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

PhD thesis

by

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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 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 (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.

2. Emerging Role of Apelin and Andrenomedullin in the Regulation of the Cardiovascular System

Apelin and andrenomedullin regulate cardiac contractility in autocrine/paracrine manner.

2.1. Characterisation and Biological Effects of Apelin

Apelin is a bioactive peptide expressed in a wide variety of tissues and exerts a broad range of biological activity.Roles have been established for the apelin-APJ (APJ is the specific receptor of apelin) 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 (Kleinz and Davenport, 2005; Newson et al., 2009; Sunter et al., 2003; Taheri et al., 2002).

2.1.1. Apelin and Inotropy

Apelin has recently been found to be a potent inotropic agent in isolated rat heart preparations 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 (Szokodi et al., 2002). They investigated the underlying intracellular mechanisms and suggested that activation of phospholipase C (PLC) and protein kinase C (PKC) were involved in the positive inotropic effect observed in the presence of apelin. Hosoya and Marsi suggested that the APJ receptor couples through inhibitory G-proteins (Hosoya et al., 2000; Masri et al., 2006). Others recommended that the modulation of the L-type Ca^{2+} channels could be involved in the positive inotropic effect of apelin (Kleinz and Davenport, 2004) which was not supported by perforated patch-clamp experiments (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 sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX) and sarcolemmal Ca^{2+}-ATPase (SERCA) but the underlying mechanism has still been unknown (Wang et al., 2008).

The apelin–APJ signaling pathway has also been identified as a potentially important mediator in the pathophysiology of chronic heart failure (Chen et al., 2003) 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. Apelin has also been implicated in the pathophysiology of arrhythmias; Ellinor et al. demonstrated that plasma apelin levels decrease in patients with lone atrial fibrillation (Ellinor et al., 2006). However the role of the APJ-apelin system in the regulation of electrophysiological parameters in the heart is still poorly understood.

2.2. Characterisation and Biological Effects of Adrenomedullin

In 1993 Kitamura et al. isolated a novel peptide called adrenomedullin (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., 1993). Substantial evidences support the perception that AM is an important regulator of the cardiovascular system (Hamid and Baxter, 2005; Kitamura et al., 1993; Szokodi et al., 2008). As a consequence of a wide distribution of AM and its receptors, AM has a remarkable range of action, from potent vaso dilatator effect through regulating cellular growth and differentiation to modulating hormone secretion, S(Samson, 1999; Szokodi et al., 2008). There are some non-cardiovascular effects of AM as well; andrenomedullin inhibits ACTH release (Parkes and May, 1997; Samson, 1999), aldosteron production (Yamaguchi et al., 1996) and influence steroid secretion. Evidence exist for a role in regulating renal blood flow and tubular function (Hinson et al., 2000) , AM attenuates and delays the insulin response to oral glucose challenge (Martinez et al., 1996).

2.2.2. 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). In isolated, perfused rat heart AM dilated coronary arteries and increased cardiac contractility in a dose–dependent manner (Szokodi et al., 1996; Szokodi et al., 1998). Moreover, AM has appeared to be among the most potent endogenous positive inoptropic substances (Szokodi et al., 2002). A dual inotropic effect was observed in isolated adult rat ventricular myocytes (Mittra et al., 2004). 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 (Saetrum et al., 2000; Stangl et al., 2000).

Number of studies suggesting that AM induce an increase in cAMP and this may be the major pathway of the signalling (Eguchi et al., 1994; Ishizaka et al., 1994; Sato et al., 1997). On the other hand compelling evidences suggests that AM enhances cardiac contractility via cAMP-independent mechanisms (Szokodi et al., 1998). However, the underlying intracellular signalling mechanisms are still largely unknown.

3. 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

4. Direct Effects of Apelin on Cardiomyocyte Contractility and Electrophysiology

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.

4.1 Materials and Methods

All animal procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Adult female Sprague Dawley rats, weighing 200 g were used for this study.

Cardiomyocytes were isolated using standard enzymatic dissociation (Terracciano and MacLeod, 1997). Failing hearts were obtained 8 weeks after left coronary artery ligation (ejection fraction (≤30%). Sarcomere length was measured by Fourier analysis of digitised myocytes images (Delbridge and Roos, 1997). Intracellular [Ca^{2+}] was monitored using two different [Ca^{2+}]-sensitive fluorescence indicators indo-1 AM or fluo-4 AM. Fluorescence emissions were acquired and F405/485 was calculated and used as a measure of $[Ca^{2+}]_i$. For measurement of intracellular pH (pH_i) cardiomyocytes were loaded with 10 µM 5-(and-6)-Carboxy-SNARF-1 AM. Data were expressed as the ratio of the emission wavelengths at 580 nm and 640 nm (F580/640). To monitor Na^+/H^+ exchanger (NHE) activity, the NH_4Cl pre-pulse method was used (Boyarsky et al., 1988).

For immunocytochemistry on isolated cardiomyocytes and cryosections of adult rat hearts two different antibodies were used to confirm the labelling patterns observed. Neonatal cardiomyocytes were plated on multi-electrode array and this setup was used to monitor the origin and spread of electrical activity in confluent neonatal cardiomyocyte monolayers, as described previously (Meiry 2001). Spontaneous electrical activity, conduction velocity and properties of the field potentials were recorded. 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.

4.2 Results

4.2.1. 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 2005). APJ receptor-like immunoreactivity was detected in a transversal striated distribution associated with T-tubules and in the intercalated disc area.

4.2.2. Effect of Apelin on Cardiomyocyte Contractility

As shown in Figure 1, 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. 1). 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 1B. 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. 1). 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 1 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.

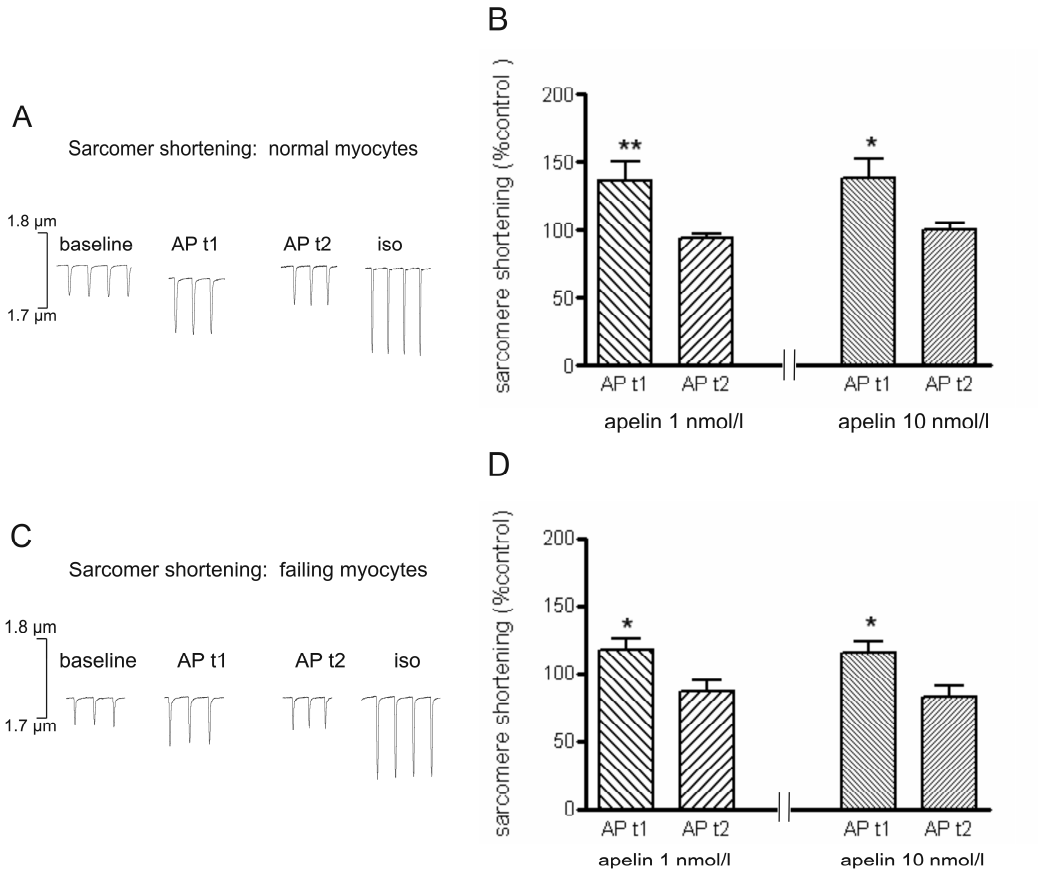

Figure 1. *Effect of apelin on sarcomere shortening of normal isolated cardiomyocyte (1A) and failing isolated cardiomyocyte (1C) at the different time point; baseline, AP t1 (1 minute in apelin,) AP t2 (after 8 minutes in apelin) and iso (30 nmol/l of isoproterenol). Graph 1B shows a significant increase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

4.2.3. 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. No effect of apelin could be observed either on the amplitude or time course of [Ca^{2+}] transients with both concentrations of apelin. To assess whether apelin affects intracellular pH and sarcolemmal NHE activity in isolated cadiomyocytes we monitored intracellular pH and we assessed the acid-extrusion ability of the NHE. 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); $p<0.01$)). 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. Apelin at both concentrations increased the speed of acid extrusion compared with control((baseline: 116.2±7.4 ratio unit (11); 1nmol/l apelin: 68.1±10.3 raatio units (11); 10 nmol/l apelin: 80±16 (11) ($p<0.05$,)), suggesting an enhanced activity of NHE.

4.2.4. Effect of Apelin on Intercellular Communication

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) ($P<0.05$)) 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) $P<0.05$). 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.

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

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). Accumulating data suggest that activation of the ERK1/2 signaling constitutes an essential adaptive mechanism int he myocardium (Lips et al., 2004; 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, 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.

5.1. Materials and Methods

All protocols were reviewed and approved by the Animal Use and Care Committee of the University of Oulu and University of Pecs. Rats were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique as described previously (Kinnunen et al., 2000; Szokodi et al., 1998). 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. 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 (U0126 :1.5 µmol/l, AG1478: 1 µmol/l, zoniporide 1 µmol/L). Westernblotting was performed as described previously (Szokodi 1998), and the following antibodies were used: anti-phospho-ERK1/2 and anti-ERK1/2 (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK). Results are presented as mean±SEM. To evaluate statistical significance two-way repeated-measures ANOVA was used for the treatment-by-time interactions. All other parameters were analyzed with 1-way ANOVA followed by Bonferroni post hoc test., followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of $P<0.05$.

5.2. Results

5.2.1. 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 2A) 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 (Szokodi et al., 2008; Tenhunen et al., 2004). Administration of U0126 (1.5 µmol/l) markedly reduced the levels of phospho-ERK1/2 both in the control and AM-stimulated hearts (Figure 2A). Infusing U0126 in combination with AM, the AM-induced inotropic effect decreased significantly, the maximal reduction being 40% ($P<0.01$; Figure 2B). Infusion of U0126 alone had no effect on contractile force (P=NS, Figure 2B).

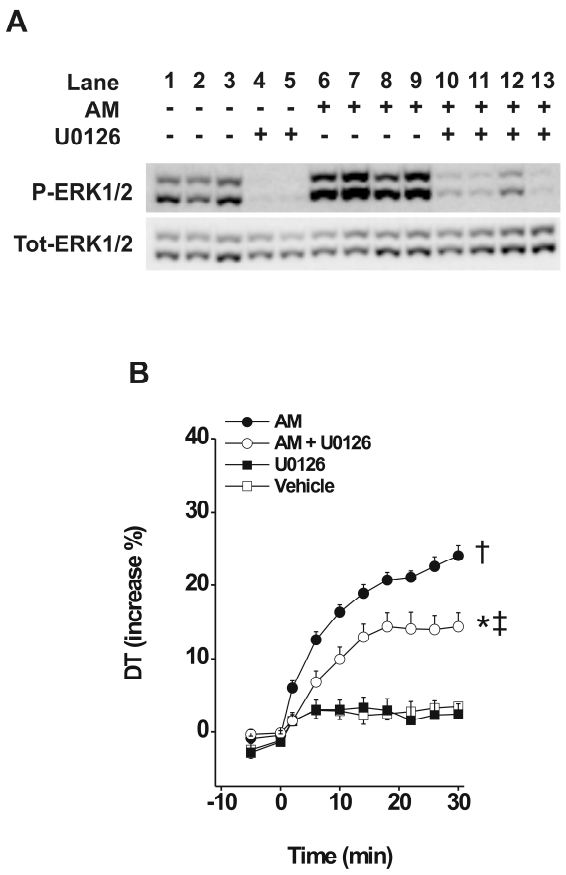

Figure 2. *ERK1/2 signaling is required for AM-mediated increase in contractility. A, Western blot analysis for ERK1/2 phosphorylation in left ventricular tissue samples. In isolated rat hearts, infusion of AM (1 nmol/l) for 30 minutes increased phospho-ERK1/2 levels and U0126 (1.5 µmol/l), a MEK1/2 inhibitor, abolished AM-induced ERK1/2 phosphorylation. B, U0126 significantly attenuated AM-enhanced contractility. DT indicates developed tension.. *P<0.01 and †P<0.001 vs control and U0126; ‡P<0.01 vs AM.*

5.2.3. 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 epidermal growth factor receptors (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. Moreover, in the presence of AG1478 the inotropic response to AM was significantly suppressed, the maximal reduction being 45% (P<0.001). Infusion of AG1478 alone had no effect on developed tension (P=NS).

5.2.4. 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 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). Infusion of zoniporide (1

μmol/L) alone had no effect on contractile force (*P*=NS). When given together with AM, zoniporide significantly attenuated the AM-induced positive inotropic effect, the maximal reduction being 46% (*P*<0.001).

6. Discussion

6.1. 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 (Ashley et al., 2005; Berry et al., 2004) 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 alkalinisation and, possibly, increased myofilament sensitivity to Ca^{2+}. In contrast to previous studies in intact hearts (Ashley et al., 2005; Berry et al., 2004; Szokodi et al., 2002) 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.

6.1.1. 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. 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 alkalinisation with subsequent sensitization of cardiac myofilaments to $[Ca^{2+}]$ can be involved in the inotropic effect of apelin.

Dai et al proposed that apelin had a predominant role in regulating cardiac contractility in the failing myocardium (Dai et al., 2006). 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.

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 (Ashley et al., 2005; Berry et al., 2004; Szokodi et al., 2002). 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 lad 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. Chamberlen 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.

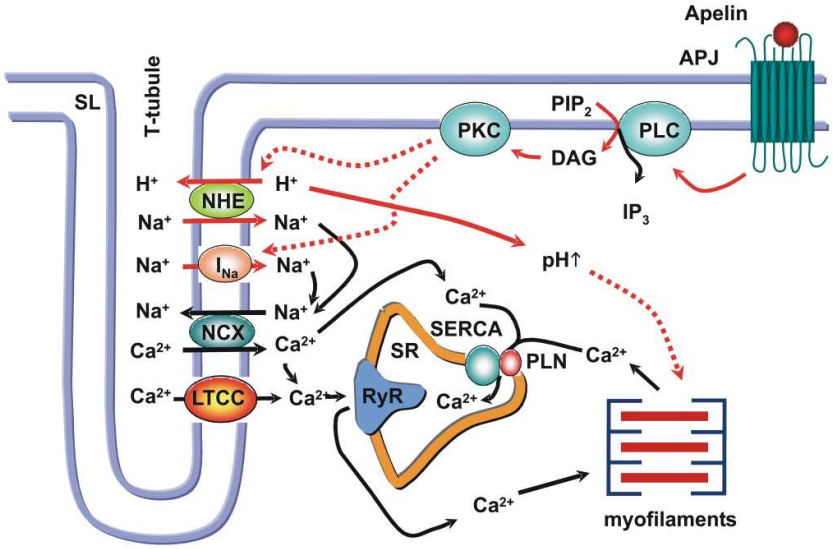

***Figure 3**. Putative signaling mechanisms of apelin in the heart. Stimulation of APJ by apelin evokes phosphorylation of PKC leading to activation of NHE, NCX and Na^+ channels. The positive inotropic effect of apelin is the result of sensitization of cardiac myofilaments to Ca^{2+} due to intracellular alkalosis and increased Ca^{2+} influx through the NCX operating in reverse mode (Chamberland et al., 2010; Szokodi et al., 2002).*

6.1.2. 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 in 2009, 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.

6.2 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.

6.2.1. Signaling Mechanism of Adrenomedullin

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).

6.2.2. 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). 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., 2008). 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; 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; Thomas et al., 2002). 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).

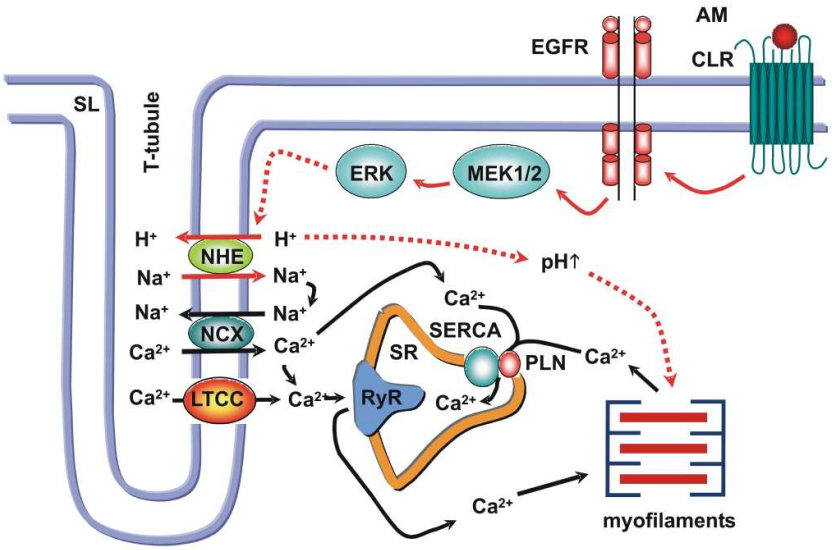

Figure 4*. Putative signaling mechanisms activated by adrenomedullin. ERK1/2 signaling serves as a key mediator of the inotropic effect of AM. EGFR acts as the upstream regulator and NHE-1 as the downstream effector of ERK1/2 in AM signaling.*

7. 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.

8. Reference list

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9. Publication

9.1. Publications Related to the Thesis

9.1.1. Publications in Refereed Journals

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]

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** [**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*. 2010;159 (1-3):9-13 ,** [**Impact factor (2008)**: 2.276]