The Role of Endogenous Peptides (Apelin, Adrenomedullin) in the Regulation of Cardiac Contractility

PhD thesis

by

Klára Farkasfalvi

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Contents

Abbreviations

ACE	angiotensin converting enzyme
ACE 2	angiotensin converting enzyme 2
AM	adrenomedullin
APJ	angiotensin-like putative receptor
Ang II	angiotensin II
AT_1-R	angiotensin II type 1 receptor
AP	action potential
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
DT	developed tension
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
GPCR	G-protein coupled receptors
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
p38-MAPK	p38 mitogen-activated protein kinase
NCX	sarcolemmal Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NHE-1	Na^+/H^+ exchanger isoform 1
PAMP	proadrenomedullin N-terminal 20-peptide
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLB vs PLN	phospholamban
RAS	renin-angiotensin system
RAMPs	receptor-activity modifying proteins
RyR	ryanodine receptors
SR	sarcoplasmic reticulum
SERCA	SR Ca^{2+} -ATPase
TnC	troponin C
TnI	troponin I
TnT	troponin T

1. Introduction

Heart failure is a serious condition, with a mortality rate greater than 50% over 5 years in severe cases. Since heart failure is a complex syndrome the therapeutic approaches are multiple, including general measures, pharmacological therapy, mechanical devices and surgical intervention (Hunt et al., 2005). The significant improvement of heart failure has been achieved by combination therapy with regard to morbidity and mortality rate however, the increasing numbers and daily doses of drugs bare the risk of potential drug interactions and more serious complications (Kappert et al., 2008). At present, the only cure for severe heart failure has a heart transplantation, which is limited by the small number of organs. Since heart failure is the fastest growing incidence within cardiovascular disease and is associated with substantial economic costs it indicates an extensive research investigating novel therapeutic strategies (Cohn et al., 2000).

The complex clinical syndrome of heart failure is characterised by circulatory congestion and progressive cardiac contractile dysfunction. This is accompanied by molecular alterations that cause remodeling of the heart, which is a self perpetuating pathological process, and a deterioration of failing heart (Cohn et al., 2000). A deeper insight into the pathophysiologic mechanisms highlight the role of various neurohormonal mechanisms and the antagonism of this system have beneficial effect on progression of heart failure (Hunt et al., 2005).

However, as the contractility of the heart is compromised, a desirable therapy would involve improvement of efficiency of the contraction-relaxation cycle, but for the time being no effective, safe, chronic positive inotropic and lusitropic therapy exists for the treatment of systolic and diastolic dysfunction in heart failure. Regulation of myocardial contractility by endogenous peptides is important in physiological and pathophysiological conditions and may be a crucial therapeutic target in the treatment of heart failure (Brutsaert, 2003). Potent positive inotropic agents apelin (Brutsaert, 2003; Szokodi et al., 2002) and andrenomedullin (Szokodi et al., 1996) act in autocrine/paracrine manner and have demonstrated cardioprotective effects (Hamid and Baxter, 2006; Jia et al., 2006; Kleinz and Baxter, 2008). Although, numerous experimental data prove the efficacy of these peptides the underlying molecular mechanisms are only partially understood.

1.1. Regulation of Cardiac Contractile Function

Contractility is defined as the intrinsic ability of the cardiac muscle fibre to contract at a given fibre length. The degree of contraction is influenced by different degrees of binding between myofilaments which depends on concentration of intracellular Ca^{2+} and the sensitivity of myofilaments to Ca^{2+}. The contractile function of the heart is regulated by a number of intrinsic and extrinsic mechanisms. Intrinsic mechanisms include the Frank-Starling mechanism and the force-frequency relation. Extrinsic mechanisms affecting cardiac function are the autonomic nervous system, hormones and regulatory peptides acting in autocrine/paracrine manner. The complex interplay between all these factors occurre continuously via both the haemodynamic state and respective feedback mechanisms, and also at the level of single cardiomyocytes (Bers, 2002; Opie, 1995).

1.1.1. Excitation-Contraction Coupling

The excitation-contraction coupling includes the events which follow the wave of excitation and lead to contraction. Initially, the wave of depolarization spreads rapidly along the myocardial sarcolemma, and also into the interior of the cells via the invaginations of the sarcolemma, the T-tubules, opening the voltage dependent L-type Ca^{2+} channels and triggering a Ca^{2+} influx (Hobai and Levi, 1999). Ca^{2+} is essential in cardiac electrical activity and is the direct activator of myofilaments, which cause contraction (Bers, 2002).

1.1.2. Ion Fluxes During Cardiac Cycle

The Ca^{2+} entering the cell through the L-type Ca^{2+} channels serves as a trigger to release Ca^{2+} from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR) (Fabiato and Fabiato, 1979). The amplified Ca^{2+} release promots free Ca^{2+} to bind to a specific site in the N-terminal domain of troponin C (TnC), resulting in a conformational change of the TnC molecule (Robertson et al., 1982; Solaro and Rarick, 1998). Cardiac troponin is a complex protein made up of three subunits: TnC, troponin I (TnI) and troponin T (TnT). TnC acts as the Ca^{2+} binding subunit, TnI inhibits the actin-myosin reaction and shuttles between tight binding to actin and tight binding to Ca^{2+}-TnC and TnT is the tropomyosin binding subunit. As a consequence of the Ca^{2+}signaling process and the conformational change in TnC, TnI moves from its diastolic state (tightly bound to actin) to its systolic state (tightly bound to TnC) (Solaro and Rarick, 1998). The interaction between TnI and TnC is followed by moving of the tropomyosin molecule to allow the crossbridges to attach

and produce force (Opie, 1995). Heads of myosins protruding from the thick filament then react with thin-filament actins in a reaction cycle that is powered by ATP (Rayment et al., 1993).

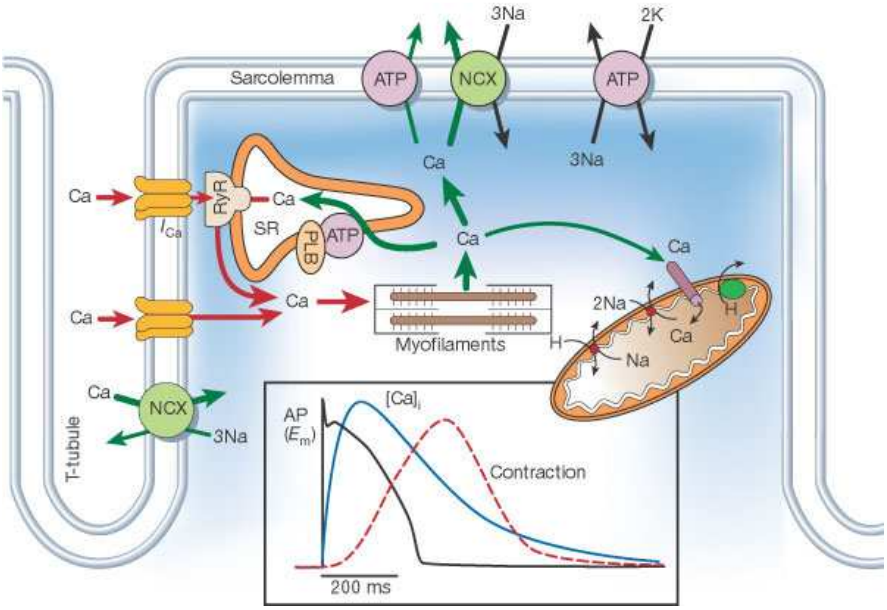

Figure 1. *The figure shows the time course of an action potential, Ca^{2+} transient and contraction measured in rabbit ventricular myocytes at 37 °C (Bers, 2002).*
(Abbreviations see at page 7)

For relaxation Ca^{2+} must be removed from cytosol which occurs through SR Ca^{2+} -ATPase (SERCA), sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX) in the largest proportion and the rest through the plasma membrane calmodulin-dependent calcium ATPase and mitocondrial Ca^{2+} uniporter (Bers et al., 1998; Rayment et al., 1993). The major regulator of SR Ca^{2+} transport is phospholamban (PLB) the endogenous inhibitor of SERCA. Phosphorylation of PLB relieves this inhibition, allowing faster twitch relaxation and decline of Ca^{2+}(Koss and Kranias, 1996) (Maclennan and Kranias, 2003).

The Ca^{2+} cycling is affected in heart failure. It was shown that Ca^{2+} cycling is reduced by approximately 50 per cent in myocytes obtained from patients with end-stage heart failure. The intracellular Ca^{2+} transient demonstrates a blunted rise with depolarization reflecting a slower delivery of Ca^{2+} to the contractile apparatus (causing slower activation) and a slower rate of fall during repolarization (causing slowed relaxation) (Beuckelmann et al., 1992; Rayment et al., 1993). In consequence of impaired Ca^{2+} cycling a lot of compensatory pathways are activated. For example

studies have shown overexpression of NCX, which allows greater Ca^{2+} influx during the action potential but generate intracellular Na^{+} overload (Pieske et al., 2002).

Another major regulator of intracellular homeostasis is the Na^{+}/H^{+} exchanger (NHE), which is a membrane protein that regulates ion fluxes. NHE extrudes one intracellular proton in exchange for one extracellular sodium thereby regulating intracellular pH. Intracellular acidosis is the primary stimulus for activation of NHE (Karmazyn et al., 1999). However, there are many pathways that may also lead to NHE activation. One of them is the α1-adrenergic receptor activation through phosphorylation of protein kinase C (PKC) (Wallert and Frohlich, 1992) causing increase in intracellular pH and Na^{+} concentration . The alkalinization is mainly responsible for the increased myofilament Ca^{2+} sensitivity and the rise in Na^{+} concentration contributes to the increase in Ca^{2+} transient via NCX. The result is a positive inotropy with modest negative lusitropy. Other factors can also exhibit stimulatory effects via phosphorylation-dependent processes. These generally represent various autocrine and paracrine as well as hormonal factors such as endothelin-1, thrombin, carbachol and angiotensin II, which probably act through receptor-signal transduction processes. The activation of myocardial NHE plays an important role in ischaemia and early reperfusion. Moreover increased activity of NHE was demonstrated in heart failure and the inhibition of NHE attenuated the functional, morphological abnormalities of the failing heart (Avkiran and Haworth, 2003).

1.1.3. The Frank-Starling Mechanism

The Frank-Starling mechanism is an important intrinsic regulatory mechanism of myocardial contractility. It was Frank who first described this observation, namely the greater the preload, the greater the force generated by frog cardiac muscle (Katz, 2002; Knowlton and Starling, 1912; Markwalder and Starling, 1914; Patterson et al., 1914).

The response of myocardium to an increased sarcomere length is biphasic (Kentish, 1999), which means a rapid increase in active force, followed by a further increase in force over several minutes (Parmley and Chuck, 1973). The underlying intracellular mechanism is still elusive. According to the most reliable theory stretching increases Ca^{2+} affinity of TnC, since no increase in intracellular Ca^{2+} level is measured. Contrary to this, the slow phase of the Frank-Starling mechanism is associated with an increase of intracellular Ca^{2+} level via NCX operating in a reverse mode (Cingolani et al., 1998) . The exact mechanism of the length-dependent changes in myofilament

Ca^{2+} sensitivity is still a matter of research interest. In recent years two theories have emerged; one of them claims that the length-dependent changes in lattice spacing determine changes in Ca^{2+} sensitivity. The other theory suggests that there is a length-sensing element (titin) in sarcomere, which can modulate actin-myosin interactions independent of changes in lattic spacing (Fuchs and Martyn, 2005).

1.1.4. The Force-Frequency Relationship

The force-frequency relationship, also known as the Treppe phenomenon was described by Bowditch, who showed that increasing stimulation frequency in frog muscle caused increased contractile force (Bowditch, 1878). There is a positive correlation to force-frequency relationship in non failing myocardium until the heart rate reaches the 170/ min value which is an important adaptive mechanism during stress in man. However, in the failing myocardium frequency potentiation of contractile force is inverse. The force-frequency relationship is explained by increased transsarcolemmal Ca^{2+} influx consequently higher intracellular Ca^{2+} level during systole (Pieske et al., 1995).

1.2. Extrinsic Factors Regulating Contractile Function

1.2.1. Adrenergic Regulation of Cardiac Contractility

Excitation-contraction coupling is affected by several extra and intracellular molecules, which enable to influence contractile strength. The main and most widely studied regulatory pathway involves the sympathetic nervous system acting through G-protein coupled receptors (GPCR) whose primary function is to transduce extracellular stimuli into intracellular signals. The effectors of the sympathetic nervous system such as epinephrine and norepinephrine act on cardiac myocytes via both β- and α-adrenergic receptors. Cardiac β-adrenergic receptors are mostly the β1, and β2 subtype is 20 percent of the total amount of β-adrenergic receptor population (Bristow et al., 1986; Steinberg, 1999). The α-adrenergic activation is also able to modulate cardiac contractility but its importance is less.

GPCRs are associated with Gs, Gi or Gq subtypes of G-proteins. The Gs activation (dominantly activated through β1- and β2-adrenergic receptors) enhances activity of adenyl cyclise producing cyclic adenosine monophosphate (cAMP). cAMP subsequently activates the multisubstrate enzyme protein kinase A (PKA) whose main targets are L-type Ca^{2+} channels, PLN, and TnI in cardiac myocytes (Katz, 1990; Walsh and Van Patten, 1994). The phosphorylation of PLN and L-type Ca^{2+} channels result in increased intracellular Ca^{2+} level inducing positive inotropic effect. Moreover, the phosphorylation of TnI decreases the sensitivity of contractile apparatus to Ca^{2+} which means increased intrinsic rate of myofibrillar relaxation (positive lusitropy) and so contribute to the shortening of cardiac twitch during β-adrenergic receptor activity (Layland et al., 2004).

In contrast to Gs, the Gi (activated through β2) pathway is responsible for inhibition of adenylyl cylase. Gi coupling qualitatively and quantitatively modifies the outcome of Gs signaling. During acute receptor stimulation, the β2-adrenergic – Gi coupling mediates compartmentalization of the Gs-cAMP signaling thereby negating the positive inotropic and lusitropic effect of β2-adrenergic activation. During prolonged recetor stimulation, Gi coupling activates multiple Gs-independent signaling pathways resulting in cardiac protective effects (Zheng et al., 2005) α-adrenergic receptors activate Gq subtype of G-proteins. The Gq pathway involves the activation of phospholipase C, hydrolysis of PIP_2 to diacylglicerol/IP_3 and activation of protein kinase C (PKC). PKC represents a large gene family with up to twelwe isoforms, which target through phosphorylation many endeffectors in the heart, including L-type Ca^{2+} channels, and myofilament proteins, accounting for the positive

inotropic actions (Kang and Walker, 2006). PKC is also capable of activating NHE leading to intracellular alkalinization and increased sensitivity of myofilaments to Ca^{2+} (Karmazyn et al., 1999).

The Gq activation pathway may play an important role in many pathophysiological conditions, too. PKC and further downstream kinases, such as extracellular-regulated kinase 1/2 (ERK 1/2), p38 mitogen-activated protein kinase (p38-MAPK) are also important to GPCR mediated regulation of hypertrophic growth and to cardioprotective mechanisms underlying ischemic preconditioning (Braz et al., 2002; Ping et al., 1999).

1.2.2. Autocrine/Paracrine Regulation

Not only the humoral but the autocrine/paracrine regulation is an important mechanism to influence contractile function. Autocrine signaling is a form of cell signaling in which the target cell is the same than the signal-releasing cell, while in case of paracrine signaling, the target cell is near to the signal-releasing cell. These locally acting mediators may be endogenous peptides, which influence different organ functions. For example, coronary endothelium releases endothelin-1 in response to elevated coronary coronary flow and endothelin-1 acting on cardiomyocytes results in positive inotropy (Piuhola et al., 2003). Regulation of myocardial contractility by endogenous peptides is important in physiological and pathophysiological conditions and may be a crucial therapeutic target (Brutsaert, 2003). Apelin, andrenomedullin are part of this system with an extensive biological effect.

1.3. Emerging Role of Apelin in the Regulation of the Cardiovascular System

Apelin is a bioactive peptide expressed in a wide variety of tissues and exerts a broad range of biological activity.

1.3.1. Characterization of the Apelin System

The gene encoding angiotensin-like putative receptor (APJ) receptor is localised in humans on the long arm of chromosome 11 and it was identified by O'Dowd in 1993. APJ receptor called angiotensin receptor-like since it shares sequency identity in 54% of angiotensin II type 1 receptor (AT_1-R) (O'Dowd et al., 1993). However, angiotensin II (Ang II) is unable to activate the APJ receptor (Lee et al., 2000) (Tatemoto et al., 1998). Apelin was found in 1998 to be an endogenous ligand for the APJ receptor. Initially, apelin was isolated as a 36 amino acid peptide from bovine stomach homogenates, that activated Chinese hamster ovary cells expressing the APJ receptor (Tatemoto et al., 1998). Further investigations clarified that apelin secreted as 77 aminoacid and it might function as a precursor with limited biological activity and required further proteolysis and post-translational modification to result in the biologically more active form, predominantly (Pyr^1) apelin-13 followed by apelin 12 and 16 (Kleinz and Davenport, 2005; Hosoya et al., 2000; Lee et al., 2000; Tatemoto et al., 1998). Apelin and APJ receptor are widely expressed throughout the body and have functional effects in both the central nervous and the cardiovascular systems. Apelin and APJ receptor are expressed in the heart (O'Carroll et al., 2000; Szokodi et al., 2002) and in human coronary artery endothelial and coronary smooth muscle cells (Kleinz and Davenport, 2005). There appears to be a lower expression in cardiomyocytes, however, there remain detectable levels of apelin and APJ receptor in these cells. A large density of apelin and its receptor was found in endocardial endothelial cells and vascular endothelial cells in human large conduit vessels and small arteries and veins (Kleinz and Davenport, 2005; Kleinz et al., 2005).

1.3.2. Biological Effects of Apelin

The broad distribution of apelin and its receptor throughout the body provide clues about the physiological functions of this novel signal-transduction system. Roles have been established for the apelin-APJ system in regulation of eating and drinking behaviour, in stress activation and as novel adipokine, but its primary effect seems to be in modulating vascular tone and cardiac contractility.

The distribution of apelin and its receptor in the hypothalamus, gastric mucosa and fat cells has led to the suggestion, that the apelin system plays a role in modulating eating behaviour, nevertheless data in the literature show controversy

(Sunter et al., 2003; Taheri et al., 2002; O'Shea et al., 2003). Furthermore, the role of apelin in regulating fluid homeostasis is discussed. The central administration of apelin to rats was associated with a reduction (Reaux et al., 2001) as well as an increase in water consumption (Taheri et al., 2002). However, in APJ and apelin knock-out mice, water intake and urine electrolyte concentration were not different from wild type mice (Kuba et al., 2007).

Apelin modulates the hypothalamic–pituitary-adrenal axis, which appears to play an important role in stress adaptation and in regulation of inflammation during septic shock through corticotrophin-relasing factor and vasopressin mediated pathway (Newson et al., 2009).

Recently published data suggest that the peripherial administration of apelin does not affect food intake but decreases body weight in dose dependent manner due to direct action of apelin on white adipose tissue lipid metabolism (Higuchi et al., 2007). In a study of both human and mouse adipocytes and in mouse model of obesity apelin has been identified as a novel adipokin that is released from fat cells and is upregulated directly by insulin (Boucher et al., 2005). Other early studies revealed that apelin and leptin secreted by adipocytes have similar insulin–regulatory properties, both agents have a negative feedback on insulin levels and action which is a novel adipoinsular axis (Heinonen et al., 2005). However, further investigations will determine their distinct roles in adipoinsular system.

1.3.3. Vascular Effects of Apelin

Apelin modulates the tone of blood vessels, which has the overall effect of lowering blood pressure without any change in heart rate (Lee et al., 2000). The apelin induced reduction in blood pressure is mediated through nitric-oxid dependent pathway (Tatemoto et al., 2001). Maguire et al. proved the endothelium-dependent vasodilatator activity of apelin via prostanoid-dependent pathway and the removal of the endothelium revealed the direct vasoconstrictor effect of apelin in arteries and veins too (Maguire et al., 2009). Moreover, Charles and co-workers found a biphasic response in mean arterial pressure and cardiac output after intravenous administration of apelin-13. The initial fall of mean arterial pressure and cardiac output was followed by a subsequent rise and then a return to baseline over 15 minutes (Charles et al., 2006). Furthermore, apelin may modulate central control of arterial pressure since intracerebroventricular injection of apelin-13 increases both arterial pressure and heart rate in conscious rats (Kagiyama et al., 2005). But in case of injection apelin 13 into the nucleus tractus solitaries and rostral

ventromedullar medulla also reveals increases in arterial pressure (Seyedabadi et al., 2002). Although the overall systemic effect of apelin appears to decrease mean arterial pressure, differences may exist among particular anatomical region. The pathophysiological significance of apelin induced vasodilatation is proved in different knockout animal models too, with the important finding that apelin provides compensatory vasorelaxation to counteract Ang II-mediated vasoconstriction (Ishida et al., 2004).

1.3.4. Apelin and the Renin-Angiotensin System

Apelin-APJ system has a high sequence homology to the AngII/AT_1-R and both systems show significant similarity in tissue distribution and activation on the same biological processes suggesting a close relation to each others. Moreover, the recently described zinc metalloproteinase angiotensin converting enzyme 2 (ACE 2) is responsible for inactivating angiotensin I, II and apelin-36, apelin-13. ACE 2 is distributed mainly in cardiac and renal tissues and insensitive to the classical ACE inhibitors (Vickers et al., 2002; Burrell et al., 2004).

Ishida et al provided further evidence to prove the interaction between apelin/APJ and AngII/AT_1-R systems. APJ knock out mice were highly sensitive to low dose Ang II treatment in contrast to the wild-type mice suggesting that the lack of apelin signaling prevents a possible counter-regulatory effect of apelin to mediate the pressor effects of AngII. These findings were further supported by another experiment in which the blood pressures of APJ/AT_1-R double knock out mice showed partial normalisation compared with AT_1-R single knock out mice (Ishida et al., 2004).

The relation between apelin/APJ and AngII/AT_1-R was investigated by Iwanaga et al in experimental model of heart failure in rats. Beside decreased ejection fraction the apelin and APJ mRNA were markedly down-regulated and the treatment with angiotensin blocker resulted in clinical improvement and increased expression of apelin and APJ in rats with heart failure. These experimental data emphasize that the beneficial effect of modulating rennin-angiotensin system in heart failure may be, at least partially, due to the restoration of apelin signaling (Iwanaga et al., 2006).

1.3.5. Apelin and Inoptropy

Apelin has recently been found to be a potent inotropic agent in isolated rat heart preparations (Szokodi et al., 2002). Szokodi et al. found a dose-dependent

positive inotropic effect in vitro due to specific activation of its receptors in the heart, which was independent of the release of cathecholamines, other vasoactive peptides (endothelin, Ang II) or nitric oxide. They investigated the underlying intracellular mechanisms and suggested that activation of PLC and PKC were involved in the positive inotropic effect observed in the presence of apelin. On the other hand researchers have found contradictory results. Hosoya and Marsi suggested that the APJ receptor couples through inhibitory G-proteins, because pertussis toxin inhibits the actions of apelin (Hosoya et al., 2000; Masri et al., 2006). It has been demonstrated that the APJ receptor is localised at the T-tubules raising the possibility that tubular Ca^{2+} channels could be involved in the positive inotropic effect of apelin (Kleinz and Davenport, 2004). This has not been supported by perforated patch-clamp experiments which have shown no evidence of modulation of Ca^{2+} flux through L-type Ca^{2+} channels following apelin administration (Szokodi et al., 2002). Wang et al. reported a double effect of apelin on intracellular Ca^{2+} concentration such as systolic increase and diastolic decrease via PKC dependent mechanism. Moreover they found that apelin enhanced the activity of NCX and SERCA but the underlying mechanism has still been unknown (Wang et al., 2008).

While the positive inotropic effect of apelin in isolated heart preparations has been repeatedly demonstrated, its effect in vivo is more complicated. For instance, it has been observed that cardiac output does not increase following acute administration of apelin. However, it causes a reduction in left ventricular enddiastolic area and increases in left ventricular elastance. Therefore, the change in loading conditions after acute apelin administration together with changes in the visco-elastic properties of the ventricle may influence the net effect of apelin on cardiac output. Chronic administration of apelin (two-week continuous infusion) resulted in significant increase in the velocity of circumferential shortening and cardiac output. Importantly, these hearts have shown no evidence of left ventricular cellular hypertrophy, which is frequently seen after chronic administration of positive inotropic substances (Ashley et al., 2005). There are experimental evidences that apelin knock out mice develop severe impairment in cardiac contractility, which becomes evident at six months of age. These data indicate as well, that apelin is important mediator to maintan cardiovascular homeostasis (Kuba et al., 2007). Moreover, the results of Dai et al suggest that apelin exerts a more pronounced positive inotropic effect in failing myocardium compared to normal trabecular muscle (Dai et al., 2006). Furthermore, apelin was found to be a cardioprotective agent too. Jia and colleagues found, that apelin improved the function of the heart

which had previously been damaged by isoproterenol (Jia et al., 2006). In an other experiment Kleinz and co-workers observed a cardioprotective effect of apelin during ischemia reperfusion injury (Kleinz and Baxter, 2008; Jia et al., 2006). These complex effects of apelin may be important when it is considered as a therapeutic agent in heart failure.

There are numerous experimental data suggesting the importance of the apelin-APJ system regulating myocardial contractility *in vivo*, the direct effects of apelin on cardiomyocyte contractility and the underlying intracellular signaling mechanisms are unknown.

1.3.6. Role of the Apelin–APJ System in Heart Failure

The apelin–APJ signaling pathway has also been identified recently as a potentially important mediator in the pathophysiology of chronic heart failure (Jia et al., 2006; Berry et al., 2004; Ashley et al., 2005). There is evidence that circulating plasma apelin levels are increase in patients in the early stage of heart failure compared with the healthy population, but in patients suffering from severe heart failure the plasma apelin levels are less than normal (Chen et al., 2003; Foldes et al., 2003). This finding may suggest that the increase in circulating apelin may be a compensatory mechanism in the early stage of heart failure to improve cardiac contractility. This theory was proved by Iwanaga et al., who demonstrated that myocardial apelin and APJ mRNA level is initially preserved in compensated ventricular hypertrophy and only downregulated when animals developed heart failure(Iwanaga et al., 2006). Foldes and coworkers have assessed left ventricular apelin and APJ receptor mRNA levels by quantitative RT-PCR and they found that apelin mRNA levels were increased and the APJ receptor mRNA was downregulated in heart failure in idiopathic dilatative cardiomyopathy (Foldes et al., 2003). The patients with heart failure due to ischaemic heart disease did not show these phenomena, suggesting that the primary disease underlying heart failure may probably influence cardiac APJ expression. However, in patients with dilated cardiomyopathy APJ polymorphism was not found in greater frequency compared to normal healthy controls (Sarzani et al., 2007).

While there is continuing debate regarding apelin and mechanisms of APJ downregulation in HF, important changes in the apelin-APJ receptor expression have been observed following left ventricle assist device insertion. Chen and colleagues have identified the APJ receptor as one of the most upregulated genes following offloading with ventricle assist device (Chen et al., 2003).

These early studies suggest that the apelin APJ axis is an important biomarker of heart failure and its upregulation is favourable regarding left ventricular remodeling. Recently published data have revealed that this includes improvement in endothelial function and decrease in cardiac fibrosis mediated via apelin/APJ and Akt/eNOS pathways (Fukushima et al., 2010). The interactions of apelin-APJ system with other neurohormonal systems involved in the pathogenesis of heart failure are not yet fully understood, as much as the effects of these modifying therapies (ACE inhibitors, beta-blockers) on apelin concentration.

1.3.7. Apelin and Arrhythmias

Apelin has also been implicated in the pathophysiology of arrhythmias; Elinor et al. demonstrated that plasma apelin levels decrease in patients with lone atrial fibrillation (Ellinor et al., 2006). In agreement with this, Kallergis et al. found that successfull cardioversion of long-lasting atrial fibrillation led to significant increase in plasma apelin levels (Kallergis et al., 2010). However the role of the APJ-apelin system in the regulation of electrophysiological parameters in the heart is still poorly understood.

1.4. The Role of Adrenomedullin in the Regulation of the Cardiovascular System

In 1993 Kitamura et al. isolated a novel peptide called andrenomedullin (AM) and since that time several hundreds of papers have been published regarding the regulation of its secretion and the wide range of its actions (Kitamura et al., 1993a). Substantial evidences support the perception that AM is an important regulator of the cardiovascular system (Kitamura et al., 1993a; Hamid and Baxter, 2005; Szokodi and Ruskoaho, 2008).

1.4.1. Characterization of the Adrenomedullin System

Human AM consists of 52 amino acids with an intramolecular disulfide bridge forming a ring structure of six residues with an amidated tyrosine at C-terminus (Kitamura et al., 1993b). Due to moderate sequence homology with calcitonin, calcitonin gene-related peptide (CGRP) an amylin, AM has six substitutions and two deletions (Sakata et al., 1993). Human AM is synthesized as a 185-amino-acid precursor. The cleavage of 21-amino-acid signal peptide from N-terminus of this prepro-AM leads to formation of a 164-amino-acid prohormone (Kitamura et al., 1993a; Kitamura et al., 1994). The prohormone is further cleaved to liberate a 20-amino-acid proadrenomedullin N-terminal 20-peptide (PAMP), which has been found to elicit biological effects independent of those of AM (Kitamura et al., 1994). The remaining prohormone is finally cleaved between positions 93 and 94 as well as 148 and 149, resulting in the formation of human AM (Kitamura et al., 1994).

Immunoreactive AM and AM mRNA were detected in numerous peripherial tissues (lung, heart, kidney, liver, intestine) as well as various regions of the central nervous system (Kitamura et al., 1994). The adrenal gland contains proportionally the largest quantity of AM, but substantial amounts were found in the cardiovascular system (Kitamura et al., 1994; Sakata et al., 1993). AM expression show close correlation with the degree of tissue vascularisation. Both endothelial cells and vascular smooth muscle cells synthesize and secret AM and it is the major source of circulating AM in the plasma (Sakata et al., 1993; Kitamura et al., 1993b; Kitamura et al., 1994). AM is present in a variety of embryonic tissues, particularly in the heart and cardiac myocytes and even further nonmol/lyocytes were found to secret AM (Jougasaki et al., 1995). Several factors may influence the secretion of AM such as inflammatory cytokines and vasoactive substances, mechanical stretching, hypoxia and oxidative stress (Chun et al., 1997; Yoshihara et al., 2002; Kawai et al., 2004; Nakamura et al., 2004) .

The characterisation of a specific AM receptor failed until in 1998 McLatchie et al described a family of receptor-activity modifying proteins (RAMPs) which are single transmembrane–domain proteins that are associated with the calcitonin receptor-like receptor (CLR) to direct its ligand binding specifity and affinity. Coexpression of RAMP1 with CLR results in a functional CGRP receptor, whereas association of CLR with RAMP2 or RAMP3 confers preferential AM binding (McLatchie et al., 1998).

1.4.2. Biological Effects of Adrenomedullin

As a consequence of a wide distribution of AM and its receptors, AM has a remarkable range of action, from potent vaso- and coronary dilatator effect through regulating cellular growth and differentiation to modulating hormone secretion (Samson, 1999) (Szokodi and Ruskoaho, 2008). There are some non-cardiovascular effects of AM as well; experimental data suggest that andrenomedullin inhibits ACTH release (Samson, 1999; Parkes and May, 1997) and aldosteron production (Yamaguchi et al., 1996). Several studies investigated the effect of AM on steroid secretion but the results were contradictory (Hinson et al., 2000). AM was found to be co-secreted with catecholamine in response to nicotine receptor stimulation; however, in vivo experiments did not support this data (Katoh et al., 1994; Masada et al., 1999). The renal effect of AM was studied widely. Evidence exist for a role of locally secreted AM in regulation of renal blood flow and tubular function but it is unlikely that circulating level of AM regulates renal function in physiological condition (Hinson et al., 2000). Furthermore, there are evidence to prove the role of AM in the pathophysiology of mesangial cell proliferation and matrix biology such as in protecting kidney glomeruli from inflammatory reactions (Chini et al., 1995; Chini et al., 1997). AM plays a role in glucose metabolism as well. AM attenuates and delays the insulin response to oral glucose challenge (Martinez et al., 1996). On other gastrointestinal effect of AM is to influence the gastrointestinal motor and secretory function. In the lung, AM inhibits bronchoconstriction induced by histamine or acetylcholine and additionally AM significantly prohibits alveolar macrophage release of neutrophil chemoattractants in response to lipopolysaccharide (Hinson et al., 2000).

1.4.3. Vascular Effects of Adrenomedullin

The most characteristic property of AM is an intensive and sustained hypotension in different species (Hinson et al., 2000; Chini et al., 1995; Chini et al., 1997). Moreover AM results in dose-dependent decrease in blood pressure in pathological conditions such as hypertension and heart failure (Rademaker et al., 1997; Shimokubo et al., 1996). The vasodilator effect of AM is mainly mediated through induction of NO release (Feng et al., 1994). In case of impaired NO production such as hypoxic condition the vasodilatation is induced by prostaglandins (Yang et al., 1996), furthermore AM also able to inhibit endothelin-1 production, which may contribute to vasorelaxation (Kohno et al., 1995). AM may act as a physiological antagonist of endothelin-1 (Kinnunen et al., 2001).

AM is indispensable for vascular morphogenesis during embryonic development and recent studies suggest that AM may be an important angiogenic factor in pathological conditions during adulthood (Nagaya et al., 2005; Ribatti et al., 2007). Experimental data suggest that AM exerts its angiogenic effect through activation of Akt and ERK independently of each other (Kim et al., 2003; Miyashita et al., 2003). On the other hand the effects of AM on proliferation of vascular smooth muscle cells are controversial. The migration of vascular smooth muscle cells into the intimal layer is the mechanism of intimal thickening consequently resulting in vascular remodeling. Some studies demonstrated antiproliferatory action (Kano et al., 1996) whereas later studies suggest that AM is a potent mitogenic factor in cultured rat vascular smooth muscle cells (Iwasaki et al., 1998).

1.4.4. Adrenomedullin and Inotropy

The effect of AM on myocardial contractility is controversial.

Systemic administration of AM results in marked haemodynamic effect such as decreased peripheral resistance, consequently increased heart rate, cardiac output and stroke volume (He et al., 1995; Parkes and May, 1997). This finding rises the question whether AM has a direct effect on contractility? In isolated, perfused rat heart AM increased cardiac contractility and dilated coronary arteries (Szokodi et al., 1996; Szokodi et al., 1998). AM has appeared to be among the most potent endogenous positive inoptropic substances since AM was active in the subnanomolar range infused into coronary arteries. Moreover, the AM–induced increase in developed tension was approximately 60% of the maximal inotropic response to the β-adrenergic agonist isoproterenol (Szokodi et al., 2002). In contrary to β-adrenergic agonist effect , AM increased the contraction force gradually with

mean time of 25-30 min to reach its maximum effect (Szokodi et al., 1998; Kinnunen et al., 2000). AM was reported to increase isometric tension in isolated rat papillary muscles (Ihara, 2000 Eu J Pharm), but other studies suggested a distinct effect of AM on cardiac contractility. A dual inotropic effect was observed in isolated adult rat ventricular myocytes, AM produced an initial increase in cell shortening followed by a negative inotropic effect on prolonged incubation (Mittra et al., 2004; Mittra and Bourreau, 2006). Former studies demonstrated a negative inotropic effect of AM on isolated rabbit cardiomyocytes (Ikenouchi et al., 1997) and human ventricular myocytes (Mukherjee et al., 2002). Other studies failed to detect any effect of AM on cardiac contractility (Stangl et al., 2000; Saetrum et al., 2000).

Number of studies suggesting that AM induce an increase in cAMP and this may be the major pathway of the signaling (Eguchi et al., 1994; Ishizaka et al., 1994; Shimekake et al., 1995; Chini et al., 1995; Sato et al., 1997), in spite of the evidence that AM enhances cardiac contractility via cAMP-independent mechanism (Szokodi et al., 1998). However, now it is clear that AM has direct effect on cardiac contractility, the underlying intracellular signaling mechanisms are still largely unknown.

2. Aims of the Study

- To evaluate the direct effect of apelin on contractile function of isolated normal and failing ventricular cardiomyocytes

- To characterize the intracellular signaling mechanism of apelin

- To investigate the effects of apelin on electrophysiological properties of cardiomyocytes

- To evaluate the intracellular signaling mechanism of the positive inotropic effect of adrenomedullin

3. Direct Effects of Apelin on Cardiomyocyte Contractility and Electrophysiology

3.1. Introduction

Regulation of myocardial contractility by endogenous peptides is important in physiological and pathophysiological conditions and may be a crucial therapeutic target (Brutsaert, 2003). An autocrine or paracrine system which potentially regulates heart function is the recently discovered APJ receptor with its endogenous ligand apelin. *Ex vivo* studies using isolated perfused rat hearts have identified apelin as one of the most potent inotropic substances recognised so far (Szokodi et al., 2002). Moreover, apelin was reported to increase left ventricular contractility *in vivo* following acute (Berry et al., 2004; Ashley et al., 2005) as well as chronic infusion in rodents (Ashley et al., 2005).

The APJ receptor and apelin have been implicated in the pathophysiology of human heart failure by a number of studies which identify this system as an attractive target for therapy (Lee et al., 2006; Kleinz and Davenport, 2005). Plasma concentration of apelin has been shown to decrease in patients with congestive heart failure (Foldes et al., 2003; Chong et al., 2006; Chen et al., 2003) and long-term cardiac resynchronization therapy could restore plasma levels of the peptide (Francia et al., 2006). Moreover, it has been demonstrated that left ventricular unloading with mechanical ventricular support increases APJ mRNA levels in patients with heart failure (Chen et al., 2003). Apelin has also been implicated in the pathophysiology of arrhythmias. Ellinor et al. demonstrated that plasma apelin levels decreased in patients with lone atrial fibrillation (Ellinor et al., 2006).

Despite recent advances in our understanding of the cardiovascular effects of the apelin-APJ system *in vivo*, the direct effects of apelin on cardiomyocyte contractility remains unknown. Therefore, the objective of the present study was to characterize the effects of apelin as well as the underlying signaling pathways, such as cytoplasmic [Ca^{2+}] and pH regulation *in vitro* using isolated adult rat ventricular myocytes. Moreover, to test the potential pathophysiological significance of apelin, we assessed the effect of the peptide on contractility in cardiomyocytes isolated from rat hearts in which chronic heart failure had been induced by coronary artery ligation. Finally, we studied the cellular localization of APJ and the effects of apelin on intercellular communication in cultured monolayers of cardiomyocytes.

3.2. Materials and Methods

3.2.1. Cell Isolation and Failing Heart Model

All animal procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Adult female Sprague Dawley rats, weighing 200 g (Harlan, UK) were used for this study. All animals were anaesthetized using 1-2% isoflurane in 100% oxygen for every procedure (excision of the heart, echocardiography, coronary artery ligation).

Cardiomyocytes were isolated using standard enzymatic dissociation (Terracciano and MacLeod, 1997). Briefly, the heart was perfused on a Langendorff apparatus with oxygenated normal Tyrode solution at 37°C for 2 minutes. The heart was then perfused with low Ca^{2+} solution for 5 minutes, followed by a 9 minutes perfusion with solution containing collagenase/hyaluronidase (see solutions). After discarding both atria and the right ventricle, the left ventricle was cut into small pieces and shaken in collagenase/hyaluronidase solution for a further 5 minutes. Cardiomyocytes were dissociated using gentle trituration, filtered through a 300 µm mesh and re-suspended in enzyme solution at room temperature until use.

Failing hearts were obtained 8 weeks after left coronary artery ligation. Briefly, the anaesthetized rats were intubated with a 16G plastic cannula and mechanically-ventilated (Harvard Apparatus, Kent, UK) at 2.5 ml tidal volume and 70 breaths per minute. A left-sided thoracotomy at the fourth intercostal space followed by pericardiectomy provided access to the heart. The left coronary artery was identified and permanently ligated at the level of the left atrial appendage using a 6-0 suture to cause myocardial infarction and subsequent heart failure. The diagnosis of heart failure was based on ejection fraction measured by using a 15 MHz probe on an Acuson Sequoia™ 256 system (Siemens Medical Systems, Germany). Transthoracic echocardiography was performed to obtain parasternal short-axis views at the mid-papillary muscle level. Ejection fraction was calculated from the systolic and diastolic 2-dimensional cross-sectional left ventricular areas, and ejection fraction less than 30% was taken as heart failure. Cardiomyocytes were isolated from the viable left ventricle of these hearts as above.

3.2.2. Measurement of Cardiomyocyte Contraction

Isolated cardiomyocytes were superfused with Krebs solution at 37°C and field-stimulated at 1Hz in a bath on the stage of an Olympus inverted microscope. Cell images were acquired with a ×60 objective at 240 frames per second and the sarcomere pattern was digitalized and analysed using Ionwizard™ software (Ionoptix Corp, CA, USA). Sarcomere shortening was measured in real time using a fast Fourier transformation of the cardiomyocyte striation pattern into a frequency power spectrum, as described previously (Delbridge 1997 J.Mol.Cell. Card.)

3.2.3. Intracellular [Ca^{2+}] Measurements

Intracellular [Ca^{2+}] was monitored using two different [Ca^{2+}]-sensitive fluorescence indicators. In initial experiments cardiomyocytes were loaded with indo-1 AM (Invitrogen, UK) and studied whilst being superfused with Krebs solution and field-stimulated, as above. Indo-1 excitation was at 385 nm, and fluorescence emissions at 405 nm and 485 nm were acquired. After subtracting background fluorescence levels, F405/485 was calculated and used as a measure of $[Ca^{2+}]_i$. F405/485 transients were analysed using Ionwizard™ software.

In another series of experiments cardiomyocyte [Ca^{2+}] was studied using fluo-4 AM (Invitrogen, UK). $[Ca^{2+}]_i$ was calculated from fluorescence using the equation:

$$[\mathrm{Ca}] = \frac{\left(k_d \, F/F_o\right)}{\left(k_d [\mathrm{Ca}]_{\mathrm{rest}}\right) + \left(1 - F/F_o\right)}$$

Where F is fluorescence, F_o is background fluorescence, k_d = 1160 nm and $[Ca^{2+}]_{rest}$ = 100 nm as previously reported (Huser et al., 1998).

For analysis of contractions and [Ca^{2+}] transients, 10-15 events were averaged with reference to the field-stimulation signal. Peak amplitude and time-to-peak (T_{peak}) were calculated from the field-stimulation signal baseline, and decay times (T_{50} and T_{90}) were calculated from T_{peak}. For the fluo-4 data the decay times (tau)

were calculated by fitting the data to a mono-exponential decay curve using pClamp™ software (Version 9.0. Axon Instruments, USA).

3.2.4. Intracellular pH Measurements and Na^+/H^+ Exchanger Activity

For measurement of intracellular pH (pH_i) cardiomyocytes were loaded with 10 µM 5-(and-6)-Carboxy-SNARF-1 AM (Invitrogen, UK) and superfused with Normal Tyrode solution at 37°C. Fluorescence excitation was at 480 nm. Data were expressed as the ratio of the emission wavelengths at 580 nm and 640 nm (F580/640).

To monitor NHE activity, the NH_4Cl pre-pulse method was used (Boyarsky et al., 1988). Briefly, the cells were perfused with normal Tyrode solution and two 5 minute pulses of 15 mM NH_4Cl were performed. Each pulse was followed by a recovery phase in normal Tyrode for 10 minutes, during which the pattern of recovery was recorded for analysis using pClamp™ software. The second NH_4Cl pulse and subsequent recovery were performed either in normal Tyrode again (control) or in normal Tyrode containing apelin. The time constant (τ) of the monoexponential curve fitted on the acid extrusion phase was normalized to the τ following the first NH_4Cl pulse. The acid extrusion rate in these conditions was taken as an index of NHE activity.

3.2.5. Immunocytochemistry

Isolated cardiomyocytes were plated onto laminin-coated slides in culture medium. 10 µm thick cryosections of adult rat hearts were mounted onto polylysine coated slides. Isolated cardiomyocytes and cryosections were fixed with acetone at −20°C, washed with phosphate buffered saline, and incubated with primary antibodies (2 hours, room temperature). Two different antibodies raised against peptides from 2 separate regions of the receptor were used to confirm the labelling patterns observed, one to the C-terminal (rabbit anti-APJ Apelin receptor IgG, Phoenix Pharmaceuticals Inc., CA, US, dilution 1:100) and one to a cytoplasmic loop (rabbit anti-APJ, affinity purified antiserum, Neuromics, MS, US, dilution 1:1000). Slides were washed with PBS, incubated with anti-rabbit IgG conjugated to AlexaFlor596 (Invitrogen, UK) for 1 hour, washed again with phosphate buffered

saline and nuclei counterstained with DAPI (Invitrogen, UK) prior to mounting. Images were recorded using a Leica TCS SP confocal microscope.

3.2.6. Cultured Neonatal Rat Cardiomyocytes

Ventricles from 3 days old Sprague-Dawley rats were dissociated, cut into small pieces, resuspended in collagenase/pancreatine solution and shaken at 37^{o}C (5 cycles, 15-25 minutes each). Fetal bovine serum was added to the mixture to inactivate enzymes and cardiomyocytes were rinsed in Fetal bovine serum. Cardiomyocytes were then filtered and plated in complete medium (10% horse serum, 5% foetal calf serum (Sigma, UK)) in Petri dishes and incubated for 45 minutes to enable fibroblasts to adhere and be removed. After assessing the viability using 0.4% trypan blue solution (Sigma, UK) neonatal cardiomyocytes were plated on multi-electrode array (MEA; Multi Channel Systems, Reutlingen, Germany) plates in 1 ml neonatal rat medium at a density of 0.5×10^{6} cells per plate, and cultured at 37°C. Neonatal rat medium was replaced every 24 hours and cardiomyocytes were grown to form a confluent monolayer.

3.2.7. Multi-electrode Array

A multi-electrode array setup (Multi Channel Systems, Reutlingen, Germany) was used to monitor the origin and spread of electrical activity in confluent neonatal cardiomyocyte monolayers, as described previously (Meiry et al., 2001). Spontaneous electrical activity, conduction velocity and properties of the field potentials were recorded. The raw data collected were filtered using a Savitzky-Golay filter, and differentiated digitally to determine the local activation time at each electrode using the Matlab™ interface (v7.0.1, Mathworks Inc, Germany) and custom software created by U. Egert (MEA-Tools, version 2.8, University of Freiburg, Germany). An activation map/isochronal map was constructed by interpolating the local activation time values for the sites between the electrodes and extrapolating the local activation time values for the four corners of the MEA matrix and were plotted using the Matlab™ standard two-dimensional tools.

The conduction velocity was calculated using the peripheral method, which utilizes the mean local activation time at each row of peripheral electrodes. Using the assumption of an uniform conduction pattern, the local activation times for each

row were averaged. The distance travelled along each external row is seven times the distance between two neighbouring electrodes, and using this information, the conduction velocity was calculated (Meiry et al., 2001). The length and shape of the field potential, a first derivative of the action potential, was analyzed by Clampfit™ software (Axon Instruments, USA).

3.2.8. Solutions

Normal Tyrode solution (in mmol/l):

NaCl 140; KCl 6; glucose 10; $MgCl_2$ 1; $CaCl_2$ 1; N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES) 10; pH 7.4.

Low [Ca^{2+}] solution (in mmol/l): NaCl 120; KCl 5.4; $CaCl_2$ 0.045; $MgSO_4$ 5; Na^+-pyruvate 5; glucose 20; taurine 20; HEPES 10; nitrilotriacetic acid 5; pH 6.96.

Enzyme solution (in mmol/l):

NaCl 120; KCl 5.4; $CaCl_2$ 0.2; $MgSO_4$ 5; Na^+-pyruvate 5; glucose 20; taurine 20; HEPES 10; pH 7.4.

Collagenase/hyaluronidase solution:

Enzyme solution with 1 mg/ml type-2 collagenase (280 u/mg, Worthington Biochemical, USA) and 0.6 mg/ml hyaluronidase (999 u/mg, Sigma, UK).

Krebs solution (in mmol/l):

NaCl 120; KCl 4.7; $MgSO_4 \cdot 7H_20$ 0.94; KH_2PO_4 1.22; $NaHCO_3$ 25, glucose 11.54.

Neonatal rat medium (NRM):

400 ml Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, UK), 100 ml 199 medium (HEPES modification), 60 ml 10% heat inactivated Horse Serum (Sigma), 27.5 ml 5% heat inactivated Foetal Bovine Serum (FBS), 13.5 ml 1M HEPES, and 6 ml penicillin/streptomycin.

Drugs

The Apelin 16-isoform was used in these experiments (Phoenix Pharmaceuticals Inc., CA, US).

3.2.9. Statistical Analysis

To assess statistical differences a one-way ANOVA with Tukey post hoc test or Bonferroni post hoc test analyses or paired t-tests were performed where appropriate. Results are expressed as mean ± standard error of the mean (n = number). $P < 0.05$ was interpreted as being statistically significant.

3.3 Results

3.3.1. Cellular Localization of the APJ Receptor

Confocal immunofluorescence microscopic imaging confirmed the presence of APJ receptor-like immunoreactivity in isolated adult ventricular myocytes and in heart tissue (Kleinz et al., 2005). APJ receptor-like immunoreactivity was detected in a transversal striated distribution associated with T-tubules (Fig. 2) and in the intercalated disc area (Fig. 2A, B, D and E).

3.3.2. Effect of Apelin on Cardiomyocyte Contractility

In isolated perfused rat hearts, apelin has been shown to induce maximal positive inotropic effect at concentrations of 1 nmol/l and 10 nmol/l (Szokodi et al., 2002). As shown in Figure 3, superfusion with 1 nmol/l and 10 nmol/l apelin increased sarcomere shortening of isolated adult ventricular myocytes, reaching a maximum after approximately 1 minute of superfusion (AP t1). This effect was transient, it lasted 1-2 minutes and sarcomere shortening returned and remained at control levels for the rest of apelin superfusion (AP t2) (Fig. 3). The maximal increases in sarcomere shortening in response to 1 nmol/l apelin (136±13 % (14), $P<0.001$) and 10 nmol/l apelin (138±14 % (14), $P<0.05$) at AP t1 are presented in Figure 3B. At the end of the experiments isoproterenol (30 nmol/l) application invariably induced a robust increase in sarcomere shortening (approximately 250 %) suggesting a maintained contractile reserve (Fig. 3).

3.3.3. Effect of Apelin on Contractility in Failing Cardiomyocytes

Subsequently, we studied the effect of apelin on sarcomere shortening in isolated cardiomyocytes from a rat model of chronic post-ischaemic heart failure. The sarcomere shortening of the failing myocytes in control conditions was significantly smaller compared to normal myocytes (Δ0.121±0.03 µm (24) $p<0.001$). As shown in Figure 3 C,D, apelin induced a transient increase in sarcomere shortening in failing cardiomyocytes (1 nmol/l apelin: 117±8.3% (12); 10 nmol/l apelin: 116±7.7% (12) $p<0.05$). Notably, the maximal responses to apelin in normal and failing cardiomyocytes did not differ significantly.

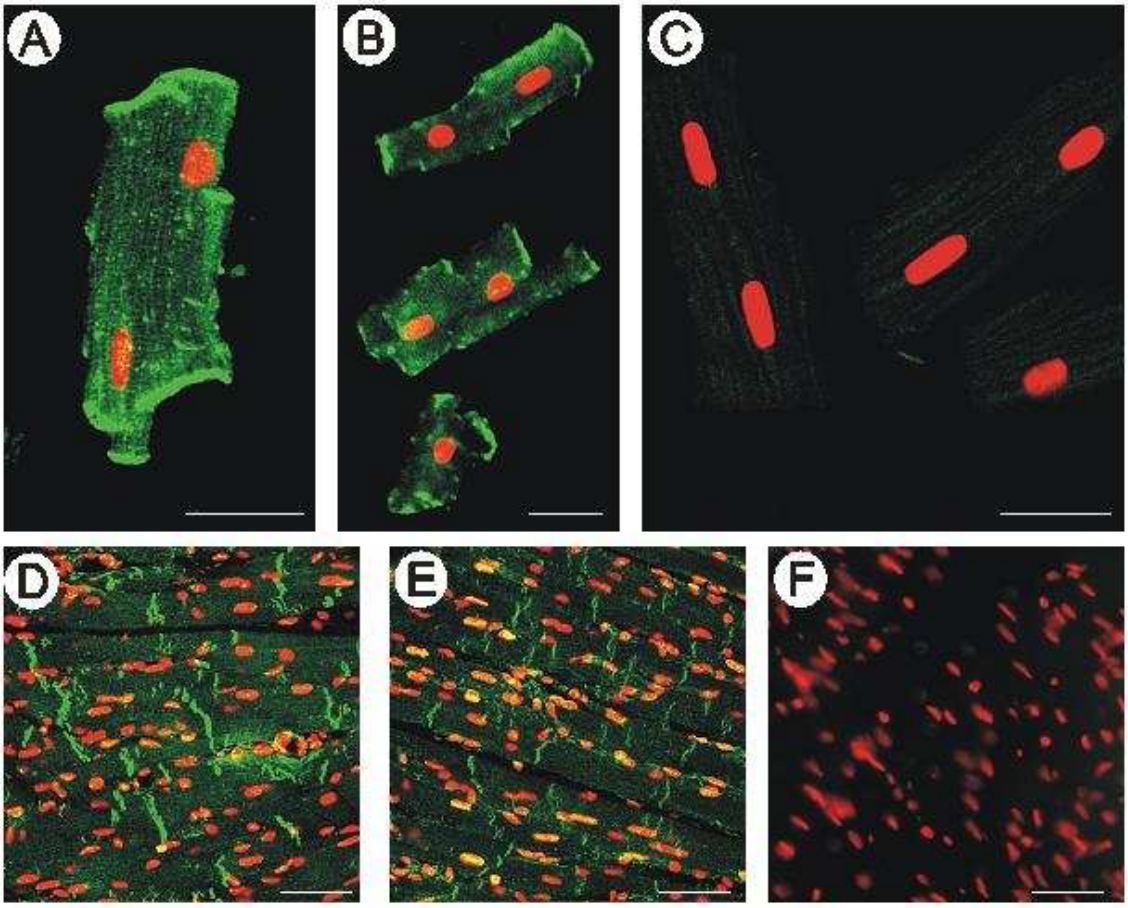

Figure 2. *Confocal images showing the localization of the APJ receptor (green label) in isolated cardiac myocytes (A, B, C) and sections of heart tissue (D, E, F). Nuclei are shown in red. Two different antibodies gave the same labeling pattern of the T-tubules and at the intercalated disc, one to a C-terminal region (A, D), and the other to a cytoplasmic loop region (B, E). No labeling was observed in the secondary antibody controls (C, F). Bar markers A, B, C, = 25 µm, D, F,E = 50 µm.*

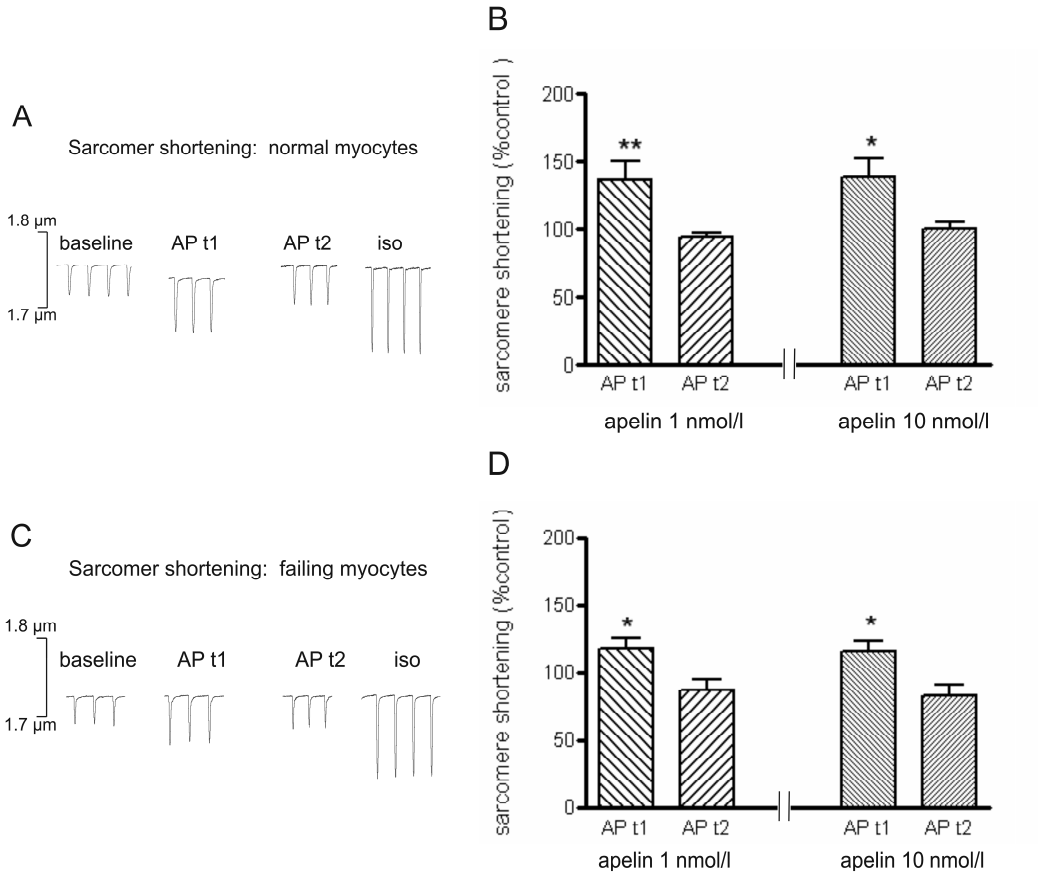

Figure 3. *Effect of apelin on sarcomere shortening of normal isolated cardiomyocyte. (3A) and failing isolated cardiomyocyte (3C) at the different time point; baseline, AP t1 (1 minute in apelin,) AP t2 (after 8 minutes in apelin) and iso 30 nmol/l of isoproterenol).*

*Graph 3B shows a significant increase in sarcomere shortening of normal and graph 3D of failing cardiomyocytes at both concentrations of apelin (1 nmol/l and 10 nmol/l l) after 1 minute (** p>0.001, * p>0.05). Failing myocytes showed a similar behaviour to normal myocytes. No statistical difference could be detected at AP t2, nor in normal or failing cardiomyocytes.*

3.3.4. Signaling Mechanisms of Apelin in Cardiomyocytes

To investigate the mechanisms underlying the effect of apelin on contractility, cytoplasmic [Ca^{2+}], the major intracellular mediator of contraction, was monitored using [Ca^{2+}]-sensitive fluorescent indicators. Initially we performed experiments using the ratiometric indicator indo-1. No effect of apelin could be observed either on the amplitude or time course of [Ca^{2+}] transients with both concentrations of apelin (1 nmol/l apelin: AP t1: 98.2±19 %, AP t2: 91.4±25 % (5) P>0.05; 10 nmol/l apelin: AP t1: 97.8±16%: AP t2: 89.6±28% (10) P>0.05). Isoproterenol, used as positive control, elicited a strong increase in [Ca^{2+}] transient amplitude. To exclude the possibility that changes in cytoplasmic [Ca^{2+}] were within the noise levels of indo-1 acquisition, we repeated the experiments with fluo-4 AM, which had a better signal-to-noise ratio. The results from these experiments are described in figure 4. There was no difference in the amplitude of [Ca^{2+}] transients upon application of apelin, it only increased in the presence of isoproterenol (Fig. 4A). There was no difference in the time course of the [Ca^{2+}] transients (Fig. 4B and 4C; [Ca^{2+}] transient amplitude in control %, 1 nmol/l apelin: AP t1: 93.79±2 % (15); 10 nmol/l apelin: AP t1: 94.47±3 %(14)) after 1 or 8 minutes of apelin superfusion.

To assess whether apelin affects intracellular pH and sarcolemmal NHE activity in isolated cadiomyocytes we monitored intracellular pH using the pH-sensitive fluorescent indicator carboxy-SNARF-1 and we assessed the acid-extrusion ability of the NHE with the NH_4Cl pre-pulse technique as previously described (Boyarsky Am J Physiol1998). Superfusion with 10 nmol/l apelin significantly decreased SNARF-1 fluorescence ratio suggesting an increase in the intracellular pH ((control: 3.01±0.1 ratio units (11); 10 nmol/l apelin: 2.64±0.1 ratio units (11); p<0.01)) (Fig. 5A, 5B). As for contractility, this effect was transitory and SNARF-1 fluorescence returned to control values after a few minutes. To assess the acid extrusion ability of NHE, 15 mM NH_4Cl in NT solution was applied for 5 minutes followed by wash out with NT for 10 minutes. Once the SNARF-1 signal returned to baseline, 1 nmol/l or 10 nmol/l apelin was added to the superfusing solution and the NH_4Cl prepulse was repeated (Fig. 5C). Apelin at both concentrations increased the speed of acid extrusion compared with control, suggesting an enhanced activity of NHE (Fig. 5D).

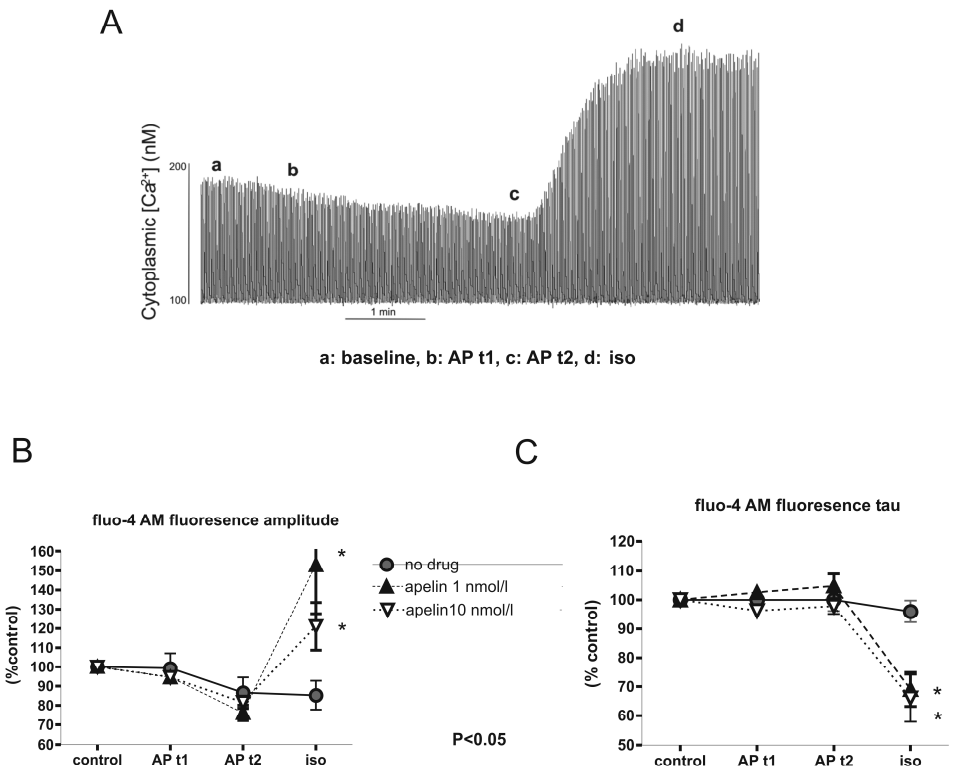

Figure 4*. The effect of apelin on [Ca^{2+}] transients measured by fluo-4 AM in normal cardiomyocytes (A). Apelin does not influence either the fluo-4 AM fluorescence amplitude (B) or the decay time (C). The nodrugl cells were superfused with normal Tyrode during the whole experiment even at the time point called isoproterenol*

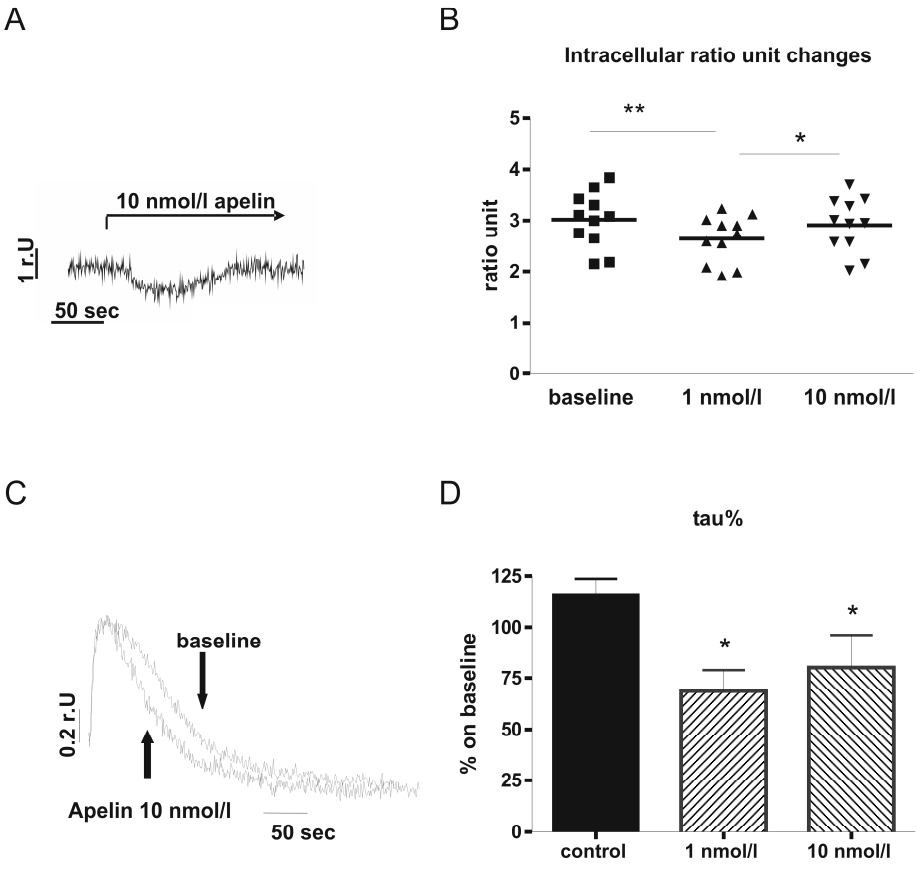

Figure 5. *Changes in intracellular pH in the presence of 10 nmol/l apelin (A). There was a significant increase in intracellular pH at both concentrations of apelin; the data are expressed in % of control values (B). A typical example of the change of NHE exchanger in normal cardiomyocytes superfused with 10 nmol/l apelin is shown in C. The acid-extrusion phase represents the activity of NHE exchanger and it was increased at both concentrations of apelin (D); the graph shows decay time as % of control (r.U=ratio unit)*

3.3.5. Effect of Apelin on Intercellular Communication

We subsequently assessed the electrophysiological properties of apelin in monolayers of cultured neonatal rat cardiomyocytes using multi electrode arrays. Recording of field potentials from spontaneously beating cultures revealed that apelin significantly increased conduction velocity (control: 18.34±1.4 cm/s; apelin: 24.1±2.2 cm/s (5) ($P<0.05$), Fig. 6A) and decreased field potential duration (apelin 1 nmol/l: 0.05±0.005 s (13); apelin 10 nmol/l: 0.048±0.005 s (15) $P<0.05$; Fig 6B). Isochronal maps showed reversible modifications in the patterns of activation (Fig. 6C). Movies of the 3D reconstructed activation of the monolayer are available as a data supplement on the homepage to the original paper [Farkasfalvi et al.,BBRC, *357*, 889-895, 2007]. File Normal Tyrode_3D.avi shows a typical activation pattern under control conditions. Apelin changed the direction of activation in monolayer cultured cardiomyocytes (file apelin_3D.avi) and this effect was reversible upon wash out.

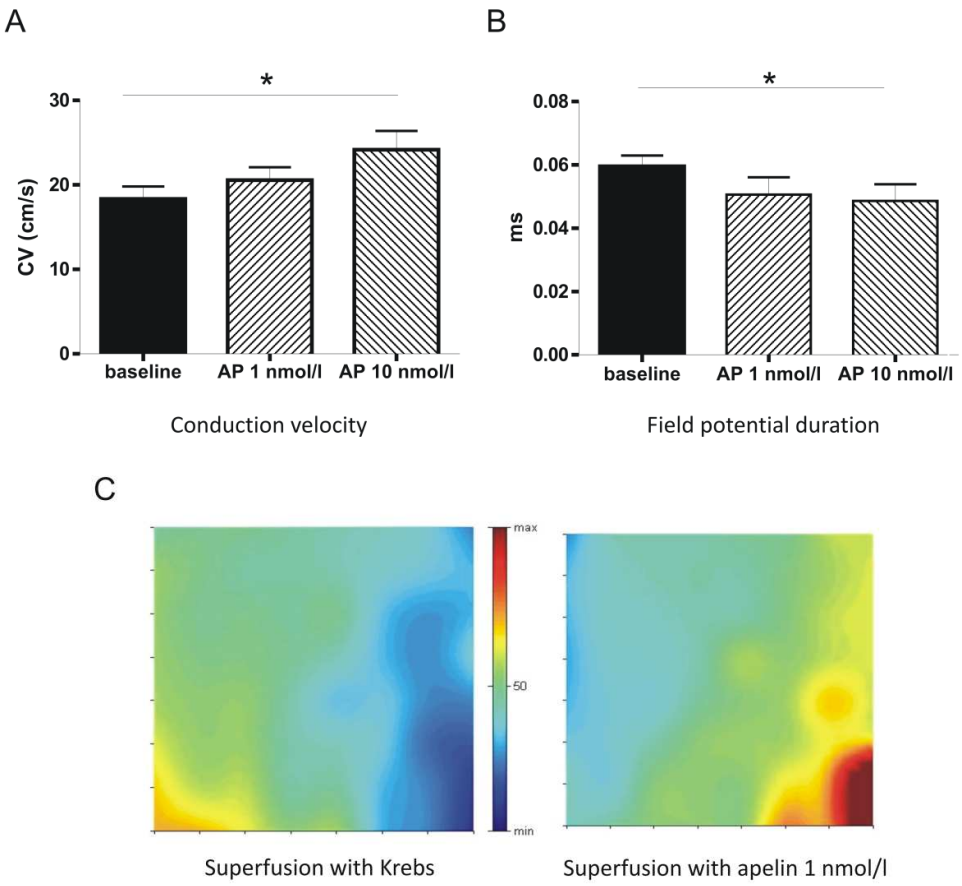

Figure 6. *In cultured neonatal cardiomyocytes conduction velocity (CV) increased (A) and field potential duration decreased with 10 nmol/l apelin (B). The isochronal map was constructed from local activation times (C); on the left the neonatal cultured cardiomyocytes were superfused with normal Tyrode and on the right with 1 nmol/lol/l apelin. Apelin changed the direction of activation in monolayer cultured cardiomyocytes and this effect was reversible upon wash out.*

3.4. Conclusion

APJ receptor-like immunoreactivity was detected in a transversely striated distribution associated with T-tubules as described previously and in the intercalated disc area, which is a novel finding.

In isolated perfused heart model apelin increased sarcomere shortening of isolated adult ventricular myocytes, but it's effect on isolated myocytes were transient and lasted 1-2 minutes, sarcomere shortening returned and remained at control levels for the rest of apelin superfusion. Apelin induced a transient increase in sarcomere shortening in failing cardiomyocytes too. Notably, the maximal responses to apelin in normal and failing cardiomyocytes did not differ significantly. As the underlying signaling mechanism no changes in $[Ca^{2+}]$ transients were established but an increase in the intracellular pH and activity of NHE proved that apelin may increase the myofilament sensitivity to Ca^{2+}.

This was the first attempt to establish the effect of apelin on electrophysiological properties. Apelin significantly increased conduction velocity and decreased field potential duration in spontaneously beating neonatal myocyte cultures and isochronal maps showed reversible modifications in the patterns of activation as well.

4. Adrenomedullin Regulates Cardiac Contractility via Extracellular Signal-Regulated Protein Kinase-Dependent Mechanisms

4.1. Introduction

Mitogen-activated protein kinase (MAPK) superfamily represents an evolutionarily conserved signal transduction system that occupies a central position in the regulation of cell growth, proliferation, differentiation, apoptosis, and transformation in all eukaryotic cells (Widmann et al., 1999). Extracellular signal-regulated kinases ERK1 and ERK2 (commonly referred to as ERK1/2) are members of the MAPK family. ERK1/2 signaling cascade is initiated in cardiac myocytes by activation of GPCRs, receptor tyrosine kinases, and by stress stimuli (Bueno and Molkentin, 2002; Wang, 2007). Accumulating data suggest that activation of the ERK1/2 signaling constitutes an essential adaptive mechanism int he myocardium, wherby it promotes cardiac myocyte survival in response to number of pathologic insults including ischemia-reperfusion injury (Lips et al., 2004) and long-term pressure overload (Purcell et al., 2007). Although the ERK1/2 pathway has been implicated in various pathological conditions, its exact physiological role in the heart is not yet understood.

AM with its cognate GPCR is widely expressed in the cardiovascular system and is emerging as an important regulator of cardiovascular homeostasis. AM, as an autocrine/paracrine factor, may protect the heart from pathological stress, e.g., AM inhibits maladaptive ventricular remodeling via reducing cardiomyocyte hypertrophy, apoptosis, and fibrosis (Ishimitsu et al., 2006). Moreover, AM is among the most potent stimulators of cardiac contractility. Although it has been demonstrated that the peptide acts independently of the classical adenylylcyclase-cAMP- PKA pathway (Szokodi et al., 1996; Szokodi et al., 1998), the precise underlying signaling mechanisms are not known. Previous studies have shown that AM increases ERK1/2 phosphorylation in various cell types including vascular smooth muscle cells (Iwasaki et al., 1998) and endothelial cells (Kim et al., 2003). In the present study, we tested wheather ERK1/2 signaling is activated by AM in the heart, and if so, whether it is involved in the inotropic response to AM.

4.2. Materials and Methods

4.2.1. Materials

Drugs used were AM (Phoenix Europe GmbH, Karlsruhe, Germany); AG1478 and U0126 (Merck Chemicals Ltd., Nottingham, UK); zoniporide (generously supplied by Dr Ross Tracey, Pfizer Global Research and Development, Groton, Conn). AM was dissolved in distilled water; AG1478, U0126, and zoniporide were dissolved in dimethyl sulfoxide. The final concentration of each solvent was <0.003%. The addition of an appropriate concentration of each solvent caused no significant change in haemodynamic variables.

4.2.2. Isolated Perfused Rat Heart Preparation

All protocols were reviewed and approved by the Animal Use and Care Committee of the University of Oulu and University of Pecs. Male 7-week-old Sprague-Dawley rats from the Center for Experimental Animals at the University of Oulu were used. Rats were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique as described previously (Szokodi et al., 1998; Szokodi et al., 2002; Szokodi et al., 2008; Kinnunen et al., 2000). The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer (see chapter 3.2.8.), pH 7.40, equilibrated with 95% O_2-5% CO_2 at 37°C. Hearts were perfused at a constant flow rate of 5.5 mL/min with a peristaltic pump (Minipuls 3, model 312). Heart rate was maintained constant (302±1 beats/min) by atrial pacing using a Grass stimulator (model S88, 11 V, 0.5 ms). Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (Grass Instruments, FT03) to the apex of the heart at an initial preload stretch of 2 g. Perfusion pressure reflecting coronary vascular resistance was measured by a pressure transducer (model MP-15, Micron Instruments, Los Angeles, Calif) situated on a side arm of the aortic cannula.

4.2.3. Experimental Design

A 40-minute equilibration period and a 5-minute control period were followed by addition of various drugs to the perfusate for 30 minutes. The concentrations of U0126 (1.5 µmol/L), AG1478 (1 µmol/L), and zoniporide (1 µmol/L) were selected because these concentrations have been demonstrated to suppress ERK1/2 (Tenhunen et al., 2004; Szokodi et al., 2008), epidermal growth factor receptor (EGFR) tyrosine kinase activity (Thomas et al., 2002) (Szokodi et al., 2008)

and inhibit Na^+/H^+ exchanger 1 (NHE1) (Knight et al., 2001; Szokodi et al., 2002; Szokodi et al., 2008), respectively. In the final stage of the experiments, the left ventricles were frozen in liquid nitrogen and stored at –80 ^oC until assayed.

4.2.4. Immunoblot Analysis

Westernblotting was performed as described previously (Szokodi et al., 1998). Left ventricular tissue was homogenized in lysis buffer containing of 20 mmol/L Tris, (pH 7.5), 10 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, supplemented with 1 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol (DTT), 1 mmol/L Na_3VO_4, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 2 µg/mL pepstatin, 2 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 20 mmol/L NaF. Total protein samples (30 µg) were loaded onto SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk and incubated with indicated primary antibody overnight. Protein levels were detected using enhanced chemiluminescence. For a second staining, the membranes were stripped for 30 minutes at 60 °C in stripping buffer (62.5 mmol/L Tris (pH 6.8), 2% SDS, and 100 mmol/L β-mercaptoethanol). The antibodies used were anti-phospho-ERK1/2 and anti-ERK1/2 (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK).

4.2.5. Statistical Analysis

Results are presented as mean±SEM. Two-way repeated-measures ANOVA was used to evaluate the statistical significance of differences among groups for cardiac contractility. When significant differences were detected for the treatment-by-time interactions, a Bonferroni post hoc test was used for specific comparisons. All other parameters were analyzed with 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of $P<0.05$.

4.3. Results

4.3.1. Extracellular Signal-Regulated Kinase1/2 and Adrenomedullin-Induced Positive Inotropic Effect

To define the role of ERK1/2 in the cardiac effects of AM, we determined the impact of AM stimulation on the activation of these kinases. Western analysis revealed that infusion of AM (1 nmol/l) for 30 minutes significantly increased left ventricular phospho-ERK1/2 levels (Figure 7A) in the rat heart preparation. To examine whether activation of ERK1/2 contributes to the positive inotropic action of AM, we assessed the effect of U0126, which is a potent specific inhibitor of MEK1/2, the upstream regulator of ERK (Tenhunen et al., 2004; Szokodi et al., 2008). Administration of U0126 (1.5 µmol/l) markedly reduced the levels of phospho-ERK1/2 both in the control and AM-stimulated hearts (Figure 7A). Infusing U0126 in combination with AM, the AM-induced inotropic effect decreased significantly, the maximal reduction being 40% (P<0.01; Figure 7B). Infusion of U0126 alone had no effect on contractile force (P=NS, Figure 7B).

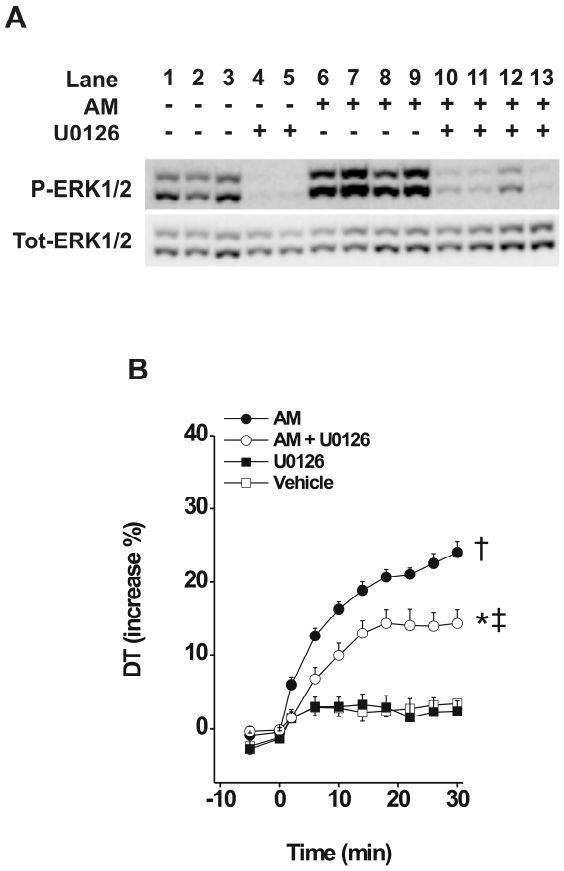

Figure 7. *ERK1/2 signaling is required for AM-mediated increase in contractility. A, Western blot analysis for ERK1/2 phosphorylation in left ventricular tissue samples. In isolated rat hearts, infusion of AM (1 nmol/l) for 30 minutes increased phospho-ERK1/2 levels and U0126 (1.5 µmol/l), a MEK1/2 inhibitor, abolished AM-induced ERK1/2 phosphorylation. B, U0126 significantly attenuated AM-enhanced contractility. DT indicates developed tension. Results are expressed as a percent change versus baseline values. Data were analyzed by 2-way repeated-measures ANOVA followed by multiple comparisons with the Bonferroni post hoc test and are reported as mean±SEM (n=4-6 for each group). *P<0.01 and †P<0.001 vs control and U0126; ‡P<0.01 vs AM.*

4.3.2. Upstream Activators of Extracellular Signal-Regulated Kinase1/2: Role of Epidermal Growth Factor Receptors

In cultured rat ventricular myocytes, agonist-stimulated ERK1/2 phosphorylation can occur via transactivation of EGFR (Thomas et al., 2002). To define the importance of EGFRs in ERK1/2 activation in the adult rat heart, we used a specific EGFR tyrosine kinase inhibitor AG1478 (Szokodi et al., 2008; Thomas et al., 2002). AG1478 (1 µmol/L) significantly reduced AM-induced increase in phospho-ERK1/2 levels (Figure 8A). Moreover, in the presence of AG1478 the inotropic response to AM was significantly suppressed, the maximal reduction being 45% (P<0.001; Figure 8B). Infusion of AG1478 alone had no effect on developed tension (P=NS, Figure 8B).

4.3.3. Downstream Targets of Extracellular Signal-Regulated Kinase1/2: Role of Na^+/H^+ Exchanger

The ERK1/2 pathway has been identified as the main regulator of NHE-1 phosphorylation in cardiac myocytes (Moor and Fliegel, 1999). To assess the contribution of NHE-1 to the effect of AM, we used zoniporide, a potent and selective inhibitor of NHE-1 (Knight et al., 2001; Szokodi et al., 2002; Szokodi et al., 2008). Infusion of zoniporide (1 µmol/L) alone had no effect on contractile force (P=NS; Figure 9). When given together with AM, zoniporide significantly attenuated the AM-induced positive inotropic effect, the maximal reduction being 46% (P<0.001; Figure 9).

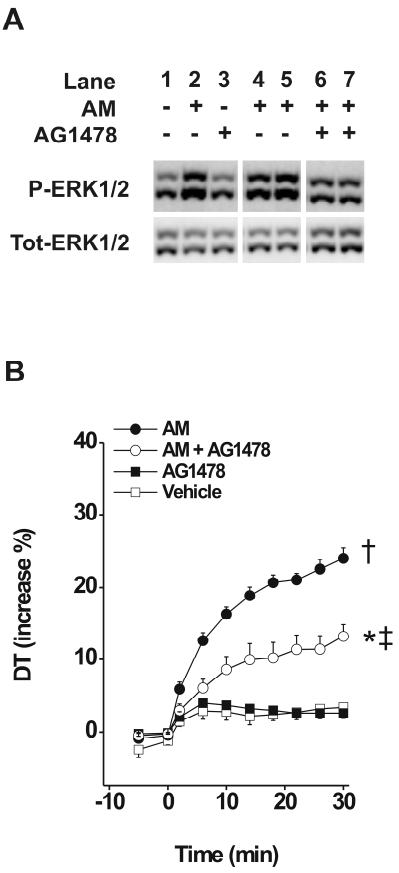

Figure 8. *EGFR transactivation contributes to AM-mediated increase in contractility. A, Western blot analysis shows that AG1478 (1 µmol/l), an EGFR tyrosine kinase inhibitor, reduced AM-induced increases in ERK1/2 phosphorylation. B, AG1478 reduced AM-induced increase in contractility. Data are mean±SEM (n=4-6 for each group). *P<0.01 and †P<0.001 vs control and AG1478; ‡P<0.001 vs AM.*

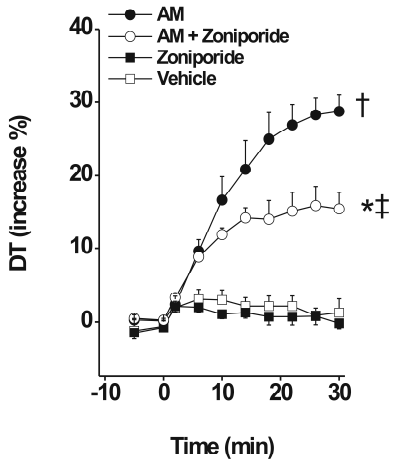

Figure 9. *NHE1 is involved in the inotropic response to AM. Zoniporide (1 µmol/l), a NHE1 inhibitor, attenuated AM-induced increase in contractility. Data are mean±SEM (n=4-6 for each group). *P<0.01 and †P<0.001 vs control and Zoniporide; ‡P<0.001 vs AM.*

4.4. Conclusion

Our data are the first to demonstrate that AM increases cardiac contractility via activation of ERK1/2 in the intact adult rat heart. Moreover, our results show that EGFR acts as the upstream regulator and NHE-1 as the downstream effector of ERK1/2 in AM signaling. Identification of novel signaling pathways that promote cardiomyocyte survival while improving contractile function may offer an attractive approach of treating patients with heart failure. Therefore, further studies are required to test the hypothesis that activation of MEK1/2–ERK1/2 signaling, possessing such beneficial effects, can eventually rescue the failing heart.

5. Discussion

5.1. Direct Effect of Apelin on Cardiomyocyte Contractility and Electrophysiological Properties

The cellular mechanisms underlying the *ex vivo* (Szokodi et al., 2002) and *in vivo* effects of apelin on left ventricular function (Berry et al., 2004; Ashley et al., 2005) required further investigation. The present study provides the first direct evidence for a positive inotropic effect of apelin in adult ventricular myocytes. Our results suggest that the positive inotropic effect of apelin is due to stimulation of the sarcolemmal NHE, leading to intracellular alkalinization and, possibly, increased myofilament sensitivity to Ca^{2+}. In contrast to previous studies in intact hearts (Szokodi et al., 2002; Berry et al., 2004; Ashley et al., 2005) where apelin induced a sustained increase in contractility, our investigation highlights the fact that apelin induced a transient increase in contractility in cardiomyocytes, suggesting that additional mechanisms are present in the whole tissue. Furthermore, our data define a previously unrecognized role of apelin in the regulation of cardiac conduction as apelin increases conduction velocity in monolayers of cultured neonatal rat cardiomyocytes.

5.1.1. Apelin and Cardiomyocyte Contractility

Apelin induced a transient increase in sarcomere shortening in adult rat cardiomyocytes, which was not accompanied by changes in cytoplasmic Ca^{2+} transients. Charo et al. confirmed our findings in apelin-APJ double knock out mice model(Charo et al., 2009). Since the NHE has been indicated as a target for apelin (Hosoya et al., 2000) and changes in intracellular pH strongly shift the $[Ca^{2+}]$-contractility curve in cardiac tissue (Kohmoto et al., 1990), we investigated the effects of apelin on intracellular pH and on NHE activity. We found that apelin increased pH and NHE activity in cardiomyocytes. Moreover, confocal immunofluorescence microscopic imaging showed that APJ is localized in the intercalated disc regions, which accommodate NHE as well (Snabaitis et al., 2006). Taking previous studies into consideration demonstrating that the apelin-induced increase in contractility was significantly attenuated by a specific inhibitor of NHE in isolated perfused rat hearts (Szokodi et al., 2002), the present data indicate that intracellular alkalinization with subsequent sensitization of cardiac myofilaments to $[Ca^{2+}]$ can be involved in the inotropic effect of apelin.

Dai et al showed that at higher concentrations of apelin right ventricular failing trabeculae increased the developed tension compared with non-failing trabeculae (Dai et al., 2006). They proposed that apelin had a predominant role in regulating cardiac contractility in the failing myocardium. We investigated the hypothesis that the increased apelin-induced inotropy in heart failure is brought about by augmented effects on cardiac myocytes. However, in our study, despite a transient increase in sarcomere shortening, there was no additional effect of apelin on failing compared with normal cardiomyocytes.

Numerous studies have demonstrated the effect of exogenous apelin in the regulation of contractility, but only the recently published data reveal the importance of the endogenous apelin-APJ pathway. Apelin and APJ knock out mice were examined. Under basal condition both of them manifested modest decrements in contractile function while with exercise stress both mutant lines demonstrated consistent and striking decrease in exercise capacity (Charo et al., 2009).

An intriguing finding of this study is the lack of a sustained effect of apelin on cell contractility. This is in obvious contrast with previous observations in intact hearts, where apelin possessed a slowly developing but sustained inotropic response (Szokodi et al., 2002; Berry et al., 2004; Ashley et al., 2005). In isolated isovolumic rat hearts apelin enhanced preload-induced increase in dP/dt_{max} only at higher levels of left ventricular end-diastolic pressure, suggesting that the peptide augments cardiac contractility along the upper part of the ascending limb of the Starling relation (Szokodi et al., 2002). If mechanical load is crucial in determining the effects of apelin, isolated unloaded cardiomyocytes would have a limited inotropic response upon application of apelin. Another explanation may be the increased sodium current due to the apelin infusion. Chamberland et al. demonstrated that apelin increases cardiac sodium current whithin 5 minutes of perfusion and reached steady state at 20 minutes (Chamberland et al., 2010). Thus; these results may suggest that increased NHE activity and enhanced cardiac sodium current underlie the positive inotropic response to apelin.

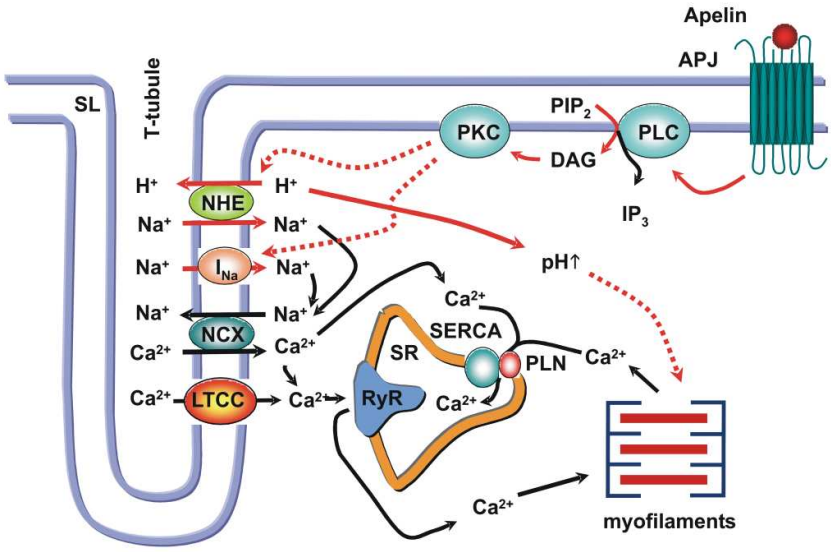

***Figure 10**. Putative signaling mechanisms of apelin in the heart. Stimulation of APJ by apelin evokes phosphorylation of PKC leading to activation of NHE, NCX and Na^+ channels. The positive inotropic effect of apelin is the result of sensitization of cardiac myofilaments to Ca^{2+} due to intracellular alkalosis and increased Ca^{2+} influx through the NCX operating in reverse mode (Chamberland et al., 2010; Szokodi et al., 2002).*

5.1.2. Apelin and Electrical Conduction

Our results demonstrate that apelin caused an increase in the frequency of spontaneous activation, conduction velocity and a decrease of the field potential duration in monolayers of cultured neonatal cardiomyocytes. The underlying mechanism, explained by Chamberland et al in2009, is due to the increase in cardiac sodium current by apelin, which accelerates the initial depolarization of ventricular action potential resulting in increased excitability of cardiac cells (Chamberland et al., 2010). The localization of APJ receptor in the intercalated disc region, the cellular structure involved in the electric coupling between cardiomyocytes, further supports the hypothesis that apelin may play an important role in intercellular communication. The effects of apelin on electrophysiological properties of cardiac tissue may explain the changes in APJ-apelin system observed in chronic arrhythmias and after cardiac resynchronization therapy (Ellinor et al., 2006). The specific role of apelin in regulating cardiac electrophysiology needs to be investigated further.

5.1.3. Summary

We have provided the first evidence that apelin has direct effects on the propagation of action potential and contractility in normal cardiomyocytes. The latter, however, possibly mediated by action on the NHE, is transitory and apelin may require other mediators and/or mechanical loading to exert its lasting inotropic action.

5.2 The Role of Adrenomedullin in the Regulation of Cardiac Contractility

Considerable evidence suggests that AM acts an autocrine or paracrine factor in regulating cardiac contractility. AM has been considered to be among the most potent endogenous positive inotropic agent, however the literature data are controversial and the underlying intracellular signaling mechanisms are still discussed.

5.2.1. Signaling Mechanism of Adrenomedullin

Experimental data suggests that activation of the adenylyl cyclase-cAMP system, which is one of the major pathways for regulation of cardiac contractility, may also mediate the cardiac effect of AM. High doses of AM augmented cardiac contractility in association with increased cAMP formation and inhibition of PKA could abolish the positive inotropic effect of the peptide in rat papillary muscles (Ihara et al., 2000). However, other experimental data indicate that cAMP is not the major second messenger of the inotropic effect of AM at physiologically more relevant concentrations. First, AM failed to to increase left ventricular cAMP content in perfused rat hearts. Second, PKA inhibition did not reduce the positive inotropic effect of AM. Finally, the response to AM could not be enhanced in the presence of phosphodiesterase inhibitor. In contrast of the cAMP mediated pathway the importance of PKC activation in the positive inotropic effect of AM was mentioned by Szokodi et al. (Szokodi et al., 1998). Activated PKC can phosphorylate a wide spectrum of cellular proteins, including L-type Ca^{2+} channel resulting in increased Ca^{2+} influx. Taken together the literature data, the signaling pathway of positive inotropic effect of AM is only partially understood and it indicates further research to find alternative signaling mechanisms.

5.2.2. Alternative Intracellular Signal Transduction Pathways

Growing body of evidence suggest that activation of the MEK1/2–ERK1/2 pathway protects the heart from various pathological insults. ERK1/2 signaling has been reported to afford cardioprotection in vivo against ischemia–reperfusion injury by reducing myocyte apoptosis (Lips et al., 2004). Recently, the requirement of ERK1/2 signaling in stress adaptation has been directly addressed using *Erk1*$^{-/-}$ and *Erk2*$^{+/-}$ mice, as well as transgenic mice with inducible expression of an ERK1/2–inactivating phosphatase in the heart (dual-specificity phosphatase 6). Although the hypertrophic response is not affected in these models after long-term pressure overload, mice with selective ablation of cardiac ERK1/2 signaling show greater

propensity towards heart failure through increased myocyte apoptosis (Purcell et al., 2007). Moreover, genetic deletion of type 5 adenylyl cyclase results in the upregulation of the MEK1/2–ERK1/2 pathway, which in turn protects the heart from aging-induced cardiomyopathy in terms of preservation of left ventricular function and resistance to myocyte apoptosis (Yan et al., 2007). Though systolic function is clearly affected in these transgenic models, it has only been established recently that ERK1/2 can directly modulate cardiac contractility. Our recent studies have provided evidence for the functional importance of ERK1/2 signaling in the acute regulation of cardiac contractility by showing that endothelin-1 increases contractile force via the ERK1/2 pathway (Szokodi et al., 1998). In the present study, the GPCR agonist AM produced a significant increase in LV phospho-ERK1/2 levels, and pharmacological inhibition of ERK1/2 activation markedly attenuated the AM–induced increase in contractile force in the intact rat heart. Previously we have demonstrated that the positive inotropic response to AM is independent of the adenylyl cyclase–cAMP–PKA pathway (Szokodi et al., 1998), and our current data indicate that ERK1/2 signaling serves as a key mediator of the inotropic effect of AM. While prolonged stimulation of the adenylyl cyclase–cAMP–PKA cascade leads to serious adverse cardiac effects, activation of the MEK1/2–ERK1/2 pathway may enhance both cardiac contractility and overall stress resistance of the myocardium.

Transactivation of EGFR has been established as a major mechanism for GPCR agonists to activate ERK1/2 (Thomas et al., 2002). Stimulation of GPCRs induces metalloproteinase-mediated ectodomain shedding of membrane-anchored proheparin-binding EGF. Soluble heparin-binding EGF then binds to and activates EGFR, triggering MEK1/2–ERK1/2 phosphorylation (Wetzker and Bohmer, 2003). Notably, pharmacological inhibition of EGFR by erlotinib provokes dilated cardiomyopathy with reduced cardiac function in the face of chronic β-adrenergic stimulation (Noma et al., 2007). Recently, we have found that transactivation of EGFR is a critical step for endothelin-1 to enhance cardiac contractility via the MEK1/2–ERK1/2 cascade (Szokodi et al., 2008). In line with these observations, inhibition of EGFR transactivation by the specific EGFR tyrosine kinase inhibitor AG1478 was accompanied by significant attenuation of AM–induced increase in phospho-ERK1/2 levels as well as the inotropic response to AM. Thus, the present data highlight the importance of EGFR in the regulation of myocardial contractility acting as an upstream signaling molecule modulating MEK1/2–ERK1/2 cascade.

Activated ERK1/2 can phosphorylate various cellular proteins including the sarcolemmal NHE1 (Moor and Fliegel, 1999). In the present study, zoniporide, a

highly selective inhibitor of NHE1, attenuated the inotropic response to AM suggesting that NHE-1 may serve as a downstream effector of ERK1/2 signaling. Stimulation of NHE1 can lead to intracellular alkalinization and sensitization of cardiac myofilaments to intracellular Ca^{2+}. On the other hand, NHE-1-mediated accumulation of intracellular Na^{+} can indirectly promote a rise in intracellular levels of Ca^{2+} via reverse mode NCX exchanger (Kentish, 1999). The finding that ≈50% of the AM–induced positive inotropic effect remained unaffected after inhibition of the EGFR–ERK1/2–NHE-1 pathway indicates the existence of additional signaling mechanisms such as PKC (Szokodi et al., 1998) or phosphoinositide 3-kinase (Kim et al., 2003).

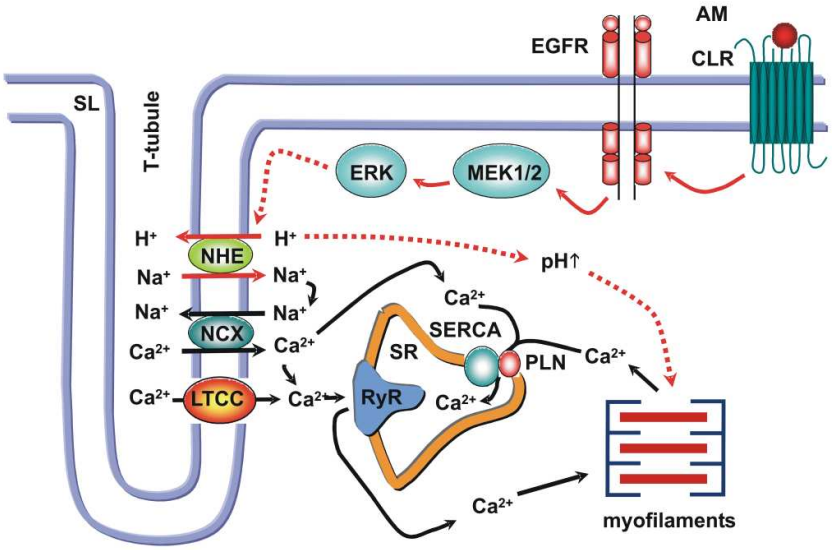

Figure 11. *Putative signaling mechanisms activated by adrenomedullin. ERK1/2 signaling serves as a key mediator of the inotropic effect of AM. EGFR acts as the upstream regulator and NHE-1 as the downstream effector of ERK1/2 in AM signaling.*

5.2.3. Summary

Our data are the first to demonstrate that AM increases cardiac contractility via activation of ERK1/2 in the intact adult rat heart. Moreover, our results show that EGFR acts as the upstream regulator and NHE-1 as the downstream effector of ERK1/2 in AM signaling. Identification of novel signaling pathways that promote cardiomyocyte survival while improving contractile function may offer an attractive approach of treating patients with heart failure. On the other hand, MEK1/2–ERK1/2 signaling is activated in most human tumors, and clinical trials of specific MEK1/2 inhibitors are underway (Sebolt-Leopold and Herrera, 2004). Our results raise the possibility that cancer therapies that target the MEK1/2–ERK1/2 pathway might cause adverse cardiac side effects.

6. Novel Findings

- Apelin increases sarcomere shortening transiently in normal and failing isolated adult ventricular myocytes.

- Apelin has no effect on the amplitude or time course of [Ca^{2+}] transients.

- Apelin increases myofilament sensitivity to Ca^{2+} due to the stimulation of sarcolemmal NHE.

- APJ receptor is localised in the intercalated disc region.

- Apelin influences the electrophysiological properties in monolayer of cultured neonatal rat cardiomyocytes.

- AM increases cardiac contractility via activation of ERK1/2 in the intact adult rat heart.

- EGFR acts as the upstream regulator and NHE1 as the downstream effector of ERK1/2 in AM signaling.

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8. References

1. Ashley,E.A., Powers,J., Chen,M., Kundu,R., Finsterbach,T., Caffarelli,A., Deng,A., Eichhorn,J., Mahajan,R., Agrawal,R., Greve,J., Robbins,R., Patterson,A.J., Bernstein,D., and Quertermous,T. (2005). The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. Cardiovasc. Res. *65*, 73-82.

2. Avkiran,M. and Haworth,R.S. (2003). Regulatory effects of G protein-coupled receptors on cardiac sarcolemmal Na^+/H^+ exchanger activity: signalling and significance. Cardiovasc. Res. *57*, 942-952.

3. Berry,M.F., Pirolli,T.J., Jayasankar,V., Burdick,J., Morine,K.J., Gardner,T.J., and Woo,Y.J. (2004). Apelin has in vivo inotropic effects on normal and failing hearts. Circulation *110*, II187-II193.

4. Bers,D.M. (2002). Cardiac excitation-contraction coupling. Nature *415*, 198-205.

5. Bers,D.M., Li,L., Satoh,H., and McCall,E. (1998). Factors that control sarcoplasmic reticulum calcium release in intact ventricular myocytes. Ann. N. Y. Acad. Sci. *853*, 157-177.

6. Beuckelmann,D.J., Nabauer,M., and Erdmann,E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. Circulation *85*, 1046-1055.

7. Boucher,J., Masri,B., Daviaud,D., Gesta,S., Guigne,C., Mazzucotelli,A., Castan-Laurell,I., Tack,I., Knibiehler,B., Carpene,C., Audigier,Y., Saulnier-Blache,J.S., and Valet,P. (2005). Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology *146*, 1764-1771.

8. Bowditch,H.P. (1878). Does the Apex of the Heart contract automatically? J. Physiol *1*, 104-107.

9. Boyarsky,G., Ganz,M.B., Sterzel,R.B., and Boron,W.F. (1988). pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO3-. Am. J. Physiol *255*, C844-C856.

10. Braz,J.C., Bueno,O.F., De Windt,L.J., and Molkentin,J.D. (2002). PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). J. Cell Biol. *156*, 905-919.

11. Bristow,M.R., Ginsburg,R., Umans,V., Fowler,M., Minobe,W., Rasmussen,R., Zera,P., Menlove,R., Shah,P., Jamieson,S., and . (1986). Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium:

coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. Circ. Res. *59*, 297-309.

12. Brutsaert,D.L. (2003). Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. Physiol Rev. *83*, 59-115.

13. Bueno,O.F. and Molkentin,J.D. (2002). Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. Circ. Res. *91*, 776-781.

14. Burrell,L.M., Johnston,C.I., Tikellis,C., and Cooper,M.E. (2004). ACE2, a new regulator of the renin-angiotensin system. Trends Endocrinol. Metab *15*, 166-169.

15. Chamberland,C., Barajas-Martinez,H., Haufe,V., Fecteau,M.H., Delabre,J.F., Burashnikov,A., Antzelevitch,C., Lesur,O., Chraibi,A., Sarret,P., and Dumaine,R. (2010). Modulation of canine cardiac sodium current by Apelin. J. Mol. Cell Cardiol. *48*, 694-701.

16. Charles,C.J., Rademaker,M.T., and Richards,A.M. (2006). Apelin-13 induces a biphasic haemodynamic response and hormonal activation in normal conscious sheep. J. Endocrinol. *189*, 701-710.

17. Charo,D.N., Ho,M., Fajardo,G., Kawana,M., Kundu,R.K., Sheikh,A.Y., Finsterbach,T.P., Leeper,N.J., Ernst,K.V., Chen,M.M., Ho,Y.D., Chun,H.J., Bernstein,D., Ashley,E.A., and Quertermous,T. (2009). Endogenous regulation of cardiovascular function by apelin-APJ. Am. J. Physiol Heart Circ. Physiol *297*, H1904-H1913.

18. Chen,M.M., Ashley,E.A., Deng,D.X., Tsalenko,A., Deng,A., Tabibiazar,R., Ben-Dor,A., Fenster,B., Yang,E., King,J.Y., Fowler,M., Robbins,R., Johnson,F.L., Bruhn,L., McDonagh,T., Dargie,H., Yakhini,Z., Tsao,P.S., and Quertermous,T. (2003). Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. Circulation *108*, 1432-1439.

19. Chini,E.N., Chini,C.C., Bolliger,C., Jougasaki,M., Grande,J.P., Burnett,J.C., Jr., and Dousa,T.P. (1997). Cytoprotective effects of adrenomedullin in glomerular cell injury: central role of cAMP signaling pathway. Kidney Int. *52*, 917-925.

20. Chini,E.N., Choi,E., Grande,J.P., Burnett,J.C., and Dousa,T.P. (1995). Adrenomedullin suppresses mitogenesis in rat mesangial cells via cAMP pathway. Biochem. Biophys. Res. Commun. *215*, 868-873.

21. Chong,K.S., Gardner,R.S., Morton,J.J., Ashley,E.A., and McDonagh,T.A. (2006). Plasma concentrations of the novel peptide apelin are decreased in patients with chronic heart failure. Eur. J. Heart Fail. *8*, 355-360.

22. Chun,T.H., Itoh,H., Ogawa,Y., Tamura,N., Takaya,K., Igaki,T., Yamashita,J., Doi,K., Inoue,M., Masatsugu,K., Korenaga,R., Ando,J., and Nakao,K. (1997). Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. Hypertension *29*, 1296-1302.

23. Cingolani,H.E., Alvarez,B.V., Ennis,I.L., and Camilion de Hurtado,M.C. (1998). Stretch-induced alkalinization of feline papillary muscle: an autocrine-paracrine system. Circ. Res. *83*, 775-780.

24. Cohn,J.N., Ferrari,R., and Sharpe,N. (2000). Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J. Am. Coll. Cardiol. *35*, 569-582.

25. Dai,T., Ramirez-Correa,G., and Gao,W.D. (2006). Apelin increases contractility in failing cardiac muscle. Eur. J. Pharmacol. *553*, 222-228.

26. Eguchi,S., Hirata,Y., Kano,H., Sato,K., Watanabe,Y., Watanabe,T.X., Nakajima,K., Sakakibara,S., and Marumo,F. (1994). Specific receptors for adrenomedullin in cultured rat vascular smooth muscle cells. FEBS Lett. *340*, 226-230.

27. Ellinor,P.T., Low,A.F., and MacRae,C.A. (2006). Reduced apelin levels in lone atrial fibrillation. Eur. Heart J. *27*, 222-226.

28. Fabiato,A. and Fabiato,F. (1979). Calcium and cardiac excitation-contraction coupling. Annu. Rev. Physiol *41*, 473-484.

29. Feng,C.J., Kang,B., Kaye,A.D., Kadowitz,P.J., and Nossaman,B.D. (1994). L-NAME modulates responses to adrenomedullin in the hindquarters vascular bed of the rat. Life Sci. *55*, L433-L438.

30. Foldes,G., Horkay,F., Szokodi,I., Vuolteenaho,O., Ilves,M., Lindstedt,K.A., Mayranpaa,M., Sarman,B., Seres,L., Skoumal,R., Lako-Futo,Z., deChatel,R., Ruskoaho,H., and Toth,M. (2003). Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. Biochem. Biophys. Res. Commun. *308*, 480-485.

31. Francia,P., Salvati,A., Balla,C., De,P.P., Pagannone,E., Borro,M., Gentile,G., Simmaco,M., De,B.L., and Volpe,M. (2006). Cardiac resynchronization therapy increases plasma levels of the endogenous inotrope apelin. Eur. J. Heart Fail.

32. Fuchs,F. and Martyn,D.A. (2005). Length-dependent Ca(2+) activation in cardiac muscle: some remaining questions. J. Muscle Res. Cell Motil. *26*, 199-212.

33. Fukushima,H., Kobayashi,N., Takeshima,H., Koguchi,W., and Ishimitsu,T. (2010). Effects of olmesartan on Apelin/APJ and Akt/endothelial nitric oxide synthase pathway in Dahl rats with end-stage heart failure. J. Cardiovasc. Pharmacol. *55*, 83-88.

34. Hamid,S.A. and Baxter,G.F. (2006). A critical cytoprotective role of endogenous adrenomedullin in acute myocardial infarction. J. Mol. Cell Cardiol. *41*, 360-363.

35. Hamid,S.A. and Baxter,G.F. (2005). Adrenomedullin: regulator of systemic and cardiac homeostasis in acute myocardial infarction. Pharmacol. Ther. *105*, 95-112.

36. He,H., Bessho,H., Fujisawa,Y., Horiuchi,K., Tomohiro,A., Kita,T., Aki,Y., Kimura,S., Tamaki,T., and Abe,Y. (1995). Effects of a synthetic rat adrenomedullin on regional hemodynamics in rats. Eur. J. Pharmacol. *273*, 209-214.

37. Heinonen,M.V., Purhonen,A.K., Miettinen,P., Paakkonen,M., Pirinen,E., Alhava,E., Akerman,K., and Herzig,K.H. (2005). Apelin, orexin-A and leptin plasma levels in morbid obesity and effect of gastric banding. Regul. Pept. *130*, 7-13.

38. Higuchi,K., Masaki,T., Gotoh,K., Chiba,S., Katsuragi,I., Tanaka,K., Kakuma,T., and Yoshimatsu,H. (2007). Apelin, an APJ receptor ligand, regulates body adiposity and favors the messenger ribonucleic acid expression of uncoupling proteins in mice. Endocrinology *148*, 2690-2697.

39. Hinson,J.P., Kapas,S., and Smith,D.M. (2000). Adrenomedullin, a multifunctional regulatory peptide. Endocr. Rev. *21*, 138-167.

40. Hobai,I.A. and Levi,A.J. (1999). Coming full circle: membrane potential, sarcolemmal calcium influx and excitation-contraction coupling in heart muscle. Cardiovasc. Res. *44*, 477-487.

41. Hosoya,M., Kawamata,Y., Fukusumi,S., Fujii,R., Habata,Y., Hinuma,S., Kitada,C., Honda,S., Kurokawa,T., Onda,H., Nishimura,O., and Fujino,M. (2000). Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. J. Biol. Chem. *275*, 21061-21067.

42. Hunt,S.A., Abraham,W.T., Chin,M.H., Feldman,A.M., Francis,G.S., Ganiats,T.G., Jessup,M., Konstam,M.A., Mancini,D.M., Michl,K., Oates,J.A., Rahko,P.S., Silver,M.A., Stevenson,L.W., Yancy,C.W., Antman,E.M., Smith,S.C., Jr., Adams,C.D., Anderson,J.L., Faxon,D.P., Fuster,V., Halperin,J.L., Hiratzka,L.F., Jacobs,A.K., Nishimura,R., Ornato,J.P., Page,R.L., and Riegel,B. (2005). ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation *112*, e154-e235.

43. Huser,J., Bers,D.M., and Blatter,L.A. (1998). Subcellular properties of [Ca2+]i transients in phospholamban-deficient mouse ventricular cells. Am. J. Physiol *274*, H1800-H1811.

44. Ihara,T., Ikeda,U., Tate,Y., Ishibashi,S., and Shimada,K. (2000). Positive inotropic effects of adrenomedullin on rat papillary muscle. Eur. J. Pharmacol. *390*, 167-172.

45. Ikenouchi,H., Kangawa,K., Matsuo,H., and Hirata,Y. (1997). Negative inotropic effect of adrenomedullin in isolated adult rabbit cardiac ventricular myocytes. Circulation *95*, 2318-2324.

46. Ishida,J., Hashimoto,T., Hashimoto,Y., Nishiwaki,S., Iguchi,T., Harada,S., Sugaya,T., Matsuzaki,H., Yamamoto,R., Shiota,N., Okunishi,H., Kihara,M., Umemura,S., Sugiyama,F., Yagami,K., Kasuya,Y., Mochizuki,N., and Fukamizu,A. (2004). Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. J. Biol. Chem. *279*, 26274-26279.

47. Ishimitsu,T., Ono,H., Minami,J., and Matsuoka,H. (2006). Pathophysiologic and therapeutic implications of adrenomedullin in cardiovascular disorders. Pharmacol. Ther. *111*, 909-927.

48. Ishizaka,Y., Ishizaka,Y., Tanaka,M., Kitamura,K., Kangawa,K., Minamino,N., Matsuo,H., and Eto,T. (1994). Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. Biochem. Biophys. Res. Commun. *200*, 642-646.

49. Iwanaga,Y., Kihara,Y., Takenaka,H., and Kita,T. (2006). Down-regulation of cardiac apelin system in hypertrophied and failing hearts: Possible role of angiotensin II-angiotensin type 1 receptor system. J. Mol. Cell Cardiol. *41*, 798-806.

50. Iwasaki,H., Eguchi,S., Shichiri,M., Marumo,F., and Hirata,Y. (1998). Adrenomedullin as a novel growth-promoting factor for cultured vascular smooth muscle cells: role of tyrosine kinase-mediated mitogen-activated protein kinase activation. Endocrinology *139*, 3432-3441.

51. Jia,Y.X., Pan,C.S., Zhang,J., Geng,B., Zhao,J., Gerns,H., Yang,J., Chang,J.K., Tang,C.S., and Qi,Y.F. (2006). Apelin protects myocardial injury induced by isoproterenol in rats. Regul. Pept. *133*, 147-154.

52. Jougasaki,M., Wei,C.M., Heublein,D.M., Sandberg,S.M., and Burnett,J.C., Jr. (1995). Immunohistochemical localization of adrenomedullin in canine heart and aorta. Peptides *16*, 773-775.

53. Kagiyama,S., Fukuhara,M., Matsumura,K., Lin,Y., Fujii,K., and Iida,M. (2005). Central and peripheral cardiovascular actions of apelin in conscious rats. Regul. Pept. *125*, 55-59.

54. Kallergis,E.M., Manios,E.G., Kanoupakis,E.M., Mavrakis,H.E., Goudis,C.A., Maliaraki,N.E., Saloustros,I.G., Milathianaki,M.E., Chlouverakis,G.I., and Vardas,P.E. (2010). Effect of sinus rhythm restoration after electrical cardioversion on apelin and brain natriuretic Peptide prohormone levels in patients with persistent atrial fibrillation. Am. J. Cardiol. *105*, 90-94.

55. Kang,M. and Walker,J.W. (2006). Endothelin-1 and PKC induce positive inotropy without affecting pHi in ventricular myocytes. Exp. Biol. Med. (Maywood.) *231*, 865-870.

56. Kano,H., Kohno,M., Yasunari,K., Yokokawa,K., Horio,T., Ikeda,M., Minami,M., Hanehira,T., Takeda,T., and Yoshikawa,J. (1996). Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. J. Hypertens. *14*, 209-213.

57. Kappert,K., Kusserow,H., and Unger,T. (2008). The pharmacological rationale behind polypharmacy in heart failure. Heart Fail. Monit. *6*, 20-27.

58. Karmazyn,M., Gan,X.T., Humphreys,R.A., Yoshida,H., and Kusumoto,K. (1999). The myocardial Na(+)-H(+) exchange: structure, regulation, and its role in heart disease. Circ. Res. *85*, 777-786.

59. Katoh,F., Niina,H., Kitamura,K., Ichiki,Y., Yamamoto,Y., Kangawa,K., Eto,T., and Wada,A. (1994). Ca(2+)-dependent cosecretion of adrenomedullin and catecholamines mediated by nicotinic receptors in bovine cultured adrenal medullary cells. FEBS Lett. *348*, 61-64.

60. Katz,A.M. (2002). Ernest Henry Starling, his predecessors, and the "Law of the Heart". Circulation *106*, 2986-2992.

61. Katz,A.M. (1990). Interplay between inotropic and lusitropic effects of cyclic adenosine monophosphate on the myocardial cell. Circulation *82*, I7-11.

62. Kawai,J., Ando,K., Tojo,A., Shimosawa,T., Takahashi,K., Onozato,M.L., Yamasaki,M., Ogita,T., Nakaoka,T., and Fujita,T. (2004). Endogenous adrenomedullin protects against vascular response to injury in mice. Circulation *109*, 1147-1153.

63. Kentish,J.C. (1999). A role for the sarcolemmal Na(+)/H(+) exchanger in the slow force response to myocardial stretch. Circ. Res. *85*, 658-660.

64. Kim,W., Moon,S.O., Sung,M.J., Kim,S.H., Lee,S., So,J.N., and Park,S.K. (2003). Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. FASEB J. *17*, 1937-1939.

65. Kinnunen,P., Piuhola,J., Ruskoaho,H., and Szokodi,I. (2001). AM reverses pressor response to ET-1 independently of NO in rat coronary circulation. Am. J Physiol Heart Circ. Physiol *281*, H1178-H1183.

66. Kinnunen,P., Szokodi,I., Nicholls,M.G., and Ruskoaho,H. (2000). Impact of NO on ET-1- and AM-induced inotropic responses: potentiation by combined administration. Am. J Physiol Regul. Integr. Comp Physiol *279*, R569-R575.

67. Kitamura,K., Kangawa,K., Kawamoto,M., Ichiki,Y., Nakamura,S., Matsuo,H., and Eto,T. (1993a). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. *192*, 553-560.

68. Kitamura,K., Kangawa,K., Kojima,M., Ichiki,Y., Matsuo,H., and Eto,T. (1994). Complete amino acid sequence of porcine adrenomedullin and cloning of cDNA encoding its precursor. FEBS Lett. *338*, 306-310.

69. Kitamura,K., Sakata,J., Kangawa,K., Kojima,M., Matsuo,H., and Eto,T. (1993b). Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. Biochem. Biophys. Res. Commun. *194*, 720-725.

70. Kleinz,M.J. and Baxter,G.F. (2008). Apelin reduces myocardial reperfusion injury independently of PI3K/Akt and P70S6 kinase. Regul. Pept. *146*, 271-277.

71. Kleinz,M.J. and Davenport,A.P. (2005). Emerging roles of apelin in biology and medicine. Pharmacol. Ther. *107*, 198-211.

72. Kleinz,M.J. and Davenport,A.P. (2004). Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells. Regul. Pept. *118*, 119-125.

73. Kleinz,M.J., Skepper,J.N., and Davenport,A.P. (2005). Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. Regul. Pept. *126*, 233-240.

74. Knight,D.R., Smith,A.H., Flynn,D.M., MacAndrew,J.T., Ellery,S.S., Kong,J.X., Marala,R.B., Wester,R.T., Guzman-Perez,A., Hill,R.J., Magee,W.P., and Tracey,W.R. (2001). A novel sodium-hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J. Pharmacol. Exp. Ther. *297*, 254-259.

75. Knowlton,F.P. and Starling,E.H. (1912). The Independence of variations in termperature and blood pressure on performance of the isolated mammalian heart. J. Physiol *44*, 206-219.

76. Kohmoto,O., Spitzer,K.W., Movsesian,M.A., and Barry,W.H. (1990). Effects of intracellular acidosis on [Ca2+]i transients, transsarcolemmal Ca2+ fluxes, and contraction in ventricular myocytes. Circ. Res. *66*, 622-632.

77. Kohno,M., Kano,H., Horio,T., Yokokawa,K., Yasunari,K., and Takeda,T. (1995). Inhibition of endothelin production by adrenomedullin in vascular smooth muscle cells. Hypertension *25*, 1185-1190.

78. Koss,K.L. and Kranias,E.G. (1996). Phospholamban: a prominent regulator of myocardial contractility. Circ. Res. *79*, 1059-1063.

79. Kuba,K., Zhang,L., Imai,Y., Arab,S., Chen,M., Maekawa,Y., Leschnik,M., Leibbrandt,A., Markovic,M., Schwaighofer,J., Beetz,N., Musialek,R., Neely,G.G., Komnenovic,V., Kolm,U., Metzler,B., Ricci,R., Hara,H., Meixner,A., Nghiem,M., Chen,X., Dawood,F., Wong,K.M., Sarao,R., Cukerman,E., Kimura,A., Hein,L., Thalhammer,J., Liu,P.P., and Penninger,J.M. (2007). Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload. Circ. Res. *101*, e32-e42.

80. Layland,J., Grieve,D.J., Cave,A.C., Sparks,E., Solaro,R.J., and Shah,A.M. (2004). Essential role of troponin I in the positive inotropic response to isoprenaline in mouse hearts contracting auxotonically. J. Physiol *556*, 835-847.

81. Lee,D.K., Cheng,R., Nguyen,T., Fan,T., Kariyawasam,A.P., Liu,Y., Osmond,D.H., George,S.R., and O'Dowd,B.F. (2000). Characterization of apelin, the ligand for the APJ receptor. J. Neurochem. *74*, 34-41.

82. Lee,D.K., George,S.R., and O'Dowd,B.F. (2006). Unravelling the roles of the apelin system: prospective therapeutic applications in heart failure and obesity. Trends Pharmacol. Sci. *27*, 190-194.

83. Lips,D.J., Bueno,O.F., Wilkins,B.J., Purcell,N.H., Kaiser,R.A., Lorenz,J.N., Voisin,L., Saba-El-Leil,M.K., Meloche,S., Pouyssegur,J., Pages,G., De Windt,L.J., Doevendans,P.A., and Molkentin,J.D. (2004). MEK1-ERK2 signaling pathway protects myocardium from ischemic injury in vivo. Circulation *109*, 1938-1941.

84. Maclennan,D.H. and Kranias,E.G. (2003). Phospholamban: a crucial regulator of cardiac contractility. Nat. Rev. Mol. Cell Biol. *4*, 566-577.

85. Maguire,J.J., Kleinz,M.J., Pitkin,S.L., and Davenport,A.P. (2009). [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. Hypertension *54*, 598-604.

86. Markwalder,J. and Starling,E.H. (1914). On the constancy of the systolic output under varying conditions. J. Physiol *48*, 348-356.

87. Martinez,A., Weaver,C., Lopez,J., Bhathena,S.J., Elsasser,T.H., Miller,M.J., Moody,T.W., Unsworth,E.J., and Cuttitta,F. (1996). Regulation of insulin secretion and blood glucose metabolism by adrenomedullin. Endocrinology *137*, 2626-2632.

88. Masada,K., Nagayama,T., Hosokawa,A., Yoshida,M., Suzuki-Kusaba,M., Hisa,H., Kimura,T., and Satoh,S. (1999). Effects of adrenomedullin and PAMP on adrenal catecholamine release in dogs. Am. J. Physiol *276*, R1118-R1124.

89. Masri,B., Morin,N., Pedebernade,L., Knibiehler,B., and Audigier,Y. (2006). The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. J. Biol. Chem. *281*, 18317-18326.

90. McLatchie,L.M., Fraser,N.J., Main,M.J., Wise,A., Brown,J., Thompson,N., Solari,R., Lee,M.G., and Foord,S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature *393*, 333-339.

91. Meiry,G., Reisner,Y., Feld,Y., Goldberg,S., Rosen,M., Ziv,N., and Binah,O. (2001). Evolution of action potential propagation and repolarization in cultured neonatal rat ventricular myocytes. J. Cardiovasc. Electrophysiol. *12*, 1269-1277.

92. Mittra,S. and Bourreau,J.P. (2006). Gs and Gi coupling of adrenomedullin in adult rat ventricular myocytes. Am. J. Physiol Heart Circ. Physiol *290*, H1842-H1847.

93. Mittra,S., Hyvelin,J.M., Shan,Q., Tang,F., and Bourreau,J.P. (2004). Role of cyclooxygenase in ventricular effects of adrenomedullin: is adrenomedullin a double-edged sword in sepsis? Am. J. Physiol Heart Circ. Physiol *286*, H1034-H1042.

94. Miyashita,K., Itoh,H., Sawada,N., Fukunaga,Y., Sone,M., Yamahara,K., Yurugi-Kobayashi,T., Park,K., and Nakao,K. (2003). Adrenomedullin provokes endothelial Akt

activation and promotes vascular regeneration both in vitro and in vivo. FEBS Lett. *544*, 86-92.

95. Moor,A.N. and Fliegel,L. (1999). Protein kinase-mediated regulation of the Na(+)/H(+) exchanger in the rat myocardium by mitogen-activated protein kinase-dependent pathways. J. Biol. Chem. *274*, 22985-22992.

96. Mukherjee,R., Multani,M.M., Sample,J.A., Dowdy,K.B., Zellner,J.L., Hoover,D.B., and Spinale,F.G. (2002). Effects of adrenomedullin on human myocyte contractile function and beta-adrenergic response. J. Cardiovasc. Pharmacol. Ther. *7*, 235-240.

97. Nagaya,N., Mori,H., Murakami,S., Kangawa,K., and Kitamura,S. (2005). Adrenomedullin: angiogenesis and gene therapy. Am. J Physiol Regul. Integr. Comp Physiol *288*, R1432-R1437.

98. Nakamura,R., Kato,J., Kitamura,K., Onitsuka,H., Imamura,T., Cao,Y., Marutsuka,K., Asada,Y., Kangawa,K., and Eto,T. (2004). Adrenomedullin administration immediately after myocardial infarction ameliorates progression of heart failure in rats. Circulation *110*, 426-431.

99. Newson,M.J., Roberts,E.M., Pope,G.R., Lolait,S.J., and O'Carroll,A.M. (2009). The effects of apelin on hypothalamic-pituitary-adrenal axis neuroendocrine function are mediated through corticotrophin-releasing factor- and vasopressin-dependent mechanisms. J. Endocrinol. *202*, 123-129.

100. Noma,T., Lemaire,A., Naga Prasad,S.V., Barki-Harrington,L., Tilley,D.G., Chen,J., Le,C.P., Violin,J.D., Wei,H., Lefkowitz,R.J., and Rockman,H.A. (2007). Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J. Clin. Invest *117*, 2445-2458.

101. O'Carroll,A.M., Selby,T.L., Palkovits,M., and Lolait,S.J. (2000). Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. Biochim. Biophys. Acta *1492*, 72-80.

102. O'Dowd,B.F., Heiber,M., Chan,A., Heng,H.H., Tsui,L.C., Kennedy,J.L., Shi,X., Petronis,A., George,S.R., and Nguyen,T. (1993). A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene *136*, 355-360.

103. O'Shea,M., Hansen,M.J., Tatemoto,K., and Morris,M.J. (2003). Inhibitory effect of apelin-12 on nocturnal food intake in the rat. Nutr. Neurosci. *6*, 163-167.

104. Opie,L.H. (1995). Regulation of myocardial contractility. J. Cardiovasc. Pharmacol. *26 Suppl 1*, S1-S9.

105. Parkes,D.G. and May,C.N. (1997). Direct cardiac and vascular actions of adrenomedullin in conscious sheep. Br. J. Pharmacol. *120*, 1179-1185.

106. Parmley,W.W. and Chuck,L. (1973). Length-dependent changes in myocardial contractile state. Am. J. Physiol *224*, 1195-1199.

107. Patterson,S.W., Piper,H., and Starling,E.H. (1914). On the mechanical factors which determine the output of the ventricles. J. Physiol 465-513.

108. Pieske,B., Kretschmann,B., Meyer,M., Holubarsch,C., Weirich,J., Posival,H., Minami,K., Just,H., and Hasenfuss,G. (1995). Alterations in intracellular calcium handling associated with the inverse force-frequency relation in human dilated cardiomyopathy. Circulation *92*, 1169-1178.

109. Pieske,B., Maier,L.S., Piacentino,V., III, Weisser,J., Hasenfuss,G., and Houser,S. (2002). Rate dependence of [Na+]i and contractility in nonfailing and failing human myocardium. Circulation *106*, 447-453.

110. Ping,P., Zhang,J., Cao,X., Li,R.C., Kong,D., Tang,X.L., Qiu,Y., Manchikalapudi,S., Auchampach,J.A., Black,R.G., and Bolli,R. (1999). PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. Am. J. Physiol *276*, H1468-H1481.

111. Piuhola,J., Makinen,M., Szokodi,I., and Ruskoaho,H. (2003). Dual role of endothelin-1 via ETA and ETB receptors in regulation of cardiac contractile function in mice. Am. J. Physiol Heart Circ. Physiol *285*, H112-H118.

112. Purcell,N.H., Wilkins,B.J., York,A., Saba-El-Leil,M.K., Meloche,S., Robbins,J., and Molkentin,J.D. (2007). Genetic inhibition of cardiac ERK1/2 promotes stress-induced apoptosis and heart failure but has no effect on hypertrophy in vivo. Proc. Natl. Acad. Sci. U. S. A *104*, 14074-14079.

113. Rademaker,M.T., Charles,C.J., Lewis,L.K., Yandle,T.G., Cooper,G.J., Coy,D.H., Richards,A.M., and Nicholls,M.G. (1997). Beneficial hemodynamic and renal effects of adrenomedullin in an ovine model of heart failure. Circulation *96*, 1983-1990.

114. Rayment,I., Holden,H.M., Whittaker,M., Yohn,C.B., Lorenz,M., Holmes,K.C., and Milligan,R.A. (1993). Structure of the actin-myosin complex and its implications for muscle contraction. Science *261*, 58-65.

115. Reaux,A., De,M.N., Skultetyova,I., Lenkei,Z., El,M.S., Gallatz,K., Corvol,P., Palkovits,M., and Llorens-Cortes,C. (2001). Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. J. Neurochem. *77*, 1085-1096.

116. Ribatti,D., Conconi,M.T., and Nussdorfer,G.G. (2007). Nonclassic endogenous novel [corrected] regulators of angiogenesis. Pharmacol. Rev. *59*, 185-205.

117. Robertson,S.P., Johnson,J.D., Holroyde,M.J., Kranias,E.G., Potter,J.D., and Solaro,R.J. (1982). The effect of troponin I phosphorylation on the Ca2+-binding properties of the Ca2+-regulatory site of bovine cardiac troponin. J. Biol. Chem. *257*, 260-263.

118. Saetrum,O.O., Hasbak,P., de,V.R., Saxena,P.R., and Edvinsson,L. (2000). Positive inotropy mediated via CGRP receptors in isolated human myocardial trabeculae. Eur. J. Pharmacol. *397*, 373-382.

119. Sakata,J., Shimokubo,T., Kitamura,K., Nakamura,S., Kangawa,K., Matsuo,H., and Eto,T. (1993). Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. Biochem. Biophys. Res. Commun. *195*, 921-927.

120. Samson,W.K. (1999). Adrenomedullin and the control of fluid and electrolyte homeostasis. Annu. Rev. Physiol *61*, 363-389.

121. Sarzani,R., Forleo,C., Pietrucci,F., Capestro,A., Soura,E., Guida,P., Sorrentino,S., Iacoviello,M., Romito,R., ssi-Fulgheri,P., Pitzalis,M., and Rappelli,A. (2007). The 212A variant of the APJ receptor gene for the endogenous inotrope apelin is associated with slower heart failure progression in idiopathic dilated cardiomyopathy. J. Card Fail. *13*, 521-529.

122. Sato,A., Canny,B.J., and Autelitano,D.J. (1997). Adrenomedullin stimulates cAMP accumulation and inhibits atrial natriuretic peptide gene expression in cardiomyocytes. Biochem. Biophys. Res. Commun. *230*, 311-314.

123. Sebolt-Leopold,J.S. and Herrera,R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat. Rev. Cancer *4*, 937-947.

124. Seyedabadi,M., Goodchild,A.K., and Pilowsky,P.M. (2002). Site-specific effects of apelin-13 in the rat medulla oblongata on arterial pressure and respiration. Auton. Neurosci. *101*, 32-38.

125. Shimekake,Y., Nagata,K., Ohta,S., Kambayashi,Y., Teraoka,H., Kitamura,K., Eto,T., Kangawa,K., and Matsuo,H. (1995). Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and Ca2+ mobilization, in bovine aortic endothelial cells. J. Biol. Chem. *270*, 4412-4417.

126. Shimokubo,T., Sakata,J., Kitamura,K., Kangawa,K., Matsuo,H., and Eto,T. (1996). Adrenomedullin: changes in circulating and cardiac tissue concentration in Dahl salt-sensitive rats on a high-salt diet. Clin. Exp. Hypertens. *18*, 949-961.

127. Snabaitis,A.K., D'Mello,R., Dashnyam,S., and Avkiran,M. (2006). A novel role for protein phosphatase 2A in receptor-mediated regulation of the cardiac sarcolemmal Na+/H+ exchanger NHE1. J. Biol. Chem. *281*, 20252-20262.

128. Solaro,R.J. and Rarick,H.M. (1998). Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. Circ. Res. *83*, 471-480.

129. Stangl,V., Dschietzig,T., Bramlage,P., Boye,P., Kinkel,H.T., Staudt,A., Baumann,G., Felix,S.B., and Stangl,K. (2000). Adrenomedullin and myocardial contractility in the rat. Eur. J. Pharmacol. *408*, 83-89.

130. Steinberg,S.F. (1999). The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. Circ. Res. *85*, 1101-1111.

131. Sunter,D., Hewson,A.K., and Dickson,S.L. (2003). Intracerebroventricular injection of apelin-13 reduces food intake in the rat. Neurosci. Lett. *353*, 1-4.

132. Szokodi,I., Kerkela,R., Kubin,A.M., Sarman,B., Pikkarainen,S., Konyi,A., Horvath,I.G., Papp,L., Toth,M., Skoumal,R., and Ruskoaho,H. (2008). Functionally opposing roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the regulation of cardiac contractility. Circulation *118*, 1651-1658.

133. Szokodi,I., Kinnunen,P., and Ruskoaho,H. (1996). Inotropic effect of adrenomedullin in the isolated perfused rat heart. Acta Physiol Scand. *156*, 151-152.

134. Szokodi,I., Kinnunen,P., Tavi,P., Weckstrom,M., Toth,M., and Ruskoaho,H. (1998). Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. Circulation *97*, 1062-1070.

135. Szokodi, I. and Ruskoaho, H. Adrenomedullin in Bader M, ed. Cardiovascular Hormone System. Bader, M. 169-190. 16-5-2008. Willey-VCH Verlag GmbH. Ref Type: Serial (Book,Monograph)

136. Szokodi,I., Tavi,P., Foldes,G., Voutilainen-Myllyla,S., Ilves,M., Tokola,H., Pikkarainen,S., Piuhola,J., Rysa,J., Toth,M., and Ruskoaho,H. (2002). Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. Circ. Res. *91*, 434-440.

137. Taheri,S., Murphy,K., Cohen,M., Sujkovic,E., Kennedy,A., Dhillo,W., Dakin,C., Sajedi,A., Ghatei,M., and Bloom,S. (2002). The effects of centrally administered apelin-13 on food intake, water intake and pituitary hormone release in rats. Biochem. Biophys. Res. Commun. *291*, 1208-1212.

138. Tatemoto,K., Hosoya,M., Habata,Y., Fujii,R., Kakegawa,T., Zou,M.X., Kawamata,Y., Fukusumi,S., Hinuma,S., Kitada,C., Kurokawa,T., Onda,H., and Fujino,M. (1998). Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem. Biophys. Res. Commun. *251*, 471-476.

139. Tatemoto,K., Takayama,K., Zou,M.X., Kumaki,I., Zhang,W., Kumano,K., and Fujimiya,M. (2001). The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regul. Pept. *99*, 87-92.

140. Tenhunen,O., Sarman,B., Kerkela,R., Szokodi,I., Papp,L., Toth,M., and Ruskoaho,H. (2004). Mitogen-activated protein kinases p38 and ERK 1/2 mediate the wall stress-induced activation of GATA-4 binding in adult heart. J. Biol. Chem. *279*, 24852-24860.

141. Terracciano,C.M. and MacLeod,K.T. (1997). Effects of lactate on the relative contribution of Ca2+ extrusion mechanisms to relaxation in guinea-pig ventricular myocytes. J. Physiol *500 (Pt 3)*, 557-570.

142. Thomas,W.G., Brandenburger,Y., Autelitano,D.J., Pham,T., Qian,H., and Hannan,R.D. (2002). Adenoviral-directed expression of the type 1A angiotensin receptor promotes cardiomyocyte hypertrophy via transactivation of the epidermal growth factor receptor. Circ. Res. *90*, 135-142.

143. Vickers,C., Hales,P., Kaushik,V., Dick,L., Gavin,J., Tang,J., Godbout,K., Parsons,T., Baronas,E., Hsieh,F., Acton,S., Patane,M., Nichols,A., and Tummino,P. (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. *277*, 14838-14843.

144. Wallert,M.A. and Frohlich,O. (1992). Alpha 1-adrenergic stimulation of Na-H exchange in cardiac myocytes. Am. J. Physiol *263*, C1096-C1102.

145. Walsh,D.A. and Van Patten,S.M. (1994). Multiple pathway signal transduction by the cAMP-dependent protein kinase. FASEB J. *8*, 1227-1236.

146. Wang,C., Du,J.F., Wu,F., and Wang,H.C. (2008). Apelin decreases the SR Ca2+ content but enhances the amplitude of [Ca2+]i transient and contractions during twitches in isolated rat cardiac myocytes. Am. J. Physiol Heart Circ. Physiol *294*, H2540-H2546.

147. Wang,Y. (2007). Mitogen-activated protein kinases in heart development and diseases. Circulation *116*, 1413-1423.

148. Wetzker,R. and Bohmer,F.D. (2003). Transactivation joins multiple tracks to the ERK/MAPK cascade. Nat. Rev. Mol. Cell Biol. *4*, 651-657.

149. Widmann,C., Gibson,S., Jarpe,M.B., and Johnson,G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. *79*, 143-180.

150. Yamaguchi,T., Baba,K., Doi,Y., Yano,K., Kitamura,K., and Eto,T. (1996). Inhibition of aldosterone production by adrenomedullin, a hypotensive peptide, in the rat. Hypertension *28*, 308-314.

151. Yan,L., Vatner,D.E., O'Connor,J.P., Ivessa,A., Ge,H., Chen,W., Hirotani,S., Ishikawa,Y., Sadoshima,J., and Vatner,S.F. (2007). Type 5 adenylyl cyclase disruption increases longevity and protects against stress. Cell *130*, 247-258.

152. Yang,B.C., Lippton,H., Gumusel,B., Hyman,A., and Mehta,J.L. (1996). Adrenomedullin dilates rat pulmonary artery rings during hypoxia: role of nitric oxide and vasodilator prostaglandins. J. Cardiovasc. Pharmacol. *28*, 458-462.

153. Yoshihara,F., Horio,T., Nishikimi,T., Matsuo,H., and Kangawa,K. (2002). Possible involvement of oxidative stress in hypoxia-induced adrenomedullin secretion in cultured rat cardiomyocytes. Eur. J. Pharmacol. *436*, 1-6.

154. Zheng,M., Zhu,W., Han,Q., and Xiao,R.P. (2005). Emerging concepts and therapeutic implications of beta-adrenergic receptor subtype signaling. Pharmacol. Ther. *108*, 257-268.

9. Publications

9.1. Publications Related to the Thesis

9.1.1. Publications in Refereed Journals

1. **Farkasfalvi K**, Stagg MA, Siedlecka U, Lee J, Soppa GK, Marczin N, Szokodi I, Yacoub MH, Terracciano CM. Apelin, the ligand for the angiotensin receptor-like 1, directly affects cardiomyocyte contractility and electrophysiology. Biochem Biophys Res Commun. 2007;357(4):889-895.
   [Impact factor (2007): 2.749; Independent citations: 27]

2. Skoumal R, **Farkasfalvi K**, Perjés Á, Kubin AM, Horváth IG, Tóth M, Ruskoaho H, Kerkelä R, Szokodi I. Adrenomedullin regulates cardiac contractility via extracellular signal-regulated protein kinase-dependent mechanisms (Submitted)

9.1.2. Abstracts

K. Farkasfalvi, MA. Stagg, U. Siedlecka, J. Lee, GKR. Soppa, N. Marczin, CMN. Terracciano: Direct effects of apelin, the ligand for the angiotensin receptor-like 1, on cardiomyocyte contractility and electrophysiology
J Mol and Cell Card 40;6 2006 p.980 ISHR poster
[**Impact factor (2006):** 4.856]

K. Farkasfalvi, MA. Stagg, U. Siedlecka, J. Lee, GKR. Soppa, N. Marczin, CMN. Terracciano: Direct effects of apelin, the ligand for the angiotensin receptor-like 1, on cardiomyocyte contractility and electrophysiology
Eur Heart J **S1,** 27 **p: 407**-407, 2006
[**Impact factor (2006)**: 7.286]

LE. Felkin, **K. Farkasfalvi**, GKR. Soppa, E. Birks, N. Marczin, PJR. Barton, MH. Yacoub, CMN. Terracciano:The apelin-angiotensin receptor-like 1 (APJ) mRNA levels closely correlate with myocardial recovery in end-stage heart failure patients treated with left ventricular assist devices (LVADs)
Eur Heart J **S1,** 27 **p:** 267-267, 2006
[**Impact factor (2006)**: 7.286]

K. Farkasfalvi, L. Felkin, N. Latif, G.K Soppa, R. George, E.Birks, P.Barton, N.Marczin, M.H Yacoub, CMN Terracciano: The Apelin Receptor mRNA Levels and Myocardial Recovery in end-stage Heart Failure Patients Treated with Left Ventricle Assist Devices (LVADs)
Circulation S 114,18 **p:** 484-484 2006
[**Impact factor (2006)**: 10.94]

K. Farkasfalvi, MA. Stagg, U.Siedlecka, J. Lee, GK Soppa, N. Marczin, I. Szokodi, MH Yacoub, CMN Terracciano: Apelin, the Ligand for the Angiotensin Receptor-like 1, Directly Affects Cardiomyocyte Contractility and Electrophysiology
Circulation S 114,18 **p:** 300-300 2006
[**Impact factor (2006)**: 10.94]

K.Farkasfalvi, MA. Stagg, SR Coppen, U. Siedlecka, J. Lee, GK. Soppa, N Marczin, I Szokodi*, MH Yacoub, C.M.N. Terracciano: Direct effects of apelin on cardiomyocyte contractility and electrophysiology
Card. Hung. 2007 37 A16

K. Farkasfalvi, L. Felkin, N. Latif, GK. Soppa, E. Birks, N. Marczin, MH. Yacoub, CM. Terracciano, I.Szokodi : APJ (the specific receptor of apelin) expression and myocardial recovery in patients treated with left ventricular assist devices (LVADs)
Card. Hung. 2008 38 B13

9.2 Other Publications

9.2.1. Publications in Refereed journals

Kónyi A, Skoumal R, Kubin AM, Füredi G, Perjés PÁ, **Farkasfalvi K**, Sárszegi Z, Horkay F, Horváth IG, Tóth M, Ruskoaho H, Szokodi I. Prolactin-releasing peptide regulates cardiac contractility. *Regul Pept*. 2009 Jul 17.
[**Impact factor (2008)**: 2.276]

Brolly M.,Melczer L.,**Farkasfalvi K**.,Radnai B.,Tekeres M.:Pacemaker diszfunkcio, -miopotenciál inhibicio
Card.Hung.23. 35-38. 1994.

Melczer L., Brolly M., **Farkasfalvi K**., Komócsi A.: Frekvencia-válaszos (rate-responsive) pacemakerek programozása impedancia kardiográfiával
Aneszteziológia és Intenzív Terápia suppl. 1. p: 33-39. 1995.

Radnai B.,Goják I**,.Farkasfalvi K**.,Vass E.,Bódis L.: Acut nitráthatás vizsgálata tartós nitrat kezelésben részesült betegeken
MBA 49.13-21. 1996.

Radnai B.,Melczer L.,**Farkasfalvi K**.,Goják I,.Bódis L.:Loss of consiousness of unusual etiology in a young patient
Orv. Hetil. 137. 79-81. 1996.

Radnai B.,Goják I,.Vass E.,Melczer L.,**Farkasfalvi K**., Bódis L.: Electromos cardioversio következményeinek megitélése echocardiographia segitségével
MBA 48. 401-409. 1996.

I.Sárosi, D.Mühl, M.Tekeres, G.Debreceni, A.Kónyi, A.Szabó, **K.Farkasfalvi**, I.Batthyányi, L.Horávth: Lifesaving thrombolysis – in the light of contraindicatio
Orv. Hetil. 138. 105-9. 1997.

Göbölös L, Hejjel L, Imre J, Lindenmol/layer-G R, Wiebe K, Foltan M, Thrum A, Ugocsai P, Tóth Z, **Farkasfalvi K**, Sipos E, Kiss R, Gyorimolnár I, Philipp A.:[Pumpless extracorporeal lung assist
Orv Hetil. 2008 Jun 29;149(26):1233-6.

9.2.2. Abstracts

Cs.Csontos, I.Schmidt, **K.Farkasfalvi**, L.Melczer, A.Cziráky, M.Tekeres:
The value of noninvasive haemodynamic monitoring in the development and progress of septic state during follow -up
Acta Anasth Scand. Suppl. 109. Vol 40. p. 231 1996.
[Impact factor (2006): 1.86]

Radnai B., Goják I., **Farkasfalvi K**., Bódis L.: The examination of cardiac effect of propafenon using non-invasive methods
ISCR 7th International Symposium on Cardiovascular Pharmacotherapy 1997. Jeruzsálem

Farkasfalvi K., Radnai B., Pintér T., Bátai I., Tekeres M.,:The impact of aesthesia on development of perioperative ischaemia in patients with coronary disease
Acta Anest. Scand. Suppl. 111 vol.: 41 p 349 1997.
[Impact factor (2006): 1.86]

L.Melczer, M. Brolly, **K.Farkasfalvi,** Cs.Csontos, B. Radnai **:**Role of the individually programmed dynamic AV delay in haemmodynamic performance of patients with VDD pacemakers at rest
Annals of Noninvasive Electrocardiology, Vol 5. No 4. Part 2. p.59,1999
[Impact factor (2006): 1.35]

T.Pintér, B. Radnai, I. Goják**, K. Farkasfalvi*,** L. Bódis: The effect of vegetative nervous system unstability on repolarisation
17th European Congress of the International Society of Non-invassive Cardiology, Szeged 1999
Annals of Noninvasive Electrocardiology, Vol 5. No 4. Part 2. p.
[Impact factor (2006): 1.35]

B. Radnai, I. Goják, T. Pintér, **K. Farkasfalvi***, Zs. Varga, L. Bódis:On the significance of peak speed of "a" wave of transmitral stream related to electrical cardioversion
17th European Congress of the International Society of Non-invassive Cardiology, Szeged 1999
Annals of Noninvasive Electrocardiology, Vol 5. No 4. Part 2. p.
[Impact factor (2006): 1.35]

B. Radnai, Zs.Varga, I. Goják, **K. Farkasfalvi** : About the necesserity
of the anticoagulant therapy after cardioversion of atrial fibrillation and flutter
Euroecho 8, Athen 2004.
Echocardiography
[Impact factor (2006): 1.05]

Goják I., Radnai B**.,** Vass E., **Farkasfalvi K.,** Bódis L.: Nitrát tolerantia vizsgálata angina pectorisban
MKT Balatonfüred, 1991. Abstracts p. 57

Radnai B., Goják I., Vass E., Dános L., **Farkasfalvi K**., Bódis L.: Iso Mack TD spray antiischaemiás hatásáról - terheléses EKG alapján
MKT, Balatonfüred, 1992 Abstracts p.

Melczer L., Brolly M., Safranko A., **Farkasfalvi K**.: Az Impedancia Kardiográfia alkalmazási lehetőségei a pacemaker therápiában.
MKT,Balatonfüred 1994 Card. Hung .suppl. 4. p.1994**.**

Farkasfalvi K., Melczer L., Csontos CS., Goják I., Radnai B.:Haemodynamic effects of different atrio-ventricular delays evaluated by Doppler echocardiography
MKT, Balatonfüred 1996. Card. Hung. 25. Suppl. p. 28, 1996**.**

Farkasfalvi K., Radnai B., Tekeres M.: Mikor várható nagyobb myocardium ischaemia (Epidurális és intubatiós narcosis hatásának összehasonlítása Holter EKG segítségével)
Anaesth. Int. Ther. 28. Suppl. 2. p. 1996

Brolly M., Melczer L., **Farkasfalvi K**., Csontos Cs., Radnai B.: Pacemaker átmeneti aszinkron müködése okozta szívritmuszavarok.
1997. Debrecen I. Arrhythmia és Pacemaker Kongresszus

Csontos Cs., Melczer L., Brolly M., **Farkasfalvi K**.: Pitvar-kamrai késleltetés optimalizálásának hatása a nyugalmi szivteljesitményre.
1997. Debrecen I. Arrhythmia és Pacemaker Kongresszus

Melczer L., Brolly M., Csontos Cs., **Farkasfalvi K:** Az élettani szivingerlés jelene és ujabb lehetőségei.
1997. Debrecen I. Arrhythmia és Pacemaker Kongresszus

Melczer L., Brolly M., Csontos Cs., **Farkasfalvi K,** Tekeres M.:
The examination of haemodynamic performance of pacemaker holders at rest by impedance cardiography
MKT, Balatonfüred 1997. Card. Hung 26.suppl. 3. p.48.1997**.**

Farkasfalvi K., Baumann J., Pintér T., Radnai B., Vermes Cs., Tekeres M.: Mit jelenthet a ritmuszavar a korai postoperativ szakban ISZB-s betegek csipő-, ill. térdprotezis implantációja kapcsán
Anaesth. Int. Ther. 28. Suppl. 2. p. 42. 1998

Radnai B., Melczer L., **Farkasfalvi K**., Csontos Cs., Goják I., Bódis L.: Holter monitorozás szerepe a pacemaker implantatio szükségességének elbírálásában és a pacemaker dysfunctio felismerésében
II.Aritmia és Pacemaker Kongresszus, Szeged 1999.
Card. Hung.

Radnai B., Goják I., **Farkasfalvi K.**: Jobb szívfél terheltségben kialakuló rhythmuszavarok és a therapia lehetőségei
Sopron 2000, Tüdőgyógyász Társaság Kongresszusa, Abstracts p.27.

Farkasfalvi K., Melczer L, Csontos Cs., Radnai B**.**: Van-e jelentősége a pitvar-kamrai késleltetési idő (avd) változtatásának normocardiás beteken ?
2001. Eger – Arrhythmia congr. , Card. Hung. 31. p.235. 2001

Radnai Béla, Goják Ilona*, **Farkasfalvi Klára****, Varga Zsuzsa: Cardioversio utáni anticoaguláns kezelésről -az echocardiogrpahia tükrében
2001. Balatonfüred Card. Hung. 31. Suppl. 2. p. 54. 2001

Varga Zs., **Farkasfalvi K**, Goják I., Pozsgai Zs., Radnai B.: A bal pitvari mechanicus functio visszatérésének jelentősége az anticoaguláns kezelés szükségességének megitélésében
MBA. 54. suppl. 2. p. 69. 2001.

Radnai B., Goják I., **Farkasfalvi K.**, Varga Zs.: Kémiai cardioversio - mégis effectiv az oralisan alkalmazott propafenon ?
2001 Eger Arrhythmia napok

Radnai B., Goják I., **Farkasfalvi K**., Vass E.: Malignant ventricular rhythm-disturbances provoked by carotis sinus stimulation
MKT. Balatonfüred, 2004. Card. Hung. 34. Suppl. C. p. 39. 2004

Győrimolnár I., Farkasfalvi K., Horváth I., Tóth Zs.., Sipos E., Simor T., Papp L.:
Efficacy of preoperativ IABP support in high risk coronary patients
2002. Szivsebészet , Card. Hung 32.vol. 3. p 179. 2002

I.Győrimolnár, F.Szedő, **K. Farkasfalvi**, G.Késmárki, L.Papp: Anticoagulation for patient with heparin-induced thrombocytopenia during coronaria revascululation surgery
2003. Szivsebészet , Card. Hung 33.suppl. 4. B 24. 2003,

K.Farkasfalvi, E.Donauer, I.Győrimolnár, R.Korontai, L.Papp: Cardiovascular monitoring of the off pump coronary revascularisation surgery using pulse contour output
2003. Szivsebészeti Kongresszus, Card. Hung 33.suppl. 4. B 23

Floderer E., Goják I., **Farkasfalvi K**., Radnai B.: Advances of pericardiocentesis directed by echocardiography
MBT DSZ. LI Vándorgyűlés, Hőgyész , MBA. 57. suppl. 1. p. 50. 2004

Radnai B., Goják I., Farkasfalvi K., Varga Zs., Floderer E., Nyárfás G.:
Bal pitvari kontrakcio és bal fülcse-áramlás összehasonlitó vizsglata pitvari arrhythmia elektromos cardioversiója kapcsán
Card. Hung.36 Suppl A76 2006

9.3. Book Chapters

Csontos Cs., Melczer L., Brolly M., **Farkasfalvi K**.,, Tekeres M.:
Pitvar kamrai késleltetés optimalizálásának hatása a nyugalmi szívteljesítményre
In: Ritmuszavarok Szerk.: Polgár P. Alföldi Nyomda, 1998.

Melczer L., Brolly M., Csontos Cs **Farkasfalvi K**.: Az élettani szívingerlés jelene és újabb lehetőségei
In: Ritmuszavarok Szerk.: Polgár P. Alföldi Nyomda, 1998.

Farkasfalvi.K. Gal J.: Echokardiográfia
1.5.6 fejezet Aneszteziologia
Szerkesztő: dr. Bogár Lajos Medicina Könyvkiadó 2008