	Cl^-	129.5 mmol/L
K^+	3.6 mmol/L	HCO_3^-	25 mmol/L
Mg^{2+}	0.6 mmol/L	$H2PO_4^-$	1.3 mmol/L
Ca^{2+}	0.2 mmol/L*	SO_4^{2-}	0.6 mmol/L

BDM-Tyrode-Buffer		**Glucose-Tyrode-Buffer****		**Pyruvate-Tyrode-Buffer**	
Reagent	**Mass [L^{-1}]**	**Reagent**	**Mass [L^{-1}]**	**Reagent**	**Mass [L^{-1}]**
NaCl	7.422 g	NaCl	7.422 g	NaCl	7.422 g
2,3-BDM	3.000 g	$NaHCO_3$	2.100 g	$NaHCO_3$	2.100 g
$NaHCO_3$	2.100 g	Glucose	2.018 g	Pyruvate	2.480 g
Glucose	2.018 g	KH_2PO_4	0.177 g	KH_2PO_4	0.177 g
KH_2PO_4	0.177 g	KCl	0.172 g	KCl	0.172 g
KCl	0.172 g	$MgSO_4 \cdot 7H_2O$	0.148 g	$MgSO_4 \cdot 7H_2O$	0.148 g
$MgSO_4 \cdot 7H_2O$	0.148 g	$CaCl_2 \cdot 2H_2O$	0.029 g	$CaCl_2 \cdot 2H_2O$	0.029 g
$CaCl_2 \cdot 2H_2O$	0.029 g	Insulin	5 IU	Insulin	5 IU

*Table 3: Composition of used buffer solutions. *: Ca^{2+} levels were increased during experiments stepwise to 2.5 mmol/L. ** Glucose-Tyrode-Buffer is equivalent to "Tyrode's solution". The unit mmol/L is identical with mM.*

2.3 Acquisition of Human Right Atrial Muscle Strips

For the majority of experiments right atrial muscle strips were used. During routinely performed cardiac surgery including coronary artery bypass graft (n = 26), aortic valve surgery (n = 8), mitral valve surgery (n = 3), surgery of an aortic aneurysm (n = 1), or a combination surgery (n = 17), the right atrial appendage is dissected and used as a venous entrance for connection catheters of the heart lung machine. Detailed characteristics of the patients is shown in table 4. Briefly after the activation of the heart lung machine, the right atrial appendage gets cut off by the surgeon and is transferred to a cup filled with a cold cardioplegic buffer containing physiological low-Ca^{2+} (0.2 mM) Tyrode's solution with 30 mM 2,3-butanedione monoxime (BDM) added. The latter substance avoids mechanical damage of myocardium during the process of isolation[156]. Next, the right atrial appendage is transferred to a laboratory in the basement of the cardiology department with minimal time delay. The muscle strips were then isolated with the use of micro-scissors (FST Fine Science Tools GmbH, Heidelberg, Germany; Aesculap AG, Tuttlingen, Germany) and a microscope (Olympus, Tokyo, Japan) in an atraumatic technique. The length of each muscle strip was aimed to be between 2.5 and 5 mm for practical reasons and the inner diameter not to exceed 0.9 mm to avoid hypoxia of the core of the muscle strip[157]. In sum, 146 atrial muscle strips were isolated and used in different experimental protocols.

2.4 Acquisition of Human Left / Right Ventricular Muscle Strips

Only one experimental series was performed with ventricular muscle strips. In contrast to atrial tissue that is commonly daily available, ventricular tissue is rare. If an organ transplantation takes place and the heart is not transplanted for any reason, it may be used for research purposes. Mostly, these hearts show preserved systolic and diastolic function and are therefore classified as non-failing hearts. However, within a year not more than ten non-failing hearts may be expected. The transplantation coordinator informs a research coordinator and this person informs the research groups.

The whole heart is transported from the operation room to a laboratory in the Zentrum für Medizinische Forschung (ZMF) of the Medical University of Graz in a cool cardioplegic solution (Custodiol®) and opened with surgical scissors. Afterwards, it is possible to carefully isolate muscle strips with the use of micro-scissors and a microscope in an atraumatic

Demographic parameter	**All patients (n = 55)**	DPP4i (n = 13)	GLP-1ra (n = 34)	SGLT2i (n = 8)
Male sex, N (%)	***41 (74 %)***	*11 (85 %)*	*24 (71 %)*	*6 (75 %)*
Female sex, N (%)	***14 (26 %)***	*2 (15 %)*	*10 (29 %)*	*2 (25 %)*
Age, years (mean ± SD)	***66.9 ± 10.4***	*66.5 ± 9.2*	*66.2 ± 10.7*	*71.6 ± 9.8*
BMI, kg/m² (mean ± SD)	***28.4 ± 5.1***	*27.8 ± 2.7*	*29.3 ± 5.6*	*25.0 ± 3.9*
CABG, N (%)	***26 (47 %)***	*6 (46 %)*	*17 (50 %)*	*3 (38 %)*
AVR, N (%)	***8 (15 %)***	*2 (15 %)*	*5 (15 %)*	*1 (13 %)*
CABG + AVR, N (%)	***17 (31 %)***	*4 (31 %)*	*10 (29 %)*	*3 (38 %)*
MVR, N (%)	***3 (5 %)***	*0*	*2 (6 %)*	*1 (13 %)*
Aortic aneurysm, N (%)	***1 (2 %)***	*1 (8 %)*	*0*	*0*
Sinus rhythm, N (%)	***52 (95 %)***	*13 (100 %)*	*33 (97 %)*	*6 (75 %)*
History of AF, N (%)	***7 (13 %)***	*0*	*4 (12 %)*	*3 (38 %)*
T2DM, N (%)	***9 (16 %)***	*0*	*8 (24 %)*	*1 (13 %)*
Hypertension, N (%)	***46 (84 %)***	*9 (69 %)*	*29 (91 %)*	*8 (100 %)*
LVEF, % (mean ± SD)	***54.3 ± 10.0***	*51.4 ± 9.8*	*54.2 ± 10.4*	*59.5 ± 6.9*
Aspirin use, N (%)	***38 (69 %)***	*10 (77 %)*	*23 (72 %)*	*5 (63 %)*
Statin use, N (%)	***38 (69 %)***	*6 (46 %)*	*26 (81 %)*	*6 (75 %)*
ACE-inhibitor use, N (%)	***31 (56 %)***	*6 (46 %)*	*23 (72 %)*	*2 (25 %)*
ARB use, N (%)	***6 (11 %)***	*2 (15 %)*	*1 (3 %)*	*3 (38 %)*
Betablocker use, N (%)	***32 (58 %)***	*8 (62 %)*	*18 (56 %)*	*6 (75 %)*

Table 4: Characteristics of patients that donated the right atrial appendage for the performed experiments. DPP4i: DPP4 inhibitors; GLP-1ra: GLP-1 receptor agonists; SGLT2i: SGLT2 inhibitors; BMI: body mass index; CABG: coronary artery bypass graft; AVR: aortic valve replacement; MVR: mitral valve replacement; AF: atrial fibrillation; LVEF: left ventricular ejection fraction; ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker

technique. The isolated ventricular muscle strips are transferred into a cup containing physiological low-Ca^{2+} (0.2 mM) Tyrode's solution with 30 mM 2,3-BDM added and can then be transported into the laboratory in the basement of the cardiology department. In sum, 30 left or right ventricular muscle strips were isolated and used in experiments with SGLT2 inhibitors.

Demographic parameter	All patients (n = 8)
Male sex, N (%)	***4 (50 %)***
Female sex, N (%)	***4 (50 %)***
Age, years (mean ± SD)	***63.6 ± 6.6***
BMI, kg/m² (mean ± SD)	***27.7 ± 3.0***
Sinus rhythm, N (%)	***7 (88 %)***
History of AF, N (%)	***1 (12 %)***
T2DM, N (%)	***3 (38 %)***
Hypertension, N (%)	***7 (88 %)***
LVEF, % (mean ± SD)	***57.8 ± 10.1***
Aspirin use, N (%)	***1 (12 %)***
Statin use, N (%)	***1 (12 %)***
ACE-inhibitor use, N (%)	***2 (25 %)***
ARB use, N (%)	***1 (12 %)***
Betablocker use, N (%)	***2 (25 %)***

Table 5: Characteristics of patients that donated ventricular tissue for the performed experiments. BMI: body mass index; AF: atrial fibrillation; LVEF: left ventricular ejection fraction; ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker

2.5 Experimental Setup for Human Muscle Strip Experiments

The isolated muscle strips were finally transferred to an organ bath using a pipette (Sarstedt, Nürnbrecht, Germany). The schematic of one single experimental channel and the whole experimental setup is shown in figure 5 and consists of the following parts:

- Measurement unit consisting of a force transducer (SI-H Force Transducers SI-KG2, World Precision Instruments Germany, Friedberg, Germany) and an organ bath
- Stimulator STM1 (SI Scientific Instruments GmbH, Gilching, Germany)
- Stand-mounted pump (Ismatec, Wertheim, Germany)
- Microscope VMT (Olympus, Tokyo, Japan)
- Thermal writing pen oscillography Linearcorder WR 3320 (Graphtec, Yokohama, Japan)
- Computer with software program Labview
- Heat pump Lauda RM 20 (LAUDA DR. R. WOBSER GMBH & CO. KG, Lauda-Königshofen, Germany)

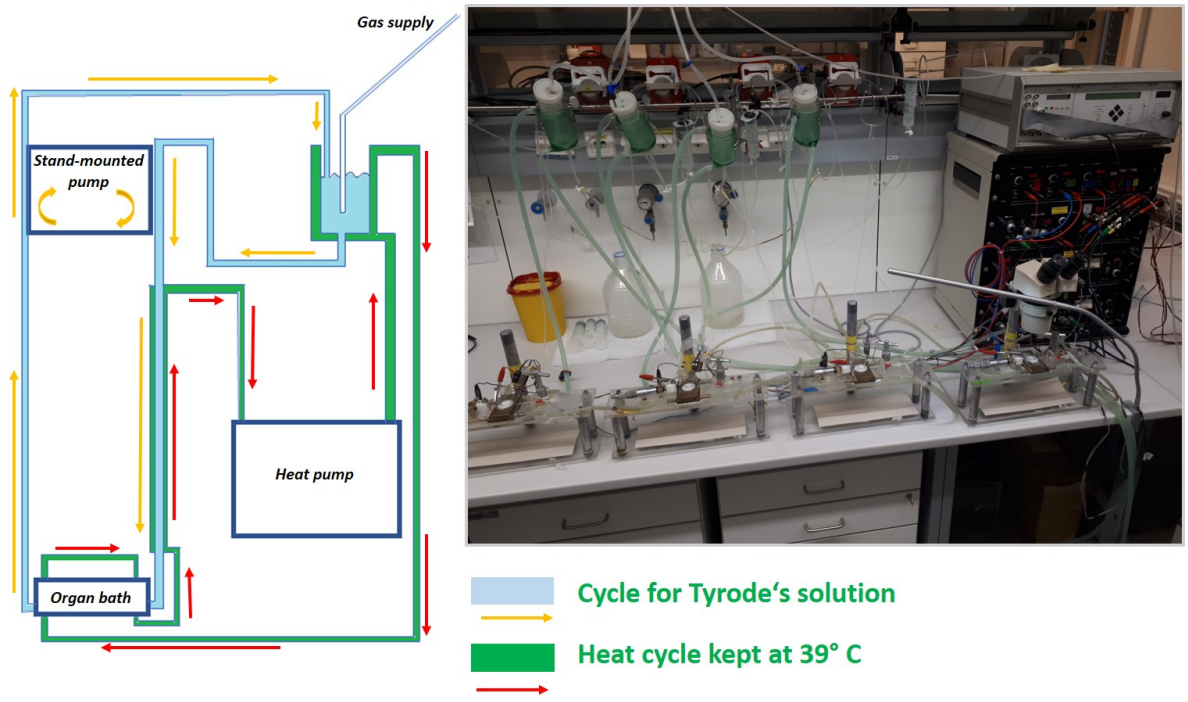

Figure 5: left side: schematic of one channel of the experimental setup. Right side: picture of the whole experimental setup with all four experimental channels and the stimulator visible.

The experimental setup consists of four separate and identical channels including an own organ bath, a reservoir for Tyrode's solution, a tube system for the transportation of the Tyrode's solution, a 50 mL syringe to rinse out Tyrode's solution, continuous gas supply with carbogen and temperature regulation. Each channel represents a closed loop with no connections between other channels; however, it is possible to add drugs or substances to each channel by pipetting into the reservoir and rinse out Tyrode's solution via the syringe. The energy supply of each organ bath is guaranteed by a permanent forward flow of the Tyrode's solution generated by a stand-mounted pump. The Tyrode's solution is permanently bubbled with carbogen gas via a frit to keep the pH value of the solution at 7.4 and sufficient volumes of O_2 solved. Moreover, the temperature of the Tyrode's solution is kept constantly at 37°C by using a heat pump, that is kept at a temperature of 39°C.

The organ bath represents the heart of the experimental setup. The organ bath itself is made of Teflon and is integrated in a thermal block. Isolated muscle strips are fixed on hooks using micro forceps and the microscope. These hooks are located inside the organ bath. One hook is connected with a force transducer that has the ability to measure beat-by-beat mechanical kinetics, the other hook is connected with a micrometer screw gauge.

Muscle strips fixed on these hooks can be stretched to an optimal length (L_{max}) in order to simulate physiological preload. According to the Frank-Starling law increased preload is associated with increased isometric contraction capacities[158]. Tyrode's solution is pumped out of the organ bath by the stand-mounted pump via a cannula and re-enters the reservoir. The whole tube system that contains Tyrode's solution is kept at a constant temperature of 37 °C.

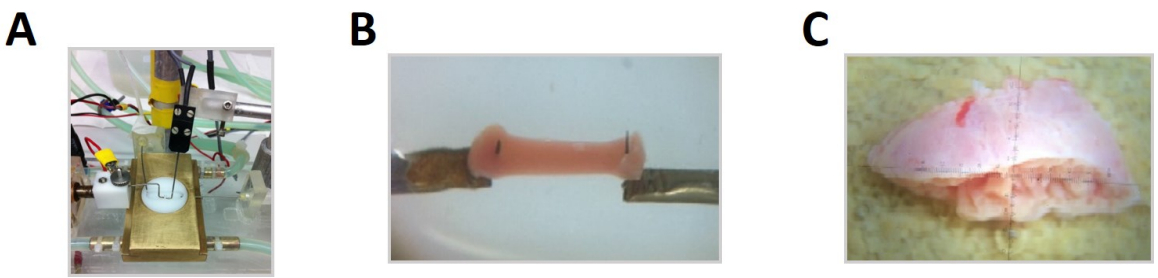

Figure 6: Photographs of the experimental setup
A: Measurement unit consisting of a force transducer (black element) and an organ bath (golden element).
B: Isolated muscle strip fixed on hooks that are connected with the force transducer and a screw gauge.
C: Right atrial appendage, view through a microscope

Muscle strips mounted on the hooks inside the organ bath can be electrically stimulated by an external stimulator. Frequency and voltage can be set manually. The voltage level is usually set 25 % above the threshold that induces a mechanical contraction. Kinetic contraction data is measured beat-by-beat by the force transducer and data are transferred to an external computer and analyzed with the software program Labview.

Before the experiment gets started, the 2,3-BDM must be eliminated. Therefore 40 mL of the initial Tyrode's solution is rinsed out before closing the loop of the tube system. Next, stimulation of the muscle strips starts, and Ca^{2+} concentrations are increased to 1.2 mM and further to 2.5 mM. Each of these Ca^{2+} concentrations are incubated for 2 minutes. Afterwards, muscle strips are stretched until no further increase of developed force can be visualized by further stretching, assuming muscle strips reached their L_{max}.

This timepoint represents the baseline of any performed experiment and afterwards different experimental protocols are performed. Atrial and ventricular muscle strips were prepared identically for an experiment. Muscle strips developing sustained arrhythmias before reaching L_{max} were excluded from further treatment

2.6 Protocols for Human Muscle Strip Experiments

2.6.1 Experiments with DPP4 Inhibitors

After a stable baseline was reached, increasing concentrations of the DPP4 inhibitors saxagliptin (0.6 nM; 2 nM; 6 nM; 20 nM; 60 nM; 200 nM; 600 nM; 2.0 µM; n = 12 muscle strips), alogliptin (0.2 nM; 0.6 nM; 2 nM; 6 nM; 20 nM; 60 nM; 200 nM; 600 nM; n = 10 muscle strips) and sitagliptin (0.6 nM; 2 nM; 6 nM; 20 nM; 60 nM; 200 nM; 600 nM; 2.0 µM; n = 9 muscle strips) were added in form of a dose-response relationship. Concentration was increased every 15 minutes. Placebo-treated muscle strips (n = 10) served as controls. Developed force, diastolic tension and the relaxation parameters RT50, and RT90 (time from peak contractility to relaxation of 50 % or 90 % of the developed force) were measured. Moreover, the number of induced arrhythmic events was assessed. If muscle strips developed sustained arrhythmias, from that time point on data was not anymore pooled to the analysis. None of the patients were pretreated with a DPP4 inhibitor. Data are presented as relative values in [%] of baseline or as absolute values of muscle strips that were treated identically.

2.6.2 Experiments with GLP-1 Receptor Agonists*

A single dose of exenatide (15 nM) was added on 12 muscle strips (substrate: glucose) and 11 muscle strips (substrate: pyruvate), GLP 1 (7-36) amide (180 nM) was added on 13 muscle strips (substrate: glucose) and 13 muscle strips (substrate: pyruvate), GLP-1(9-36) amide (200 nM) was added on 6 muscle strips (substrate: glucose) and 6 muscle strips (substrate: pyruvate). To assess the role of GLP-1 receptor and supposed translocation of GLUT1, isoproterenol (100 nM) was added on 6 muscle strips (substrate: glucose) and 6 muscle strips (substrate: pyruvate). Another 6 muscle strips (substrate: glucose) and 7 muscle strips (substrate: pyruvate) were treated with increasing concentrations of Ca^{2+} (4.0 mM and 7.2 mM) to assess the inotropic response of the muscle strips without activation of downstream mechanisms that influence Ca^{2+} regulating enzymes in cardiomyocytes. All chosen concentrations of GLP-1 receptor agonists were based on previous data from dose-response experiments and selected in order to achieve maximal positive inotropic effects[90]. Developed force and diastolic tension were recorded and analyzed at BL, the time point of developed force maximum, and again at steady state conditions (= 25 minutes after the intervention). Muscle strips that developed arrhythmic events were excluded from analysis. None of the patients were pretreated with a GLP-1 receptor agonist. In the experiments with isoproterenol, patients receiving beta blockers as a premedication were excluded. Data are presented as relative values in [%] of baseline or as absolute values of muscle strips that were treated identically[159].

2.6.3 Experiments with SGLT2 Inhibitors

Atrial and ventricular tissue was treated with the selective SGLT2 inhibitors empagliflozin and dapagliflozin, while the combined SGLT1/SGLT2 inhibitor T-1095 was only investigated in ventricular myocardium. The experimental protocol was identical for all mentioned substances in both, atrial and ventricular myocardium. After a stable baseline was reached, increasing concentrations (200 nM; 2 µM; 10 µM) of empagliflozin (atrial muscle strips: n = 5; ventricular muscle strips: n = 9), dapagliflozin (atrial muscle strips: n = 7; ventricular muscle strips: n = 8), and T-1095 (ventricular muscle strips: n = 6) were added in form of a dose-response relationship. Placebo treated muscle strips served as controls in atrial (n = 7) and ventricular (n = 7) myocardium. Developed force, diastolic tension and the relaxation parameter RT50 were measured. None of the patients was pretreated with a SGLT2 inhibitor. Data are presented as relative values in [%] of baseline or as absolute values of muscle strips that were treated identically.

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018) DOI: 10.1007/s10989-018-9706-9.

2.7 Diastolic Tension and Developed Force

The diastolic tension represents the tension of the resting muscle strips. The muscle strips are put slackly on hooks, afterwards a zero adjustment of the diastolic tension was performed. Stretching of the muscle strips leads to an increase in diastolic tension. Figure 7A

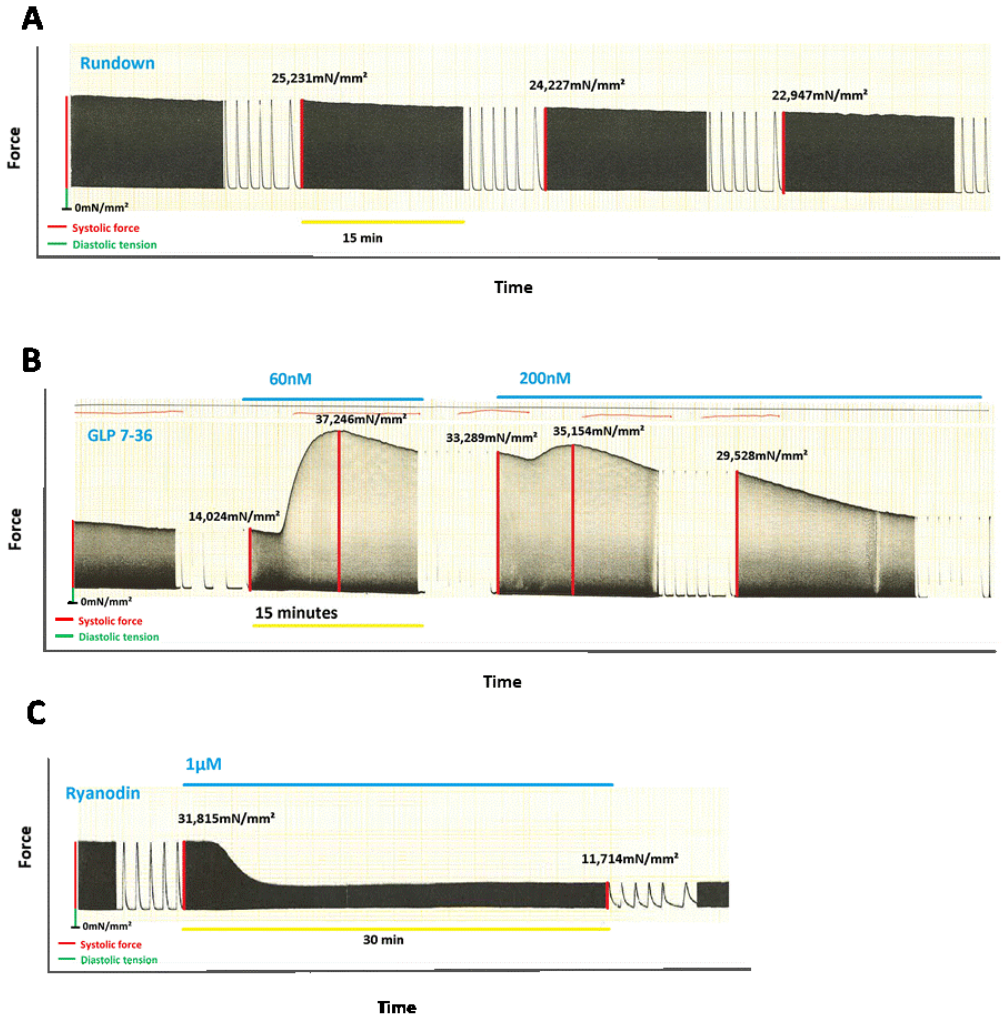

Figure 7: Original registration of the experiment performed on the 10th April 2014 (A), 6th August 2014 (A) and 5th December 2013 (B). Each experiment at 1 Hz and 37 °C. Paper feed: black bar at 5 mm per minute, single beats at 5 mm per second. 1 Hz; 37 °C.

A: No intervention, the decline in developed force and diastolic tension represents rundown.

B: Administration of 60 nM GLP-1(7-36) amide induce a strong positive inotropic effect with transient character. After 15 minutes, a cumulative dose of 200 nM GLP-1(7-36) amide was added and another small positive inotropic effect is visible

C: Administration of 1 µM ryanodine induces a strong negative inotropic effect.

shows the diastolic tension and the developed force in relation to the zero line (0 mN/mm²) in an original registration. A muscle strip, that is stretched to its L_{max} undergoes a physiological rundown. The developed force and the diastolic tension decrease slowly but steadily over time and can also been seen in figure 7A.

2.8 Inotropic Effects

A pharmacological intervention may exert three possible effects: firstly, no inotropic effect with a continuation of the physiological rundown (see figure 7A; BL: 25.2 mN/mm²; after 30 minutes: 22.9 mN/mm²). Secondly, a positive inotropic effect with an increase in developed force (see figure 7B; BL: 14.0 mN/mm²; after approximately 8 minutes of incubation with 60 nM GLP-1(7-36) amide: 37.3 mN/mm²). And thirdly, a negative inotropic effect with a decrease in developed force (see figure 7C; BL: 31.8 mN/mm²; after 30 minutes of incubation with ryanodine: 11.7 mN/mm²).

2.9 Patch-Clamp Experiments

Saxagliptin-treatment on human atrial muscle strips led to unexpected findings with regard to observed arrhythmias. Therefore, an electrophysiological assessment was performed in the laboratory of Gottfried Schatz Research Center, Biophysics of the Medical University of Graz in collaboration with Brigitte Pelzmann and Chintan Koyani. The following methods have been partially published[160].

2.9.1 Animals*

The experimental procedure and number of used animals were approved by the ethics committee of the Federal Ministry of Science, Research and Economy of the Republic of Austria (guinea pigs (GPs); BMWF-66.010/0110-WF/V/3b/2016). The experiments were conducted according to the Directive of the European Parliament and of the Council of September 22, 2010 (2010/63/EU). Dunkin Hartley GPs (6-10 weeks old) of either sex were used[160].

* parts of this chapter have been published in Biochem. Pharmacol. (2017) DOI: 10.1016/j.bcp.2017.08.021.

2.9.2 Isolation of Primary Guinea Pig Ventricular Myocytes*

GP ventricular (GPV) myocytes were isolated as described previously[161]. Briefly, GP were euthanized, hearts were quickly excised and mounted on a Langendorff apparatus and afterwards the coronary system was perfused with a buffer solution containing 126 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.49 mM $NaHCO_3$, 0.5 mM HEPES/Na^+, 0.45 mmol/L glucose, and 100 IU/mL collagenase (Worthington CLS 2) at a temperature of 37°C (pH was adjusted to 7.4 using NaOH). After enzymatic digestion, cells were isolated from ventricles and Ca^{2+} concentrations were raised stepwise to 1.8 mM. Cells were transferred to cell culture medium M199 containing penicillin 50 IU/ml, streptomycin 50 µg/ml, and maintained at 37°C under 5% CO_2. All experiments were performed on the day after isolation. In sum, 16 GPV myocytes were used derived from 3 GPs[160].

2.9.3 Electrophysiological Recordings and Analysis*

Action potentials (APs) and transmembrane currents were recorded in the whole cell configuration of the patch clamp technique using the amplifiers List L/M-EPC 7 (LIST Electronics) and Axopatch 200B (Molecular Devices), and the A/D - D/A converters Digidata 1322A and Digidata 1200 (Molecular Devices). Patch pipettes with a tip-resistance of 2-3 MΩ were used. The pCLAMP software (Molecular Devices) was used for data acquisition and analyses. Only quiescent rod-shaped cardiomyocytes with clear cross-striations were used for electrophysiological experiments. Cell membrane capacitance was determined by integration of the capacitive transient elicited by a 10 mV hyperpolarizing step from -50 mV. Ion currents were normalized to cell membrane capacitance and expressed as pA/pF. In order to allow equilibration of pipette solution with the cytosol, current recordings were started 4 min after rupture of the membrane patch. All ion currents and APs were recorded at 36-37°C.

To record APs and steady state current (I_{ss})-voltage relation, cardiomyocytes were superfused with the standard external solution containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 2.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM HEPES/Na^+, 5.6 mM glucose, (pH 7.4 adjusted with NaOH) and pipettes were filled with an internal solution containing 110 mM KCl, 4.3 mM ATP/K^+, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, and 10 mM HEPES/K^+ (pH 7.4 adjusted with KOH; estimated free Ca^{2+} concentrations < 10 nM). For AP recordings cells were stimulated with minimal suprathreshold current pulses (5 ms) at a frequency of 1 Hz, as previously described[162]. In

* parts of this chapter have been published in Biochem. Pharmacol. (2017) DOI: 10.1016/j.bcp.2017.08.021.

order to exclude any initial transient behavior, the first 10 APs were excluded from analysis. The pCLAMP data were imported into MATLAB® (R2016a) and the AP parameters of 10 consecutive APs were algorithmically analyzed and averaged. I_{ss} recordings were performed using voltage ramps from -100 to +60 mV (20 s duration).

Data were collected after GPV myocytes were superfused for 5 minutes with either saxagliptin at a concentration of 2 µM or standard external solution as described above. Myocytes were considered as controls for themselves at baseline conditions. If no intervention was performed, physiological rundown occurred[160].

2.10 Substances

Saxagliptin, sitagliptin, and alogliptin were purchased from Selleckchem (Munich, Germany); empagliflozin, dapagliflozin, and T-1095 were purchased from AdooQ Bioscience (Irvine/California, United States of America); exenatide and GLP-1(9-36)NH_2 were purchased from Tocris (Bristol, United Kingdom), NaCl, $NaHCO_3$, KH_2PO_4, NaH_2PO_4, NaOH, KOH sodium-pyruvate, $MgCl_2$, 2,3-BDM, penicillin, streptomycin, M199, EGTA, HEPES/Na^+, and HEPES/K^+ were purchased from Sigma-Aldrich (Taufkirchen, Germany), KCl, $MgSO_4$, $CaCl_2$, (+)D-glucose were purchased from Merck (Darmstadt, Germany), Custodiol® was purchased from Dr. Franz Köhler Chemie GmbH (Bensheim, Germany), collagenase CLS 2 was purchased from Worthington Biochemical Corporation, (New Jersey, United States of America). Distilled water (Milli-Q Integral 5 System; Billerica/Massachusetts, United States of America) with an electric conductance of < 125 nS was used.

2.11 General Data Analysis & Statistics

Within each experimental group, identically treated muscle strips or myocytes were pooled and mean values ± standard error of the mean (SEM) were calculated. In human muscle strip experiments, developed force as percent of BL and diastolic tension as absolute values were analyzed. In the experiments with DPP4 inhibitors, additionally the relaxation parameters RT50 and RT90 were analyzed as percent of BL, while in the experiments with SGLT2 inhibitors only the relaxation parameter RT50 was analyzed. Moreover, the differences in developed force after an intervention are presented as the change in the relative values (delta of developed force) in percent of BL in the experimental series with

GLP-1 receptor agonists. In the patch-clamp experiments, APD and outward I_{ss} was analyzed.

Differences between factors (between and within factors) were tested by using a two-way ANOVA for repeated measures with Sidak`s multiple comparison post hoc test. To compare groups, missing values were assumed as mean values of the corresponding group. To compare groups at one time point, Student's unpaired t-test was used. Normal distribution was tested by using the Shapiro–Wilk-Test. In case the assumption of normality was violated, a non-parametric equivalent was used instead. If not otherwise declared, data are expressed as mean ± SEM. Values of $p < 0.05$ (two-sided) were considered statistically significant. To calculate statistics, IBM SPSS Version 22.0 was used (IBM Corporation, Armonk/New York, United States of America).

3. Results

3.1 Functional Effects of Sitagliptin on Human Atrial Myocardium

Increasing concentrations of sitagliptin after 120 minutes at a concentration of 2 µM reduced the developed force in the same extent as under rundown conditions (sitagliptin: 50.6 % ± 13.2 % of BL; controls: 71.7 % ± 9.2 % of BL; p = 0.14) as shown in figure 8A.

Diastolic tension did not change after sitagliptin administration (sitagliptin at baseline: 7.3 mN/m² ± 2.3 mN/m²; at 2 µM: 4.7 mN/m² ± 1.9 mN/m²; controls at baseline: 6.0 mN/m² ± 0.9 mN/m²; after 120 minutes: 5.9 mN/m² ± 1.0 mN/m²; p = 0.91) as shown in figure 8B.

Although numerically increased, there was no significant change in the relaxation parameters RT50 (sitagliptin: 124.2 % ± 18.3 % of BL; controls: 100.0 % ± 8.1 % of BL; p = 0.59) and RT90 (sitagliptin: 112.7 % ± 8.3 % of BL; controls: 103.2 % ± 2.6 % of BL; p = 0.19) due to a high standard deviation as shown in figure 8C and 8D.

Unsuspectedly, 5 muscle strips (56 % of all) in the sitagliptin group developed sustained arrhythmias at different concentrations of sitagliptin.

3.2 Functional Effects of Alogliptin on Human Atrial Myocardium

Administration of increasing concentrations of alogliptin revealed similar effects as treatment with sitagliptin: there was no change in developed force (figure 9A; alogliptin: 58.0 % ± 12.3 % of BL; controls: 71.7 % ± 9.2 % of BL; p = 0.30), diastolic tension (figure 9B; alogliptin at baseline: 6.3 mN/m² ± 1.7 mN/m²; at 600 nM: 4.1 mN/m² ± 1.9 mN/m²; controls at baseline: 6.0 mN/m² ± 0.9 mN/m²; after 120 minutes: 5.9 mN/m² ± 1.0 mN/m²; p = 0.70) or the relaxation parameter RT50 (figure 9C; alogliptin: 95.6 % ± 5.2 % of BL; controls: 100.0 % ± 8.1 % of BL; p = 0.53).

On the other hand, there was a remarkable and significant increase in the relaxation time RT90 (figure 9D; alogliptin: 125.2 % ± 14.6 % of BL; controls: 103.2 % ± 2.6 % of BL; p = 0.038) compared to placebo-treated muscle strips.

Interestingly, muscle strips treated with alogliptin developed sustained arrhythmias in a similar extend as observed after sitagliptin-administration.

In sum, sustained arrhythmias occurred in 50 % of alogliptin-treated muscle strips. Especially these muscle strips showed a reproducible increase in the relaxation parameters RT50 and

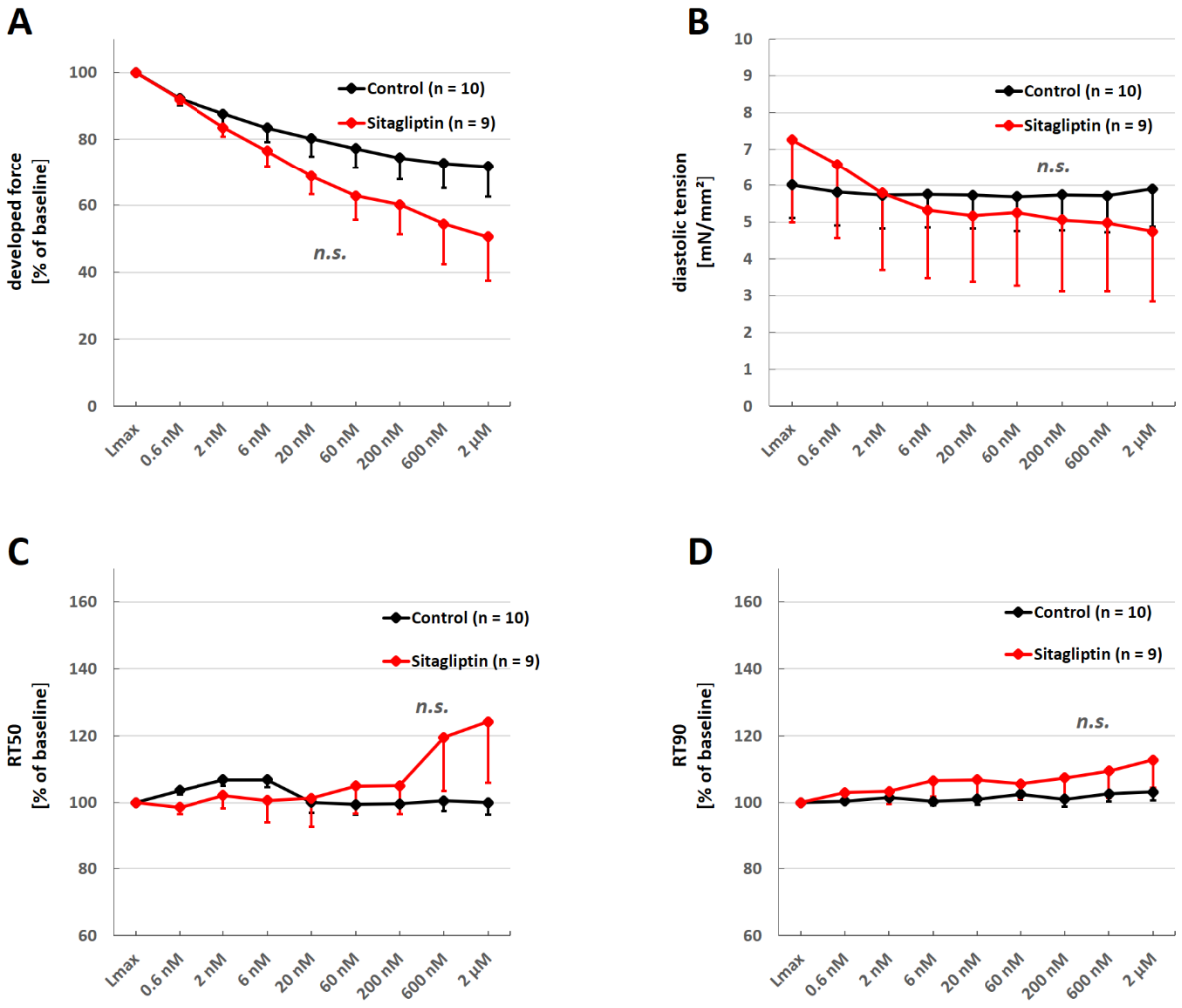

Figure 8: Effects of sitagliptin on human atrial myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of sitagliptin on developed force (red line; n = 9; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

B: Effect of sitagliptin on diastolic tension (red line; n = 9; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

C: Effect of sitagliptin on RT50 (red line; n = 9; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

D: Effect of sitagliptin on RT90 (red line; n = 9; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

RT90, however, no more data on relaxation could be collected during the running arrhythmia. The arrhythmic events occurred at different concentrations, as in the sitagliptin-treated muscle strips. Therefore, the calculation of a clean statistical analysis was impossible.

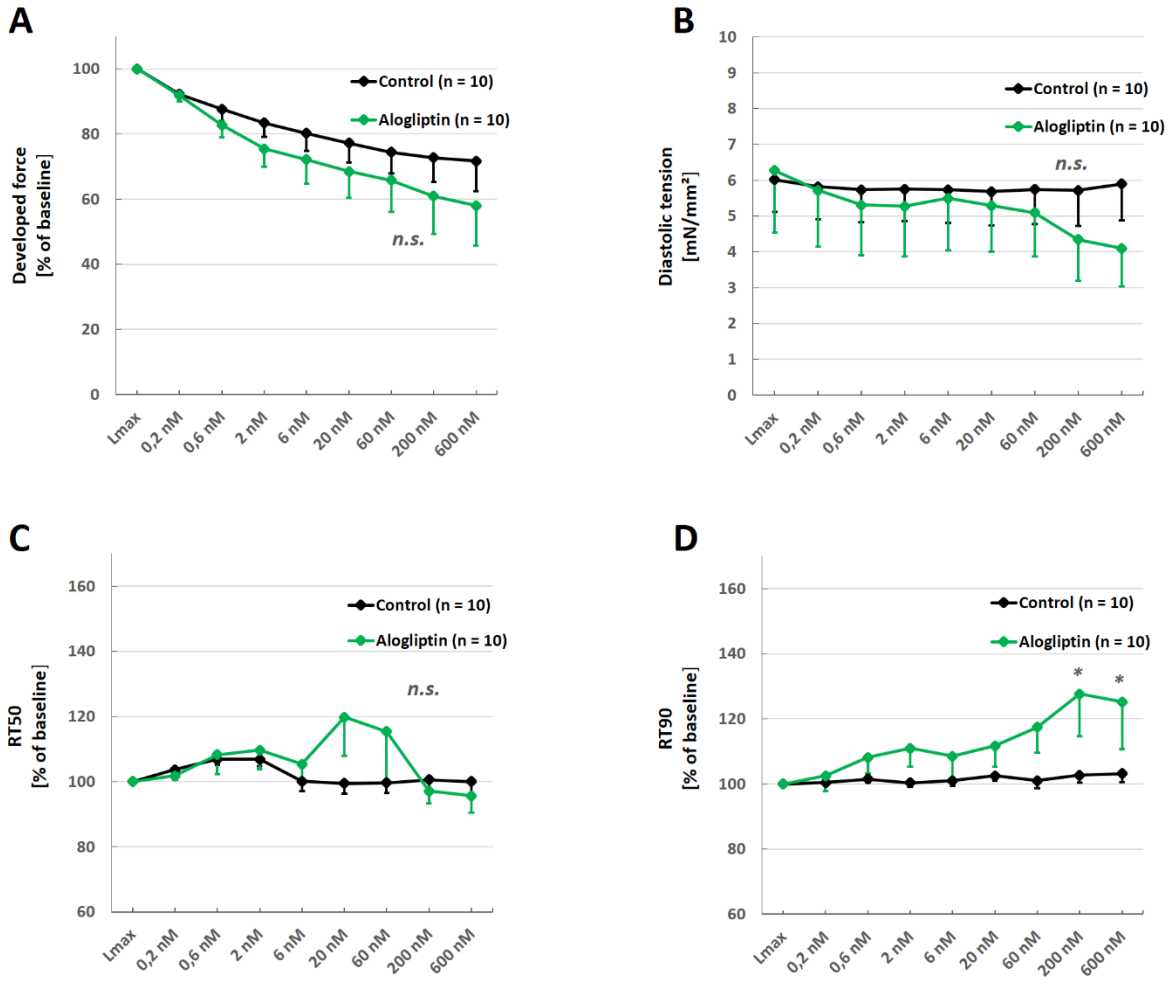

Figure 9: Effects of alogliptin on human atrial myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of alogliptin on developed force (green line; n = 10; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

B: Effect of alogliptin on diastolic tension (green line; n = 10; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

C: Effect of alogliptin on RT50 (green line; n = 10; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

*D: Effect of alogliptin on RT90 (green line; n = 10; [%] of BL ± SEM). Alogliptin induces a significant increase in the relaxation parameter RT90 (black line; n = 10). *: $p < 0.05$.*

3.3 Functional Effects of Saxagliptin on Human Atrial Myocardium*

In contrast to sitagliptin and alogliptin, administration of increasing concentrations of

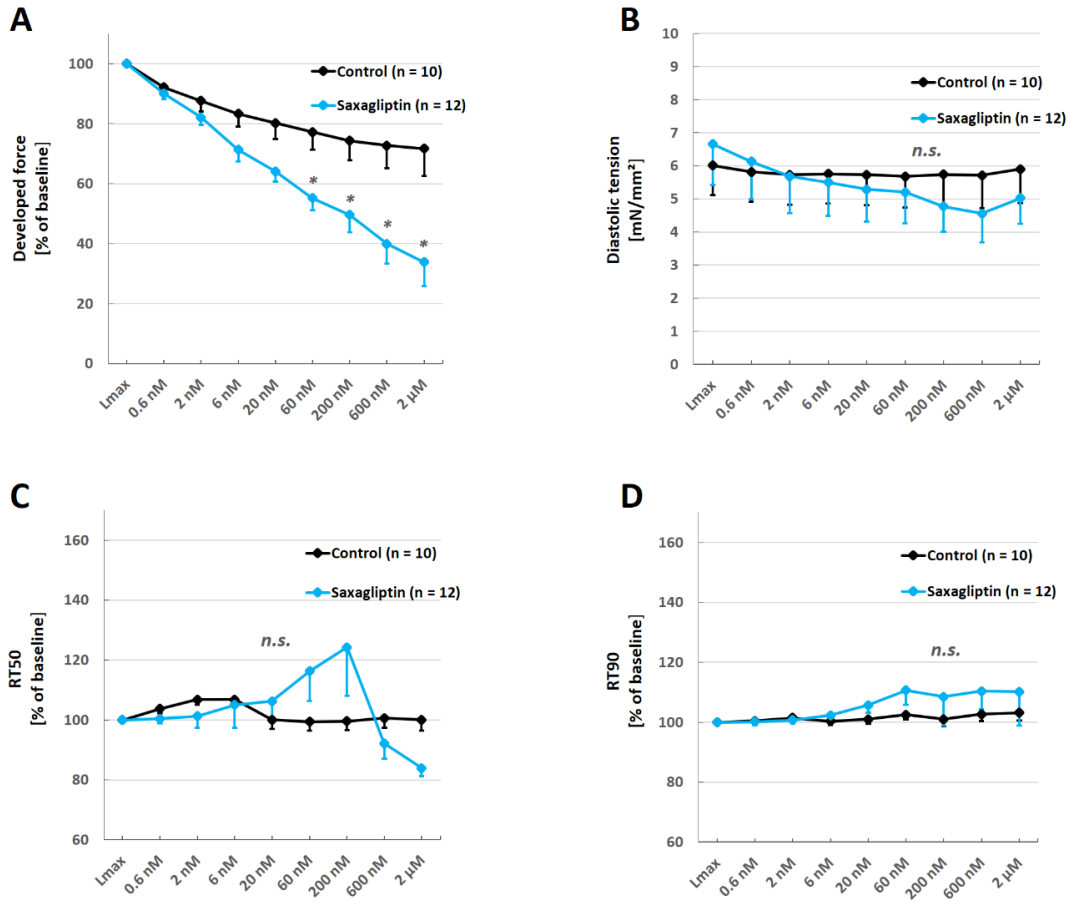

Figure 10: Effects of saxagliptin on human atrial myocardium, dose-response-relationship. 1 Hz; 37°C. This figure has been modified from the publication in the Biochemical Pharmacology journal; 2017. DOI: 10.1016/j.bcp.2017.08.021.

*A: Effect of saxagliptin on developed force (blue line; n = 12; [%] of BL ± SEM). Significant negative inotropic effect compared to control muscle strips (black line; n = 10). *: $p < 0.05$.*

B: Effect of saxagliptin on diastolic tension (blue line; n = 12; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

C: Effect of saxagliptin on RT50 (blue line; n = 12; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

D: Effect of saxagliptin on RT90 (blue line; n = 12; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

saxagliptin revealed a significant negative inotropic effect compared to placebo-treated muscle strips (figure 10A; saxagliptin: 33.8 % ± 8.1 % of BL; controls: 71.7 % ± 9.2 % of BL; saxagliptin revealed a significant negative inotropic effect compared to placebo-treated p = 0.0045). However, no effect on diastolic tension (figure 10B; saxagliptin at baseline: 6.7 mN/m² ± 1.3 mN/m²; at 2 µM: 5.0 mN/m² ± 0.8 mN/m²; controls at baseline: 6.0 mN/m² ± 0.9 mN/m²; after 120 minutes: 5.9 mN/m² ± 1.0 mN/m²; p = 0.77) or the relaxation parameters RT50 (figure 10C; saxagliptin: 84.0 % ± 2.6 % of BL; controls: 100.0 % ± 8.1 % of BL; p = 0.84) and RT90 (figure 10D; saxagliptin: 110.2 % ± 11.3 % of BL; controls: 103.2 % ± 2.6 % of BL; p = 0.14) could be observed. The relaxation times tended to get prolonged in the presence of saxagliptin like observed in the experiments with the other DPP4 inhibitors. However, similarly to sitagliptin and alogliptin treatment, saxagliptin induced sustained arrhythmias in 58 % of all treated muscle strips. These arrhythmias occurred at different concentrations; thus, statistical evaluation of the relaxation parameters was impossible since data derived from affected muscle strips could not be analyzed properly[160].

3.4 Proarrhythmogenic Effects of DPP4 Inhibitors

The assessment of functional effects of different DPP4 inhibitors on human atrial

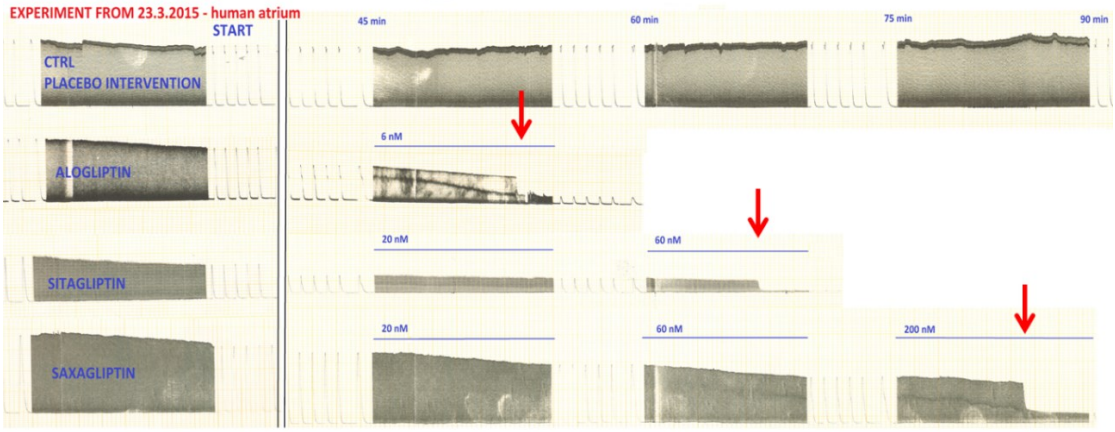

Figure 11: Original registration of the experiment performed on the 23rd March 2015. 1 Hz; 37 °C. Paper feed: black bar at 5 mm per minute, single beats at 5 mm per second

All tested DPP4 inhibitors induce sustained arrhythmias (red arrows). Experimental channels from the top to the lowest: control (rundown; no arrhythmia induced), alogliptin (induces the arrhythmia at a concentration of 6 nM), sitagliptin (induces the arrhythmia at a concentration of 60 nM), and saxagliptin (induces the arrhythmia at a concentration of 200 nM).

myocardium revealed unexpected results: sitagliptin, alogliptin, and saxagliptin induced arrhythmogenic events in approximately half of all tested muscle strips (see figure 12A). A representative original registration from an experiment performed on the 23rd March 2015 is shown in figure 11. Shortly before the arrhythmia occurred, a prolongation of the relaxation properties of affected muscle strips could be observed every time (see figure 12B and 12C).

Additionally, saxagliptin exerted a significant negative inotropic effect compared to physiological rundown. These findings caused our research group to go deeper into the mechanistic of observed effects. The common results of different laboratories have been published by Koyani & Kolesnik et al. in 2017[160].

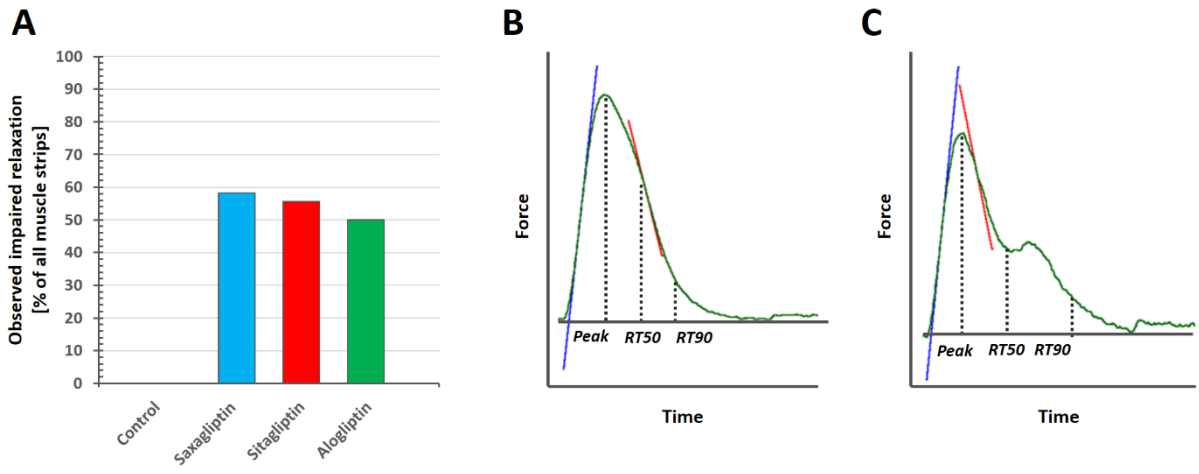

Figure 12: Analysis of arrhythmic events occurring after treatment with different DPP4 inhibitors. Representative original recordings of the experiment performed on the 15th May 2015. 1 Hz; 37 °C

A: All tested DPP4 inhibitors induce sustained arrhythmias in a similar extend (blue bar: saxagliptin-treatment, arrhythmias occurred in 58 % of all tested muscle strips; red bar: sitagliptin-treatment, arrythmias occurred in 56 % of all tested muscle strips; green bar: alogliptin-treatment, arrhythmias occurred in 50 % of all tested muscle strips; no bar: control-muscle strips, no arrhythmias occurred after placebo-treatment). Arrhythmic events occur mainly due prolongation of relaxation properties.

B: Original recording of one muscle strip with normal kinetic properties at L_{max} derived from the experiment performed on the 15th May 2015. RT50 = 86 ms; RT90 = 136 ms.

C: Same muscle strip after 20 nM alogliptin treatment. Disturbed relaxation and prolongation of the relaxation parameters are visible. RT50 = 106 ms; RT90 = 200 ms.

3.5 Saxagliptin Impairs the Electrophysiological Function of Myocytes*

As saxagliptin impaired cardiac excitation-contraction coupling, as a next step the cardiac cellular electrophysiology was explored. Therefore, patch-clamp experiments with GPV myocytes were performed. A representative action potential shows that rundown prolongs APs of cardiomyocytes (figure 13B left side). However, saxagliptin-superfusion for 5 min

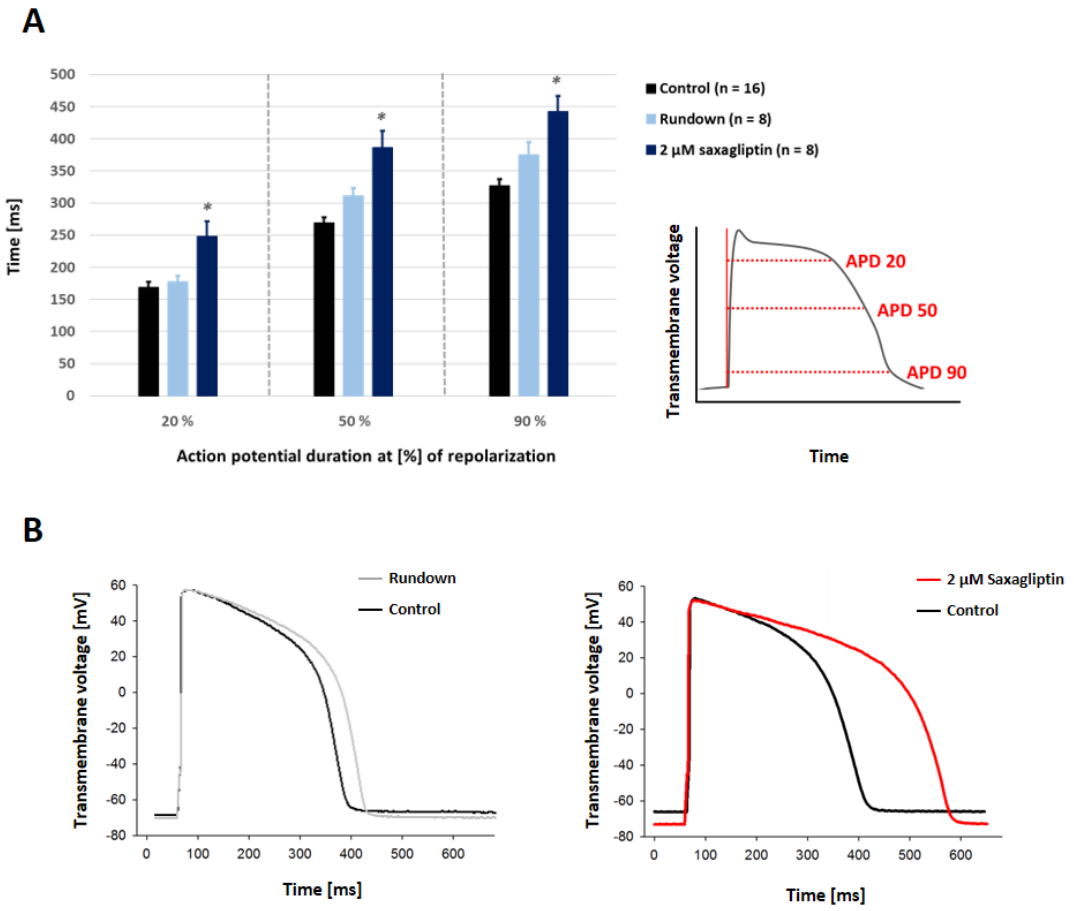

Figure 13: Analysis of the acute impact (5 minutes) of saxagliptin on GPV myocytes. This figure has been modified from the publication in the Biochemical Pharmacology journal; 2017. DOI: 10.1016/j.bcp.2017.08.021.

*A: APD at 20 %, 50 %, and 90 % of repolarization in [ms] ± SEM. Black bar: control myocytes at the beginning of the experiment (n = 16); bright blue bar: placebo-treated myocytes (n = 8); dark blue bar: saxagliptin-treated myocytes (n = 8). *: $p < 0.05$ vs. rundown and control.*

B: Representative APs. Original registration from the experiment performed on the 27th August 2015. Left side: rundown leads to a slight prolongation of the AP. Right side: saxagliptin-treatment leads to a more pronounced prolongation of the AP.

* parts of this chapter have been published in Biochem. Pharmacol. (2017) DOI: 10.1016/j.bcp.2017.08.021.

further prolonged APs (figure 13B right side). Analysis of APs of GPV myocytes showed that saxagliptin significantly prolonged APD (figure 13A) at 20 % (control: 169 ± 9 ms; rundown: 177 ± 9 ms; saxagliptin: 248 ± 24 ms; $p < 0.05$), 50 % (control: 269 ± 8 ms; rundown: 311 ± 12 ms; saxagliptin: 386 ± 26 ms; $p < 0.05$), and 90 % (control: 327 ± 10 ms; rundown: 375 ± 20 ms; saxagliptin: 424 ± 25 ms; $p < 0.05$) of repolarization. In parallel, a significant reduction in outward I_{ss} density between +10 to +60 mV membrane potentials could be observed representing mainly K^+ outward currents (Fig 14B; A shows a representative recording in the change of I_{ss}). This may account for the observed APD prolongation[160].

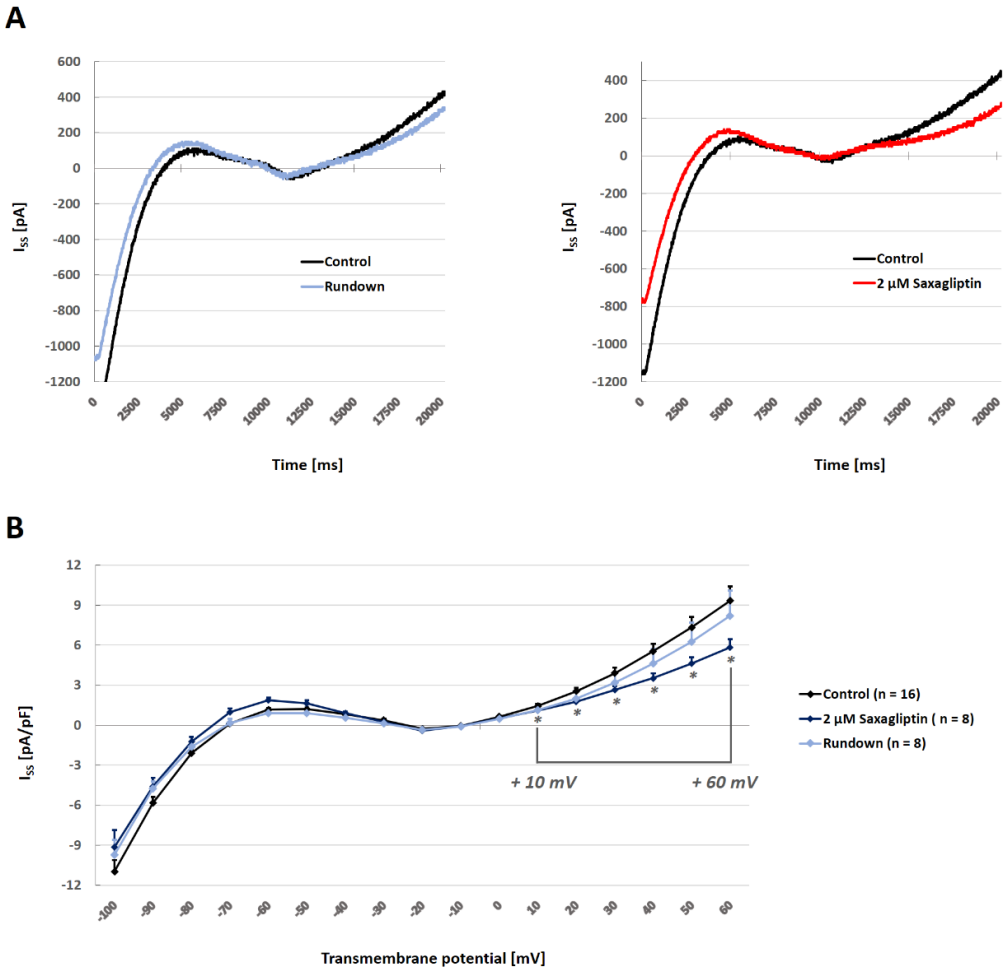

Figure 14: Analysis of the acute impact (5 minutes) of saxagliptin on GPV myocytes. This figure has been modified from the publication in the Biochemical Pharmacology journal; 2017. DOI: 10.1016/j.bcp.2017.08.021.

A: Representative current traces of I_{ss}. Original registration from the experiment performed on the 26th August 2015. Left side: rundown. Right side: 2 µM saxagliptin.

*B: I_{ss} before (black line; control n = 16) and after 5 min of superfusion with either external solution alone (bright blue line; rundown, n = 8) or 2 µM of saxagliptin (dark blue line; n = 8). *: $p < 0.05$.*

3.6 The Inotropic Effect of Exenatide – Role of Energy Substrate*

The acute, positive inotropic abilities as well as molecular downstream mechanisms have already been published in a previous work. A significant translocation of the GLUT1 has been shown after exenatide administration, however, so far it remains unclear whether GLUT1 translocation and consecutive improved cellular glucose uptake is involved in the positive inotropic effect.

The main results of the previous publication could be confirmed with the new experiments: administration of a single dose of 15 nM exenatide led to a transient positive inotropic effect. This effect occurred in the presence of glucose and pyruvate. Irrespective of the energy substrate, after exenatide administration the human atrial myocardium showed a strong increase in developed force within minutes followed by a decline to stable steady state conditions that were reached after 25 minutes. As shown in figure 15A, the positive inotropic effect tends to be more pronounced in glucose treated muscle strips compared to pyruvate-treated muscle strips ($p = 0.055$) at the time of maximum developed force (194.1 % ± 27.0 % in glucose versus 141.2 % ± 10.5 % in pyruvate) and at steady state conditions after 25 minutes (156.5 % ± 15.7 % in glucose versus 116.1 % ± 5.1 % in pyruvate). Figure 15B shows the change in developed force between baseline and steady state conditions after 25 minutes. In glucose-treated muscle strips the increase in developed force was significantly higher (56.5 % ± 15.7 %) compared to pyruvate treated muscle strips (16.1 % ± 5.1 %, $p = 0.03$).

Interestingly, there were some non-responding muscle strips that did not show any change in developed force besides physiological rundown (33 % of the glucose-treated muscle strips and 18 % of the pyruvate-treated muscle strips).

Within the non-responding muscle strips, there were no obvious similarities in the patients' medical records such as diabetes, comorbidities or medications. Diastolic tension (figure 15C; pyruvate at baseline: 6.2 mN/m² ± 1.4 mN/m²; at 15 nM exenatide: 6.5 mN/m² ± 1.4 mN/m²; glucose at baseline: 7.4 mN/m² ± 1.0 mN/m²; at 15 nM exenatide: 5.9 mN/m² ± 1.0 mN/m²; $p = 0.86$) and the relaxation parameter RT50 (figure 15D; pyruvate: 95.8 % ± 1.7 % of BL; glucose: 95.0 % ± 7.6 % of BL; $p = 0.92$) did not differ between glucose and pyruvate-treated muscle strips after exenatide-administration.

In contrast to DPP4 inhibitors, exenatide-administration did not induce any arrhythmias[159].

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018) DOI: 10.1007/s10989-018-9706-9.

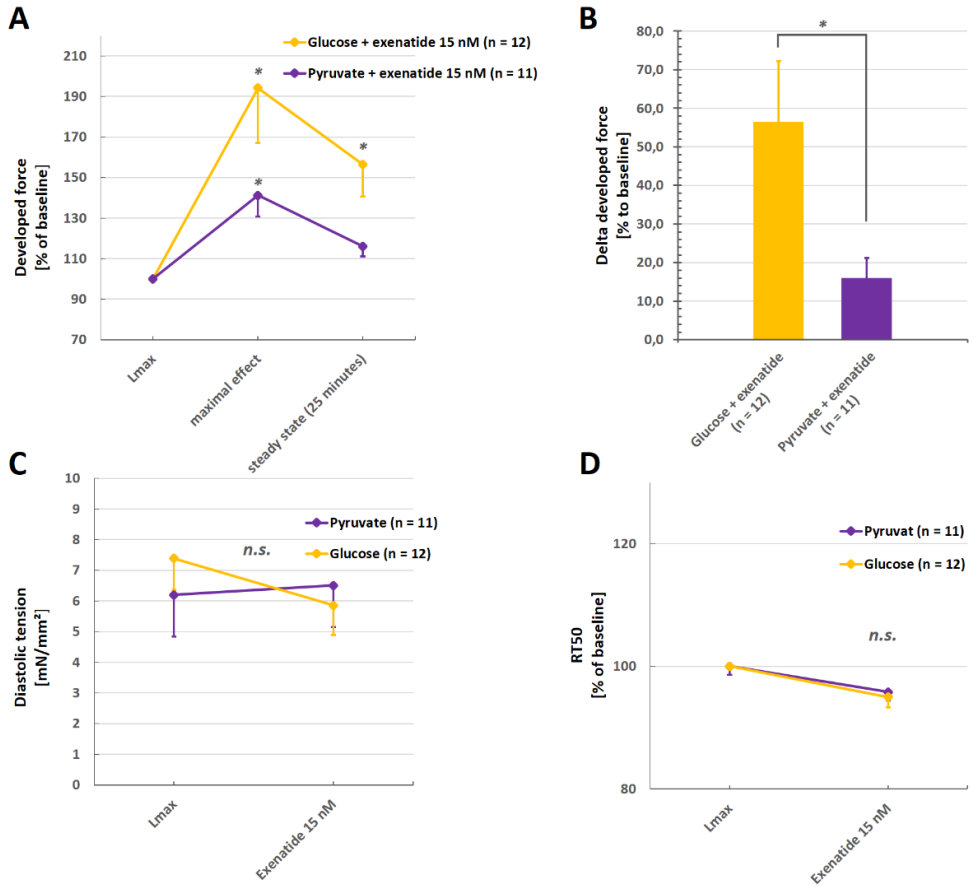

Figure 15: Substrate-dependent inotropic effect of exenatide on human atrial myocardium. 1 Hz; 37°C This figure has been modified from the publication in the International Journal of Peptide Research and Therapeutics; 2018. DOI: 10.1007/s10989-018-9706-9

*A: 15 nM exenatide exerts a positive inotropic effect in the presence of both substrates (purple: pyruvate, n = 11; yellow: glucose, n = 12; [%] of BL ± SEM). There was a stronger effect in the presence of glucose. *: p < 0.05 versus baseline. No statistical difference between groups (borderline level; p = 0.055)*

*B: Analysis of developed force delta to baseline at time point of steady state conditions for glucose-treated (yellow bar; n = 12; [% of delta to baseline]) and pyruvate-treated (purple bar; n = 11) muscle strips. *: p < 0.05 between groups*

C: Effect of 15 nM exenatide on diastolic tension of muscle strips pretreated with pyruvate (purple line; n = 11; [mN/mm²] ± SEM) and glucose (yellow line; n = 12). No difference between groups. n.s.: not significant.

D: Effect of 15 nM exenatide on RT50 of muscle strips pretreated with pyruvate (purple line; n = 11; [%] of baseline] ± SEM) and glucose (yellow line; n = 12). No difference between groups. n.s.: not significant.

3.7 The Inotropic Effect of GLP-1(7-36) Amide – Role of Energy Substrate*

To further evaluate the role of the energy substrate and possibly involved GLUT1 translocation, the positive inotropic effect of synthetic GLP-1 (7-36) amide was evaluated. This hormone is identical to the physiological, human GLP-1 receptor agonist responsible for the incretin effect. In contrast to shown GLUT1 translocation after exenatide administration in the previous publication of our research group, tests for GLUT1 translocation after exposure to GLP-1 (7-36) amide were never performed and translocation of GLUT1 and thereby a stronger positive inotropic effect in the presence of glucose after GLP-1 (7-36) amide administration was only assumed.

A single dose of 180 nM GLP 1 (7-36) amide revealed similar effects on developed force as observed after exenatide treatment. Figure 16A shows a rapid increase in developed force that occurred within a few minutes in the presence of glucose and pyruvate (198.6 ± 34.6 % in glucose versus 110.0 ± 4.4 % in pyruvate; $p < 0.05$). This effect was followed by a decrease in developed force to steady state conditions after 25 minutes (138.7 ± 18.8 % in glucose versus 99.9 ± 3.3 % in pyruvate; $p = 0.25$). The increase in developed force at steady state was higher in muscle strips superfused with glucose-enriched solution (38.7 ± 18.8 %) compared to pyruvate-enriched solution (-0.1 ± 3.3 %, $p = 0.052$), with a borderline significant difference (figure 16B).

Interestingly, also after GLP-1(7-36) amide administration, a significant portion of muscle strips did not respond with a positive inotropic effect (31 % of the glucose-treated muscle strips and 54 % of the pyruvate-treated muscle strips) with no obvious explanation given by the patient's medical history. Like observed after treatment with exenatide, GLP1 (7-36) amide administration did not influence diastolic tension (figure 16C; pyruvate at baseline: 5.1 mN/m² ± 2.2 mN/m²; at 180 nM GLP-1(7-36) amide: 5.1 mN/m² ± 2.1 mN/m²; glucose at baseline: 5.2 mN/m² ± 0.4 mN/m²; at 180 nM GLP-1(7-36) amide: 4.5 mN/m² ± 0.4 mN/m²; $p = 0.71$) or the relaxation parameter RT50 (figure 16D; pyruvate: 94.7 % ± 3.2 % of BL; glucose: 88.0 % ± 3.0 % of BL; $p = 0.14$), irrespective of the energy substrate.

In line with the findings of exenatide, no arrhythmias were induced by GLP-1(7-36) amide administration. Overall, GLP-1(7-36) amide and exenatide had similar functional effects[159].

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018) DOI: 10.1007/s10989-018-9706-9.

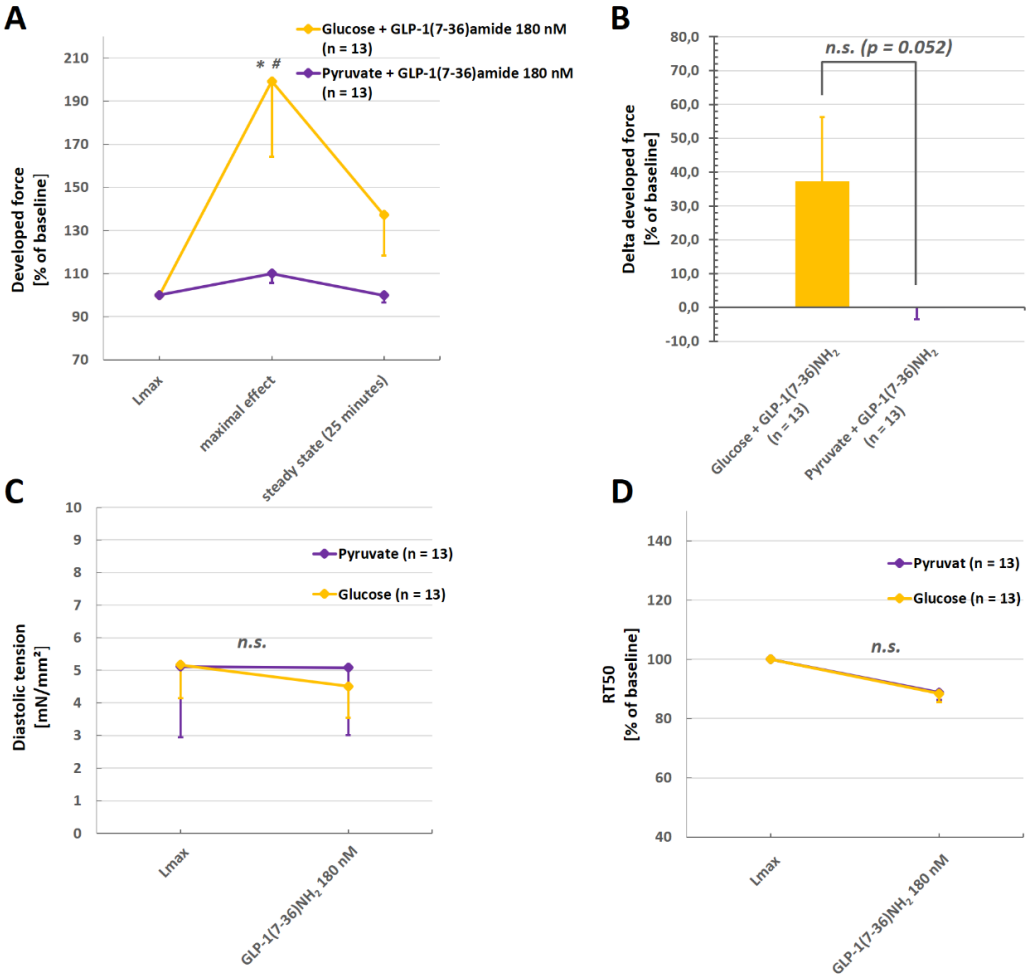

Figure 16: Substrate-dependent inotropic effect of GLP-1(7-36) NH_2 on human atrial myocardium. 1 Hz; 37°C. This figure has been modified from the publication in the International Journal of Peptide Research and Therapeutics; 2018. DOI: 10.1007/s10989-018-9706-9

*A: 180 nM GLP-1(7-36) NH_2 exerts a positive inotropic effect in the presence of glucose (yellow: glucose, n = 13; [%] of BL ± SEM); while in the presence of pyruvate only a minor increase of developed force was induced (purple: pyruvate, n = 13. *: $p < 0.05$ versus baseline. #: $p < 0.05$ between groups.*

B: Analysis of developed force delta to baseline at time point of steady state conditions for glucose-treated (yellow bar; n = 13; [% of delta to baseline]) and pyruvate-treated (purple bar; n = 13) muscle strips. n.s.: not significant (borderline level: p = 0.052 between groups)

C: Effect of 180 nM GLP-1(7-36) NH_2 on diastolic tension of muscle strips pretreated with pyruvate (purple line; n = 13; [mN/mm²] ± SEM) and glucose (yellow line; n = 13). No difference between groups. n.s.: not significant.

D: Effect of 180 nM GLP-1(7-36) NH_2 on RT50 of muscle strips pretreated with pyruvate (purple line; n = 13; [%] of baseline] ± SEM) and glucose (yellow line; n = 13). No difference between groups. n.s.: not significant.

3.8 The Impact of GLP-1(9-36) Amide on Atrial Tissue*

For the GLP-1 receptor antagonist GLP-1 (9-36) amide no inotropic properties were reported and translocation of GLUT1 was not tested by our research group previously. Muscle strips superfused with either glucose or pyruvate enriched solution were treated with a single high dose of GLP-1 (9-36) amide (200 nM).

Figure 17A shows the functional effects on developed force 25 minutes after the intervention. In glucose treated muscle strips, the decrease in developed force after administration of GLP-1 (9-36) amide was more pronounced (85.7 % ± 1.7 % of BL) compared to muscle strips that were treated with pyruvate (96.2 % ± 2.4 % of BL; $p < 0.05$). Additionally, no difference in diastolic tension (figure 17B; pyruvate at baseline: 7.1 mN/m² ± 0.9 mN/m²; at 200 nM GLP-1(9-36) amide: 5.9 mN/m² ± 0.8 mN/m²; glucose at baseline: 6.0 mN/m² ± 0.6 mN/m²; at 200 nM GLP-1(9-36) amide: 6.2 mN/m² ± 1.1 mN/m²; $p = 0.72$) could be observed between different energy substrates[159].

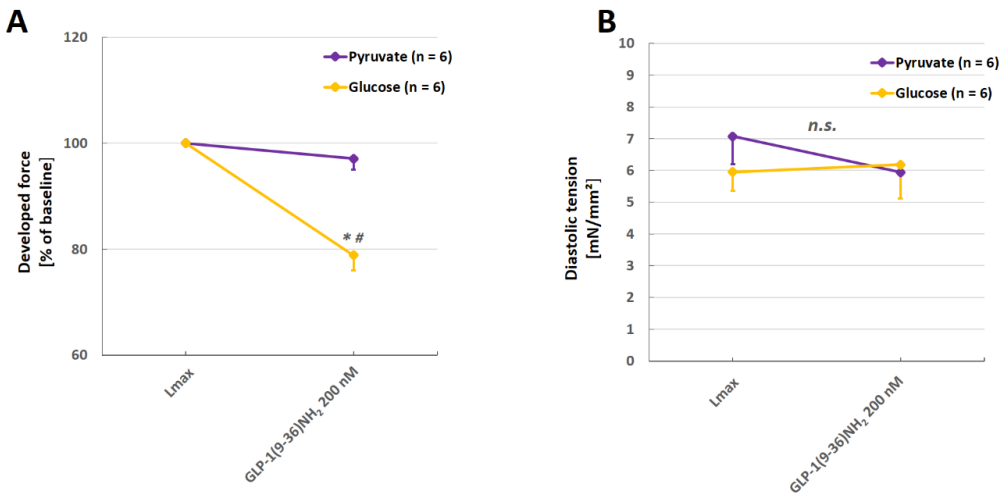

Figure 17: Substrate-dependent inotropic effect of GLP-1(9-36) NH_2 on human atrial myocardium. 1 Hz; 37 °C. This figure has been modified from the publication in the International Journal of Peptide Research and Therapeutics; 2018. DOI: 10.1007/s10989-018-9706-9

*A: 200 nM GLP-1(9-36) NH_2 has no impact on contractility in the presence of glucose (yellow: glucose, n = 6; [%] of BL ± SEM); or pyruvate (purple: pyruvate, n = 6). *: $p < 0.05$ vs. baseline. #: $p < 0.05$ between groups.*

B: Effect of 200 nM GLP-1(9-36) NH_2 on diastolic tension of muscle strips pretreated with pyruvate (purple line; n = 6; [mN/mm²] ± SEM) and glucose (yellow line; n = 6). No difference between groups. n.s.: not significant.

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018) DOI: 10.1007/s10989-018-9706-9.

3.9 Beta Receptor Activation in Atrial Tissue*

To further investigate the observed variations of the GLP-1 receptor dependent positive inotropic effect using different energy substrates, muscle strips superfused with either glucose or pyruvate enriched solution were treated with a single dose of 100 nM of the beta receptor agonist isoproterenol.

Figure 18A shows the increase in developed force between baseline and steady state condition force 25 minutes after the intervention. Although the positive inotropic effect expressed as the change of developed force was stronger in the presence of glucose (179.1 % ± 68.0 %) compared to pyruvate (121.8 % ± 32.9 %), there was no statistical difference between the groups (p = 0.47). Additionally, isoproterenol did not exert a different effect on diastolic tension (figure 18B; pyruvate at baseline: 5.6 mN/m² ± 0.7 mN/m²; at 100 nM isoproterenol: 4.5 mN/m² ± 1.0 mN/m²; glucose at baseline: 5.3 mN/m² ± 0.4 mN/m²; at 100 nM isoproterenol: 4.8 mN/m² ± 0.6 mN/m²; p = 0.98) suggesting that beta receptor activation is followed by a similar response in the presence of each used energy substrate[159].

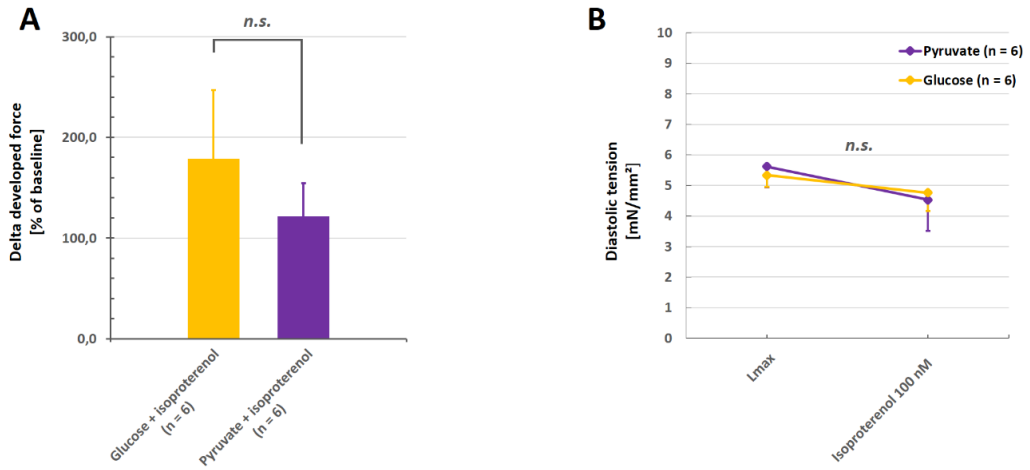

Figure 18: Substrate-dependent inotropic effect of isoproterenol on human atrial myocardium. 1 Hz; 37°C. This figure has been modified from the publication in the International Journal of Peptide Research and Therapeutics; 2018. DOI: 10.1007/s10989-018-9706-9

A: Analysis of developed force delta to baseline after addition of 100 nM isoproterenol for glucose-treated (yellow bar; n = 6; [% of delta to baseline]) and pyruvate-treated (purple bar; n = 6) muscle strips. n.s.: not significant

B: Effect of 100 nM isoproterenol on diastolic tension of muscle strips pretreated with pyruvate (purple line; n = 6; [mN/mm²] ± SEM) and glucose (yellow line; n = 6). No difference between groups. n.s.: not significant.

3.10 Calcium Response in the Presence of Pyruvate and Glucose*

Complementary to the findings of beta receptor activation, myocardial contractility was measured at different Ca^{2+} concentrations in the presence of glucose and pyruvate. A stepwise increase of the Ca^{2+} concentrations (from baseline 2.5 mM to 4.0 mM and finally 7.2 mM) led to a similar positive inotropic effect in the presence of glucose and pyruvate.

As shown in figure 19A, the effect was slightly more pronounced in the presence of glucose. However, no significant difference to pyruvate-treated muscle strips could be observed at the concentrations of 4.0 mM Ca^{2+} (146.6 % ± 14.8 % of BL in glucose versus 136.7 % ± 13.2 % of BL in pyruvate) and 7.2 mM Ca^{2+} (187.1 % ± 19.5 % of BL in glucose versus 156.7 % ± 21.9 % of BL in pyruvate; p = 0.4 between groups). Additionally, increasing concentrations of Ca^{2+} did not exert a different effect on diastolic tension (figure 19B; pyruvate at baseline: 6.9 mN/m² ± 1.3 mN/m²; at 7.2 mM Ca^{2+}: 5.3 mN/m² ± 1.0 mN/m²; glucose at baseline: 5.9 mN/m² ± 0.5 mN/m²; at 7.2 mM Ca^{2+}: 5.2 mN/m² ± 1.0 mN/m²; p = 0.56). These results suggest a comparable myocardial contractility in response to calcium in the presence of glucose or pyruvate[159].

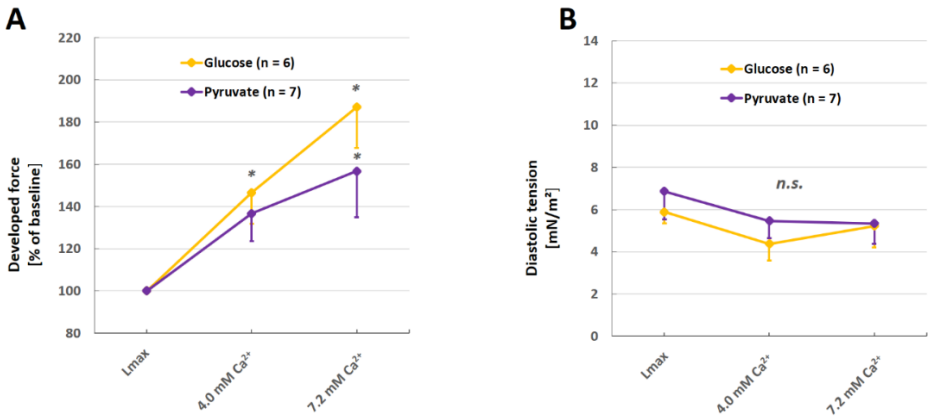

Figure 19: Substrate-dependent inotropic effect of Ca^{2+} on human atrial myocardium. 1 Hz; 37°C. This figure has been modified from the publication in the International Journal of Peptide Research and Therapeutics; 2018. DOI: 10.1007/s10989-018-9706-9

*A: Ca^{2+} exert the same impact on contractility in the presence of glucose (yellow: glucose, n = 6; [%] of BL ± SEM); or pyruvate (purple: pyruvate, n = 7). *: p < 0.05 vs. baseline. No significant difference between groups.*

B: Effect of Ca^{2+} on diastolic tension of muscle strips pretreated with pyruvate (purple line; n = 6; [mN/mm²] ± SEM) and glucose (yellow line; n = 6). No difference between groups. n.s.: not significant.

3.11 Effects of Empagliflozin on Human Myocardium

As the third modern antidiabetic concept, functional effects of SGLT2 inhibitors were assessed. Experiments in atrial and ventricular tissue were performed with empagliflozin.

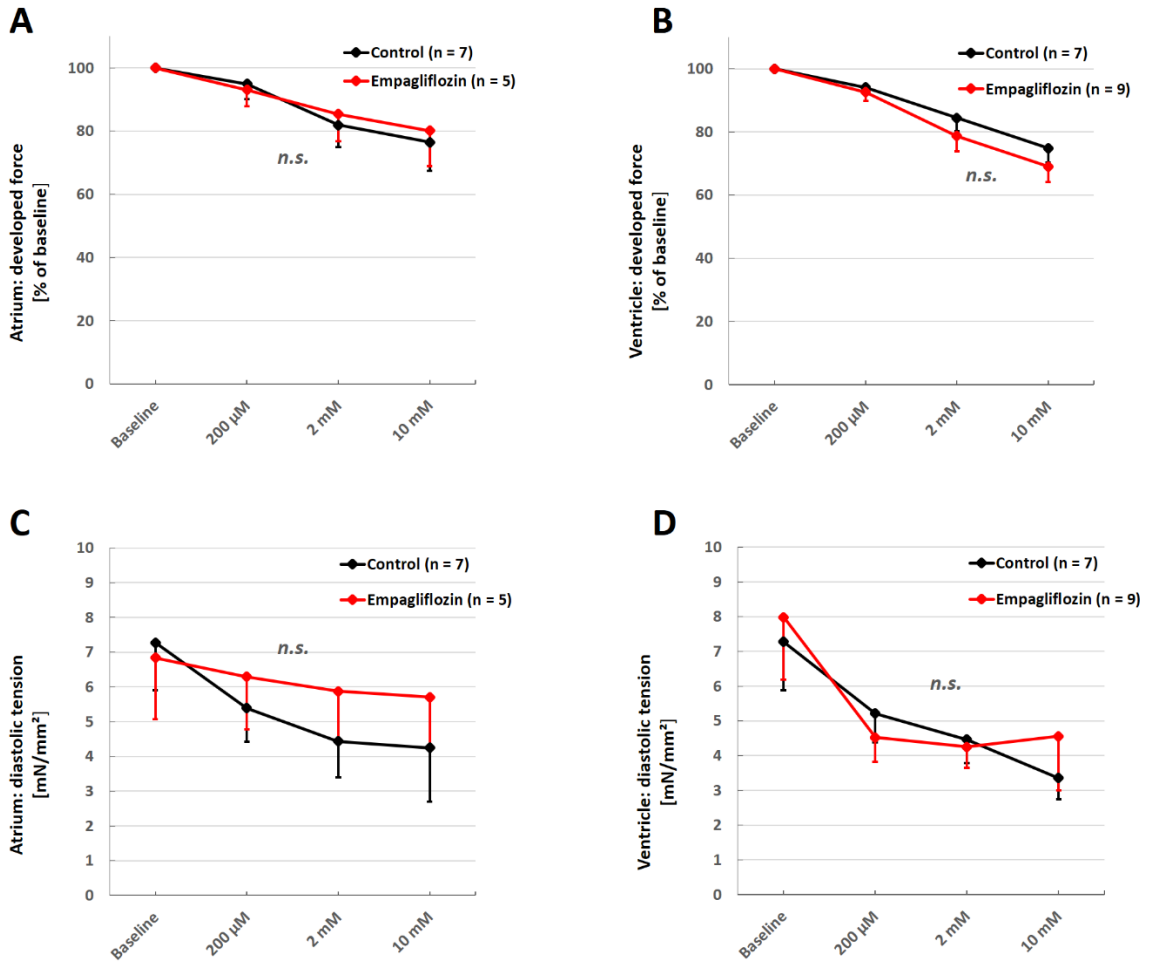

Figure 20: Effects of empagliflozin on human myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of empagliflozin on developed force (red line; n = 5; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

B: Effect of empagliflozin on developed force (red line; n = 9; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

C: Effect of empagliflozin on diastolic tension (red line; n = 5; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

D: Effect of empagliflozin on diastolic tension (red line; n = 9; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

In atrial tissue, administration of increasing concentrations of empagliflozin had no effect on developed force (figure 20A; empagliflozin at 10 µM: 80.1 % ± 11.3 % of BL; controls: 76.5 % ± 9.0 % of BL; p = 0.87), diastolic tension (figure 20C: empagliflozin at BL: 6.8 mN/m² ± 1.8 mN/m²; at 10 µM: 5.7 mN/m² ± 1.4 mN/m²; controls at baseline: 7.3 mN/m² ± 1.4 mN/m²; after 90 minutes: 4.2 mN/m² ± 1.5 mN/m²; p = 0.67), or the relaxation parameter RT50 (figure 21A; empagliflozin at 10 µM: 104.0 % ± 5.8 % of BL; controls: 111.5 % ± 6.4 % of BL; p = 0.24). In ventricular myocardium, empagliflozin had also no effect on developed force (figure 20B; empagliflozin at 10 µM: 69.1 % ± 5.0 % of BL; controls: 74.9 % ± 4.5 % of BL; p = 0.41), diastolic tension (figure 20D: empagliflozin at BL: 8.0 mN/m² ± 1.8 mN/m²; at 10 µM: 4.6 mN/m² ± 1.6 mN/m²; controls at baseline: 7.3 mN/m² ± 1.4 mN/m²; after 90 minutes: 3.4 mN/m² ± 0.6 mN/m²; p = 0.85), or the relaxation parameter RT50 (figure 21B; empagliflozin at 10 µM: 103.8 % ± 7.4 % of BL; controls: 108.7 % ± 8.9 % of BL; p = 0.34). No arrhythmic events could be detected. Overall, no acute functional effects on atrial and ventricular myocardium could be observed after administration of high concentrations of empagliflozin.

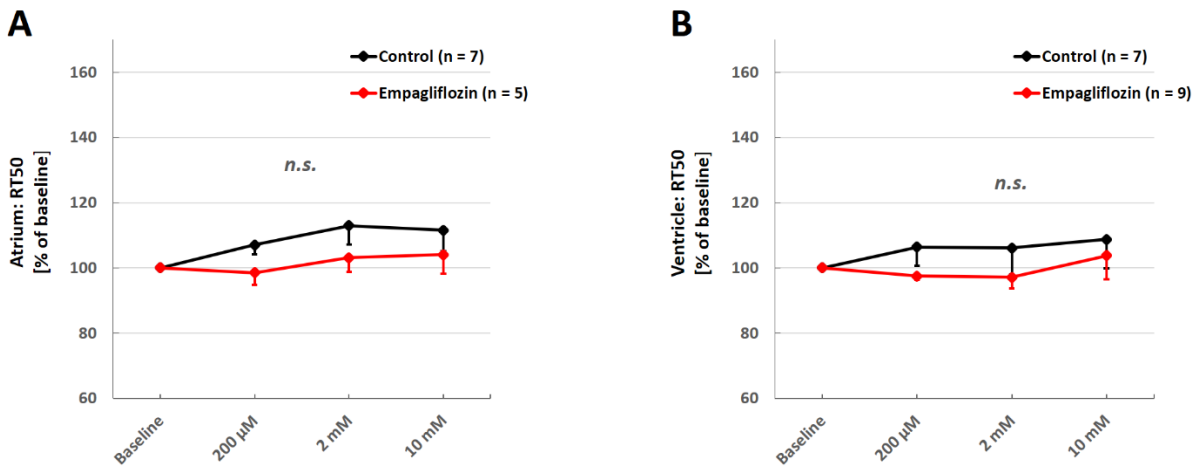

Figure 21: Effects of empagliflozin on human myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of empagliflozin on RT50 (red line; n = 5; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

B: Effect of empagliflozin on RT50 (red line; n = 9; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

3.12 Effects of Dapagliflozin on Human Myocardium

In order to gather more information on class-specific as well as molecule-specific effects, the SGLT2 inhibitor dapagliflozin was investigated in the same way as empagliflozin in atrial and

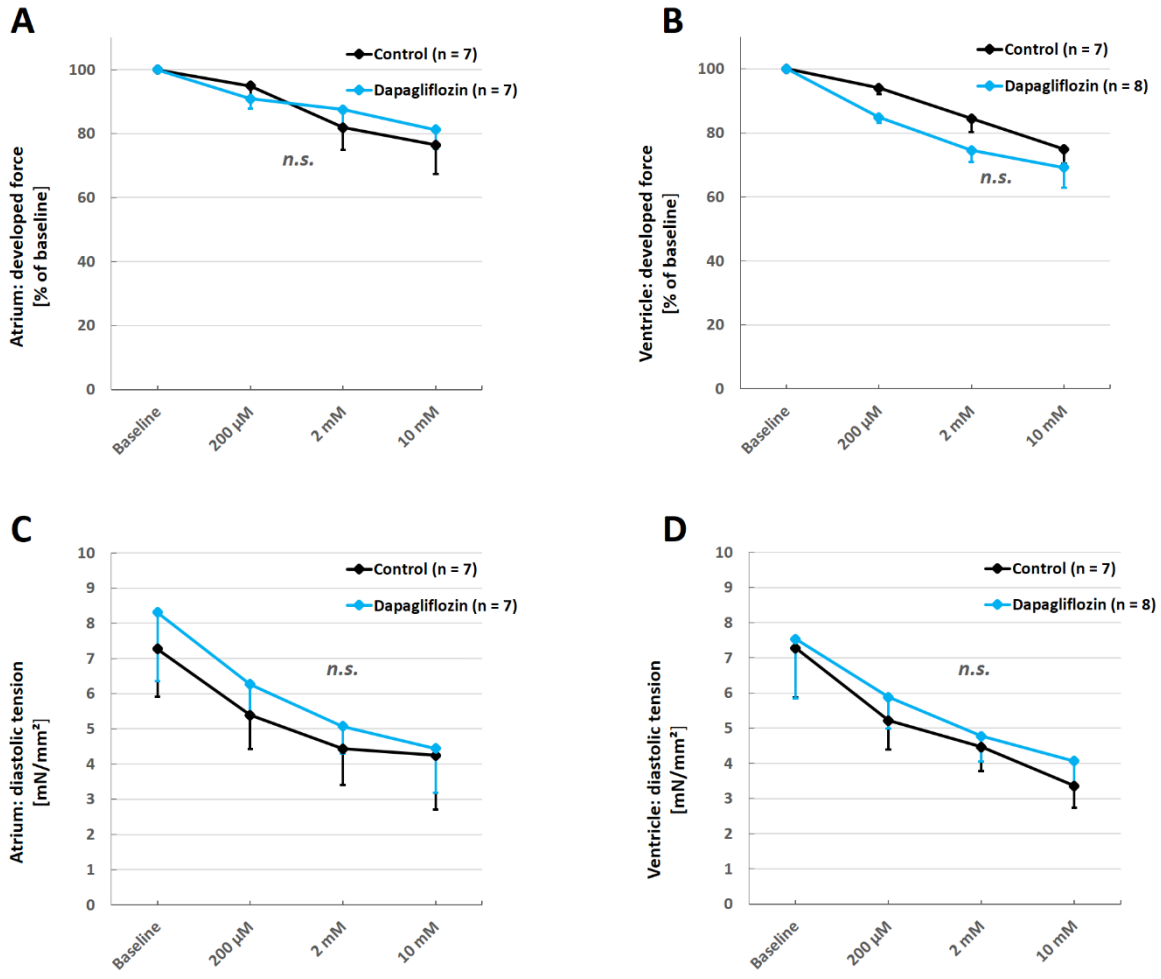

Figure 22: Effects of dapagliflozin on human myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of dapagliflozin on developed force (blue line; n = 7; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

B: Effect of dapagliflozin on developed force (blue line; n = 8; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

C: Effect of dapagliflozin on diastolic tension (blue line; n = 7; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

D: Effect of dapagliflozin on diastolic tension (blue line; n = 8; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

ventricular myocardium. In atrial tissue, administration of increasing concentrations of dapagliflozin had no effect on developed force (figure 22A dapagliflozin at 10 µM: 81.3 % ± 5.9 % of BL; controls: 76.5 % ± 9.0 % of BL; p = 0.78), diastolic tension (figure 22C: dapagliflozin at BL: 8.3 mN/m² ± 2.0 mN/m²; at 10 µM: 4.4 mN/m² ± 1.3 mN/m²; controls at baseline: 7.3 mN/m² ± 1.4 mN/m²; after 90 minutes: 4.2 mN/m² ± 1.5 mN/m²; p = 0.64), or the relaxation parameter RT50 (figure 23A; dapagliflozin at 10 µM: 110.4 % ± 3.1 % of BL; controls: 111.5 % ± 6.4 % of BL; p = 0.36). In ventricular myocardium, dapagliflozin had also no effect on developed force (figure 23B; dapagliflozin at 10 µM: 69.2 % ± 6.2 % of BL; controls: 74.9 % ± 4.5 % of BL; p = 0.11), diastolic tension (figure 22D: dapagliflozin at BL: 7.5 mN/m² ± 1.7 mN/m²; at 10 µM: 4.1 mN/m² ± 0.7 mN/m²; controls at baseline: 7.3 mN/m² ± 1.4 mN/m²; after 90 minutes: 3.4 mN/m² ± 0.6 mN/m²; p = 0.69), or the relaxation parameter RT50 (figure 23B; dapagliflozin at 10 µM: 111.2 % ± 7.4 % of BL; controls: 108.7 % ± 8.9 % of BL; p = 0.47).

No arrhythmic events could be detected. In summary, high concentrations of dapagliflozin do not induce acute functional effects on atrial and ventricular myocardium. Moreover, the behavior of dapagliflozin on human myocardium is similar to empagliflozin.

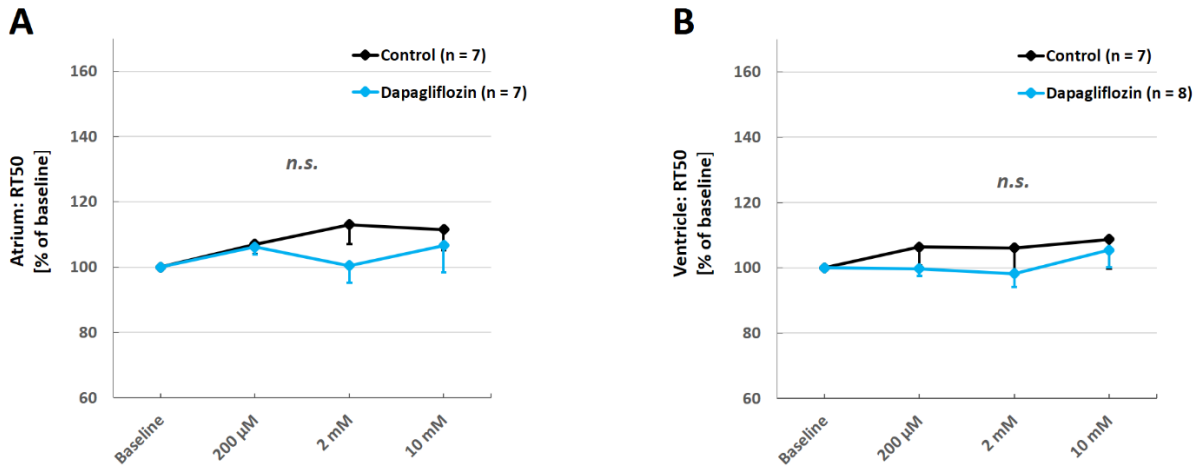

Figure 23: Effects of dapagliflozin on human myocardium, dose-response-relationship. 1 Hz; 37°C

A: Effect of empagliflozin on RT50 (blue line; n = 7; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in atrial myocardium. n.s.: not significant.

B: Effect of dapagliflozin on RT50 (blue line; n = 8; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 7) in ventricular myocardium. n.s.: not significant.

3.13 Effects of T-1095 on Human Ventricular Tissue

To evaluate possible inotropic actions of SGLT2 inhibitors via the SGLT1 receptor, which is expressed in human cardiomyocytes and plays an important role in the glucose uptake and

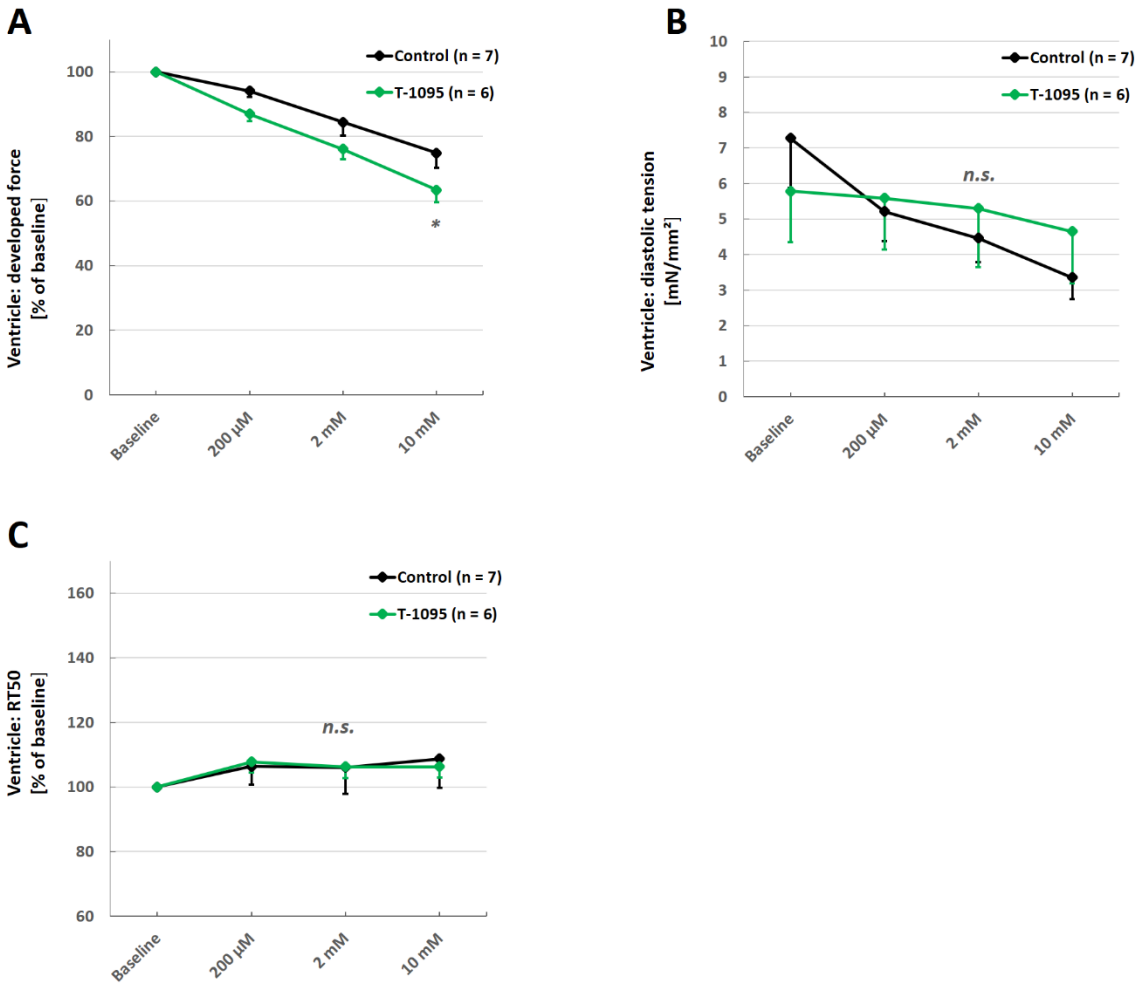

Figure 24: Effects of T-1095 on human ventricular myocardium, dose-response-relationship. 1 Hz; 37°C

*A: Effect of T-1095 on developed force (green line; n = 6; [%] of BL ± SEM). T-1095 induces a significant negative inotropic effect compared to control muscle strips (black line; n = 7). *: $p < 0.05$.*

B: Effect of T-1095 on diastolic tension (green line; n = 6; [mN/m²] ± SEM). No difference compared to control muscle strips (black line; n = 7). n.s.: not significant.

C: Effect of T-1095 on RT50 (green line; n = 6; [%] of BL ± SEM). No difference compared to control muscle strips (black line; n = 10). n.s.: not significant.

further energy supply of cardiomyocytes, the SGLT1 and SGLT2 inhibitor T-1095 was administrated on human ventricular muscle strips. As expected and already shown[51], SGLT1 inhibition resulted in a significant negative inotropic effect (figure 24A; T-1095 at 10 µM: 63.4 % ± 3.9 % of BL; controls: 76.5 % ± 9.0 % of BL; p = 0.027). However, diastolic tension (figure 24B: T-1095 at BL: 5.8 mN/m² ± 1.4 mN/m²; at 10 µM: 4.7 mN/m² ± 1.5 mN/m²; controls at baseline: 7.3 mN/m² ± 1.4 mN/m²; after 90 minutes: 4.2 mN/m² ± 1.5 mN/m²; p = 0.88) and the relaxation parameter RT50 (figure 24C; T-1095 at 10 µM: 106.3 % ± 3.3 % of BL; controls: 108.7 % ± 8.9 % of BL; p = 0.83) were not influenced.

Like in the experiments with empagliflozin and dapagliflozin, after administration of increasing dosages of T-1095, no arrhythmic events were detected.

Taken together the findings of the selective SGLT2 inhibitors empagliflozin and dapagliflozin with the SGLT1 and SGLT2 inhibitor T-1095, it seems unlikely that the SGLT2 inhibitors relevantly block the SGLT1 receptor with respect to the selected supraphysiological concentrations.

4. Discussion

In this thesis the functional effects of modern antidiabetic concepts on human myocardium and GPV myocytes were analyzed. The main results are that (1) DPP4 inhibitors induced arrhythmias in human atrial myocardium most likely due to an impairment of relaxation; moreover, saxagliptin exerted a negative inotropic effect; (2) saxagliptin impairs the electrophysiological function of GPV myocytes and prolongs APD most likely due to a reduction of the K^+ outward current; (3) the positive inotropic effect of the GLP-1 receptor agonists exenatide and GLP-1(7-36) amide on human atrial myocardium was more pronounced in the presence of glucose compared to pyruvate indicating an involvement of GLUT1 translocation; and (4) SGLT2 inhibitors exerted no functional effects on human ventricular and atrial myocardium.

4.1 DPP4 Inhibitors

The results reported in this thesis caused a further investigation of saxagliptin and sitagliptin. For alogliptin, no further experiments were performed.

4.1.1 Discrepancies in Cardiovascular Outcome Trials*

Despite demonstrated overwhelming positive effects of all DPP4 inhibitors in different in vivo and in vitro experiments that are summarized in the corresponding section of the introduction, the SAVOR-TIMI53 trial surprisingly failed to demonstrate a significant reduction in cardiovascular endpoints in the saxagliptin arm. Moreover, there was an unexpected significant increase in the rate of hospitalization for heart failure in the saxagliptin-treated patients. The results of the trial were extendedly discussed with no adequate mechanistic explanation so far[98]. In the EXAMINE trial, a numeric increase – yet not significant due to a smaller number of participants compared to the SAVOR-TIMI trial – of the rate of hospitalization for heart failure was reported after alogliptin-treatment. Interestingly in this context, the TECOS trial that investigated sitagliptin showed no increase in the rate of hospitalization for heart failure compared to placebo[163]. This leads to the question if DPP4 inhibitors should be regarded as a “substance class” or as drugs that inhibit DPP4 amongst other effects.

* parts of this chapter have been published in BioMed. Res. Int. (2017). DOI: 10.1155/2017/1253425.

4.1.2 Pleiotropic Effects of DPP4 Inhibitors

The discrepancy between the results of cardiovascular outcome trials may be a consequence of a different chemical structure of DPP4 inhibitors: sitagliptin, alogliptin, and saxagliptin share no stereochemical similarities with the exception that all mentioned substances represent small molecules with a molecular weight of less than 410 g/mol[164-166] giving them the potential to induce pleiotropic effects.

With respect to the antidiabetic effect of these drugs, the active site of the enzyme DPP4 is located in the center of a structural “cave” or “pouch” with a diameter of approximately 2 nm[167], large enough that a potential inhibitor does not need to have a certain stereochemical structure. Moreover, the active site is composed of multiple binding subsites, that all may inhibit the activity of DPP4; in fact, different DPP4 inhibitors act on different binding subsites[168]. This serves as adequate explanation why DPP4 accepts inhibitors of different shape.

Due to the difference in the chemical structure of saxagliptin, alogliptin, and sitagliptin, potential pleiotropic effects may be of different nature depending on the substance. These effects may contribute to explanation models of the different outcomes of the clinical trials.

4.1.3 Further Investigation of Saxagliptin and Sitagliptin

With respect to myocardial contractility in atrial muscle strips, the results of this thesis show a discrepancy between different DPP4 inhibitors too. The reported negative inotropic effect in saxagliptin-treated muscle strips as well as the prolongation of RT90 in alogliptin-treated muscle strips and the observation of arrhythmic events in both provide a hint on potential harmful direct effects on human myocardium. Administration of sitagliptin did not induce negative inotropy but arrhythmic events in the same extend as saxagliptin and alogliptin. Since saxagliptin showed the most potent effects on developed force, while sitagliptin showed the weakest effects overall, further experiments in GP hearts and ventricle myocytes were performed with both substances. In this thesis, an unexpected prolongation of the APD after saxagliptin-treatment could be observed. Moreover, the K^+ outward current was significantly reduced after saxagliptin-treatment. Therefore, the substance was analyzed in cooperation with different laboratories of the Medical University of Graz and the University of Graz.

The negative inotropic effect of saxagliptin could be confirmed in a Langendorff perfusion model in GP hearts. DPP4 is only expressed in capillaries but not myocytes of the corresponding cells and tissues of different species. Therefore, the mechanism of action of saxagliptin is very likely independent of DPP4. In turn, it could be demonstrated that saxagliptin gets internalized in GPV cells. Here, saxagliptin inhibits NCX function and decreases the phosphorylation of PLB and CAMKII in a DPP9 dependent mechanism without affecting the total expression levels of both proteins. Since PLB serves as SERCA2a inhibitor in its unphosphorylated form, these results suggest an impairment of Ca^{2+} removal during diastole due to a compromised SERCA2a and NCX function. In line with these findings, the saxagliptin-treated hearts analyzed in the Langendorff setup showed an increase in diastolic tension. However, such an increase in diastolic tension could not be observed in the atrial muscle strips.

Lastly, saxagliptin-treatment lead to an increase of the QTc time interval in guinea pig hearts. This fits well to the observed prolongation of the APD. The levels of active PKC were reduced after saxagliptin-treatment. Downregulated PKC leads to reduction of the K^+ outward current and thereby explains the findings of the patch-clamp experiments. The latter mechanism is dependent on DPP9 too.

Sitagliptin internalizes into guinea-pig cardiomyocytes like saxagliptin; however, sitagliptin does not inhibit DPP9 and subsequently exerts no effect on PLB, SERCA2a, NCX, or PKC in contrary to saxagliptin [160,169].

These results may explain the induction of arrhythmic events in atrial muscle strips for saxagliptin. However, the cause for arrhythmic events in sitagliptin-treated muscle strips remains unclear.

4.1.4 Cardiac Dysfunction Links Saxagliptin to Heart Failure*

During development and progression of heart failure, the myocardium undergoes alterations in electrophysiological and Ca^{2+} handling properties that lead to contractile dysfunction and increase the propensity for arrhythmia[170]. Out of the known candidates, multifunctional protein kinases contributing to the development of heart failure, CAMKII[171] as well as PKC[172] are strongly affected by saxagliptin.

Loss of CAMKII auto-phosphorylation impairs PLB-mediated SERCA2a function in SR Ca^{2+} reuptake during cardiac relaxation[173,174]. Reduced SERCA2a expression[175] and/or its

* parts of this chapter have been published in Biochem. Pharmacol. (2017). DOI: 10.1016/j.bcp.2017.08.021.

activity[176,177] play a major role in the initiation and progression of heart failure[178]. This phenomenon reduces SR Ca^{2+} content, lowers the amount of Ca^{2+} released during cardiomyocyte contraction, and thereby leads to systolic dysfunction at organ level[179,180]. On the other hand, impaired SERCA2a function reduces the amount and rate of Ca^{2+} removal from cytoplasm, hence elevating diastolic Ca^{2+} levels, a key trigger for diastolic dysfunction[181]. In addition to SERCA2a, NCX also plays a pivotal role in the maintenance of cardiac relaxation as well as diastolic Ca^{2+} levels[182,183]. Activity of SERCA2a and PLB decreases while that of NCX increases in failing human hearts[184,185].

These findings imply that saxagliptin adversely affects hearts with normal as well as reduced contractility, although these effects may be critical for hearts with pre-existing contractile dysfunction, as evident in heart failure. This notion supports clinical outcome where the highest rate of hospitalization for heart failure was observed in saxagliptin-treated patients suffering from heart failure[98,160].

4.1.5 Saxagliptin Impairs Electrophysiological Function of Myocytes*

The data of the patch clamp experiments and the further investigations demonstrate that saxagliptin prolongs AP duration in cardiomyocytes and thereby increases QTc interval. The latter represents a most common electrophysiological abnormality observed in the failing heart[186]. At molecular level, saxagliptin reduced PKC activity that lead to impairment of K^+ outward currents (I_K), yet the upstream signaling and involvement of specific PKC isoform(s) remain unknown. The role of PKC during myocardial ischemia has been well established in various experimental models[187]. Currently, limited information is available on saxagliptin-mediated effects on QT interval in patients. One safety and pharmacokinetic study based on two small trials consisting 40 healthy volunteers and 50 patients exposed to various doses of saxagliptin for two weeks showed no significant impact on QTc interval[188]. Moreover, within the SAVOR-TIMI53 trial, no follow-up ECGs were performed after saxagliptin administration[97]. Sitagliptin[189] and linagliptin[190] showed no significant effect on QT interval during clinical studies. On the contrary, teneligliptin was associated with QTc interval prolongation at high doses[191]. This would be consistent with our data showing inhibition of PKC at high concentrations (2 µM) of saxagliptin that lead to prolongation of APD and thereby QTc interval. Monitoring of QTc interval may be prudent in patients at the risk of accumulation of saxagliptin – like in conditions of renal failure[160,192]. However, teneligliptin and saxagliptin show a pretty different stereochemical structure.

* parts of this chapter have been published in Biochem. Pharmacol. (2017). DOI: 10.1016/j.bcp.2017.08.021.

Taken together, these results provide the first direct link between the observation of the SAVOR-TIMI53 trial regarding hospitalization for heart failure and a myocardial dysfunction induced by saxagliptin. As a consequence, saxagliptin should only be used as an antidiabetic agent with caution.

4.2 GLP-1 Receptor Agonists

The findings reported in this thesis demonstrated that the GLP-1 receptor agonists exenatide and GLP-1(7–36) amide, but not the GLP-1 receptor antagonist GLP-1(9–36) amide, exerted positive inotropic effects in the presence of both glucose and pyruvate. This effect was more pronounced in the presence of glucose. However, beta receptor activation and increasing Ca^{2+} concentrations were followed by a similar positive inotropic effect without significant differences between the energy substrates.

4.2.1 Translocation of GLUT1*

Glucose and pyruvate incubated muscle strips showed comparable patterns of positive inotropy: first, an acute phase with a significant increase in developed force was observed, which was followed by a decline until steady state was reached (about 25 min after drug administration). This is consistent with the findings of a previous report that demonstrated a transient positive inotropic effect, which is most likely the result of GLP-1 receptor desensitization. Furthermore, it was shown that incubation with 2 nM exenatide for 3 hours induced increased translocation of glucose transporter GLUT1 from the cytosol to the membrane in atrial cardiomyocytes[90].

So far, there is no data available regarding the downstream mechanism that induces the GLUT1 translocation after GLP-1 receptor activation. In the current experiments, steady state conditions were reached 25 min after adding the drugs. At this time point, glucose incubated muscle strips showed a stronger positive inotropic effect than pyruvate incubated muscle strips. This effect may be a consequence of improved glucose metabolism via enhanced translocation of GLUT1, with subsequent generation of more ATP. GLUT1 expression plays a major role in basal glucose uptake in the heart, however the most important glucose transporter for the adult heart is GLUT4[193]. Pools of both GLUT1 and GLUT4 are located inside cardiomyocytes within compartments of endosomes and may be recruited if the appropriate signals reach these pools[194]. Pathophysiological processes can lead to an

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018). DOI: 10.1007/s10989-018-9706-9.

upregulation of GLUT1, which is observed in conditions of hypertrophy of the heart or chronic hypoxia/ischemia[193,195]. However, in contrast to GLUT4 translocation, the underlying mechanisms of the GLUT1 translocation are poorly understood and interactions with the incretin system have yet to be studied more in depth. To be mentioned in this context, myocardial glucose uptake via translocation of GLUT4 amplifies the positive inotropic effect of insulin independent of Ca^{2+} [51,159].

4.2.2 Pyruvate and Glucose as Energy Substrates*

All pyruvate-treated muscle strips showed a much stronger basal contractility compared to those treated with glucose. Noted at this point, data in the results section only show relative values as [%] of baseline. It may be possible that the observed weaker positive inotropic effect of GLP-1 receptor agonists in the presence of pyruvate is a consequence of a higher contractility at BL and a subsequent partial inotropic incompetence, while the glucose pretreated muscle strips could react properly to GLP-1 receptor activation.

For pyruvate, positive inotropic abilities have been reported[54,196], which may also explain the results of the experiments performed with GLP-1(9–36) amide by counteracting the physiological rundown. The concentration of pyruvate used was twice as high as the concentration of glucose because one molecule of glucose is degraded into two molecules of pyruvate during glycolysis. The main part of the energy metabolism and synthesis of ATP takes place in the citrate acid cycle. However, the direct effect on contractility by pyruvate was underestimated. The small positive inotropic effect observed after GLP-1(7–36) amide administration to the pyruvate-treated muscle strips is also a consequence of a rather high number of non-responders with no adequate explanation so far.

Pyruvate and glucose treated muscle strips showed a similar response to beta receptor activation in contrast to GLP-1 receptor activation. This implicates that a comparable inotropic competence is preserved in response to downstream targets of the PKA. In line with this finding, increasing only the concentration of Ca^{2+} caused a similar positive inotropic effect in the presence of glucose and pyruvate. The latter observation suggests a similar myocardial Ca^{2+} metabolism. Given that Ca^{2+}-regulatory proteins of cardiomyocytes are partly dependent on ATP availability[155], both energy substrates used may provide a comparable amount of ATP in atrial muscle strips and furthermore, the BL condition of the muscle strips is similar in the presence of glucose and pyruvate. The observed slightly

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018). DOI: 10.1007/s10989-018-9706-9.

stronger positive inotropic effects in the presence of glucose may still be caused by the difference in basal contractility[159].

4.2.3 The Positive Inotropic Effect on Atrial Myocardium*

Left ventricular filling is the result of both left ventricular relaxation and left atrial contraction. The latter action takes place in late diastole and contributes up to 30% of left ventricular filling. Patients with type-2 DM are at an increased risk of left atrial dysfunction and chronic heart failure[197,198].

In conditions of systolic and diastolic heart failure, backwards transmission of increased left ventricular end-diastolic filling pressures causes an enlargement and remodeling of the left atrium. This in turn strengthens the role of the left atrium in the process of left ventricular filling[199,200] and an increase in the contractility of the left atrium may be helpful in such conditions[201]. Given that improved atrial function is associated with beneficial outcomes in heart failure patients, preservation of atrial function may therefore be particularly important in patients with type-2 DM. In fact, decreased left atrial ejection fraction (LAEF) was associated with decreased LVEF, lower functional capacity, and higher rates of hospitalizations for heart failure in patients with manifest heart failure[202,203].

Also, in patients with coronary artery disease or myocardial infarction, left atrial dysfunction was an independent determinant of heart failure hospitalizations and mortality[204,205]. As a consequence, atrial fibrillation, which is equal to a loss of atrial function, significantly increases the risk of mortality in both symptomatic and asymptomatic heart failure patients[159,206,207].

4.3 SGLT2 Inhibitors

Experiments with the SGLT2 inhibitors empagliflozin and dapagliflozin in human atrial and ventricular myocardium revealed no inotropic or any other functional effect. The combined SGLT1/SGLT2 inhibitor T-1095 exerted a significant negative inotropic effect.

* parts of this chapter have been published in Int. J. Pept. Res. Ther. (2018). DOI: 10.1007/s10989-018-9706-9.

4.3.1 SGLT2 Inhibitors – Interactions with SGLT1*

The human heart does not express SGLT2, but SGLT1 is expressed[52,208]. In murine hearts, where SGLT1 is expressed too, inhibition of SGLT1 after myocardial injury lead to a significant reduction of glucose uptake and further to a decrease of ATP content[209]. This is of importance because under stress induced by physical activity or diseases as heart failure, the value of glucose as the main energy substrate increases due to an insufficient oxygen supply of the myocardium[210]. Under resting conditions however, the main energy supply of the heart is generated by the metabolism of fatty acids which in fact is more oxygen-consuming compared to glycolysis[211]. Therefore, inhibition of SGLT1 may be harmful for injured myocardium. With the experiments performed in this thesis, a possible interaction of SGLT2 inhibitors with SGLT1 seems unlikely considering the used supraphysiological concentrations of empagliflozin and dapagliflozin. Only the combined SGLT1 and SGLT2 inhibitor T-1095 lead to a slight but significant negative inotropic effect suggesting a relevant impact of SGLT1 on myocardial energy supply in non-failing human myocardium, which is in line with a previous study[52,58].

4.3.2 Role of SGLT2 Inhibitors on Ca^{2+}-Homeostasis*

Baartscheer et al. showed that empagliflozin inhibits the NHE1 pathway. NHE1 pumps Na^+ into the cell in exchange for H^+. Inhibition of the NHE1 leads to a decrease in cellular Na^+. This in turn potentially reduces the activity of NCX that pumps Na^+ out of the cell in exchange for Ca^{2+}. As a result, cellular Ca^{2+} decreases. Moreover, Baartscheer et al showed that mitochondrial Ca^{2+} ($[Ca^{2+}]m$) significantly increases upon empagliflozin administration. $[Ca^{2+}]m$ signaling is critical for energy production as well as for the activation of cell death pathways which are implicated in the development of heart failure[151]. Decreased intracellular Ca^{2+} is likely to result in a negative inotropic effect, however, this is not necessarily the case if both, systolic and diastolic Ca^{2+} decreases and the Ca^{2+} transient remains stable. These three changes in intracellular ion homeostasis counteract the alterations typically seen in heart failure models – exempli gratia elevated levels of intracellular Na^+ and Ca^{2+} and reduced levels of mitochondrial Ca^{2+} in heart failure. Elevated diastolic Ca^{2+} also results in impaired relaxation and therefore diastolic dysfunction. Interestingly, empagliflozin significantly improved diastolic function in a rodent model of diabetes and reduced the expression of pro-fibrotic and pro-hypertrophic proteins[212] as well as in rat, mouse, and human cardiomyocytes by reduced myofilament stiffness and increased phosphorylation levels of myofilaments without altering the cellular Ca^{2+} homeostasis[213]. These effects could

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not be explained by reduced blood pressure levels as reported in several other models after SGLT2 treatment.

In sum, there is strong evidence towards a direct myocardial effect of SGLT2 inhibitors; however, the muscle strip experiments of this thesis did not elucidate any effect of empagliflozin or dapagliflozin on diastolic tension in atrial and ventricular myocardium. On the other hand, weak effects on a functional basis cannot be excluded since the muscle strip setup is not appropriate for the detection of weak effects. Nevertheless; regulatory effects on the Ca^{2+} homeostasis might explain at least in part beneficial effects as seen in the EMPA-REG-Outcome trial. Direct cardiac involvement is supported by the fact that the Kaplan-Meier-curves of hospitalization for heart failure separated rapidly in the empagliflozin-arm of the EMPA-REG Outcome trial, which suggests a mechanism occurring quickly after initiation of the SGLT-2 inhibitor treatment[58].

*4.4 Limitations and Strengths**

A major limitation of the performed experiments is the fact that they only provide functional data and give no mechanistic insight.

In the experiments with DPP4 inhibitors, arrhythmic events could not be induced in all tested muscle strips, either arrhythmias occurred at different concentrations or timepoints, or the experiment stayed stable to the end. For these experiments, another major limitation is caused by the analysis tools: due to technical issues, data analysis could only be performed at certain timepoints. Therefore, the prolongation of the relaxation time could not be plotted in a reasonable way.

In the experiments with the GLP-1 receptor agonists, the observed stronger positive inotropic effect of GLP-1 receptor agonists in the presence of glucose should be noted critically. A translocation of GLUT1 after exenatide administration was not shown and only assumed based on recently published data. In this context, experiments that show a translocation of GLUT1 after GLP-1(7–36) amide administration were never performed. Additionally, the importance regarding the difference in basal contractility of both energy substrates remains unclear[159].

In the experiments with the SGLT2 inhibitors, potential interactions with SGLT1 were not assessed directly. The role of SGLT1 as an energy supplier for cardiomyocytes increases in diseased myocardium. The hearts of the donors of the ventricular muscle strips however

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were considered as nonfailing hearts due to a prior examination with a transthoracic echocardiography. Experiments with failing human hearts were not feasible to perform due to a very low availability at the Medical University of Graz.

The strength of this thesis is the use of human atrial and ventricular tissue. Furthermore, myocardium was stimulated at a frequency of 1 Hz in bicarbonate buffered solutions, recapitulating physiological conditions within this in-vitro model. The functional properties on myocardial kinetic parameters were assessed carefully and the results serve as first insight of interactions of various antidiabetic drugs on human myocardium or at least to demonstrate the absence of such effects. Further, an approach that combines experiments on different organic levels (full human atrial or ventricular myocardium; GP myocytes) and different analysis-techniques (functional kinetic data and electrophysiology data) was chosen and their results support each other. Moreover, the results acquired in this thesis served as the base for experiments that partially elucidated their molecular mechanisms. In this way, interaction of DPP4 inhibitors with myocardium independent of DPP4 could be successfully revealed. This is of particular interest because all used drugs except T-1095 are already in clinical use as anti-diabetic therapeutics. Moreover, the demonstration of direct functional effects on human myocardium helps to understand the results of the cardiovascular outcome trials.

4.5 Conclusion

With new and emerging primarily antihyperglycemic drugs, the intersection of antidiabetic treatment and cardiovascular therapy is progressing. Besides modulating diabetes as a cardiovascular risk factor several new antidiabetic drugs imply direct cardiovascular effects and at least for some DPP4 inhibitors, GLP-1 receptor agonists, and SGLT2 inhibitors these effects seem to directly affect myocardial tissue. The enlightenment of the interaction of antidiabetic drugs with myocardium is essential for the understanding of the outcomes of large clinical outcome trials, ranging from very beneficial to potential harmful effects. Therefore, antidiabetic concepts must be perceived from a translational point of view in order to determine personalized clinical treatment. This can only be achieved by an in-depth examination of cardiovascular outcome data in conjunction with basic science data. This thesis gives insight in various not yet known effects of these antidiabetics on human myocardium. However, molecular mechanisms and their interpretation remain mostly unknown. *What we know is a drop, what we don't know is an ocean (Sir Isaac Newton)*.

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