Effects of inhibitory G protein upon muscarinic and β-adrenergic signaling in the failing heart

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# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**

**1 ABBREVIATIONS**

**2 PAPERS INCLUDED**

**3 BACKGROUND**

3.1 Introduction
3.2 *Nanos gigantum humeris insidentes*
3.3 Heart failure
3.4 Remodeling
3.5 Evolving concepts of a neurohormonal model
3.6 G protein-coupled receptors
3.7 Cardiac β-adrenoceptors
   3.7.1 Compartmentation of cardiac β-adrenergic signaling
   3.7.2 β-adrenoceptor signaling in heart failure
3.8 Muscarinic receptors
   3.8.1 Cardiac muscarinic receptors
3.9 The cardiac contractile apparatus
3.10 cAMP-dependent and cAMP-independent contractility
3.11 Rationale for this thesis

**4 AIMS**

**5 METHODS**

5.1 Animal model
5.2 Papillary muscles
5.3 Receptor binding assay
5.4 MLC-2 Phosphorylation
5.5 Membrane preparations and AC activity
5.6 Measurement of cAMP accumulation in left ventricular cardiomyocytes

**6 SUMMARY OF RESULTS**

**7 DISCUSSION**

7.1 Primary findings of this thesis
7.2 An energy efficient inotropic response – an alluring concept in heart failure
7.3 The functional role of Gi – a compartmented inhibitor of cAMP signaling

**9 FUTURE PERSPECTIVES**

9.1 MLC-2 – from a preclinical peculiarity to potential clinical therapy
9.2 The functional role of Gi – shifting the paradigm

**10 REFERENCE LIST**
“The heart is the beginning of life, for it is by the heart the blood is moved...the source of all action”

*William Harvey, 1673*
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1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<td>AChR</td>
<td>acetylcholine-receptor</td>
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<td>AKAP</td>
<td>A kinase anchoring protein</td>
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<td>AR</td>
<td>adrenoceptor</td>
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<tr>
<td>cAMP</td>
<td>3’,5’-cyclic adenosine monophosphate</td>
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<td>CCh</td>
<td>carbachol</td>
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<td>CHF</td>
<td>congestive heart failure</td>
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<td>CRC</td>
<td>contraction relaxation cycle</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>GPCR</td>
<td>G-protein-coupled receptors</td>
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<td>G protein</td>
<td>guanine nucleotide regulatory binding protein</td>
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<td>HF</td>
<td>heart failure</td>
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<tr>
<td>IP₃</td>
<td>inositol 1,4,5 trisphosphate</td>
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<td>LV</td>
<td>left ventricle</td>
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<td>MLC-2</td>
<td>myosin light chain-2</td>
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<tr>
<td>MLCK</td>
<td>MLC-kinase; myosin light chain kinase</td>
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<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
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<tr>
<td>PKA</td>
<td>protein kinase A/cAMP dependent protein kinase</td>
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<td>PLB</td>
<td>phospholamban</td>
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<td>PTX</td>
<td>pertussis toxin</td>
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<tr>
<td>RAAS</td>
<td>the renin-angiotensin-aldosterone system</td>
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<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SERCA</td>
<td>sarcoplasmatic reticulum Ca²⁺ ATPase</td>
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<td>SR</td>
<td>sarcoplasmatic reticulum</td>
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<tr>
<td>TM</td>
<td>tropomyosin</td>
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<td>TnC</td>
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<td>TnT</td>
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2 Papers included

PAPER I
Activation of muscarinic receptors elicits inotropic responses in ventricular muscle from rats with heart failure through myosin light chain phosphorylation.
*Br J Pharmacol.* 2009;156:575-86

Hussain RI, Qvigstad E, Birkeland JA, Eikemo H, Glende A, Sjaastad I, Skomedal T, Osnes JB, Levy FO, Krobert KA.

PAPER II
Cyclic AMP-dependent inotropic effects are differentially regulated by muscarinic G_i-dependent constitutive inhibition of adenylyl cyclase in failing rat ventricle.
*Br J Pharmacol.* 2011;162:908-16

Hussain RI, Afzal F, Mørk HK, Aronsen JM, Sjaastad I, Osnes JB, Skomedal T, Levy FO, Krobert KA.

PAPER III
The functional activity of inhibitory G protein (G_i) is not increased in failing heart ventricle.
*J Mol Cell Cardiol.* 2013;56:129-38

Hussain RI, Aronsen JM, Afzal F, Sjaastad I, Osnes JB, Skomedal T, Levy FO & Krobert KA

PAPER IV
Non-classical regulation of β_1- and β_2-adrenoceptor-mediated inotropic responses in rat heart ventricle by the G protein G_i.
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* Both authors contributed equally to the work
3 BACKGROUND

3.1 Introduction

The subject of this thesis was to elucidate the contractile effects of cardiac muscarinic signaling with a special emphasis on the role of the inhibitory G-protein (G\textsubscript{i}) activated by the muscarinic M\textsubscript{2} receptor, its interplay with other receptor systems (mainly β\textsubscript{1}- and β\textsubscript{2}-adrenoceptors and serotonin receptors) and its regulation of adenylyl cyclase (AC) in the normal and failing heart. The majority of the work was conducted in beating ex-vivo myocardial tissue from rat ventricle with supplementary experiments measuring cAMP-accumulation, adenylyl cyclase activity, receptor binding and Western blotting. The overall aim was to advance our understanding of the functional effects of the muscarinic system and especially the role of G\textsubscript{i} to regulate cardiac contractility. The data indicate that the role of G\textsubscript{i} is more complex than currently thought and that some of the changes occurring to muscarinic signaling in the failing heart might be of a compensatory character rather than deleterious.

3.2 Nanos gigantum humeris insidentes

Is Latin for “dwarfs standing on the shoulders of giants”, words often attributed to Sir Isaac Newton expressing the view that new discoveries are often built on previous knowledge. Our current understanding of the heart and cardiovascular disease results from a long evolution of medical knowledge, in this respect, before addressing the specific topics of this thesis, I would like to give a short presentation of the main lessons within cardiovascular medicine from the past.

From the earliest descriptions of the cardiovascular system, the heart has been an organ of prime importance. From Egyptian hieroglyphs dated 2500-1600 BC we know that the heart was believed to be the center of consciousness and knowledge [1] whereas in ancient Greece and in the medical texts of “Corpus Hippocraticum” by Hippocrates of Cos (460-370 BC) and his disciples, different cardiovascular presentations and symptoms (pitting edema, cachexia,
dyspnea) were described [2]. By dissection of monkeys and pigs, Claudius Galenus (129-200) described the difference in color of venous and arterial blood and believed that venous blood was generated in the liver whereas arterial blood originated from the heart [3]. The function of heart being a pump was introduced by the Persian physician Ibn-Sīnā (980 – 1037, also known as Avicenna) who in his medical encyclopedia called “Qanon of Medicine” described the heart as a pump responsible for pulse. Avicenna also described the process of clotting of vessels (atherosclerosis) as a result of poor choice of diet and the beneficial effects of exercise [4]. The relationship between heart and lung was introduced by Ibn Al-Nafis (1213-1288, Damascus) a physician who described the pulmonary circulation and also alluded to the existing of pulmonary capillaries [5]. This was further elaborated by William Harvey (1578-1657, England) who postulated that the main organ responsible for circulation was the heart and not the liver thus laying the foundation of modern concepts of cardiovascular circulation [6]. Harvey also recognized that edema and swelling observed in heart failure could be a result of obstruction of the venous return to the heart. Consequently, this understanding lead to the simple but logical medieval treatment of heart failure; bloodletting [6]. More precise understanding in the cardiac anatomy was contributed by the scholars at the University of Padua, most prominently Andreas Vesalius (1514-1564) and his student Realdo Colombo (1516–1559) [6]. A treatment still used today is the discovery of the therapeutic effect of digitalis by William Withering (1741-1799) which represented a important milestone in the management of heart failure [7]. The “modern era of heart disease” however was introduced by Corvisart when he in 1806 published a detailed report on the distinction of hypertrophy and dilatation [8]. Slowly, the concept of heart failure shifted from an anatomy oriented field towards the importance of hemodynamics, pressure and flow. This view was foremost pioneered by the esteemed cardiac physiologists; Ernst Starling and Carl J. Wiggers [9].

As the medical knowledge increased and new research tools within molecular biology were introduced, the focus shifted towards a more mechanistic understanding of the disease. The research from the 1970s to the present time has focused on the neurohormonal changes,
remodeling and the intracellular signaling that occur during heart failure (topics that will be discussed later). However, traces of the evolution in our understanding of heart failure can still be found in the way physicians approach a patient with a cardiac disorder. From the “giants” of ancient Egypt and Greece to Arabic and renaissance Italian scholars, we have inherited the importance of clinical examination and can recognize the clinical presentation of heart failure. Due to research from the seventeenth to twentieth century we learned more about cardiac physiology and pathophysiology which helps us explain the underlying mechanisms mediating the signs and symptoms of heart failure, which has lead us to conduct a more targeted physical examination. Finally, to confirm the diagnosis or to specify the pathogenesis, we take advantage of the advances derived from present day molecular biology and advanced physics ultimately leading to better and targeted treatment for the heart failure patients. In a microscopic scale, the fundaments of this thesis is also based on a long line of work and theories made by others, many of which we build on and some that we question. Thus, *Nanos gigantum humeris insidentes* is a descriptive analogy and gives a humbling perspective of one’s few drops of contributions in the waste seas of medical knowledge.

### 3.3 Heart failure

Heart failure is one of the main causes for hospitalization and drug administration in the western world; in Europe its prevalence varies from 1-2% and rises to ≥10% for people above 70 years old [10]. Heart failure is a common end stage condition in a plethora of cardiovascular diseases (see table 1a) and due to its diverse etiology and different clinical presentations, heart failure is more precisely a clinical syndrome rather than one single medical disease. The definition of heart failure has been redefined along with our increased understanding of the syndrome. In the 2012 guidelines from the European Society of Cardiology (ESC) heart failure is defined as an abnormality of cardiac structure or function leading to failure of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues, despite normal filling pressures (or only at the expense of increased filling pressures) [10].
Heart failure is a chronic progressive syndrome intersected by episodes of acute decompensation (figure 1). People who develop heart failure have markedly reduced quality of life [10] and the prognosis of patients with chronic heart failure is poor; five-year survival rate is about 50% [11] and in patients with severe heart failure it is reported a ~50% mortality within one year [12]. Clinically, the functional classification of progression and severity of heart failure is often categorized in the New York Heart Associations heart failure-classification, NYHA functional class (Table 1b).

**Table 1a. Common causes of heart failure**

- Ischemic heart disease
- Hypertension
- Valvular heart disease
- Cardiomyopathies e.g. alcohol, genetic, infectious
- Endocrine disorders (e.g. diabetes, hypo/hyperthyroidism, Cushing’s syndrome)
- Congenital
- Others (e.g. illicit drugs, sarcoidosis, rheumatic heart disease, HIV, lupus)

**Table 1b. The New York Heart Association’s functional classification of heart failure.**

<table>
<thead>
<tr>
<th>NYHA Class I</th>
<th>NYHA Class II</th>
<th>NYHA Class III</th>
<th>NYHA Class IV</th>
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<tr>
<td>The patient has no limitations of activities. No symptoms from ordinary daily activity.</td>
<td>Slight mild limitation of activity, comfort by resting.</td>
<td>Marked limitation of activity, which is comfortable only at rest.</td>
<td>Symptoms at rest, recommended to be in rest.</td>
</tr>
</tbody>
</table>

*The NYHA classification that primarily functions to measure the severity of symptoms and loss of function in patients with heart failure. Modified from American Heart Association [13].*

*Figure 1. Disease progression as a function of quality of life and mortality in patients with chronic heart failure. Figure adapted from Gheorghiade et al., 2005 [14].*
3.4 Remodeling

From the original writings of Corvisart dated 1806 the notion that “two types of dilatation, active with thick walls and increased force of contraction, and passive with thinning of the walls and a decreased force of contraction” [8] are today recognized as left ventricular hypertrophy (or concentric hypertrophy) and left ventricular dilatation (or eccentric hypertrophy) – two types of the pathological cardiac remodeling that occur in the course of heart failure. The term remodeling is somewhat general and it encompasses the changes that occur in genome expression and on the molecular-, cellular- and interstitial level that altogether clinically manifests as changes in size, shape and function both of the heart and vasculature after an injury or stressor [15]. In heart failure, remodeling is mainly used as a pathological term describing the transitory compensatory but long term maladaptive mechanisms that follow a deleterious hemodynamic, metabolic and/or inflammatory stimulus (i.e. myocardial infarction, aortic stenosis, various cardiomyopathies etc.) and the accompanied neurohormonal stimulation. However, remodeling can also occur in physiological settings like pregnancy or increased long term exercise, in these cases, the remodeling functions as a necessary adaptive and compensatory mechanism facilitating cardiac stroke volume.

Pathophysiologically, cardiac maladaptive remodeling is causally linked to activation and up-regulation of a series of neurohormonal cascades (including catecholamines, angiotensin, aldosterone, endothelin etc.) as a response to cardiac wall stress or injury (discussed more below). This neurohormonal activation is accompanied by, and causes, a plethora of changes that are characteristic to maladaptive remodeling. Although not fully elucidated, maladaptive remodeling includes a re-expression of cardiac fetal genes, cardiomyocyte apoptosis, inflammation, alternations in the cardiac excitation-contraction coupling, changes in the signal transduction (discussed below) and changes in the energetic and metabolic state of the cardiomyocytes [15]. The cardiac remodeling affects not only cardiomyocytes, normally, cardiomyocytes make up approximately two thirds of the myocardial mass but comprise only approximately one-third of the total cell number in the heart [16]. In addition, the extracellular
matrix (ECM) plays an important role in pathological remodeling. As part of the remodeling process, gene expression in the heart switches into a profibrotic state, increasingly transitioning the ECM from cardiac fibroblasts into myofibroblasts. This causes a shift in the balance between the matrix metalloproteases (MMPs), proteolytic enzymes responsible for myocardial extracellular protein degradation and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) [17]. As a result, there is a promotion of fibrosis causing impaired contractility, stiffness and increased incidence of atrial fibrillation [18].

With improved imaging techniques, cardiac remodeling in the failing heart can, macroscopically, be characterized in three distinct forms; 1) concentric remodeling which is echocardiographically recognized by normal LV mass index and increased relative wall thickness, 2) concentric hypertrophy with increased LV mass index and increased relative wall thickness and 3) eccentric hypertrophy with increased LV mass index and normal relative wall thickness [19, 20]. Whereas concentric remodeling is believed to be a part of the normal aging process, both concentric and eccentric hypertrophies are pathological processes [21].

Concentric hypertrophy is best described as a consequence of pressure overload leading to increased myocardial wall thickness with little or no change in left ventricular chamber size, increased wall stiffness, especially during diastole, and a normal left ventricular ejection fraction – in the clinic this is known as heart failure with preserved ejection fraction (HFpEF) [22]. On the molecular level, this is characterized by; increased width of the individual cardiomyocytes combined with apoptosis, an increase and reassembly of the contractile proteins into more parallel alignment and an impaired calcium handling associated with impaired relaxation [23]. Lately much attention has also been given to the comorbidities associated with concentric hypertrophy (e.g. obesity, diabetes, hypertension, pulmonary disease) and their pathophysiological contribution to the development of HFpEF. It is hypothesized that the aforementioned comorbidities cause a systemic pro-inflammatory state that induces coronary microvascular endothelial inflammation which eventually reduces nitric oxide bioavailability.
leading to reduced cyclic guanosine monophosphate (cGMP) production and reduced protein kinase G (PKG) activity in adjacent cardiomyocytes [23]. PKG is known to function as an innate brake on myocardial hypertrophy [24-26] and a deficient NO-cGMP-PKG signaling from endothelium to myocardium is shown to impair cardiac relaxation [27-29]. Consistent with this hypothesis that comorbidity causing inflammation plays a central role in the development of concentric hypertrophy. Concentric hypertrophy can also progress to eccentric hypertrophy and then, if the loss of cardiomyocytes progresses and fibrosis increases, the myocardium undergoes dilatation.

On the other hand, eccentric hypertrophy in itself is best characterized by volume overload (such as aortic or mitral valve regurgitation) causing a reassembly of cardiomyocytes and their contractile units in series, resulting in a relatively greater increase in the length of the cardiomyocytes (vs. parallel alignment and thickening of the cardiomyocytes in concentric hypertrophy) [30]. The restructuring of the contractile units into series can contribute to preservation of the ventricular function. However, as the process progresses, this elongation of the cardiomyocytes is also the hallmark of the transition from compensated hypertrophy to decompensated heart failure with reduced ejection fraction (HFrEF) [30].

3.5 Evolving concepts of a neurohormonal model

Until the 1980’s, heart failure was viewed primarily as a hemodynamic disorder, characterized by increased venous pressure, impaired pump performance and thereby reduced cardiac output. This view also directed the development of treatment options for heart failure. Pharmacological therapy focused on the altered hemodynamics. In addition to diuretics, positive inotropic drugs along with directly acting vasodilators were recognized as the best medical treatment [31]. Clinical trials done in the 80’s and early 90’s however, indicated that the current understanding of heart failure at that time, the disorder of hemodynamics, was insufficient and could not account for the outcomes of the trials. First, it was discovered that the positive inotropic drugs and the directly acting vasodilators, although improving short-term function
often worsened the long time prognosis [32]. Accompanied with these findings, it was also revealed that β-adrenoceptor blockers, in spite of their negative inotropic function, actually reduced mortality [33, 34], a finding that was later extensively investigated and confirmed in landmark randomized clinical trials (US CARV [35], CIBIS II [36], MERIT-HF [37], COPERNICUS [38]). In addition to the reports on the beneficial effect of β-adrenoceptor blockers, there was also increasing evidence on the positive effects of angiotensin-converting enzyme (ACE), as first shown in the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) I [36] and later also by the study groups of SOLVD-T [39] and SAVE [40]. Following, about a decade later, the beneficial effects of angiotensin II receptor blockers (ARB) were verified (CHARM [41], Val-HeFT [42]) as were the effects of mineral corticoid receptor antagonists (MRA) in RALES [43] and EMPHASIS-HF [44].

These discoveries from the mid-1980s to 2000 initiated a shift in attention from impaired hemodynamics to the importance of the neuroendocrine systems involved in the development and progression of heart failure. Today, standard care of heart failure treatment relies on blocking the deleterious effects of the sympathetic nervous system with β-adrenoceptor blockers and the renin-angiotensin-aldosterone system (RAAS), with ACE-inhibitors/ARBs and MRA [10]. In addition, diuretics and nitrates still play an important treatment role for symptomatic relief. In contrast, there are peptides, such as endothelin-1 and vasopressin [45], both shown to play a contributing part in the progression of heart failure, but trials so far blocking these peptides have disappointingly failed [46]. In addition to inhibiting the detrimental cascades occurring during heart failure (blocking the sympathetic nervous system and renin-angiotensin-aldosterone system) augmenting some of the positive compensatory signaling pathways accompanying the course of heart failure have also been in focus as potential therapeutic approaches. Such an example is natriuretic peptides (NP), endogenous hormones produced by, amongst other tissues, the heart and released in response to myocardial wall stress and/or overload [47]. Although the complete actions of the NP release in heart failure is probably not fully elucidated, many mechanistic studies have shown that NPs have natriuretic, diuretic,
vasodilating and possibly abilities to reduce mal-adaptive cardiac remodeling [47-51]. Based on this knowledge, it has been conceptually appealing to augment the levels of NPs thus utilizing their “compensatory” effects as heart failure therapy. Intravenous infusion of BNP (nesiritide) did show a small reduction in dyspnea in a large randomized trial in acute heart failure patients, but such treatment did however not reduce death or hospitalization [52, 53]. Likewise, serial infusions of nesiritide in heart failure patients in ambulatory care also failed to show an impact on outcome [54]. Other ways to increase the levels of NPs, than exogenous administration of NPs or their analogues, is by inhibition of the membrane-bound enzyme neprilysin (NEP), a endopeptidase responsible to hydrolyze, amongst others peptides, ANP, BNP and CNP [55, 56]. The most recent clinical advancement in this field is the PARADIGM-HF trial [57], were a NEP inhibitor (sacubitril) combined with valsartan was investigated head to head in a double blind fashion against enalapril on top of standard medical treatment in patients with HFrEF. The results of PARADIGM-HF showed a highly statistically significant 20% reduction in the primary composite endpoint of cardiovascular death or heart failure hospitalization and a 16% reduction in the risk of death from any cause in favor of sacubitril/valsartan on top of standard of care compared to enalapril [57]. As NEP also hydrolyses other peptides, the precise mechanisms behind the positive outcomes observed in PARADIGM-HF is yet to be elucidated. Additionally, which role sacubitril/valsartan will have in the heart failure treatment algorithm is currently not decided. Other recent advancements in our knowledge of heart failure are in the role of the body’s inflammatory response. Several reports have demonstrated enhanced expression and release of different inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 etc., and also shown that inflammation may play an important role in the advancement of heart failure [58]. However targeted anti-inflammatory therapy has yet to be proven beneficial in randomized clinical trials.
3.6 G protein-coupled receptors

There exist many ways of intercellular communication in a multicellular organism. Whereas the small hydrophobic molecules, such as steroid hormones, diffuse across the plasma membrane of a cell, the vast majority of hydrophilic signaling molecules bind to their receptors on the plasma membrane. There are three large families of cell surface receptors, namely ion channel-linked receptors, enzyme-linked receptors and the G protein-coupled receptors (GPCRs) [59]. Drugs targeting adrenergic and angiotensin GPCRs alone accounted for the majority of prescriptions for cardiovascular disease in mid-1990’s and mid 2000’s [60].

All GPCRs are made up by a structure of seven transmembrane α-helices with an extracellular amino terminus and an intracellular carboxy terminus. The heterotrimeric G proteins consist of three subunits (α, β and γ). Upon GPCR activation by an agonist, the G protein exchanges its GDP with GTP resulting in a break-up of the G protein into an activated α subunit and a βγ complex. The G protein subunits (Gα and the Gβγ) then propagate signals inside the cell by modulating the activity of one or more effector molecules, e.g. adenylyl cyclase (AC), phospholipase, and ion channels etc. (figure 2) [59]. In turn, the activity of these effector molecules regulates the production of second messengers including cyclic adenosine monophosphate (cAMP), inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (figure 2).

![Image of G protein-coupled receptor](image)

Figure 2. Seven transmembrane domain G protein-coupled receptor with an interacting G protein (α, βγ). Activation of the receptor by e.g. a neurotransmitter or hormone replaces GDP with GTP on Gα, followed by a dissociation of the Gα and Gβγ subunits [61].

3.7 Cardiac β-adrenoceptors
Activation of the sympathetic nervous system (mediated by noradrenaline and adrenaline) causes positive inotropic, chronotropic and lusitropic effects on the heart primarily through β-adrenergic G protein-coupled receptors (β-AR). The dominant subtype of the β-receptors in the heart is the β₁-AR, however, the presence of β₂- and β₃-ARs are also of significance.

The functional contractile effects of β₁-ARs (figure 3) starts by the activation of the membrane bound enzyme adenylyl cyclase (AC) through the Gₐₛ subunit. The activated AC will subsequently increase the formation of the second messenger cAMP which again activates protein kinase A (PKA). PKA in turn mediates phosphorylation of a variety of cellular effector proteins. In cardiomyocytes, the activated PKA phosphorylates the L-type Ca²⁺ channels, ryanodine receptor (RyR), phospholamban (PLB), troponin I (TnI) and myosin binding protein C (MyBP-C). This coordinated signaling pathway will result in the positive inotropic, lusitropic and chronotropic effect observed after activating the β₁-ARs. In more detail, as figure 3 illustrates, the phosphorylation of the L-type Ca²⁺ channels and the RyRs results in an increase in both the sarcolemmal Ca²⁺ entry and the release of sarcoplasmatic Ca²⁺. Unphosphorylated PLB has an inhibitory effect on the sarcoplasmatic reticulum Ca²⁺ ATPase (SERCA) which is responsible for the re-uptake of Ca²⁺ into the sarcoplasmatic reticulum (SR). The PKA-dependent phosphorylation of PLB attenuates PLB’s inhibitory effects on SERCA resulting in faster re-uptake and subsequently increased release of Ca²⁺ into the cytosol, for review see Bers [62]. Lastly, phosphorylation of TnI decreases the myofilament Ca²⁺-sensitivity promoting a more rapid Ca²⁺ release from the myofilaments and re-uptake into SR during the diastole [63]. In addition to the direct contractile effects, β-ARs, especially the β₁-subtype also play a fundamental role in the pathological remodeling process during HF (discussed later).
3.7.1 Compartmentation of cardiac β-adrenergic signaling

To maintain specificity, efficiency and speed of the intracellular response to receptor activation, the activated intracellular signal pathways are often stringently regulated and compartmented. A classic example of such a compartmented signaling is evident in the difference between activation of β-ARs by catecholamines and activation of prostanoid EP receptors by prostaglandin E₁ (PGE₁). Although both receptor activations result in a similar increase in intracellular cAMP [64], only β-ARs elicit increased contractility in isolated perfused hearts. Similarly, glucagon-like peptide-1 receptor (GLP1-R) increases cAMP levels comparable to β-ARs, but the functional effect of GLP1-R is a negative inotropic effect [65] in contrast to the positive inotropic and lusitropic response by β-ARs. These differences in functional effect are attributed to compartmented intracellular signaling, regulating intracellular targets and effectors in the signaling pathway despite having the same second messengers early in the signaling cascade.
For the cardiac β-AR response it is absolutely imperative to have control over the localization, duration and the amplitude of the cAMP formation. This control is achieved by the AC enzymes which are responsible for catalyzing the production of cAMP by conversion of adenosine triphosphate (ATP) to cAMP and pyrophosphate and, by the cyclic nucleotide phosphodiesterases (PDEs), enzymes that are responsible for degrading the phosphodiester bond in cAMP (and cGMP) and hence resulting in the reduction of cAMP and restricting its signaling [59]. The spatial compartmentation of cAMP signaling is facilitated by A-kinase anchoring proteins (AKAPs); scaffolding proteins that can bring together cAMP effector proteins like AC, PKA, RyR, PDEs, protein phosphatases, and sometimes also GPCRs into signaling complexes [66], this is especially evident in the specific invaginations of the plasma membrane named caveolae or lipid rafts [67].

To date, there are nine transmembrane and one soluble isoform of AC described [68]. In cardiomyocytes, AC5 and AC6 are the two main isoforms responsible for the synthesis of cAMP. Although sharing many similarities e.g. both are activated by Gs and inhibited by Gi and submicromolar concentrations of Ca^{2+}, there is evidence that AC5 and AC6 exert opposite effects; AC6 is hypothesized to have beneficial effects on cardiac contractility [69], cell survival and Ca^{2+} handling whereas AC5 is thought to be detrimental [70].

Eleven PDE families and 50 PDE isoforms have been identified, five of these eleven families are responsible for cAMP degradation in the heart; namely PDE1, PDE2, PDE3, PDE4 and PDE8 [71, 72]. Whilst PDE1 and PDE2 can hydrolyze both cGMP and cAMP, PDE3 prefers cAMP and PDE4 and PDE8 are specific for cAMP [71]. Depending upon the species, the role of these different PDEs vary, i.e. in mice it is reported that PDE4 contributes up to 30% of the total cAMP-hydrolytic activity [73] and is especially linked to the β-AR pathway, whereas in human myocardium PDE4 is reported to account for only 10% [74-76] and the most significant PDE isoform is purportedly PDE3 [77, 78].
3.7.2 β-adrenoceptor signaling in heart failure

In heart failure patients; elevated plasma levels of adrenaline and noradrenaline correlates with increased LV dysfunction and mortality. Chronic increased β-AR stimulation is known to cause harmful effects and blocking β-ARs is standard heart failure therapy. The harmful effects appear to arise specifically from β₁-AR signaling. In animal models, overexpression of the β₁-AR is reported to cause hypertrophy, cardiac dysfunction and interstitial fibrosis [79] and activation of PKA and calcium/calmodulin-dependant protein kinase is thought to play a key as in a pro-apoptotic signaling cascade [80, 81].

The increased sympathetic nerve activity in heart failure, as quantified by noradrenaline spillover techniques in heart failure patients is reported to be as much as 50 times above normal [82, 83], sand this causes several changes to the β-AR signaling pathway. One fundamental hallmark is the reduced contractile response to activation of β-AR in the cardiomyocytes. The reduced β-AR-mediated contractile response is in part attributed to a selective reduction of β₁-AR levels, an upregulation of the inhibitory G₁ protein (see paper II, figure 1) and an increased expression and activity of the β-AR kinases [84]. Important alterations to the β₁-AR compartmentation are also described; the normal association between PKA and AKAPs is reported to be dramatically reduced, and furthermore, a decreased expression and activity of PDE3 and PDE4 is reported [85].

The functional relevance and changes occurring to the β₂-AR during heart failure are less known. Some studies indicate that β₂-ARs play a cardioprotective role in heart failure; in mice lacking β₂-ARs, chronic isoproterenol stimulation resulted in increased myocyte apoptosis and mortality compared with wild-type mice [86]. In a post-infarction model of heart failure, selective stimulation of β₂-ARs and blocking β₁-ARs improved cardiac performance and reduced cardiac apoptosis compared to selective β₁-AR blockade alone [87]. As β₂-ARs are dually coupled, both to G₁ and G₃z, the cardioprotective effects of β₂-ARs are hypothesized to
derive from the Gi signaling component of β2-ARs, which inhibits the Gs signaling from βARs [88].

3.8 Muscarinic receptors

The muscarinic receptors are the predominant target for the parasympathetic neurotransmitter acetylcholine (ACh) and cholinomimetic drugs. Five different subtypes of muscarinic receptors (AChR) have been pharmacologically characterized; M1, M2, M3, M4, M5 [89, 90]. Whereas the receptors of subtypes M1, M3 and M5 are coupled to Gq leading to activation of phospholipase C resulting in formation of inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG), the M2 and M4 subtypes are coupled to pertussis toxin (PTX) sensitive Gi proteins and hence elicit a direct inhibition of AC, resulting in decreased formation of intracellular cAMP [91].

3.8.1 Cardiac muscarinic receptors

The parasympathetic nervous system innervates the heart through the vagal nerve. The M2 receptor subtypes are termed “cardiac” muscarinic receptors because the M2-AChR subtype is the dominant muscarinic receptor subtype expressed in the heart [92]. The main function of the M2 receptor subtype is to regulate the pacemaker activity, atrioventricular conduction and to regulate contractility in the atria and the ventricles [93].

Although M2 is the dominant muscarinic receptor subtype expressed in the heart [92, 93], the presence of other muscarinic receptor subtypes has been reported in the heart [94]. The cardiac M2 receptors are coupled to pertussis toxin (PTX) sensitive Gi proteins and elicit a direct inhibition of AC, resulting in decreased formation of intracellular cAMP (figure 3) [91]. The general consensus is that in the atria, the M2 receptors elicit a direct negative inotropic effect [92]. This is different in the ventricles where the negative inotropic effects are only observed when AC is pre-stimulated (indirect inotropic effect) [93]. However, stimulation of the muscarinic receptors in human, rat and guinea pig atria is also reported to produce a positive
inotropic response [95, 96]. This positive inotropic effect is assumed to be mediated by hydrolysis of inositol phospholipids [95, 96]. A similar muscarinic receptor mediated positive inotropic response has also been reported in the normal human and guinea-pig ventricle [97, 98], but the signaling pathway of this effect has not been elucidated. Although there are reports suggesting that stimulation of the muscarinic receptors in the heart can increase the myofilament sensitivity to Ca$^{2+}$ [99, 100], a muscarinic receptor-mediated positive inotropic effect derived from altered myofilament Ca$^{2+}$ sensitivity has never been reported.

3.9 The cardiac contractile apparatus

Regardless of the signaling pathway mediating an inotropic response, cardiac contractility ultimately involves the cardiac contractile apparatus. This apparatus constitutes of myofibrils formed by repeating units of sarcomeres. The sarcomeres are in turn composed of parallel filaments of two sorts; cardiac thin and thick myofilaments (figure 4). During a contraction, the thin and thick filaments slide on each other and the sarcomere length shortens. This process is Ca$^{2+}$ dependent as Ca$^{2+}$ initiates and regulates the series of complex protein interactions that results in the formation of the force-generating crossbridging between the two filaments [63].

![Figure 4: Illustration of the essential construction of the cardiac sarcomere. Adapted from Hwang & Sykes, Nature Drug Discovery 2015 [101]](image)
The cardiac thick myofilaments (figure 5) are made up by titin, myosin binding protein-C (MyBP-C) and myosin [101]. The myosin is the “motor” molecule generating force and motion by its cyclic interaction with actin. Myosin is a hexameric protein comprised of one pair of myosin heavy chain (MHC), one pair of essential light chain (ELC or MLC-1) and one pair of the regulatory light chain (RLC or MLC-2) [102]. The phosphorylation level of MLC-2 in the heart is regulated by two enzymes; Ca\textsuperscript{2+}/calmodulin (Ca\textsuperscript{2+}/CaM)-dependent myosin light chain kinase (MLCK) and dephosphorylated by myosin light chain phosphatase (MLC-phosphatase) [102]. The degree of total MLC-2 phosphorylation is determined by the balance of the activities of these two enzymes (figure 6). MLC-2 with its location in the fulcrum of the lever arm of MHC (figure 6), together with the MyBP-C, is believed to both have important modulating roles in cardiac myosin contractility and stiffness [103]. More specifically, whereas MyBP-C phosphorylation is thought to enhance relaxation [104], phosphorylation of MLC-2 by MLCK has been shown to increase the rates of crossbridging and thus generating force [105] by enhancing the cardiac filament response to Ca\textsuperscript{2+} [106]. In contrast to smooth muscle where phosphorylation of MLC-2 is essential for activation of contraction, in cardiac tissue, MLC-2 phosphorylation plays a modulatory role on contraction which is enabled by Ca\textsuperscript{2+} binding to troponin C. Studies in transgenic mice have tried to further dissect the role of cardiac MLC-2. When phosphorylatable Ser-14/15/19 of MLC-2 was replaced by non-phosphorylatable Ala (TG-RLC(P-)), cardiac contractility was significantly depressed [105], as measured both by the blunted β\textsubscript{1}-AR-mediated inotropic response and the lack of complete ejection to a normal end-systolic volume in transgenic hearts as compared to non-transgenic hearts [105, 107]. Regulation of Ca\textsuperscript{2+}-sensitivity via MLC-2 phosphorylation is also hypothesized to potentially play a compensatory role contributing to maintain contractility in the failing heart [108], as more discussed later.
3.10 cAMP-dependent and cAMP-independent regulation of contractility

Cardiac receptor-mediated changes to contractility occur by two distinct mechanisms, cAMP-dependent and cAMP-independent pathways (see figure 7 for illustration). Classically, cAMP-dependent regulation of contractility is mediated through stimulation of Gs coupled
receptors such as the cardiac β₁-ARs. When these receptors are stimulated by agonist, the G_{αs}-
subunit activates the membrane-bound AC enzyme which increases the formation of cAMP with
subsequent activation of protein kinase A (PKA). PKA in turn phosphorylates a myriad of
proteins such as the L-type Ca^{2+} channel, RyR, PLB, TnI (see section 3.8 and figure 3 for
details). The phosphorylation of these key proteins orchestrates a sharp increase in the
intracellular Ca^{2+} transient resulting in many of the key features seen in a β-AR-mediated
contractility. As depicted in figure 7 (left side), the contraction-relaxation cycle of the cAMP
dependent contractility displays the characteristic pattern of increasing the speed of force
generation that is accompanied by a shortened time to peak force and an increase in relaxation
speed (lusitropic effect). This highly efficient pathway elicits a rapid increase in cardiac
contractility at the expense of high energy consumption due to its ATP-dependent processes
(e.g. ion-pumps). In the failing heart, chronic use of this high energy consuming cAMP-
dependent inotropic pathway to maintain stroke volume is detrimental (as demonstrated by the
failure of PDE inhibitors) and provides the rationale for the use of β-blockers as first line
treatment for heart failure patients [109]. On this basis, increasing contractility in the failing
heart through stimulation of the more energy efficient cAMP-independent pathways is arguably
more likely to provide a clinical benefit. Although increasing contractile force in itself requires
energy, the hallmark of an energy efficient inotropic mechanism would require increasing force
generation without increasing the Ca^{2+} transient and thus avoiding the expenditure of ATP to
handle the increase in Ca^{2+} handling. This alluring concept is probably best described for the
cardiac α₁-ARs where the early of work of Lee and Downing [110, 111] reported that α₁-AR-
mediated inotropic responses did not significantly increase cardiac oxygen consumption.
Accordingly, Aoyagi et al [112] reported that α₁-AR-mediated inotropic response is more
energy efficient compared to β-AR-mediated. The mechanism for such an energy-efficient,
cAMP-independent increase in contractile force mediated by the cardiac α₁-ARs is shown to be
mediated by phosphorylation of MLC-2 which in turn increases the rate of cardiac myofilament
crossbridging in a Ca^{2+} sensitizing manner [113]. After that this was shown by Andersen et al
[113], this mechanism to increase contractility by MLC-2 phosphorylation is also been
described for other cardiac receptor systems; 5-HT\textsubscript{2A} serotonin receptors [114], prostanoid F receptors [115] and both urotensin-II- [116] as well as apelin evoked effects [117].

![Diagram of cAMP-dependent and independent pathways of contractility and the corresponding contraction-relaxation cycles.](image)

Figure 7. Illustration of the cAMP-dependent and independent pathways of contractility and the corresponding contraction-relaxation cycles. Modified from: Levy et al.2008 [118].

### 3.11 Rationale for this thesis

As portrayed above, our understanding of heart failure has evolved from purely anatomical observations, to a mechanistic and pathophysiological understanding and has now advanced to focusing on the molecular interactions and alterations occurring in the failing myocardium. One key protein in the heart is the inhibitory G protein, Gi. Of the four families of G proteins (namely Gi/o, Gs, Gq/11, and G12/13), Gi is the most highly expressed in the heart. It is found to be present at 1-5 pmol/mg protein in cardiac tissue [119] and in comparison, the density of most receptors ranges between 50 to 500 fmol/mg protein [84]. Furthermore, a quantification of GPCRs in the four chambers of the heart also revealed that Gi-coupled GPCRs are most abundant in the heart [120]. Classically, the most important functional effect of cardiac Gi is to inhibit AC, as revealed by the indirect negative inotropic response elicited by M\textsubscript{2}
receptor activation after pre-stimulation with a β1-AR agonist – a functional effect termed “accentuated antagonism” of inotropy [92]. Knowing that sustained β1-AR stimulation plays a deleterious role in heart failure and Gi being the “most predominant brake”, Gi undoubtedly has an important role in heart failure. However, relative to its counter G protein, the widely studied stimulatory Gs, the functional effects and alterations to Gi signaling are relatively poorly understood in heart failure. In recent years, experimental enhancement of vagal tone stimulation has emerged as a potential therapeutic approach in heart failure [121]. In a rodent model of post-infarction heart failure, controlled stimulation of the vagal nerve during 140 days achieved a 73% reduction in relative risk of death in the intervention animals compared to sham operates [122] and, similar results have been reported in other animal models of heart failure [123]. This potential therapeutic approach was also recently tested in the clinical study NECTAR-HF [124]. However, despite being based on sound preclinical data, NECTAR-HF failed to show a significant effect on its primary endpoint; left ventricular remodeling. However, the study did reveal a modest improvement in quality of life measurements. Consideration should be given to the fact that the study had a relatively small sample size, short duration and consisted of a selection of heart failure patients in a stable phase of their disease, all of which may have been contributing factors to the lack of a significant positive result on the primary endpoint. Nevertheless, the recent attention to the role of the parasympathetic nervous system in heart failure warrants more research in the field. At the time the research for this thesis was undertaken, the functional role of Gi in heart failure was contradicting with reports indicating Gi to have a negative impact on desensitizing β-ARs as well as reports unable to replicate such finding, see El-Armouche et al [84] for a thorough review. Based on this unresolved role of Gi in the failing heart, we wanted to characterize the functional effects of Gi activation in heart failure, both its contractile role with and without β-AR pre-stimulation (paper I) as well as elucidate Gi’s interplay with AC and different AC-coupled receptor systems (paper II-IV). With the data presented in this thesis, we hope to increase the understanding of the role of Gi in the failing myocardium.
4. AIMS

The overall aim of this thesis was to elucidate the role of signaling via muscarinic M2 receptors and Gi in normal and failing myocardium. More specifically, the aims were to:

1. Determine whether activation of muscarinic receptors can mediate an inotropic effect in ventricle analogous to what has been previously reported in other species and if so, to elucidate the signaling pathway(s) and mechanism of the muscarinic receptor-mediated inotropic effect (paper I).

2. Determine whether muscarinic receptors constitutively inhibit adenylyl cyclase and if so, whether they modify the β-AR- or 5-HT4-mediated cAMP-dependent inotropic responses in the normal or failing heart (paper II).

3. Determine if increased Gi activity in the failing heart contributes to the decreased β-AR-mediated increase in contractility and if it correlates with a reduction of adenylyl cyclase activation (paper III).

4. Clarify the role of Gi to regulate the β1- and β2-mediated inotropic effect in the normal and failing myocardium (paper IV).
5. METHODS

5.1. Animal model

Our investigations conform to the *Guide for the Care and Use of Laboratory Animals* by the U.S National institutes of Health (NIH Publication No. 85-23, revised 1996). Two animals per cage in a temperature-regulated room on a 12:12 h day/night cycle were given access to food and water *ad libitum*. As described by Sjaastad *et al* [125], an extensive myocardial infarction was induced in 320 gram male Wistar rats under anesthesia (68% N₂O/ 29% O₂/ 2-3% isoflurane) by proximal ligation of the left coronary artery. Six weeks later the rats were again anaesthetized and intubated, and left ventricular pressures were measured as described by Sjaastad *et al* 2000 [126]. The left ventricles were cut open, and the posterior papillary muscle was excised. Then the left ventricle was pinned to a plate, and the endocardial surface was digitally photographed and infarct size (% of inner surface) was traced on screen (KS 100, Kontron Electronics, Germany).

Due to availability of tissue, the possibilities to pre-treat the animals, success rate of surgery and reproducibility of experiments, the post-infarction rat model was chosen for all our experiments. A non-rodent animal model such as pig, dog or rabbit might have been better, since due to larger size, they probably are more comparable to humans. However, the ease of use, combined with in-house knowhow as well as cost-related factors related to using rats outweighed the use of models from larger animals. Using a mouse model would also be an option, but due to the smaller size, higher heart rate and more difficulty to ligate the left coronary artery as well as getting viable ventricular strips or papillary muscles, we preferred to use rat. Post-infarction heart failure is one of the main etiologies behind HFrEF, which also was the heart failure type we wanted to investigate. As figure 3 in paper I illustrates, some of our findings and responses are closely correlated to the increase in end diastolic pressure in the left ventricle and hence more causally linked to the development of heart failure *per se* rather than just an infarction. For research in diastolic dysfunction, aortic banding or trans-aortic
constriction models, both eliciting more hypertrophy and interstitial fibrosis than the post-
infarction model, would have been more suited. If we wanted to mimic hypertension caused
heart failure, high diet salt fed Dahl salt-sensitive rats would have been a better option to use.
Similar, if a more co-morbid HFpEF model was our main interest, salt-sensitive mice cross-
breed with diabetes models are available.

5.2. Papillary muscles

Isometrically contracting left ventricular posterior papillary muscles from rat ventricles
six weeks after a transmural myocardial infarction were used in this thesis. Papillary muscles are
multicellular preparations containing most cell types present in the ventricle. Thus, the structural
composition of the heart muscle tissue is intact in this preparation with preserved cell to cell
connections and extracellular matrix present. Furthermore, in contrast to cardiomyocyte
preparations the papillary muscles are not exposed to enzymatic digestion of the tissue which
might damage cells and select a subpopulation of surviving cardiomyocytes. Accordingly, this
might represent a more physiological in vitro model system to study contractile function in the
heart compared to isolated cardiomyocytes although extrapolation to in vivo conditions must be
done with caution. Despite the presence of non-cardiomyocytes in the papillary muscles the
functional mechanical response measured in these preparations is cardiomyocyte specific.
Another advantage of using the beating papillary muscle preparation is that they can be clamp-
frozen, during the experiments, for the use in other assays such as RT-PCR (after RNA
preparation) and Western blotting. In this way it is possible to correlate the results of these
assays with the functional data obtained from the contracting preparations.

The papillary muscles were excised from perfused rat hearts and subsequently placed in
an organ bath with an oxygenated (95% O2, 5% CO2) salt solution containing in mM: NaCl
118.3; KCl 3.0; CaCl2 0.2; MgSO4 4.0; KH2PO4 2.4; NaHCO3 24.9; glucose 10.0; mannitol 2.2
(pH 7.4, 31 °C). The Ca^{2+}: Mg^{2+} ratio of 1:20 was used in order to avoid contracture during the
preparation and mounting of the muscles. After stabilization under these conditions the salt
solution was changed to a similar one except the $\text{Ca}^{2+}: \text{Mg}^{2+}$ ratio was changed to 3:2 (with $[\text{Ca}^{2+}] = 1.8 \text{mM}$) and Na$^+$ changed accordingly in order to perform contraction experiments. Based on numerous stability experiments the beating muscles seemed to obtain a satisfactory stability within 90 minutes after initiation of electrical stimulation with only an insignificant decay in basal developed force over the following hours. Stable basal conditions are essential in order to get reliable results when performing lengthy experiments with respect to time, e.g. concentration-response experiments. The papillary muscles were equilibrated in the presence of ascorbate (100$\mu$M), the $\alpha_1$-adrenoceptor antagonist prazosin (0.1$\mu$M) and the $\beta$-AR antagonist timolol (1$\mu$M) when appropriate.

The posterior papillary muscle was chosen in all experiments because the anterior muscle was often a part of the myocardial infarction in the failing animals and often displayed substantial fibrosis. The papillary muscles were electrically field-stimulated by platinum electrodes parallel to the muscles in the organ baths. The electrical stimulation was delivered as square pulses of 5 ms duration and the current was adjusted to about 20% above the individual threshold for each muscle. Stimulation frequency was set at 1 Hz in all experiments.

### 5.3 Receptor binding assay

Papillary muscles snap frozen in liquid nitrogen were crushed to powder and placed into a microcentrifuge tube containing ~0.5 ml of ice cold 50 mM Tris-HCl (pH 7.4 at 20 °C), 1 mM EDTA, 10 mM MgCl$_2$ with protease inhibitors (100 $\mu$M phenylmethylsulfonyl fluoride and 100 $\mu$M phenanthroline) and homogenized with an Ultra-Turrax homogenizer (5x10 s bursts). An equal volume of ice cold 1M KCl was added to the homogenate, mixed and placed on ice for 10 min. The homogenate was centrifuged at 20000 rpm for 12 min at 4 °C in a Beckman J2-MC Centrifuge (Beckman Instruments Inc.). The membrane pellets were resuspended in ice cold 50 mM Tris-HCl (pH 7.5 at 20 °C), 1 mM EDTA buffer containing protease inhibitors and mixed with an Ultra-Turrax at maximum speed (this procedure was repeated 2x). The membrane preparation was then filtered through a nylon mesh (60$\mu$M pore size) and used immediately for
the binding assay. Affinity (pKₐ) and receptor density (Bₘₐₓ) were determined from equilibrium binding analysis of the non-selective muscarinic antagonist 1-quinacolininyl[phenyl-4³H] benzilate ([³H]QNB) (specific activity of 42Ci/mmol; GE Healthcare, Buckinghamshire, England) binding to each membrane preparation. Membranes were incubated with increasing concentrations of [³H]QNB in the absence (total binding) or presence (non-specific binding) of 1 μM atropine for 2 h at 24 ºC [127].

5.4 MLC-2 Phosphorylation

Phosphorylation of MLC-2 in clamp frozen papillary muscles was determined as previously described [128]. In brief, proteins were separated using charged gel electrophoresis that separated phosphorylated and non-phosphorylated MLC-2 by difference in charge. MLC-2 was identified by using anti-ventricular MLC-2 monoclonal antibody and phosphorylated MLC-2 was identified by staining with a phospho-specific anti-MLC-2 (Ser19) antibody. MLC phosphorylation was quantified by densitometric scanning and is reported as phosphorylated MLC-2 in percent of total MLC-2.

Due to variability in Western blotting increasing the risk of error such as in sample processing, loading and quantification of protein, transfer of protein to membrane, immunoblot detection etc., all samples were measured in triplicates and each experiment was performed at least three times.

5.5 Membrane preparation and AC activity

Membranes were prepared as described by Krobert et al 2001[129]. AC activity was measured and analyzed by determining conversion of [α-32P]-ATP to [32P]-cAMP in membranes as detailed in Krobert et al 2001 [129]. Increases in AC activity induced by isoprenaline or forskolin (experiments performed in triplicates) are reported as % increase over basal or control (stimulated in the absence of atropine).
5.6 Measurement of cAMP accumulation in left ventricular cardiomyocytes

Adult left ventricular cardiomyocytes were isolated from the excised Sham and HF rat hearts by retrograde aortic perfusion with a nominally Ca\textsuperscript{2+}-free JOKLIK-MEM solution (Sigma-Aldrich) and enzymatic digestion using collagenase type 2 (Worthington Lakewood, NJ) (90 U/ml) as described by Andersen et al. 2004 [130]. Left ventricular cardiomyocytes were incubated for 20 h with either 1 \( \mu \text{g/ml} \) PTX or saline of equal volume in the incubation media (1.2-ml reaction volume). Experiments were conducted either in the presence of the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich; 0.5mM) or the selective PDE3 and PDE4 inhibitors cilostamide (Tocris Bioscience, Bristol, UK; 1 \( \mu \text{M} \)) and rolipram (Tocris Bioscience; 10 \( \mu \text{M} \)), respectively, as indicated. cAMP accumulation was measured with a radioimmunoassay as previously described by Skomedal et al. 1980 [131]. Protein was measured with the Coomassie Plus Protein Assay (Pierce, Rockford, IL) according to the manufacturer’s protocol, and cAMP accumulation was normalized to the amount of protein in each sample.
6. SUMMARY OF RESULTS

Paper I

- The muscarinic receptor agonist carbachol (10 μM) elicited a sustained positive inotropic effect in papillary muscles from rats with post-infarction chronic heart failure and not in sham operates.
- The carbachol-induced inotropic effect correlated well with parameters indicative of congestion, not infarction size.
- The carbachol-mediated inotropic response appeared to be mediated through the M₂ muscarinic receptor subtype.
- The inactivation of Gᵢ by pertussis toxin attenuated the carbachol-induced inotropic effect.
- The carbachol-induced inotropic effect is dependent on myosin light chain phosphorylation and regulated by myosin light chain kinase and myosin light chain phosphatase.

Paper II

- The maximal β-adrenergic inotropic effect is reduced despite a large increase in the potency of isoproterenol in failing left rat ventricle.
- AC activity induced by stimulation of β-ARs and forskolin is reduced in HF.
- Muscarinic Gᵢ-mediated constitutive inhibition of forskolin-stimulated AC is increased in the failing myocardium compared to Sham. However, muscarinic Gᵢ mediated constitutive inhibition of β-AR stimulated AC remains the same in failing as in Sham myocardium.
- Muscarinic receptor constitutive activity modulates only 5-HT₄-mediated contractility, but not β-AR mediated contractility.
Paper III

- The $\beta$-AR-mediated inotropic effect is attenuated but the muscarinic accentuated antagonism remains unaltered in the failing ventricle.
- Inactivation of $G_i$ does not restore the attenuated $\beta$-AR-mediated inotropic response in HF, but increases the potency of isoproterenol in Sham.
- $G_i$ inactivation does not restore the reduced basal AC activity or the reduced $\beta$-AR-mediated AC activity in HF.
- Simultaneous inactivation of PDE3 and PDE4 produces a robust cAMP-dependent inotropic response only in myocardium with prior inactivation of $G_i$.

Paper IV

- $G_i$ inactivation increased both $\beta_1$-AR- and $\beta_2$-AR-evoked cAMP accumulation.
- Inactivation of $G_i$ did not restore the attenuated $\beta_1$-AR or $\beta_2$-AR-mediated inotropic responses in HF but increased agonist potencies.
- The $\beta_2$-AR functional compartment is regulated by $G_i$, PDE3 and PDE4.
- Inhibition of both PDE3 and PDE4 is necessary to reveal increased basal cAMP after inactivation of $G_i$. 
DISCUSSION

7.1 Primary findings of this thesis

As detailed in this thesis, we have investigated the functional role of inhibitory G protein (Gᵢ) in a post-infarction rat model of heart failure. This research project was designed to elaborate upon the current understanding and functional relevance of Gᵢ in normal and failing heart. Paper I reports that activation of muscarinic receptors, classically known to inhibit the β-AR-mediated inotropic response, is sufficient to elicit a sustained positive inotropic response in the absence of β-AR activation only in failing myocardium. The data indicate that muscarinic receptor activation increases myofilament Ca²⁺ sensitivity through enhancing myosin light chain phosphorylation, by regulating activity of myosin light chain kinase and myosin light chain phosphatase. Furthermore, we provide evidence that the constitutive activity of Gᵢ is increased in the failing heart and that this constitutive activity seems to functionally modulate 5-HT₄-mediated, but not β-AR-mediated inotropic responses, indicative of compartmentation of Gᵢ signaling (paper II). In contrast to previous reports [132, 133], our data do not support the hypothesis that inactivation of increased Gᵢ protein activity in failing heart can restore the blunted β-AR-mediated inotropic response observed in the failing heart (paper III). Our data also indicate that Gᵢ together with PDE3 and PDE4 regulate β₂-AR-compartmentation (paper IV). Taken together, the data of this thesis extend the functional role of Gᵢ beyond that of classical “accentuated antagonism” in the failing heart. In particular, the data are consistent with a role of Gᵢ functioning as a compensatory protein that can mediate an more energy efficient inotropic response, as explained below. In addition, the data are not consistent with previous reports proposing an enhancement of Gᵢ-mediated inhibition of the β-AR-mediated inotropic response in the failing heart [110, 133]. In fact, our data suggest the inhibitory capacity of Gᵢ is reduced in the failing heart.
7.2 An energy efficient inotropic response – an alluring concept in heart failure

In chronic heart failure, especially HFrEF, impairment of excitation-contraction coupling (figure 3) leads to a significant reduction in myocardial contractility. Chronic elevated levels of catecholamines stimulating β-ARs, although initially functioning as a compensatory mechanism to improve contractility, increase the cardiac energy demand and promotes pathological remodeling, eventually resulting in a vicious self-reinforcing deleterious cycle. Maintaining and/or even improving cardiac contractility in heart failure without promoting pathological remodeling is an appealing therapeutic concept. Since preclinical and clinical research has provided strong evidence that stimulating the high energy demanding pathway involving the cAMP/PKA system (β1-AR agonists and PDE inhibitors) is in fact deleterious, there has been an emphasis to develop drugs that can increase contractile function by an more energy-efficient mechanism; eliciting a positive inotropic response without significantly increasing the Ca\(^{2+}\) amplitude and oxygen consumption. Calcium sensitizers, agents that increase contractility by increasing Ca\(^{2+}\) sensitivity of the myofilaments, are one type of “energy-efficient” inotropes that may prove to be therapeutically beneficial. However, such clearly defined “energy-efficient” Ca\(^{2+}\) sensitizing inotropes are currently lacking in the clinic. Levosimendan; a calcium sensitizer thought to stabilize the binding conformation between Ca\(^{2+}\) and TnC [134], thus allowing a more efficient utilization of the Ca\(^{2+}\) has not in a convincing manner proven improved survival. In fact, a recent study by Ørstavik et al [135] provided evidence that the inotropic effects of levosimendan is probably mediated by its active metabolite OR-1896 through PDE3 inhibition thus enhancing cAMP-mediated effects and not by a Ca\(^{2+}\) sensitizing mechanism. In similar, omecamtiv mecarbil, a so-called myosin activator, purportedly increases contractility by selectively binding to the S1 domain of cardiac myosin thereby reducing the activation threshold for cardiac myosin ATPase [136], has also yet to proven to be beneficial in the clinic. The drug is currently in development and recently, a phase II safety study indicated that the drug might increase myocardial ischemia at high plasma levels [137] thus it remains to see if the drug will be pursued in a mortality and morbidity trial. In this respect, it is interesting that with the exception of William Withering’s digitalis, no inotropic agent to date is
recommended as standard therapy for patients with chronic heart failure according to the ESC guidelines.

Although the functional utility of drugs increasing calcium sensitivity of the myofilaments remains a subject of discussion, receptor-mediated inotropic responses eliciting increased calcium sensitivity of myofilaments is well described [110-118, 138]. As depicted in figure 7, the inotropic response derived by this mechanism of calcium sensitization involves a small elongation of the contraction-relaxation cycle in contrast to classical lusitropy evoked by cAMP-dependent inotropic responses, as seen in the β1-AR elicited contractility. Prior to publishing paper I, our group reported the emergence of a novel 5-HT2A serotonin receptor-mediated inotropic response only in the failing heart that purportedly increased calcium sensitization of myofilaments [114]. Based on the knowledge that cAMP-dependent contractility was deleterious, we hypothesized that during heart failure, to sustain adequate generation of contractile force, the heart undergoes adaptive compensatory changes that favor the emergence of energy efficient signaling pathways with the capacity to increase generation of contractile force. Using this theoretical perspective, together with the report that muscarinic receptor stimulation modestly increases calcium sensitivity (but not sufficient enough to elicit an inotropic response) in normal hearts [99, 100], we wanted to determine if muscarinic receptor stimulation in the failing heart was also enhanced to such an extent that it could elicit increase in contractility through calcium sensitization. As detailed in paper I, we observed a sustained carbachol-evoked positive inotropic response only in failing rat myocardium. This inotropic response was mediated specifically by the muscarinic M2 receptor, since the relatively specific M2 specific antagonists AF-DX 116 and AF-DX 384 shifted the carbachol concentration-response curve to higher concentrations (paper I, fig 4). The positive inotropic response was nearly abolished by pertussis toxin, indicating that the response was mediated through Gi signaling, the G protein activated by M2 receptors. The time base of the contraction relaxation cycle of the carbachol-mediated inotropic response was unchanged, similar to that of responses through both α1-AR and 5-HT2A receptors. And similar to the responses mediated by α1-AR and
5-HT$_{2A}$ receptors, downstream on the filament level, we determined that the M$_2$-elicited inotropic response was mediated by increasing myosin light chain 2 (MLC-2) phosphorylation by a combination of increasing myosin light chain kinase activity and/or decreasing myosin light chain phosphatase activity, as determined by the effects of the appropriate inhibitors.

Recent reports indicate that MLC-2 plays a key role in adaptation to heart failure and cardiac stress [139] as well as serving a protective rescue mechanism in hypertrophic cardiomyopathy [140]. In fact, MLC-2 phosphorylation also seems to play a fundamental role in sustaining basal contractility in the heart [141], an observation also supported by us as we observed a 19% reduction of basal contractility after pretreatment with the MLCK inhibitor ML-9 and a nearly 30% reduction after pretreatment with the Rho-kinase inhibitor Y-27632 (paper I). In this regard, receptor-mediated inotropic responses acting through the mechanism of increasing MLC-2 phosphorylation in the failing heart, in our opinion, may prove to be a good alternative for maintaining adequate contractile force generation compared to activation of the more detrimental cAMP/PKA pathway. Adding to this hypothesis, a growing body of studies indicate that $\alpha$-ARs, in the diseased heart, might counteract the deleterious effects of $\beta$-ARs by evoking MLC-2-dependant energy efficient positive inotropy, physiological hypertrophy as well as ischemic preconditioning (see Jensen et al [142] for review). The emergence of the M$_2$ receptor-mediated inotropic response reported in paper I, is also consistent with the paradigm claiming that heart failure represents a state of imbalance between the sympathetic and parasympathetic nervous system, where the former is up-regulated and the latter down-regulated [143]. It has been shown that chronic vagus nerve stimulation (and hence muscarinic receptor activation) is cardioprotective and improves left ventricular function in pre-clinical models of heart failure [122, 144, 145]. In fact, this hypothesis has recently been resuscitated to the extent that a small explorative phase II trial, NECTAR-HF, was initiated whereby chronic vagal stimulation was given to heart failure patients. However, NECTAR-HF failed to meet its primary endpoint, although with some positive signals as measured on quality of life. Irrespective of a clear positive phase II study, investigators have initiated a larger hard end-point
driven trial, INOVATE-HF. INOVATE-HF is 80% powered to detect differences in the primary efficacy endpoint of all-cause mortality and heart failure hospitalization [143] and this should be sufficient to reveal decisive data if the role of vagal stimulation has a potential beneficial role as heart failure therapy [143]. If INOVATE-HF manages to demonstrate improved left ventricular function through increased inotropy, our data on the positive inotropic response mediated by M2 through MLC-2 could shed a mechanistic light on the mechanism of action for such a tentative inotropic effect.

7.3 The functional role of $G_i$ – a compartmented inhibitor of cAMP signaling

A characteristic feature of chronic heart failure is the reduced contractile responsiveness to $\beta$-AR stimulation. This attenuation of the $\beta$-AR response seems to be specific to alterations in the receptor signaling pathways as the failing heart reportedly preserves calcium activated force generation [146-149]. The mechanisms contributing to the reduced $\beta$-AR-mediated contractile response, although not fully elucidated, likely result in part from a selective reduction of $\beta_1$-AR density, increased expression and activity of $\beta$-AR kinases and an increase in $G_i$ protein levels [84]. The increase of $G_i$ protein levels in failing heart was first described in the early 1990’s and there are reports indicating that the increase in $G_i$ seems to be paralleled by a corresponding impairment of the $\beta$-AR system to activate AC and evoke an inotropic response in various animal models of heart failure [133, 150-152]. In addition, decreased levels of $G_i$ are reported to correlate with increased positive inotropic and arrhythmogenic effects of $\beta$-AR stimulation [153]. In a study in guinea-pig cardiomyocytes chronically treated with noradrenaline, inactivating $G_i$ signaling through pertussis toxin (PTX) treatment indicate that the $\beta$-AR inotropic response can be “rescued” [132]. Similar results were reported from the same investigators in human cardiomyocytes from heart failure patients where PTX restored the maximal fractional cell shortening to $\beta$-AR stimulation [132]. In a study directly measuring contractile force, PTX treatment partially restored the attenuated $\beta$-AR inotropic response in both left atria and ventricular papillary muscles in a post-infarction rat model of heart failure [133]. These two aforementioned reports have provided the primary basis for the prevailing
inference that increased $G_i$ protein activity contributes to the reduced $\beta$-AR responsiveness in heart failure. Pathophysiologically, such a $G_i$-mediated inhibition and desensitization of $\beta$-AR makes sense; as previously discussed, $\beta$-AR-dependent signaling is high energy consuming and has shown long term maladaptive remodeling properties. Therefore, intrinsic inhibition of $\beta$-AR dependent signaling during heart failure development is expected to be of benefit and may serve as a compensatory response mimicking the effect of $\beta$-blockers in the treatment of heart failure. Although these aforementioned studies imply a role of increased $G_i$ activity in the attenuation of $\beta$-AR-mediated contractility, not all studies are consistent with this interpretation and therefore, the role of $G_i$ in the failing heart remains somewhat controversial. Inhibitory G proteins are widely expressed in the mammalian heart with $G_{\alpha i}$ being the quantitatively predominant $G_{\alpha i/o}$ isoform both in human atrium and ventricle [154, 155] and rat ventricle [133, 152] and its primary function is to inhibit AC. In the heart, the role of $G_i$ to antagonize a prior $\beta_1$-AR-mediated inotropic response through muscarinic $M_2$ receptor activation (accentuated antagonism) is well established [92]. For the failing heart, a causal link between increased $G_i$ levels, as observed in heart failure, to directly contribute to the reduction of $\beta_1$-AR contractility is not intuitively obvious as $G_i$ does not directly take part in the inate $\beta_1$-AR signaling. However, one possible hypothesis is that muscarinic receptors are constitutively active sustaining a tonic $G_i$ inhibition upon AC activation that will counteract AC activation by the $\beta_1$-AR system. In fact, in normal rat ventricular cardiomyocyte membranes, muscarinic receptors are reported to exert a mild inhibitory constitutive activity upon AC [156]. Thus, we hypothesized the increase in $G_i$ protein levels in heart failure would increase constitutive activity of muscarinic receptors, which in turn would contribute to the reduced $\beta_1$-AR responsiveness in heart failure. In contrast, $\beta_2$-ARs, which are purportedly dually coupled to both $G_s$ and $G_i$, the heart failure associated increase in $G_i$ might play a more direct inhibitory role upon a $\beta_2$-AR activation. Based on this hypothesis and the paucity of functional data regarding the role of $G_i$ in heart failure, the purpose of papers II-IV was to further investigate the possible contribution of both constitutive muscarinic receptor activity and $G_i$ in the reduction of $\beta$-AR-mediated responsiveness in failing myocardium. This project was divided into three parts. First, we wanted to investigate if the
increased expression of Gi coupled with the increased muscarinic receptor level in heart failure (reported in paper I) increased muscarinic constitutive inhibition of the AC system subsequently modifying cAMP-mediated regulation of contractility (paper II). Secondly, we wanted to investigate if increased Gi activity in the failing heart contributed to the decreased β-AR-mediated inotropic response (paper III) and if it correlated with a reduction in the ability to activate AC (paper III). Thirdly, since β2-ARs dually couple to both Gi and Gs, we investigated if the increased Gi levels differentially regulated β1-AR versus β2-AR-mediated inotropic responses and AC activation.

As hypothesized, constitutive muscarinic receptor activity was increased in the failing rat ventricle compared to normal myocardium [156] (paper II). However, unexpectedly, the increased constitutive muscarinic activity only affected forskolin- and serotonin-evoked responses but not the β-AR (paper II). Consistent with this finding, PTX inactivation of Gi signaling failed to restore or “rescue” the β-AR-mediated inotropic response (paper III) as reported in prior in vitro studies [151, 152]. In fact, in our study, the potency to isoproterenol was significantly increased in failing ventricle and PTX inactivation of Gi exerted no additional effect upon isoproterenol potency. In contrast, in sham operates, PTX pretreatment increased the potency of isoproterenol to evoke an inotropic response by a magnitude similar to that observed in failing ventricle (paper III). The interpretation of these data is more consistent with the idea that Gi inhibitory activity upon the β-AR-mediated inotropic response is reduced in failing heart as opposed to increased, at least in rat ventricle. Additionally, and in accordance with effects upon contractility, inactivation of Gi did not restore the reduced β-AR- or forskolin (direct AC activator)-evoked AC activity in failing ventricular membranes to Sham levels but unexpectedly decreased β-AR-evoked AC activity (paper III). Dissecting the effects of Gi inactivation in the failing heart upon β1- versus β2-AR, PTX restored both β1-AR and β2-AR-evoked cAMP accumulation levels in cardiomyocytes to Sham values (paper IV). However, it is important to note that the PTX-mediated increase in failing heart was significantly blunted compared to PTX enhancement observed in Sham ventricle. Inactivation of Gi significantly increased the maximal
β₂-AR inotropic response only in Sham, consistent with the dual coupling of β₂-AR to both Gs and Gi. However, PTX treatment did not restore either the β₁-AR or β₂-AR-evoked inotropic response in failing heart to Sham levels (rather both were reduced) despite the PTX-mediated increase in cAMP levels (paper IV). Taken together, our results are not consistent with some prior studies reporting that inhibition by Gi contributes to the reduced β-AR-mediated inotropic response. However in contrast, our studies seem to indicate that the mechanism underlying the reduced β₁-AR responsiveness resides in alterations in the β₁-AR signaling pathway downstream of Gi rather than from an increase in tonic inhibition of Gi upon AC. Possibly, the divergence in our data from some of the others results from the fact that some of the other studies were performed in in vitro systems artificially up-regulating Gi whereas our model system is an ex vivo system. Alternatively, as discussed extensively in paper III, we propose that the divergence of our data from several prior studies [132, 133] resides in the interpretation of the actual data, whereby we conclude the data from these prior studies [132, 133] are actually consistent with our data. In conclusion, our data taken together with prior studies do not convincingly support a role for increased Gi activity in the mediation of the reduced β-AR-mediated inotropic response.

During our experiments characterizing the role of Gi, we uncovered an unforeseen and peculiar finding that simultaneous inhibition of PDE3 and PDE4 elicited a large inotropic response in both normal and failing rat ventricle only after prior inactivation of Gi with PTX (paper III & IV). PDE3,4 inhibition in the absence of the non-selective β-AR antagonist timolol also elicits a modest inotropic response that is completely blocked by timolol. These data indicate that PDE3,4 inhibition alone does not elicit its effect in PTX-treated tissue by simply potentiating constitutive activity of another (non β-AR) Gs-coupled receptor. In addition, the effect of PTX does not result from removing constitutive muscarinic receptor activity since the muscarinic inverse agonist atropine does not evoke an inotropic response in PTX-treated tissue. Together, these data indicate the PDE3,4-evoked inotropic response is independent of constitutive receptor activation of Gs and Gi. Further, the response is cAMP-dependent as the inotropic response was accompanied by a lusitropic effect. We have proposed that these data
indicate that Gi is critical for maintaining a low receptor-independent basal AC activity in a signaling compartment capable of mediating a cAMP-dependent inotropic response. The further implication of these data is that AC itself has a relatively high intrinsic constitutive activity with Gi functioning as a “tonic” brake directly on AC, rather than upon the β-AR system specifically. Although the mechanism by which ADP-ribosylation of Gi (which presumably only removes receptor activation of Gi) can alter AC activity remains unknown. We have hypothesized that the balance of receptor-independent constitutive activity of Gs and Gi activity upon AC is shifted in favor of Gs by PTX removal of available free constitutively active Gi that is in large excess to the amount of Gs. Assuming this is the case, whereby PTX treatment shifts the balance towards Gs, the response to all Gs-coupled receptors or direct activators of AC (e.g. forskolin) should also be enhanced. This mechanism would account for the fact that β1-AR-mediated responses are enhanced by PTX treatment. Consistent with this hypothesis, the 5-HT4 receptor-mediated inotropic response in the failing ventricle was amplified by PTX (paper II). Recently it is also reported that PTX enhances the forskolin-evoked inotropic response and AC activation [157]. The finding from this latter study is particularly relevant since forskolin activation of AC is independent of receptor activation but is in part dependent upon the level of available Gs. That PDE3,4 inhibition was required to unmask this PTX-dependent increase in constitutive AC activity indicates that PDE breakdown of cAMP is sufficient and necessary to compensate for increased basal (constitutive) cAMP production when Gi activity is impeded by PTX. In summary, we propose that inactivation of Gi removes a tonic intrinsic receptor-independent Gi inhibition upon spontaneous (intrinsic) Gs activation of AC.
8  FUTURE PERSPECTIVES

The two primary topics investigated in this research project were 1) the importance of receptor-mediated regulation of MLC-2 activity to enhance contractility in failing heart and 2) understanding the functional role of Gi to regulate compartmentation of AC signaling (β-AR) and its subsequent effect upon cardiac contractility. Some potential future implications of our findings are discussed below.

8.1 MLC-2 – from a preclinical peculiarity to potential clinical therapy

Increasingly in recent years, a number of publications have documented the relevance of MLC-2 phosphorylation in cardiac function and heart disease. Significantly reduced MLC-2 phosphorylation is observed in patients with heart failure [108, 158] as well as in different animal models of heart disease [107, 159, 160]. A possible cardioprotective role of MLC-2 is also emerging; Warren and co-workers reported that overexpressing cardiac-specific MLCK and thus increasing the phosphorylation of MLC-2, protected mice from developing severe heart failure [139]. In parallel, some studies have investigated the role of MLC-2 in the development of cardiomyopathy, whereby specific mutations within MLC-2 are associated with development of certain cardiomyopathies [161, 162]. Yuan and co-workers [140] reported that constitutive phosphorylation of MLC-2 prevented the development of a pathological phenotype of hypertrophic cardiomyopathy in mice. In addition to recognizing the role of MLC-2 in the etiology of cardiac disease, numerous studies indicate that different cardiac receptors can evoke inotropic responses through increasing MLC-2 phosphorylation, sensitizing the myofilaments to Ca$^{2+}$ [113-117]. Arguably, inotropic responses elicited through MLC-2 is preferred compared to β1-ARs-elicited cAMP-mediated inotropic responses which are more energy consuming. It is particularly interesting that the muscarinic M$_2$ receptor-mediated inotropic response occurs only in failing myocardium (paper I). This change in the functional response to M$_2$ activation may be a compensatory response offering inotropic support in the failing heart. Consistent with this
hypothesis, 5-HT$_{2A}$ receptors in rat ventricle mediate a MLC-2-dependent inotropic response only in failing heart [114].

More recently, apelin, the endogenous ligand for the apelin-receptor system (a GPCR also known as APJ) can also elicit a sustained and potent inotropic effect mediated through increasing MLC-2 phosphorylation [117]. Interestingly, apelin and the APJ system are also emerging as a potential therapeutic target in heart failure. Purportedly, apelin or APJ agonists decrease renin-angiotensin levels, evoke vasodilation and mediate a sustained inotropic effect [36, 117, 163]. Although apelin and the APJ system is reportedly down-regulated in advanced heart failure [164-166], studies treating with apelin or stimulating APJ have reported promising cardioprotective effects (see [163] for review).

Chronic vagus nerve stimulation has also shown promising preclinical results that may be analogous to those with apelin. In a post-infarction rat model of heart failure, chronic vagus nerve stimulation significantly improved the survival rate (relative risk reduction for mortality ~70% compared to Sham) as well as improved cardiac function accompanied by a normalization of biventricular weight compared to sham operates [122]. These results provided the basis for conducting a clinical investigation assessing the possible beneficial role of chronic vagus nerve stimulation in heart failure. Recently, a small randomized trial indicated an improvement in quality of life in the patients that received vagus stimulation [124]. However, unfortunately this study failed to significantly show improvement in cardiac remodeling and function which were its primary endpoints [124]. More conclusive data are expected from the larger INOVATE-HF study, which will determine if chronic vagus nerve stimulation reduces heart failure hospitalization and/or all-cause mortality [143]. If INOVATE-HF shows beneficial effects in the intervention group, some findings in this thesis may prove relevant for understanding the mechanisms mediating the beneficial effects. Possibly, chronic vagus nerve stimulation may improve ventricular function through 1) stimulation of M$_2$ receptor-mediated contractility by MLC-2 phosphorylation hence improving cardiac stroke volume, 2) increased cardiac G$_i$
activation reducing basal AC activity and 3) antagonism of the β1-AR system as well as modulating the receptor mediated potassium current [167] both mechanism reducing heart rate which by default would result in reduced oxygen consumption [168] and thus better ventricular efficiency.

8.2 The functional role of Gᵢ – shifting the paradigm

The results of studies reported in papers II-IV lead us to the hypothesis that Gᵢ alone has a high constitutive activity functioning as a “tonic” inhibitor or brake to maintain a basal AC activity in the signaling compartment capable of mediating a cAMP-dependent inotropic response. This hypothesis extends the functional role of Gᵢ beyond its established primary and classical role of counteracting β₁-AR-mediated activation of AC (accentuated antagonism).

Subsequent studies conducted in our group [157], reported that PTX amplified both the R,R and R,S fenoterol stereoisomer (purportedly coupled to Gₛ only and dually coupled to Gₛ and Gᵢ respectively)-evoked maximal inotropic response and cAMP accumulation. That PTX enhanced the Gₛ-selective (R,R)-fenoterol-mediated response likely suggests that Gᵢ regulates AC activity independent of receptor coupling to Gᵢ protein. Therefore, the data from Melsom et al [157] provided additional support that Gᵢ tonically exerts an inhibition upon AC activation. What remains to be determined is the mechanism by which PTX mediates this enhancement of AC activity, since PTX is thought to only block or interrupt receptor-mediated activation of Gᵢ. In Melsom et al [157] there are several intriguing hypothesis 1) the βγ-sink model whereby PTX-inhibition of Gᵢ not only inactivates Gᵢ but also sequesters a significant amount of a shared βγ-pool, thereby indirectly increasing receptor-independent spontaneous activation of Gₛ leading to increased basal AC activity and 2) the nucleoside diphosphate kinase B-model (NDPK) whereby NDPK-activation of Gᵢ dominates in the absence of PTX treatment due to an excess of Gᵢ protein levels over Gₛ, resulting in low basal cAMP production readily degraded by PDEs (as first described by Hippe et al [169]. In the presence of PTX, Gᵢ is irreversibly inactivated due to ADP-ribosylation, thus NDPK could then predominantly activate Gₛ subsequently stimulating AC and increasing cAMP levels. Additional studies are needed to determine the mechanism
mediating the generalized PTX enhancement of all cAMP signaling inotropic factors.

Regardless, the data presented in this thesis, follow-up work from our group and that of others indicates a role of Gi extending well beyond antagonism of Gs coupled receptor activation of AC. As such, it will be interesting to determine if increasing Gi activity has beneficial effects for the treatment of heart failure. In fact, a “protective” effect of Gi is recently reported in an elegantly study by Keller et al [170] where Gαi2 deficiency combined with cardiac β1-AR overexpression strongly impaired survival and cardiac function but β1-AR overexpression alone did not induced cardiac hypertrophy. Thus we argue that there is more to Gi than previously believed and we hope the future evolvement of this field will provide additional clarity in regards to the functional role of Gi in the failing heart.


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