The calcium-sensing receptor in heart physiology and development of endothelin-1-dependent hypertrophy

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Table of contents

1. Abbreviations

AC	Adenylate cyclase
Ang II	Angiotensin II
ATPase	Adenosinetriphosphatase
cAMP	Cyclic adenosine monophosphate
CaR	Calcium-sensing receptors
CEC	Chelerythrine chloride
DAG	Diacylglycerol
ECD	Extracellular domain
ECE	Endothelin-converting enzyme
ET-1	Endothelin-1
ETA	Endothelin receptors type A
ETB	Endothelin receptors type B
Gd	Gadolinium
GPCR	G protein-coupled receptors
I/R	Ischemia-reperfusion
ICD	Intracellular domain
IGF II	Insulin-like growth factor II
IP3	Inositol triphosphate
IP3R	Inositol triphosphate receptor
MAPK	Mitogen-activated protein kinase
MLP	Myocarial LIM protein
NO	Nitric oxide
NOS	Nitric oxide synthase
ODC	Ornithine decarboxylase
PI4K	Phosphatidylinositol 4-kinase
PKC	Protein kinase C

PLB	Phospholamban
PLC	Phospholipase C
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
RAMP	Receptor activity-modifying protein
RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
TGF-β	Transforming growth factor-β
TMD	Transmembrane domain

2. Introduction

2.1. Heart failure

In developed countries, heart failure is the main reason of hospitalization of the people aged above 65 years and takes first place among factors, leading to death. These patients also suffer from side events, such as arrhythmia, tachycardia, oedema, dyspnea, and different violations in blood circulation. Many cardiovascular diseases lead to the heart failure development later on [Guha et al., 2013].

The definition of the heart failure by European society of Cardiology says: “Heart failure is a clinical syndrome in which patients have typical symptoms and signs resulting from an abnormality of cardiac structure and function” [McMurray et al., 2012]. Thus, heart failure is a state in which cardiac output is not able to cover body needs. Many reasons can lead to loss of heart muscle efficiency via its damage or overloading such as, - myocardial infarction, hypertension, diabetes, and amyloidosis. Patients with heart failure used to have reduced contraction force of ventricles. In normal healthy hearts, increased filling leads to an increased contraction force (Frank-Starling law of the heart) and this way enhances cardiac output. During heart failure, these mechanisms break down and ventricles overloaded with blood are not further able to increase required cardiac output. A reduced stroke volume can be a result of systolic or diastolic dysunction. Systolic heart failure is usually easier to recognize and diagnose. It is a failure of pump activity of the heart muscle and usually leads to an increased heart volume. Heart failure caused by diastolic dysfunction is more difficult to identify. It is

not always caused by heart hypertrophy and usually happens due to violation of relaxation phase.

2.2. Cardiac hypertrophy

Hypertension and hypertrophy are one of the events leading to the heart failure development [Mann et al., 2005]. Heart muscle hypertrophy is an adaptive response of the heart to pressure overload. Left ventricular hypertrophy is the most common event, nevertheless, enlargement also occurs in atria and right ventricle.

Ventricular hypertrophy is known as an increase in the heart muscle mass. Two types of cardiac hypertrophy can be distinguished: healthy and pathological. Healthy cardiac hypertrophy is the normal response of the heart muscle to exercise or pregnancy [Mone et al., 1996], which results in an increase in the heart muscle mass and pumping ability. For instance, trained athletes have hearts with left ventricular mass 60% greater than untrained individuals. This type of hypertrophy leads to an increase in chamber radius and is called eccentric hypertrophy.

Unhealthy or pathological hypertrophy is a response to stress, heart muscle injury, hypertension or hormones. In this case ventricular mass is increasing, but pumping capability remains insufficient to cover body needs. This type of hypertrophy shows an increase in wall thickness but not in the radius or capacity of the ventricle. It is termed concentric hypertrophy. As a response to pressure, cells add new sarcomeres in-parallel to existing ones.

Nowadays many factors and agents are known to induce hypertrophy:

Mechanical factors. Mechanical stretching is able to induce expression of specific genes, leading to the development of hypertrophy. Cytoskeletal myocardial LIM protein (MLP) is a mechanical sensor, triggering hypertrophic events in cardiomyocytes [Gehmlich et al., 2008]. Stretching initiates protein kinase phosphorylation, which, in turns, activates transcription factors and thus regulates expression of corresponding genes in cardiac cells [Knöll et al., 2002].

Peptides and catecholamines. Cardiotrophin and endothelin-1 (ET-1), angiotensin II (Ang II), insulin-like growth factor-II (IGF II), transforming growth factor-β (TGF-β), interleukin-1 and catecholamines are contributing to the myocardial hypertrophy development [Horio et al., 2010; Harada et al., 1999; Rosenkranz et al., 2002]. Mitogen-activated protein kinase (MAPK) cascade, activated by Ang II or catecholamines, transfers in subsequent steps the signal right into the cell nucleus [Takano et al., 1996; Simm et al., 1998; Nigmatullina et al., 2004].

Calcium. Increased Ca^{2+} inside the cell can also trigger hypertrophy. Initial intracellular Ca^{2+}-increase can be due to chronic pressure overload. Enhanced Ca^{2+} inside the cell activates a Ca^{2+}-dependent phosphatase called calcineurin. Calcineurin in turns dephosphorylates the transcription factor NFAT3, which then enters the nucleus and activates genes involved in hypertrophic processes [Nigmatullina et al., 2004].

On the cellular level, patients with hypertrophy and heart failure-dependent loss of heart muscle function show changes in mechanisms of temporal $[Ca^{2+}]_{in}$ increase and in expression of contractile proteins.

$[Ca^{2+}]_{in}$ alterations suggest changes in the functioning of L-type Ca^{2+}-channels or in mechanisms of intracellular Ca^{2+}-release from sarcoplasmic reticulum (SR) [Missiaen et al., 2000]. For instance, in hypertrophic animal models decreased ability of L-type Ca^{2+}-channels to activate Ca^{2+}-dependent-Ca^{2+} release was shown.

Thus, being an important signalling molecule in the heart tissue, Ca^{2+} not only contributes to the cellular contractility and activation of different signalling cascades and pathways, but also plays a role in the development of different heart diseases, among which are heart failure and hypertrophy. Therefore, normal and abnormal homeostasis of Ca^{2+}, and its role in the physiological and pathophysiological conditions in the heart are crucial for investigation. Although, much has been done in this direction, still a lot of questions remain elusive.

2.3. Ca^{2+} as a first messenger

Ca^{2+} is a crucial molecule in our organism. Ca^{2+} ions are well known to be important second messengers. They trigger contractility, signal transduction, activation of enzymes. Intracellular calcium concentration is strictly regulated and can be changed on a short-term by opening of the calcium channels, its release from intracellular stores and work of Ca^{2+}-pumps and adenosinetriphosphatases (ATPases). Extracellular Ca^{2+}-concentration under physiological conditions is also quite stable. This can be achieved by strict regulation of Ca^{2+}-homeostasis by different mechanisms. One of them is functioning of parathyroid glands. Via so-called calcium-sensing receptors (CaR), the parathyroidea detects Ca^{2+} levels outside the cell. If Ca^{2+}-concentrations are low, it releases the parathyroid hormone (PTH) [Chattopadhyay et al., 2006]. PTH in turns

activates bones osteoclasts, leading to Ca^{2+}-reabsorption. Vice versa, high extracellular Ca^{2+} levels, detected by CaR, inhibit PTH release.

So thus, Ca^{2+} acts as an important first messenger via CaR, first shown in parathyroidea [Brown et al, 1993]. However, not only parathyroidea cells express these receptors. CaR were found in many organs involved in Ca^{2+} homeostasis, like kidneys, bones, intestines [Yamaguchi et al., 1998; Chattopadhyay et al., 1998; Chattopadhyay et al., 2004; Riccardi et al., 1995] and also nervous and cardiovascular systems [Tfelt-Hansen et al., 2006; Yano et al., 2004].

2.4. CaR: structure and function

The CaR belong to the family C of the G protein-coupled receptors (GPCRs). GPCRs are seven transmembrane receptors which are known as the largest group of cell surface membrane receptors and they have become the most important drug targets [Tfelt-Hansen et al., 2003].

CaR have three structural domains: large amino-terminal or extracellular domain (ECD, 612 amino acid residues), a seven transmembrane domain (TMD, 250 amino acid residues) and a C-terminal or intracellular domain (ICD, 216 amino acid residues). The receptor is modified by N-glycosylation [Ray et al., 1998] and it is presented in homodimeric configuration on cell surface, which is crucial for its normal function [Bai et al., 1999]. The main extracellular Ca^{2+} binding site is ECD, but it was also shown that TMD takes part in Ca^{2+}-sensing [Ray et al., 2002].

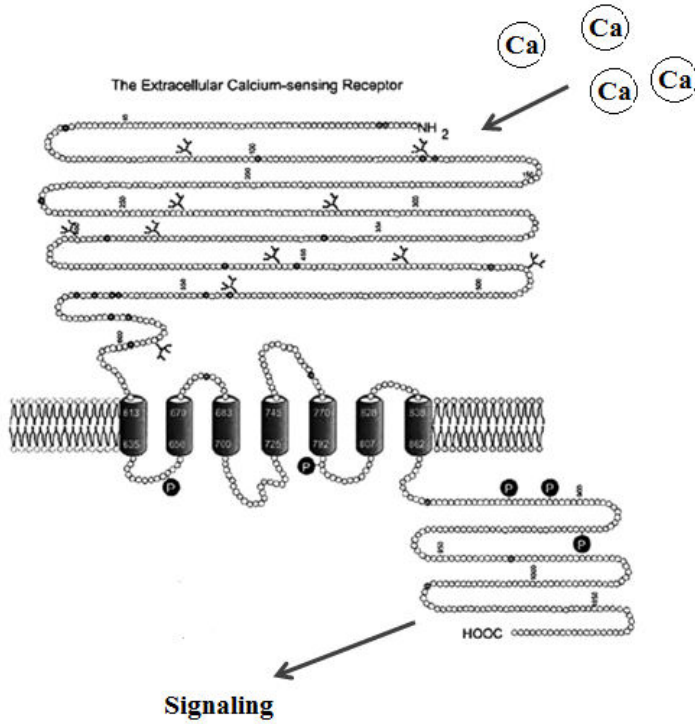


Figure 1. CaR structure [modified from Smajilovic et al., 2008]

The main ligand of the receptor is Ca^{2+}. However, it has other ligands which belong to two different groups: direct agonists and allosteric modulators. Allosteric modulators change the affinity of the receptor to Ca^{2+} and other direct agonists. For example, CaR activation with Ca^{2+} is faster in the presence of calcimimetic AMG 073, which is a positive allosteric modulator of the receptor [Holstein et al., 2004]. Direct agonists have higher ability to bind to the receptor if they have high

positive charges. Moreover, CaR sense changes in ionic strength and pH [Quinn et al., 1998; Quinn et al., 2004] (Table 1).

Direct agonists	**Positive Allosteric Modulators**	**Negative Allosteric Modulators**
Cations (Ca^{2+}, Mg^{2+}, Gd^{3+}, etc)	L-type amino acids	Calcilytics
Polyamines (putrescine, spermine, spermidine)	Calcimimetics	N-tricyclo[3.3.1.13,7]dec-1-yl-2-quinoxalinecarboxamide (NPS2390)
Antibiotics (neomycin, etc)		
pH		
Ionic strength		

Table 1. Agonists and antagonists of CaR

CaR have a low-affinity, but their Hill coefficient is three-four (measured in bovine parathyroid cell) [Quinn et al, 1997]. Due to this property, the receptor is able to detect very small changes in extracellular Ca^{2+}.

Activation of the receptor leads to different intracellular events, depending on the cell type where it is expressed. In most of the cells CaR activation consistently leads to a phospholipase C (PLC)-dependent

formation of inositol triphosphate (IP_3) which in turns releases Ca^{2+} ions form SR. This effect is mediated by an activation of receptor subunit Gαq [Tfelt-Hansen et al., 2006; Kifor et al., 1997]. These events also result in protein kinase C (PKC) mobilization, which in turns modulates activity of the CaR by negative feedback [Bai et al., 1998]. CaR activate via PLC phosphatidylinositol 4-kinase (PI4K) enzyme, which contributes to the first step in inositol lipid biosynthesis [Huang et al., 2002].

CaR also interact with Gαi which leads to the inhibition of adenylate cyclase (AC) and a drop in cellular cyclic adenosine monophosphate (cAMP) [Holstein et al., 2004].

The CaR are supposed to be linked to several signalling pathways of different MAPKs. These pathways are responsible for proliferation, differentiation and regulation of peptide secretion and ion channel activity. [Kifor et al., 2001; Tfelt-hansen et al., 2005; Tfelt-Hansen et al., 2004 ; Tfelt-Hansen et al., 2003].

2.5. CaR in Ca^{2+}-homeostasis and their expression in different organs

CaR play an important role in Ca^{2+}-homeostasis in the body. As mentioned previously, their expression was shown in parathyroid glands for the first time. Here they regulate the release of PTH. A decreased Ca^{2+} level in plasma results in CaR-mediated PTH release from parathyroid cells. PTH promotes Ca^{2+} reabsorption and bone resorption which leads to an increase in plasma Ca^{2+} levels [Chattopadhyay et al., 2006]. $1.25(OH)_2$ vitamin D_3 in kidney proximal tubular cells is being

synthesised under PTH and low Ca^{2+}-level. Vitamin D_3 metabolites trigger Ca^{2+} absorption in the intestine. CaR also modulate secretion of calcitonin from thyroid C cells, preventing inhibition of bone resorption by calcitonin under high plasma Ca^{2+}. [Copp, 1994].

Except for parathyroid glands, CaR expression has been demonstrated to be crucial for Ca^{2+}-homeostasis in other organs, too, namely in kidneys, bones and the intestine [Riccardi et al., 1996; Yamaguchi et al., 1998; Chattopadhyay et al., 1998; Chattopadhyay et al., 2004].

CaR in kidney

In the kidneys, the receptor is being expressed in almost all nephron segments including the ascending limb, distal and proximal tubules, collecting duct and glomeruli. The highest levels of CaR were found in the ascending limb [Graca et al., 2016]. Depending on the function of the specific tubular tracts, CaR inhibit the reabsorption of Ca^{2+}, K^+, Na^+ and water. It also modulates signalling pathways of tubulocytes, which activate electrolyte or water reabsorption. In proximal tubules it is antagonising the effect of PTH by increasing phosphate reabsorption [Vezzoli et al., 2009].

CaR in gastrointestinal tract

CaR are expressed in many segments of gastrointestinal tract, such as stomach, esophagus and colon [Magno et al., 2011; Chattopadhyay et al., 1998]. In the esophagus, CaR stimulation increases phosphorylation of extracellular signal-regulated kinase secretion of interleukin-8 (IL-8) and intracellular Ca^{2+} mobilization [Justinich et al., 2008]. In the stomach CaR are expressed in different cell types and accordingly lead to

different responces and events. Pathways activated by CaR also differ in these cells. CaR in G-cells activate PLC pathways and modulate gastrin production [Buchan et al., 2001]. It was also shown that CaR activation in human mucous epithelial cells results in increased proliferation and intracellular Ca^{2+}-levels [Rutten et al., 1999]. In parietal cells, the receptor regulates H^{+}-K^{+}-ATPase activity, which is necessary for gastric acid secretion [Dufner et al., 2005].

In the colon CaR agonists led to the activation of PLC induced pathways and also to cAMP inhibition [Cheng et al., 2002; Cheng et al., 2004]. It inhibits the forskolin-stimulated fluid secretion in colonic crypt cells [Cheng et al., 2004] and up-regulates expression and secretion of bone morphogenetic protein in myofibroblasts [Peiris et al., 2007]. Despite the CaR's influence to proliferation, they negatively regulate cell proliferation and differentiation markers in cultured intestinal cell lines [Chakrabarty et al., 2003]. In conclusion, CaR regulate proliferation and differentiation processes in tissue specific manner.

CaR in bones

The first evidence of the presence of CaR in bone appeared in 1997 after experiments with osteoblastic MC3T3-E1 cell line [Yamaguchi et al., 1998]. They have been shown to be expressed in osteoblastic [Chang et al., 1999] as well as in osteoclastic cells [Kameda et al., 1998]. In osteoblasts CaR induce cell proliferation [Dvorak et al., 2004], but mechanisms are still remain unclear. In osteoclasts, stimulation of the receptor results in the inhibition of a bone resorbing activity [Chang et al., 1999]. It also plays a role in differentiation and apoptosis of osteoclastic cells in PLC-dependent manner [Mentaverri et al., 2006].

CaR in nervous system

CaR are also presented in different parts of the central nervous system [Yano et al, 2004]. The highest level of CaR expression was detected in the subfornical organ, which is exposed to systemic fluid due to an absence of a blood brain barrier [Yano et al., 2004]. Stimulation of CaR in the neurons of the subfornical organ leads to a subthreshold, hyperpolarization activated inward current [Washburn et al., 2000]. There is also high expression of CaR in the hippocampus, where they regulate the opening of Ca^{2+}-permeable, non-selective cation channels and Ca^{2+}-activated K^{+}-channels [Ye et al., 1997 A]. It has also been found that in hippocampus CaR can be activated by amyloid peptides. They are excessively produced in patients with Alzheimer's disease [Ye et al., 1997 B]. Another protein, alipoprotein E, linked to Alzheimer's disease, is also able to activate CaR [Conley et al., 2009]. Furthermore, CaR were shown to affect synaptic transmission [Phillips et al., 2008].

CaR are not only expressed in neurons. Their presence was shown in microglia [Chattopadhyay et al., 1999], oligodendrocytes [Chattopadhyay et al., 1998] and astrocytes [Chattopadhyay et al., 2000]. There they regulate Ca^{2+}-activated K^{+}-chanels and promote parathyroid hormone-related protein (PTHrP) secretion respectively [Chattopadhyay et al., 1999; Chattopadhyay et al., 1998].

Other groups showed that the receptor is also expressed in mammary glands [Cheng et al., 1998], epidermis [Bikle et al., 1996] and some other organs.

In summary, CaR were detected in many different tissues where its role also varies. They are responsible for an activation of many different pathways leading to many biological actions in the body. Thus they are interesting therapeutic targets. Nevertheless, more investigation on their role and expression should be done.

2.6. CaR in cardiovascular system

The crucial role of Ca^{2+}-ions in the regulation of different physiological functions in the heart is known since long ago. Now it is well accepted that Ca^{2+}-ions are very important second messengers in the heart tissue. Increased Ca^{2+}-concentration inside the cardiomyocyte, via Ca^{2+}-dependent Ca^{2+}-release form SR, is required for the electromechanical coupling of heart cells. With every heart beat intracellular Ca^{2+}-concentration is elevated approximately 10-fold from basic level. Therefore, intracellular Ca^{2+}-concentration is important to be regulated. Several mechanisms exist to meet this demand such as a regulation of the activity of Ca^{2+}-channels, pumps, proteins and exchangers.

As explained above, Ca^{2+} is also able to act as a first messenger in cardiac tissue via CaR on the cardiomyocytes plasma membrane.

First results indicating CaR expression in the heart appeared in 2003 when Wang et al. identified CaR in rat heart tissue by real-time polymerase chain reaction (RT-PCR) and immunoblot analysis [Wang et al., 2003]. In the cardiovascular system CaR are expressed in neonatal and adult heart muscle cells [Tfelt-Hansen et al., 2006; Wang et al., 2003] as well as in vascular smooth muscle cells [Smajilovic et al.,

2006] and endothelial cells [Weston et al., 2005]. A group of scientists has also reported a presence of CaR in perivascular nerves of isolated rat arteries and showed that increased $[Ca^{2+}]_{out}$ causes nerve-dependent relaxation of pre-contracted arteries [Bukoski et al., 1997]. In vascular smooth muscle cells CaR are involved in modulation of vessel tone and thus blood pressure regulation [Smajilovic et al., 2008]. CaR in endothelial cells adjust opening of Ca^{2+}-sensitive K^+-channels [Weston et al., 2005] and thus, also contributing to the blood pressure control.

mRNA and protein of the CaR were detected in both atrial and ventricular cardiomyocytes of Sprague–Dawley rats [Wang et al., 2003]. It is Gαq coupled. Intracellular Ca^{2+} and IP_3 levels were elevated after activation of cardiovascular CaR [Tfelt-Hansen et al., 2006]. This finding indicates PLC-linked CaR activation in the heart. CaR agonists also led to MAPK signalling pathways activation [Holstein et al., 2004].

2.7. CaR and receptor activity-modifying proteins interaction

CaR were reported to require activity of the receptor activity modifying proteins (RAMPs) to be presented on cell surface [Bouschet et al., 2005]. RAMPs are single transmembrane proteins which are expressed in many cells [McLatchie et al., 1998; Morfis et al., 2003]. Three types of these receptors were shown: RAMP1, RAMP2 and RAMP3 respectively [McLatchie et al., 1998]. Bouschet at al demonstrated that RAMP1 and RAMP 3 are important to stimulate CaR traffic to plasma membrane in HEK293 cells [Bouschet et al., 2005]. CaR were the first members from its class (C class, GPCRs family) shown to require RAMPs activity. RAMP1 and RAMP3 levels determine the presence or absence of functional CaR. Interestingly the expression of both RAMPs was

increased in cardiac tissue and myocytes in rats with chronic heart failure [Cueille et al., 2002].

2.8. CaR in heart disease

Ca^{2+} control is very important for the physiological function of the heart. Therefore, plasma Ca^{2+} levels as well as intracellular Ca^{2+} are strictly controlled by various mechanism mentioned above (Ca^{2+}-uptake, Ca^{2+}-release and plasma Ca^{2+}-homeostasis). During the development of heart failure, these mechanisms are being violated. Dysfunction of the intracellular Ca^{2+}-cycle is crucial for the pathogenesis of heart failure [Orrenius et al., 2003]. The sarcoplasmatic reticulum (SR), as an intracellular Ca^{2+}-storage organelle, is sensitive to intracellular Ca^{2+}-alterations and disturbances. The exhaustion of Ca^{2+} from the SR can disrupt its function and lead to SR stress, which in turns, leads to cell damage and dysfunction. Ultimately, this can result in apoptosis [Kaufman, 2002]. SR stress is also involved in several heart diseases, namely hypertension, myocardial ischemia and dilated cardiomyopathy [Ni et al., 2011]. Main mechanism leading to an increased intracellular Ca^{2+} is Ca^{2+}-dependent-Ca^{2+}-release via ryanodine receptors (RyRs). Nevertheless, IP_3-receptors (IP_3R) on SR membrane play a role in Ca^{2+}-release [Mackenzie et al., 2002]. Some studies have demonstrated that IP_3R expression was enhanced in patients with end-stage chronic heart failure, and RyRs, in contrast, were downregulated [Go et al., 1995]. These findings lead to the possible role of CaR in the development of different heart pathologies, as CaR are known to trigger IP_3-dependent Ca^{2+}-release.

It was also shown that IP_3R play a role in SR-mitochondria cross-talk, leading to an increased Ca^{2+}-uptake by mitochondria [Seguchi et al., 2005]. This SR-mitochondria interaction is observed in failing hearts and inducing programmed cardiomyocytes death [Pacher et al., 2001]. Lu et al suggested that CaR signalling play key role in cardiomyocyte apoptosis via interplay between SR and mitochondria in the progression of heart failure. This effect of the receptor is IP_3R mediated [Lu et al., 2013].

Development of apoptosis was also reported in neonatal rat ventricular cardiomyocytes under CaR stimulation. This effect is promoted by MAPK and caspase 9 signalling pathways activation [Sun et al., 2006]. Participation of CaR in neonatal rat cardiomyocytes apoptosis induced by ischemia-reperfusion (I/R) injury was also shown [Jiang et al., 2008]. I/R injury induced CaR enhanced expression and increased rate of apoptosis.

Interestingly, inhibition of CaR during post-conditioning of the heart significantly improved function and decreased apoptosis rate after I/R injury [Gan et al., 2012].

Other events that may involve CaR activation are hypertension and hypertrophy development. Group of scientists demonstrated involvement of the receptor in Ang II-induced hypertrophy [Wang et al., 2008]. But so far not many data indicated the functional role of the receptor during cardiac hypertrophy development.

2.9. ET-1 and cardiac hypertrophy development

ET-1 is a potent vasoconstrictor peptide consisting of 21 amino acids. In cardiovascular system it is produced by vascular endothelial cells, endocardial endothelial cells and cardiomyocytes [Yanagisawa et al., 1988; Rubanyi and Polokoff, 1994; Mebazaa et al., 1993; Cingolani et al., 2006] with the help of endothelin-converting enzyme 1 (ECE1) as a respond to hypertension, stress, diabetes, etc. Its action is provided via G-protein-coupled receptors. There are two main isoforms of ET-receptors: ET receptors type A (ET_A) and ET receptors type B (ET_B) [Fareh et al., 1996]. ET-1 induces positive inotropic effect in acute way, negative inotropic and growth stimulation effects in long-term exposure [Hinrichs et al., 2011].

High plasma levels of ET-1 and increased expression of ET-receptors have been found in many models of heart failure [Omland et al., 1994].

Interestingly, two subtypes of ET_B receptors were recently investigated, that are PD142893 sensitive (ET_{B1}) or resistant (ET_{B2}). It is supposed that ET_B receptors participate in the development of cardiac hypertrophy [Lee et al., 2004].

Group of scientistsShiraishi et al. showed the relation between CaR and increased ET-1 expression in kidney [Shiraishi et al., 2003]. This leads to the centre questions addressed in this study.

3. Aims

The aims of the study was to identify the role of CaR in cardiac physiology and the pathophysiology of heart hypertrophy. Three aims were addressed:

- to investigate CaR expression in isolated rat cardiomyocytes and cardiac tissue under physiological and pathophysiological conditions.
- to determine the functional role of the CaR in basal electromechanical coupling of the cardiac muscle cells.
- to study whether CaR coupling is modified in the development cardiac hypertrophy
- to clarify mechanisms, involving CaR in these events.
- to identify the relationship between ET-1, RAMP-1, and CaR during cardiac hypertrophy.

4. Material and Methods

4.1. Buffers and solutions

4.1.1. Powell Medium (All Chemicals provided by Carl Roth, Germany):

NaCl	110.0 mM	6.43 g/l
KCl	2.6 mM	0.19 g/l
KH_2PO_4	1.2 mM	0.16 g/l
$MgSO_4$	1.2 mM	0.30 g/l
HEPES	25.0 mM	5,96 g/l
Glucose	11.0 mM	2.18 g/l

4.1.2. Collagenase solution:

- 5 ml of warmed up and oxygenated Powell Medium
- 12.5 µl of 0.1 M $CaCl_2$ solution
- 25 mg collagenase (type 2, CLS II, Biochrom), the amount is batch addicted

4.1.3. CCT Medium (10L):

- 2 packs M199 (Biochrom, Germany) dissolved in 10L dist. H_2O

- 36 g/l HEPES (Carl Roth, Germany)
- 6.56 g 5мM Creatine (Sigma Aldrich, Germany)
- 3.95 g 5 мM Carnitin (Sigma Aldrich, Germany)
- 6.26 g 5 мM Taurine (Sigma Aldrich, Germany)
- 10 mg Arabinofuranosyl-cytosine (Sigma Aldrich, Germany)

pH= 7.4

4.1.4. Cell lysis buffer (2 ml):

- 1770 µl lysis buffer (self-made: 50mM Tris with HCl ph 6,7 + 2% SDS)
- 20 µl protease inhibitor (Sigma Aldrich, Germany)
- 10 µl phosphotase inhibitor (Sigma Aldrich, Germany)
- 200 µl Dithiothreitol (DDT) (Roche Life Sciences, USA)

+ Benzonase (1:30) 5 µl per plate after 1st rotation step (Merck, Germany)

4.1.5. Tissue lysis buffer (1 ml):

- 100 µl lysis buffer (Cell Signaling Technology, Germany)
- 900 µl H_2O
- 10 µl Phenylmethane sulfonyl fluorid, PMSF (0.1 M) (Life Technologies, USA)

4.1.6. Contraction buffer (to measure Ca^{2+}-transients and load-free cell shortening under different Ca^{2+}-concentrations)**:**

NaCl	125 mM
KH_2PO_4	1.2 mM
KCl	2.6 mM
$MgSO_4$	1.2 mM
Glucose	10 mM
HEPES	10 mM

pH = 7.4

4.1.7. FURA-2 AM loading buffer:

- 5µl FURA-2 AM (50mg FURA-2AM (Life Technologies, USA) in 300µl DMSO)
- 1 ml contraction buffer

4.1.8. MOPS Running buffer (1x):

- 50 ml MOPS SDS Running Buffer (20x) (NuPAGE, Life Technologies, USA)
- 950 H_2O

4.1.9. Transfer Buffer (500 ml):

- 374.5 ml H_2O
- 25 ml transfer buffer (Life Technologies, USA)
- 100 ml methanol (Carl Roth, Germany)
- 500 µl antioxidant (contains N,N-Dimethylformarmide) (Life Technologies, USA)

4.1.10. Blocking buffer (for one membrane):

- 10 ml 1% TBS (AppliChem, GmbH, Germany)
- 0.5 g BSA (Albumin Fraction V, Roth, Germany)

4.1.11 Primary antibody solution (for one membrane):

- 10 ml 1% TBS (AppliChem, GmbH, Germany)
- 0.5 g BSA (Albumin Fraction V, Roth, Germany)
- Primary antibody according to company protocol (Table 1)
- 10 µl Tween (AppliChem, GmbH, Germany)

4.1.12. Secondary antibody solution (for one membrane):

- 10 ml 1% TBS (AppliChem, GmbH, Germany)
- 0.5 g BSA (Albumin Fraction V, Roth, Germany)

- Secondary antibody according to company protocol (Table 2)
- 10 µl Tween (AppliChem, GmbH, Germany)

4.1.13 PKC Kinase Activity Assay Kit (Assay Designs, Ann Arbor, USA), amount per well:

- PKC Substrate Microtiter Plate, precoated with substrate peptide for PKC (1 plate – 96 wells)
- 40 µl Phosphospecific Substrate Antibody (1 µg/ml solution of rabbit polyclonal antibody specific for phosphorylated PKC substrat)
- 40 µl Anti-Rabbit IgG: HRP Conjugate (1 µg/ml solution solution of horseradish peroxidase conjugated goat antirabbit IgG) diluted in Antibody Dilution Buffer (1mg/ml)
- 50 µl Kinase assay dilution buffer (for ATP and samples dilution)
- 10 µl ATP
- 40 µl Active PKC (20 µg/ml)
- 100 µl 20x Wash buffer
- 60 µl TMB Substrate (Stabilized tetramethylbenzidine substrate)
- 20 µl Stop Solution (Acid solution to stop color reaction)

4.1.14 DNA mix (for 1 sample):

- 2 µl 5*RT-Buffer

- 1 µl Oligo d tang
- 1 µl dNTP's
- 0.5 µl DTT (0.1 M) (Invitrogen, Canada)
- 0.2 µl RNAsin (40 U/µl) (Promega, USA)
- 0.3 µl M-MLV reverse transcriptase (200 U/µl) (Invitrogen, USA)

4.1.15. Primer mix (for 1 sample):

- 10 µl IQ Cyber Green Supermix (BIO-RAD, Germany)
- 0.6 µl Primer (Table 2)
- 6.4 µl H2O

4.1.16. Agarose-gel loading buffer:

- 1 ml 10xTAE buffer (BioLine, Germany)
- 0.5 ml Glycerin (Karl Roth, Germany)
- Bromphenolblue (add a little bit untill solution is blue) (Merck, Germany)

4.1.17. Loading marker (for 1 well):

- 5 µl 100 bp DNA ladder (Fermentas, GmbH, Life Sciences, Germany)
- 10 µl Loading buffer
- 58 µl 1XTAE-buffer

4.2 Methods

4.2.1. Isolation of cardiomyocytes

Cells were isolated from male Wistar rats aged three to four moths *according to the guideline of animal welfare.* Rats were killed under deep 4-5% Isofluran (Baxter GmbH, Germany) anesthesia, hearts with intact aorta were excised from the opened chest and placed into ice-cold salt solution (0.9% NaCl). Lung, esophagus, trachea and thymus were removed. Aorta was shortened at the second vessel.

Subsequently, the heart was rinsed to the Langendorff perfusion system which was filled with 80 ml The Powell Medium had been warmed up and oxygenated with carbogen (95%O_2, 5%CO_2). The heart was rinsed, putting cannula of the perfusion system inside aorta lumen and fixing it in this position with a clip and a rope after.

The hearts were washed from blood with Powell Medium until 45 ml remained in Langendorff system. Afterwards, the system was closed and 5 ml of prepared collagenase solution were added.

Perfusion time was 25 min. After the hearts (without atria) had been removed from system, the ventricles were separated from the atria and cut into small pieces using a chopper (0.7 mm). The ventricles were transferred on a watch glass and chopping continued with two scalpels.

The resulting homogenate was exposed to collagenase solution for five more minutes and then filtered using nylon mesh.

Subsequently, three centrifugation steps were performed (Allegra 21 centrifuge, Beckman Coulter, Germany):

- 400 rpm 3 min (250 µM $CaCl_2$)
- 400 rpm 2 min (500 µM $CaCl_2$)
- 300 rpm 1 min (1 mM $CaCl_2$)

Afterwards, the cells were plated (with CCT medium or contraction buffer with 0.5 mM, 1 mM, 1.2 mM, 2 mM or 4 mM $CaCl_2$) on FCS-precoated culture dishes (precoated with 4% (v/v) FCS (Fetal Cow Serum, PAA Laboratories GmbH, Austria) and 2% (v/v) penicillin and streptomycin (Gibco, Life Technologies, Germany)). They were incubated for one hour (incubator BBD 6220 Heraeus, Thermo Scientific, Germany) and then exposed to different analysis.

4.2.2. Determination of load-free cell shortening

Cell contraction was measured at room temperature and analyzed using cell-edge-detection system as described before [Langer et al., 2003]. The cells were stimulated via two AgCl electrodes with biphasic electrical stimuli, composed of two equal but opposite rectangular 50-V stimuli of 5 ms duration. Cardiomyocytes were exposed to 2 Hz stimulation. Contraction signals were recorded every 15 s. The mean of these four measurements were used to define the shortening of each cell. A special black and white linear camera was used to measure the cells' lengths (data recording at 500 Hz). The cells were situated in CCT Medium with 1.25 mM extracellular Ca^{2+}-concentration. In some series of experiments the effects of different extracellular Ca^{2+}-concentrations were analyzed.

In this part of experiments, cells were incubated in a cell culture buffer as described previously [Piper et al., 1989]. Cells were then situated in contraction buffer with 0.5, 1.0, 1.25, 2.0 or 4.0 mM Ca^{2+}-concentrations respectively. Data is presented as cell shortening normalized to cell diastolic length (dL/L(%)).

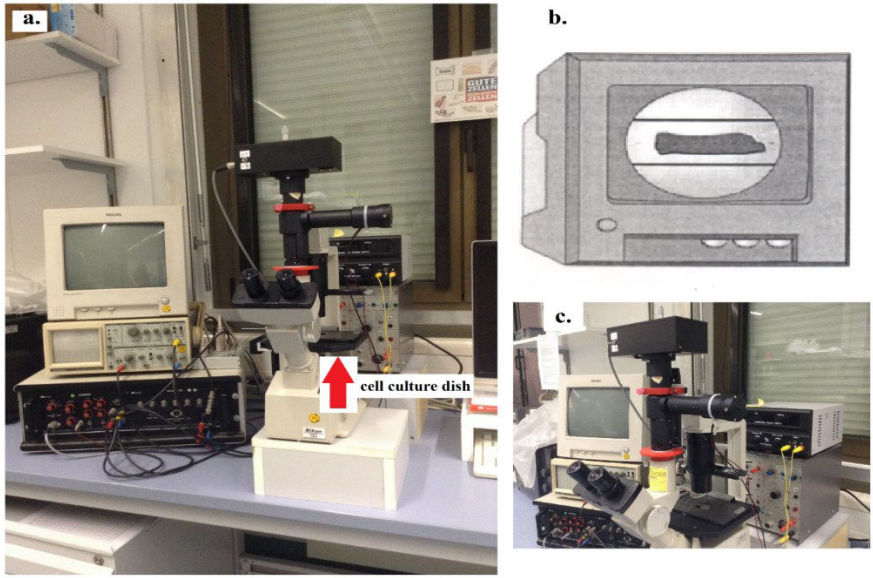


Figure 2. a. Cell-edge-detection system (SH Heidelberg, Germany). **b.** screening of cardiomyocyte via black and white linear camera. **c.** Linear camera. Cell edges detected by linear camera calculated by software Cell (Heidelberg, Germany) and expressed as difference between systolic and diastolic cell lengths in percent (dL/L,%)

4.2.3. Quantification of Ca^{2+}-transients

To investigate further the effects of CaR activation on cell shortening, systolic Ca^{2+}-transients were measured. Fluorescent dye FURA-2 acetoxymethyl ester (FURA AM – see Buffers and Solutions) was used [Ladilov et al., 2003]. Cells were isolated as described previously (see cardiomyocytes isolation). Isolated cardiomyocytes were placed onto laminin-precoated glass cover-slips (Roth, Germany) in M199. Immediately before systolic Ca^{2+} measurements, M199 was replaced by FURA-2 AM loading buffer (see buffers and solutions) for 30 min at 37 ^{o}C. After loading, the cells were washed with contraction buffer (see buffers and solutions) to allow hydrolysis of acetoxymethyl esters within the cells. When added to the cell, FURA-2AM crosses cell membrane and once inside the cell, acetoxymethyl is removed by cellular esterases. Cover slips with loaded cells were introduced into a gas-tight, temperature-controlled (37^{o}C), transparent perfusion chamber positioned in the light path of an invert microscope (Nikon eclipse TS100). Alternation excitation of the fluorescence dye at wavelength of 340/380 nm was performed with an AR-caution system (ION optix Corp.) adapted to microscope. ION Optix Corp. system was used to capture light emitted (500-520 nm) from the single fluorescent cell and background area. The data was analyzed as the ratio of light emitted at 340-to-380 nm wavelength. Background area and non-stimulated sarcomeres of the cardiomyocyte were measured first (fluorescence of cells were 10-20 times higher that background fluorescence), followed by measurements of 1 Hz Myo Pacer (ION Optic Corp., Germany) stimulated cell sarcomeres.

4.2.4. Tensometry

Functional properties of rat myocardium were examined on heart muscle strips *in vitro*. BiopacSystems setup (USA) with isometric transducer TSD 125C was used (Fig. 3).

Rats were narcotized with Isofluran (4-5%). The heart was excised from the chest and then placed into Powell Medium with 1,5 mM $CaCl_2$. Ventricles and atria were cut in small strips (4-6 mm length, 0.8-1 mm diameter) and put into the 20 ml bath with oxygenated (95% O_2, 5% CO_2) Powell Medium at room temperature. Upper edge of the strips preparations were connected to tense transducer via a metal lath (plank). Lower edge was fixed to the bath base.

The strips were stimulated with 2 silver plated electrodes (stimulant ESL-2, Russia) with 0.1 Hz frequency, 40 mV amplitude, and 5 ms duration.

Strips were placed to bath Powell Medium and elaboration during 40-60 min was performed. When signals were stable, control parameters were registered with original software (Elf, A.V. Zaharov, Russia). Tested substances were added directly to the baths with muscle strips preparations. Drug effects were calculated in percent comparison to the control values. Signals were calculated using Elf, Origin and Excel.

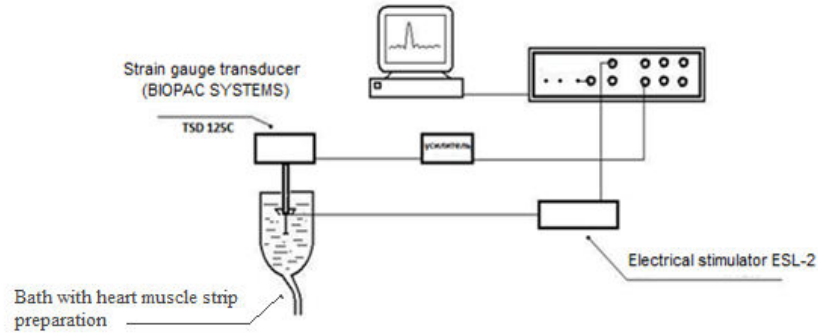


Figure 3. Tensometric setup scheme

4.2.5. Rat model of nitric oxide deficiency

The experimental model of nitric oxide-deficiency (NO-deficiency) was used to investigate left ventricular CaR and ET-receptors expression. Three months old Wistar rats were divided into four groups and kept in individual cages. Animals had unlimited access to water and food. Non-specific NO-synthase (NOS) inhibitor L-NAME (*L-nitro-argenine methyl ester hydrochlorid*, Sigma Aldrich, Switzerland) was added to drinking water (7.5mg/day) during 1 month in the first animal group and during 6 months in the second animal group. Group 3 and 4 consisted of animals, not treated with L-NAME in corresponding age with 1 and 2 group [Schreckenberg et al., 2015]. Heart tissue samples were collected thereafter for qRT-PCR and Western Blot analysis.

These experiments have been described before in greater details [Schreckenberg et al., 2015]. Tissue samples from these experiments have been used for this study.

4.2.6. Tissue lysis

To prepare tissue samples for immunoblot analysis isolated hearts were cut in small pieces (0.1 g), collected to the tubes and supplied with 500 µl tissue lysis buffer (10 times dilution) provided by Cell Signaling Technology (Germany). Samples were then homogenized (6,000 upm - Homogenisator Precellys24, Peqlab Biotechnology, GmbH Germany) in Precell-Tubes 2 times for 20 sec. After 10 min cold homogenates exposed to 30 s sonication (60% amplitude, 0.7 cycle) using Ultra Sonic Processor (UP100H, Hielscher, Germany) and 10 min spin in centrifuge (14,000g, 4^{o}C, Allegra 25R centrifuge, Beckman Coulter). Supernatants were collected and diluted in the ratio 1:50 with water. In the end 40 µl of Laemmli buffer (Sigma, USA) were added to each 100 µl of samples.

4.2.7. Cell lysis

Cardiomyocytes were isolated as described before and collected for protein analysis. Cell culture medium was removed from plates and cells were washed with ice-cold PBS. Each culture dish was supplied with 100 µl of lysis buffer (see Buffers and Solutions) and placed onto the Vortex (Unimax 1010, Heidolph Instruments) for 30 min. After 15 min Benzonase were added to each culture dish and rotation continued. In the end cell suspension was scraped with cell scrapers (Sarstedt Inc, USA) and collected to 1.5 ml tubes (Sarstedt Inc, USA). Samples were frozen at -20^{o}C or Western Blot analysis was directly performed.

4.2.8. Western Blot analysis

To perform Immunoblot analysis tissue or cell lysates were diluted with 20 µl of Bromphenol Blue (BPB, Merck, Germany) and warmed up in Thermostat (5 min, 95°C; Techne DRI-BLOCK DB-20, Labtech International). The protein samples were separated using gel electrophoresis. Samples with BPB (20 µl each) were loaded onto the wells of the 15% SDS-PAGE gel (Invitrogen, USA) which was placed into the chamber with MOPS running buffer (Buffers and Solutions). First well of each gel was loaded with Marker (Spectra Multicolor, Thermo Scientific, USA). 200 V was applied along the gel for one hour. Proteins were spread according to their molecular weight.

To be able to detect and quantify proteins, loaded gels were next exposed to the second electroblotting step of 30 V together with Nitrocellulose Transfer Membranes (Whatman GmbH, Germany) in the chamber containing transfer buffer (Buffers and Solutions). After 60-75 min proteins together with marker were transferred onto membrane. To avoid subsequent non-specific bindings of antibodies, transfer membranes were incubated with blocking buffer (Buffers and Solutions) for 1 hour under gentle agitation.

To detect targeted proteins, special antibodies against them were used (Table 2). After blocking membranes with transferred proteins were exposed to the dilute solution of primary antibodies (Buffers and Solutions) over night at 4°C under agitation. To wash unbound probes of primary antibodies away 1% TBS together with 0.1% Tween were used. Secondary antibodies solution directed at a species-specific portion (Table 2) of the primary antibody were used thereafter. Secondary

antibodies are able to bind chemiluminescent agents, and thus visualize certain protein level. Several secondary antibodies bind to one primary antibody which enhances the signal.

To visualize the targeted protein level Chemiluminescent Substrate (Thermo Scientific, USA) and chamber (Peqlab, Biotechnologie, Germany) were used. *Quantity One* software detected and calculated protein levels (BioRad, Germany).

Name	Catalog number	Source
Anti-CaR	SAB4503369, Sigma Aldrich, Germany	Antibody produced in rabbit
Anti- RAMP1	Sc-11379, Santa Cruz Biotechnology, Inc, Germany	Rabbit polyclonal antibody
Anti-GAPDH	CB1001, Merck, Germany	Mouse monoclonal antibody
Anti-β-actin	A2668, Sigma Aldrich, Germany	Antibody produced in rabbit
Anti-Phospholamban (FL-52)	Sc-30142, Santa Cruz Biotechnology, Inc, Germany	Rabbit polyclonal antibody
Anti-P-Phospholamban, phosphorylated form of phospholamban (Ser 16)	Sc-12963 Santa Cruz Biotechnology, Inc, Germany	Goat polyclonal antibody

Table 2. Antibodies used in the study

4.2.9. Determination of PKC activity

In order to quantify PKC activity the nonradioactive activity assay (Assay Designs Ins, Ann Arbor, USA) was used. The concept is based on a solid phase enzyme-linked immune-absorbent assay that utilizes a specific substrate for PKC and a specific antibody against the phosphorylated form of the substrate. Isolated cadriomyocytes, exposed to the cell shortening measurements and CaR agonists stimulation, were thereafter collected and lysed as described before. The clear supernatant was used to measure the entire protein concentration and the PKC activity according to the manufacturer suggestions (PKC Activity Assay Kit, Assay Designs, Ann Arbor, USA).

PKC Substrate Microtiter Plate wells were soaked with Kinase Assay Dilution Buffer for 10 min at room temperature right before sample addition. 40 µl of samples and control (blank) were added to appropriate wells thereafter at 30^{o}C. Subsequent procedure was made in room temperature. Reaction in each well was initiated by ATP supplement. After 90 min reaction was stopped by emptying the wells and phosphospecific substrate antibody was added for more 60 min. Wash buffer was used four times to wash wells after antibody incubation. Next, samples were incubated with HRP Conjugate (30 min, room temperature) and again washed four times with washing buffer. TMB substrate was added to the samples for substrate stabilization with subsequent Stop solution incubation. Sample analysis was performed using ELISA reader (TECAN, Germany) at 450 nm. Results were transferred and calculated in Excel. Relative kinase activity was taken as:

$$\frac{\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})}}{\text{Quantity of crude protein used per assay}}$$

4.2.10. Collection of cells for the PCR analysis

Cell isolation procedure was performed as described before. Isolated cells were plated onto 2 ml Petri dishes precoated with FCS. Cultures were stimulated according to requested conditions during 4 h. After incubation, cells were washed twice with ice-cold PBS, scrapped from plates and collected for the qRT-PCR analysis.

4.2.11. RNA isolation and quantification

Total cellular RNA was extracted using *Trizol* reagent (Invitrogen, USA). Heart tissue was cut in small pieces and put to Precell-Tubes. 1 ml Trizol was added to each tube and homogenization step was performed twice (5000 rpm, 20 s).

In case of isolated cells, 1 ml of Trizol was added to each sample and gently mixed for 5 min at room temperature.

For RNA purification 200 µl of chloroform was added to each sample, carefully shacked and spinned in centrifuge (12000 g, 4^{o}C; Allegra 25R, Beckman Coulter, Germany) for 15 min. Colorless aqueous phase was collected from each tube after centrifugation. 500 µl isopropanol was added to each tube and stored at room temperature for 20 min and then centrifugation step was repeated. Supernatant was gently removed and RNA precipitate was washed with 75% ethanol under centrifugation.

RNA samples then were air-dried for 1 h and dissolved with water (50 µl). RNA concentration was quantified with NanoDrop ND-2000 (peqLab Biotechnologie GmbH). Quantity of total RNA and hydrolyzed RNA was determined with the ND-2000 software. According to RNA quantity, water was added to each sample to a final 15 µl volume for the 3-fold dilution. cDNA then was synthetized using DNA-mix (see buffers and solutions) and cDNA thermal cycler (Cyclogene, Techne, Germany). Obtained cDNA samples were diluted with water in ratio 1:10 and then qRT-PCR was performed.

4.2.12. qRT- PCR

3 µl of dissolved (1:10) cDNA samples were supplied with 17µl primer mix (see Buffers and Solutions) and exposed to qRT-PCR analysis using amplificator. cDNA fragments were amplified by 45 cycles of PCR (denaturing, annealing and extension) according to primer protocol. The gene products were quantified using SYBR Green assays (BioRad, Germany). Melting curve analysis was performed to quantify certain PCR product. To find difference in expression, samples were normalized to the expression of the reference gene and related to the control treatment. GAPDH and B2M were used as reference genes (Table 3). The data was analyzed by relative quantitation $2^{-\Delta\Delta CT}$ method.

Gene	Forward	Reverse
CaR	AAGTGCCCGGATGACTTCTG	GGTTGGTGGCCTTGACGATA
ET_A	ATTTGGCCCTGCCTAGCA	CCCACCATTCCCACGATGA

receptor	AT	A
ET_B receptor	GCTAGCCATCACTGCGATCT	TGTCTTGGCCACTTCTCGTC
ECE-1	TCTGGCCAACATCACCATCC	TAGACCACGATGGGCTCAGA
RAMP-1	AGCATCCTCTGCCCTTTCATT	GACCACCAGGGCAGTCATG
B2M	GCCGTCGTGCTTGCCATTC	CTGAGGTGGGTGGAACTGAGAC
HPRT	CCAGCGTCGTGATTAGTGAT	CAAGTCTTTCAGTCCTGTCC

Table 3. Primer sequences used in the study

4.2.13. Agarose gel electrophoresis of cDNA fragments

To visualise cDNA amplificated fragments Agarose gel electrophoresis was performed after RT-PCR. Agarose gel was created by mixing 1 g of Agarose with 1xTAE-Buffer (see Buffers and Solutions) and boiling them together in microwave up to 60^{o}C. Mixture was supplied by 3.75 µl SYBR Safe dye (BioRad, Germany) and placed to a gel electrophoresis camera for 30 min until it was solid. Cuts for gel wells were made.

10 µl of samples were mixed with 2 µl of Loading Buffer (see Buffers and Solutions) and loaded into the wells. First well was filled by Loading Marker (see Buffers and Solutions). 75 V-voltage was applied

for the samples during 30 min. cDNA fragment bands were detected under UV- light of 366 nm (UV-chamber, PeqLab, Biotechnologie, GmbH, Germany) and captured by camera (Olympus SP-500UZ)

4.3. Statistics

Data are expressed as raw data points or means ± SD/SEM as indicated in the legend to the figures. ANOVA and the Student-Newman-Keuls test for post hoc analysis were used to analyze experiments in which more than one group was compared. In cases in which only two groups were compared, Student's t-test or Mann-Whitney test was employed, depending on a normal distribution of samples (Levene test). P levels are indicated as expressed in the legend to the figures.

5. Results

5.1. The role of the CaR in physiological heart functioning

5.1.1. Expression of CaR in adult rat cardiomyocytes

Expression of CaR in neonatal rat cardiomyocytes and cardiac tissue was reported previously in Sprague–Dawley rats [Tfelt-Hansen et al., 2006]. So here we start with confirming these findings for cardiomyocytes derived from adult Wistar rats. In different preparations we were able to detect stable mRNA level of CaR (Fig.4a). Moreover, CaR protein level is consistently presented in isolated heart cells (Fig.4b). By downregulation of CaR with small inhibitory RNA (siRNA) specificity of the protein band was confirmed.

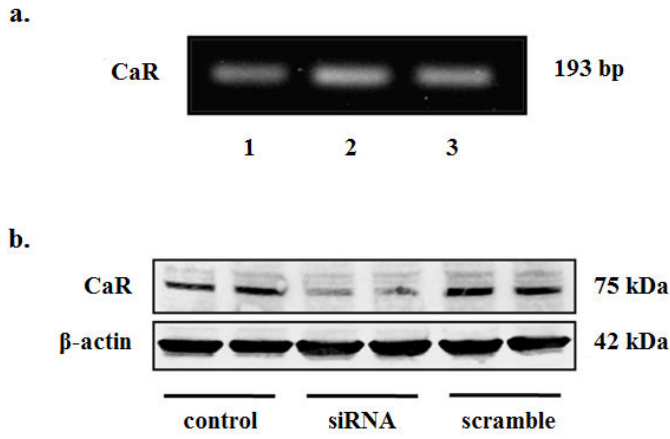


Figure 4. CaR expression in ventricular cardiomyocytes. **a.** mRNA level of CaR in 3 different samples of isolated cardiomyocytes detected by RT-PCR, n=3 **b.** CaR protein level detected by Western Blot in cardiomyocytes cultured for 24 h in presence of siRNA vs CaR (0.05 μM), scramble RNA (0.05 μM) or control, n=3.

It is worth noting, that CaR expression was shown in left and right ventricular and also atrial tissues (Fig. 5). There seems to be a tendency to lower expression in right ventricles and atria.

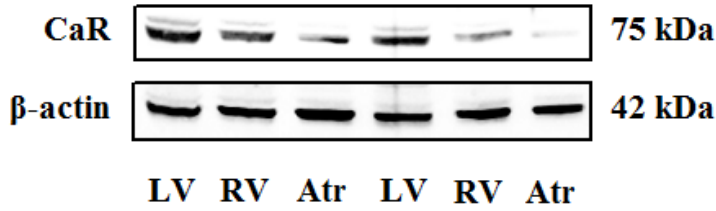


Figure 5. CaR protein expression in left ventricular (LV), right ventricular (RV) and atrial (Atr) tissue.

5.1.2. Ca^{2+}-concentration-dependent CaR activity

Ca^{2+} ions are main natural agonists of the CaR. However, Ca^{2+} can also affect cell shortening in CaR-independent way. To distinguish between the action of CaR and the effect of Ca^{2+} up-take on cell shortening, extracellular Ca^{2+}-concentration on NPS-sensitive cell shortening was determined. At near physiological extracellular Ca^{2+}-concentrations (1-1.25 mM) non-specific inhibitor of CaR NPS2390 significantly

decreased load-free cell shortening (Fig. 6). At higher Ca^{2+}-concentrations the effect of NPS2390 was diminished.

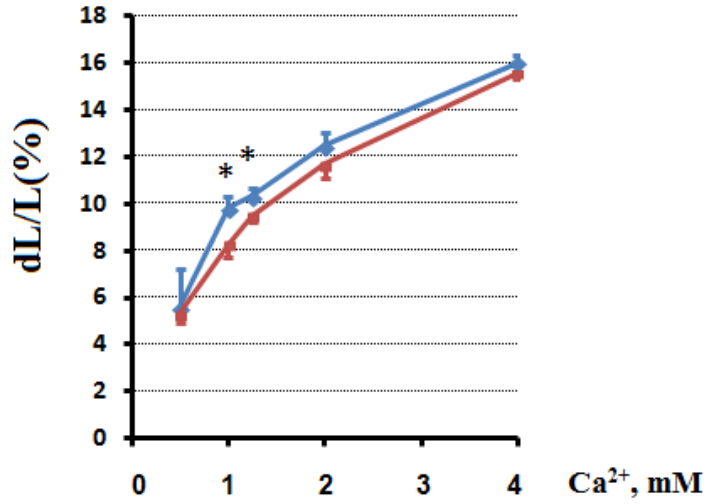


Figure 6. Effect of extracellular Ca^{2+}-concentration on NPS-sensitive cell shortening. Extracellular Ca^{2+}-concentration was set to 0.5-4 mM. NPS – NPS2390 (10 μM). Load-free cell shortening is expressed as shortening amplitude normalized to diastolic cell lengths (dL/L%). Data are means ± SEM from n=27, *p<0.05 vs untreated controls. Blue graph is indicating control values, red – cell shortening under NPS2390.

5.1.3. Effect of CaR acute inhibition on cardiac performance

To find out whether CaR contribute to the basal heart function, we blocked receptors by administration of NPS2390. NPS2390 significantly reduced contraction force of tissue stripes derived from rat heart ventricles (Fig. 7a). Impaired contractile activity of atria was less pronounced (Fig. 7b).

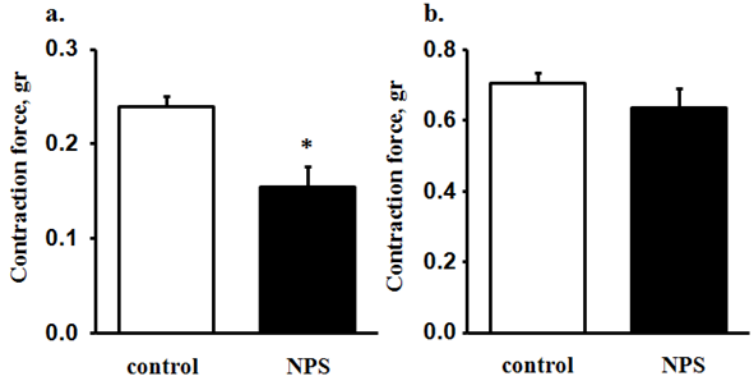


Figure 7. Effects of CaR inhibition on isolated heart muscle strips. **a.** CaR inhibition with NPS2390 (100μM) on the heart ventricular strips **b.** NPS2390 (100μM) effect on the atrial heart muscle strips. Contraction force was measured in grams. Data are means ± SEM from n=7 strips; *$p<0.05$ vs control.

5.1.4. Effects of CaR activation on cardiomyocytes function and cardiac tissue contractility

Other CaR agonists are also able to increase CaR-dependent effect on cell shortening. These experiments were performed again on isolated cardiomyocytes and heart muscle strips. Isolated cardiomyocytes with physiological Ca^{2+}-concentration were exposed to polyamine putrescine and gadolinium (Gd) incubation at physiological Ca^{2+}-concentration. Putrescine increased isolated cell shortening by approximately 12%. Cells incubated with Gd showed a bit higher increase in shortening (approximately 20% comparison to control) (Fig. 8a). In the same time, relaxation velocity had tendency to growth under CaR selective stimulation (Fig. 8b).

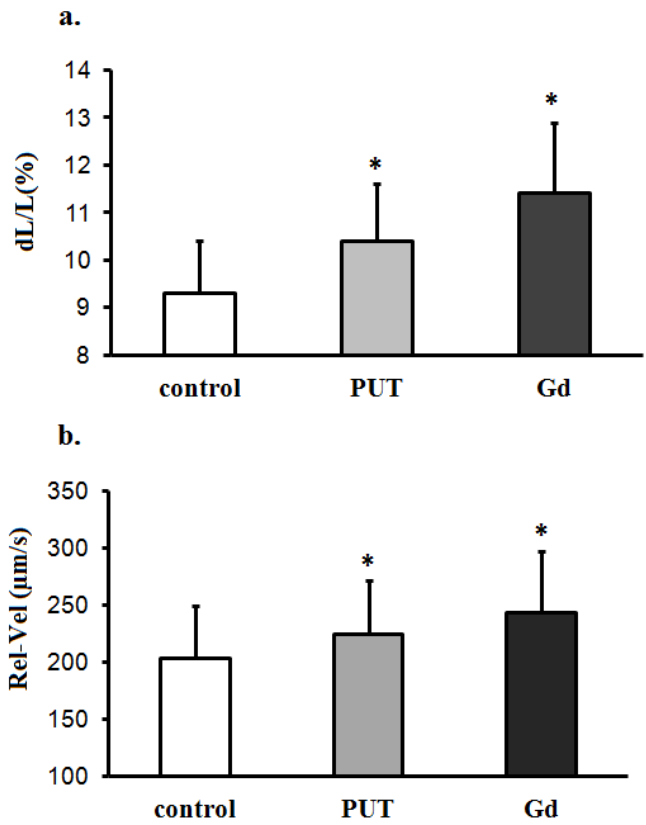


Figure 8. Effects of CaR stimulation on the cell function. **a.** Load free cell shortening under CaR stimulation. **b.** Relaxation velocity of cardiomyocytes under CaR stimulation; putrescine (10 µM), Gd (1 mM). Data are means ± S from n=36 cells; *p<0.05 vs control values.

Similar to the experiments performed on isolated cardiomyocytes, CaR agonists putrescine and another polyamine spermine, increased contraction force on left ventricular heart muscle strips (experiments were performed using tesometry equipment) but effects of putrescine

was stronger (Fig. 9a, 9b). Same activation was accomplished for atrial tissue (Fig. 9c, 9d). Both polyamines had only a minor effect on atria tissue.

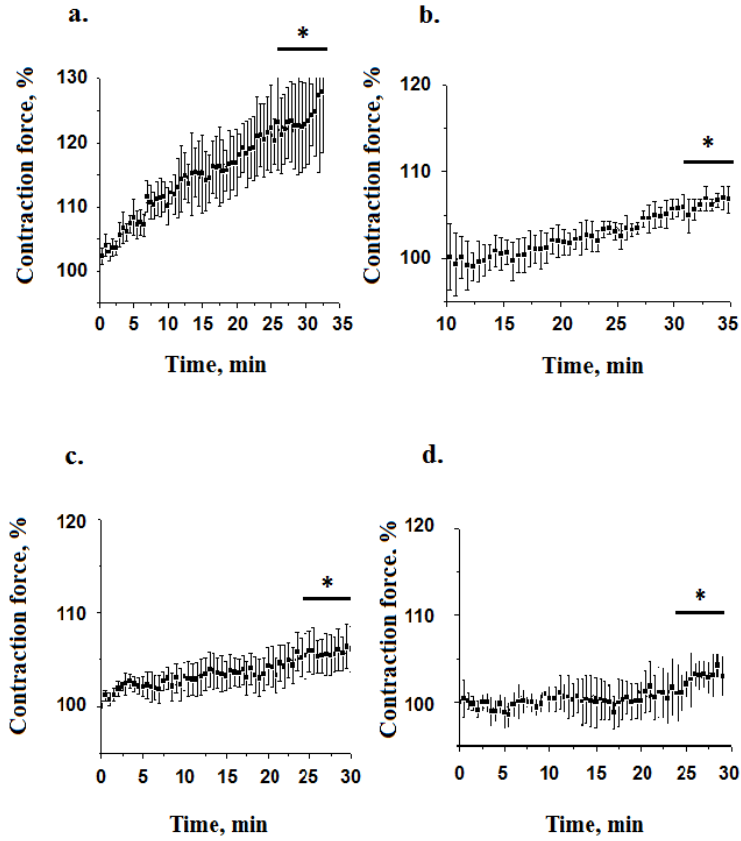


Figure 9. Effects of CaR activation on heart muscle strips contraction force. **a.** Putrescine effect on ventricular heart muscle strip contractility **b.** Heart ventricular tissue contractility under spermine incubation (initial

signals were recorded starting from 10 min after signal got stable) **c.** Effects of putrescine on atrial contraction force **d.** Spermine effect on heart atrial contraction force; Putrescine – 300 μM, Spermine – 300 μM. Control values (0 min) were taken as 100%. Data are means ± SEM from n=10 stripes; *p<0.05 vs control values in the beginning of stimulation.

5.1.5. Acute effects of putrescine and Gd on the Ca^{2+}-transients of isolated cardiomyocytes

To check whether CaR activation leads to changes in systolic and diastolic Ca^{2+}, Fura-2 AM-loaded isolated cardiomyocytes were exposed to putrescine and Gd for 10 min. Ion optics system, used for these purposes, gives a possibility to measure any variations in Ca^{2+}-transients.

Similar to the increased cell shortening, putrescine (Fig. 10a dotted line) and Gd (Fig. 10c dotted line) showed an increase in systolic Ca^{2+}. This effect was observed during first 10 min and then went to plateau phase. In the same time, diastolic Ca^{2+} did not show any changes in the presence of CaR agonists (Fig. 10a, 10c solid line). Previously obtained increase in relaxation velocity correlates to a decreased time of Ca^{2+} presence in sarcolemma of cardiomyocytes, because the time required for the Ca^{2+}-uptake back to SR significantly reduced after cells were incubated with CaR agonists (Fig. 8b).

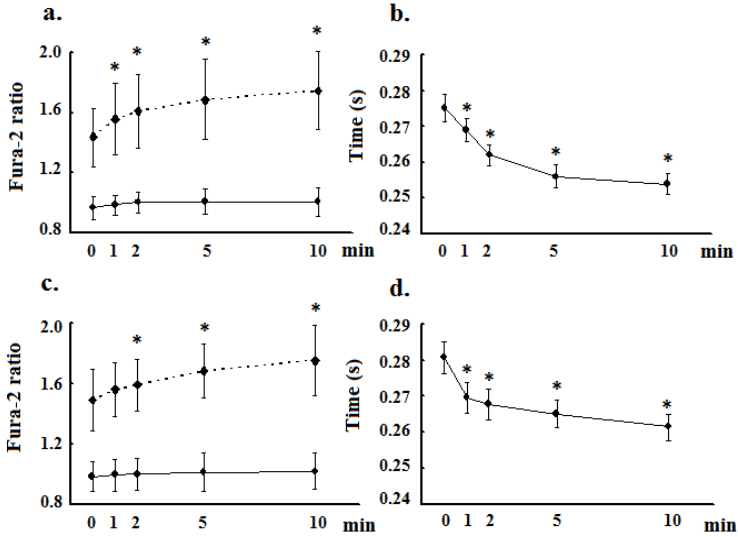


Figure 10. Ca^{2+}-transients under CaR activation. **a.** Systolic (dotted line) and diastolic (solid line) Ca^{2+} load under putrescine stimulation **b.** Ca^{2+}-uptake to intracellular stores under putrescine; *x axis* indicating experimental time, *y axis* indicating time required for Ca^{2+}-uptake **c.** Effects of Gd on the systolic (dotted line) and diastolic (solid line) Ca^{2+} **d.** Ca^{2+}-uptake under Gd stimulation; *x axis* indicating experimental time, *y axis* indicating time required for Ca^{2+}-uptake. Putrescine – 10 μM, Gd – 1 mM. Data are means ± SD from n=36 cells. *p<0.05 vs control (0 min).

5.1.6. Load-free cell shortening under chronic inhibition of CaR

More convincing evidence of CaR role in basal cell shortening would be an effect of downregulated CaR. siRNA directed against CaR (24h) and receptor antagonist NPS2390 (24h) were used for these purposes. CaR

expression was reduced in cardiomyocytes under NPS2390 and siRNA chronic stimulation (Fig. 11a). Similarly to the acute effects (Fig. 11b), NPS2390 reduced cell shortening in over-night stimulation. siRNA directed against CaR also lowered contractile activity (Fig. 11c).

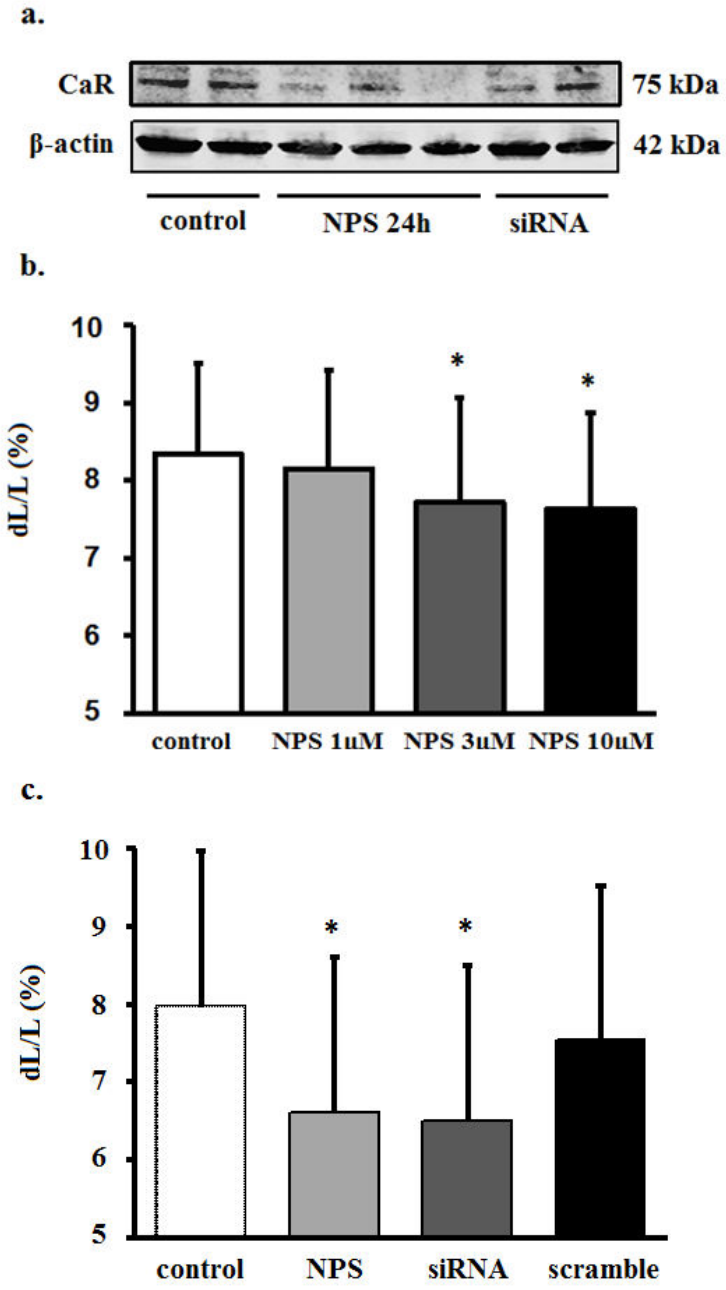
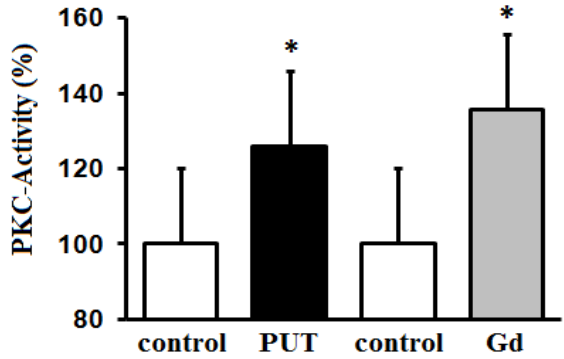

a.
CaR
β-actin
75 kDa
42 kDa
control
NPS 24h
siRNA
b.
dL/L (%)
10
9
8
7
6
5
*
*
control
NPS 1uM
NPS 3uM
NPS 10uM
c.
dL/L (%)
10
9
8
7
6
5
*
*
control
NPS
siRNA
scramble

Figure 11. Effects of CaR downregulation **a.** Representative Western Blot indicating CaR protein expression in control and under NPS2390 (10 μM) and siRNA vs CaR (0.05 μM) 24 h stimulation. **b.** Load free cell shortening of cardiomyocytes after overnight incubation with NPS2390 in concentration-dependent manner. **c.** Effect of NPS2390, siRNA vs CaR and scramble (neg.siRNA, 0.05 μM) on cell shortening. Data are means ± SD from n=100 cells, *p<0.05 vs control.

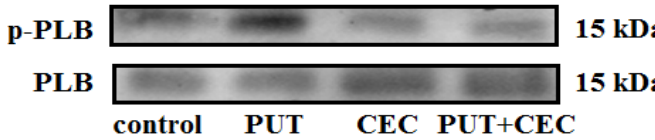
5.1.7. CaR agonists-dependent PKC activation

CaR activation is supposed to promote PLC-dependent IP_3 formation. In parallel diacylglycerol (DAG) production takes place and leads to PKC activation. PKC in turns phosphorilates phospholamban (PLB) at positions 10 and 16 [Shintani-Ishida et al., 2011]. PLB is an established PKC target in cardiomyocytes [Simmerman et al., 1986]. Total PKC activity was detected in isolated cardiomyocytes incubated with putrescine and Gd and compared with untreated controls. Both agonists increased PKC activity (Fig. 12a). A Western Blot confirmed PKC activation by determining an enhanced phosphorylation of PLB by CaR agonists (Fig. 12b). Levels of phosphorylated PLB under putrescine increased by approximately 26% comparison to control. Chelerythrine chloride (CEC), used to antagonise PKC, cancelled an effect of putrescine by reducing phosphorylated PLB level by approximately 13% (Fig. 12c).

a.


PKC-Activity (%)
160
140
120
100
80
*
*
control
PUT
control
Gd

b.


p-PLB
15 kDa
PLB
15 kDa
control
PUT
CEC
PUT+CEC

c.

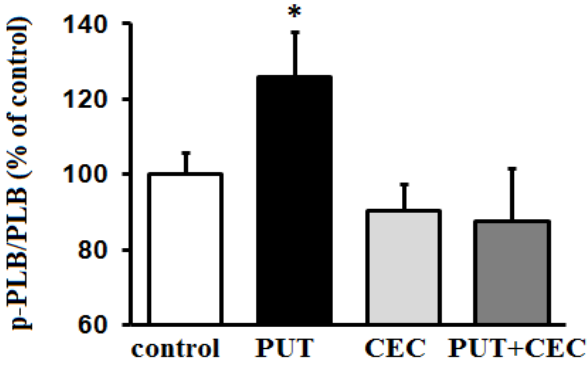

p-PLB/PLB (% of control)
140
120
100
80
60
*
control
PUT
CEC
PUT+CEC

Figure 12. PKC activity and phospholamban phosphorylation. **a.** Determination of PKC activity and PKC-dependent phosphorylation of PLB. Cells were exposed to putrescine (PUT, 10 μM) or Gd (1 mM) for 20 mins and PKC activity was measured thereafter. **b**. Representative immunoblot indicating phosphorylated PLB (p-PLB) immunoreactivity in cardiac myocytes after 20-min putrescine and CEC (10 μM) stimulation. p-PLB values were normalized to total PLB levels. **c.** Densitometric analysis of immunoblots. The fraction of p-PLB to total PLB after 20 min putrescine and CEC stimulation. Data are means ± SEM of n=4 cell preparations, *$p<0.05$ vs control.

5.1.8. Effects of PKC on the CaR-dependent functional increase

PKC effects on contractile ability of heart muscle cells were investigated with the cell edge detection system. Determination of PKC effect was checked by CaR agonist putrescine. Cells were paced at 2 Hz frequency and pre-incubated with CEC for 30 min. Putrescine alone led to an increased cell shortening and relaxation velocity as it was shown previously. But its effect was abolished by PKC inhibitor (Fig. 13a, 13b).

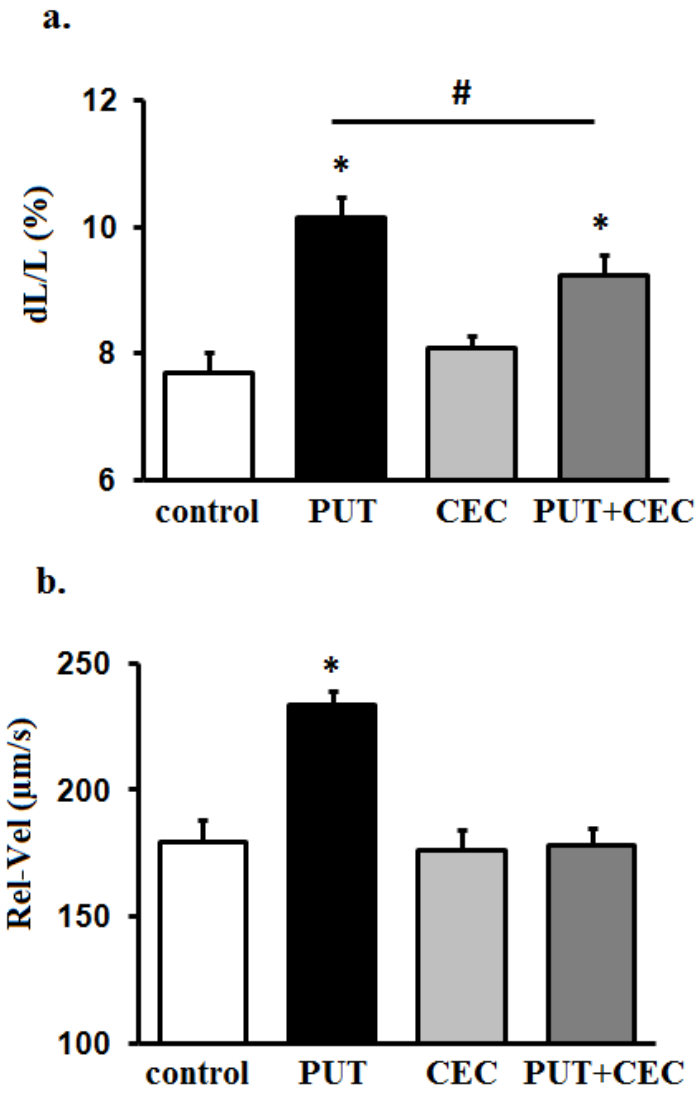

a.
12
10
8
6
dL/L (%)
#
*
*
control
PUT
CEC
PUT+CEC
b.
250
200
150
100
Rel-Vel (µm/s)
*
control
PUT
CEC
PUT+CEC

Figure 13. Effects of PKC inhibitor on CaR-dependent increase in cell shortening. **a.** Load free cell shortening after 20 min of putrescine (PUT, 10 μM) and CEC (10 μM) incubation. b. Relaxation velocity under putrescine and CEC 20 min stimulation. Data are means ± SEM from n= 36 cells, *p<0.05 vs control.

5.1.9. CaR and RAMP1 interaction

RAMP1 is required for trafficking and proper functioning of CaR. So RAMP1 is supposed to influence cardiomyocytes function via mediating CaR protein activity. To check this hypothesis, rat ventricular cardiomyocytes were incubated with siRNA directed against RAMP1 and load-free cell shortening was analysed thereafter. Putrescine was used to activate CaR and to check its responsiveness under downregulated RAMP1. CaR agonist increased shortening and relaxation velocity of the cells exposed to scramble RNA, but this effect was not observed in cells treated with siRNA vs RAMP1 (Fig. 14b, 14c). Immunoblot analysis was performed to confirm the lack of the RAMP1 protein under siRNA vs RAMP1 stimulation (Fig. 14a). RAMP1 expression was reduced by 37.0±4.8 % (p=0.022) under siRNA vs RAMP1 stimulation.

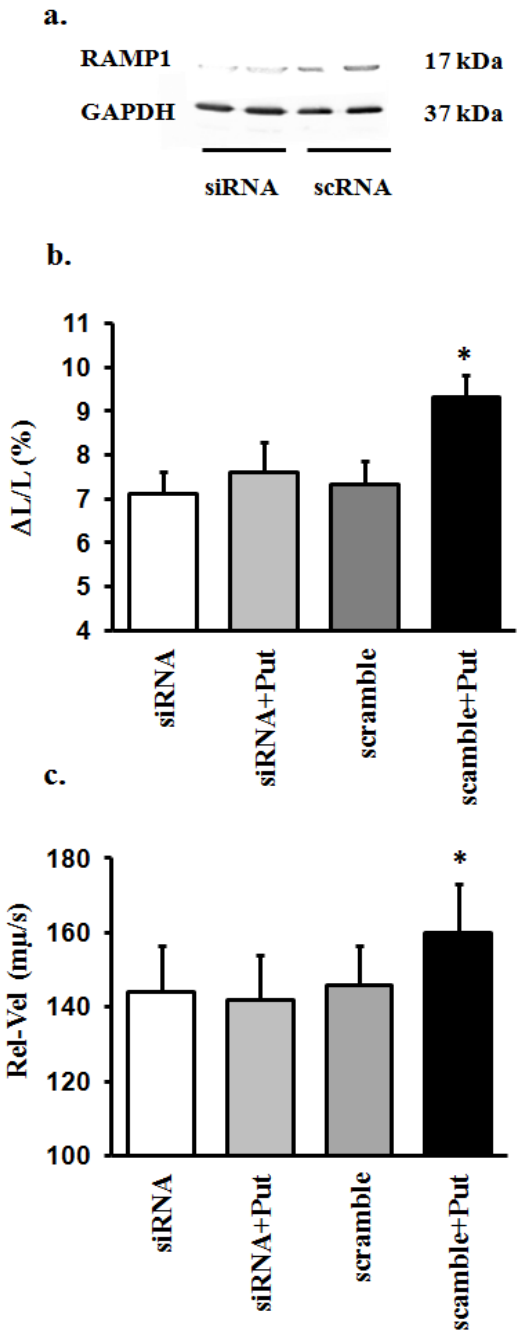

a.
RAMP1
17 kDa
GAPDH
37 kDa
siRNA
scRNA
b.
ΔL/L (%)
11
10
9
8
7
6
5
4
*
siRNA
siRNA+Put
scramble
scamble+Put
c.
Rel-Vel (mμ/s)
180
160
140
120
100
*
siRNA
siRNA+Put
scramble
scamble+Put

Figure 14. Role of RAMP1 in CaR functioning. **a.** Representative immunoblot indicating lack of RAMP1 protein under siRNA vs RAMP1 24 h treatment. **b.** Load-free cell shortening under PUT (10 μM), siRNA vs RAMP1 (0.05 μM) and scramble RNA (0.05 μM). **c.** Relaxation velocity under PUT (10 μM), siRNA vs RAMP1 (0.05 μM) and scramble RNA (0.05 μM). Data are means ± SEM, from n=36, *$p<0.05$ vs siRNA.

CaR protein expression did not change under siRNA vs RAMP1 stimulation comparison to scramble RNA (Fig. 15a, 15b).

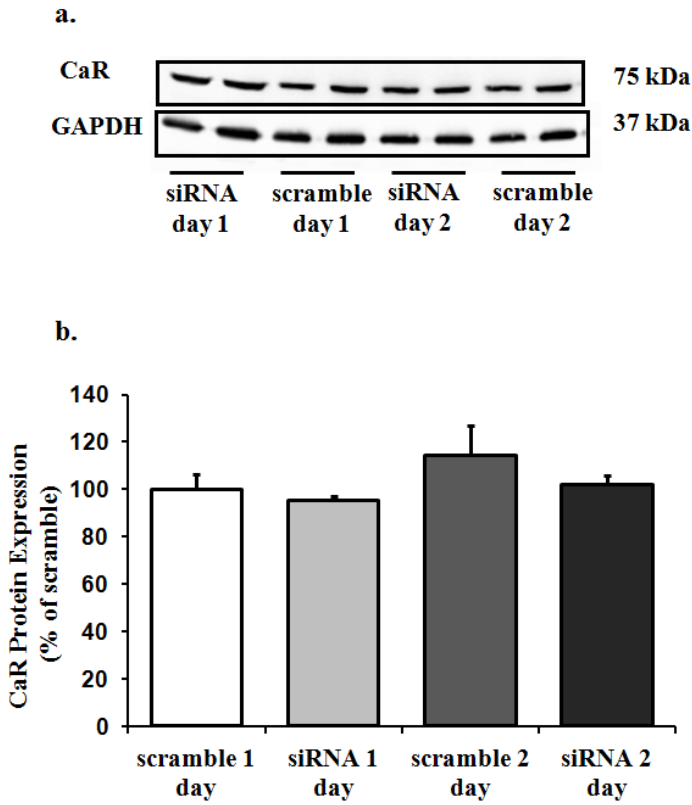


Figure 15. Role of RAMP1 in CaR expression. **a.** Representative immunoblot indicating CaR protein expression under siRNA vs RAMP1 (0.05 μM) and scramble RNA (0.05 μM). b. Quantitative analysis of CaR protein expression. Data are means ± SEM, from n= 4 cultures.

5.2. CaR and hypertrophy

To define the role of CaR in hypertrophy *in vivo* and *in vitro* hypertrophic heart models were investigated.

5.2.1. Hypertrophy *in vivo*. Expression of CaR, ET-receptors and RAMP1

As an *in vivo* hypertrophic model, the rats with NO-deficiency were used. After four weeks these rats develop high blood pressure and increased left ventricular mass [Schreckenberg et al., 2015]. mRNA levels of CaR, RAMP1, ET_A, ET_B and ECE-1 were analysed in tissue samples from these animals. CaR and RAMP1 mRNA was upregulated under NO-deficiency (Fig. 16d, 16e). Vasoconstrictor peptide ET-1, produced in vascular endothelial cells, endocardial endothelial cells and cardiac myocytes with the help of ECE-1, was reported to be increased in those rats [Brunner et al., 1995]. As it was mentioned in Introduction, ET-1 acts via two main types of the receptors – ET_A and ET_B [Fareh et al., 1996]. Interestingly, enhanced ET_B receptors mRNA expression was observed (Fig. 16b) in L-NAME treated rats, whereas, ET_A and ECE-1 did not show any changes (Fig. 16a, 16c).

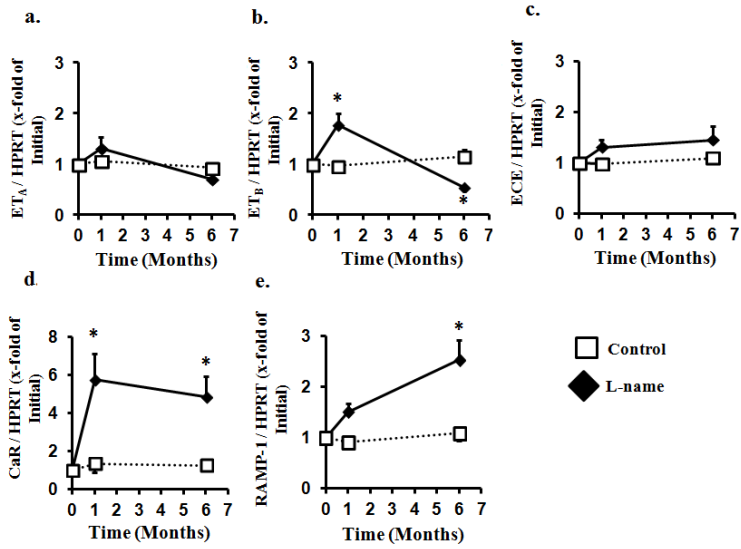


Figure 16. CaR and ET-receptor levels under NO-deficiency *in vivo.* **a.** ET_A; **b.** ET_B; **c.** ECE; **d.** CaR; **e.** receptor RAMP1 mRNA levels after 1 and 6 months L-NAME treatment. Amplification of cDNA is normalized to HPRT. Data are means ± SD from n=8 rats, *p<0.05.

5.2.2. Hypertrophy *in vitro*. Role of ET-1 and phenylephrine in CaR-dependent cardiomyocytes shortening

To develop hypertrophy *in vitro* hypertrophic stimuli such as β-adrenergic stimulator phenylephrine (PE) and peptide ET-1 were used. PE and ET-1 in chronic stimulation both decreased cell shortening and relaxation velocity but the effect of ET-1 was potentiated by CaR inhibitor NPS2390 (Fig. 17a, 17b). PE effect did not show difference under NPS2390 stimulation.

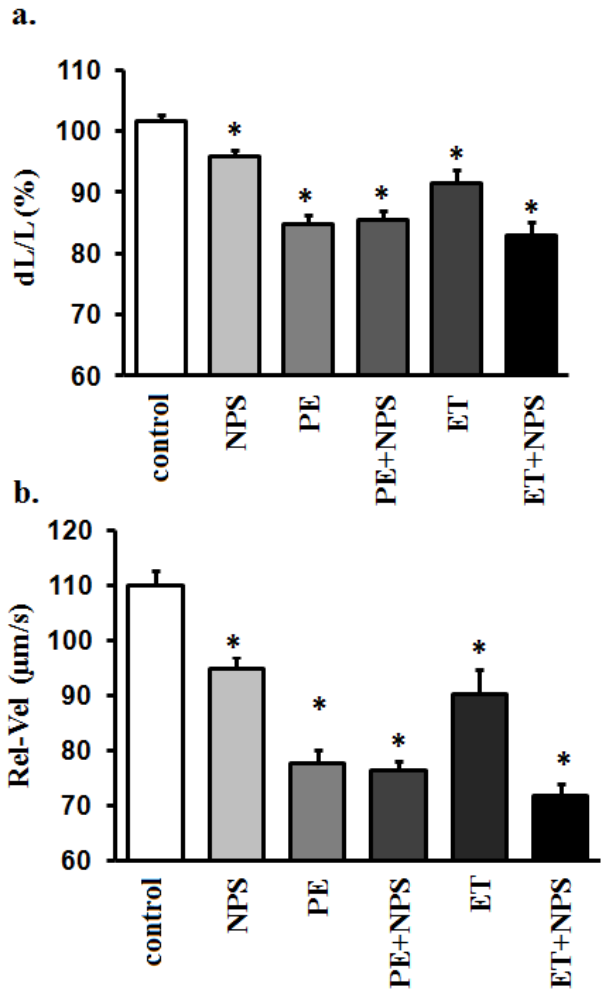


Figure 17. Effects of CaR inhibition on cellular function during hypertrophy development *in vitro.* **a.** Load free cell shortening under ET-1 (100 nM), PE (10 µM) and CaR inhibitor NPS2390 (10 µM) 24 h stimulation. **b.** Relaxation velocity of cardiac myocytes under ET-1 (100

nM), PE (10 μM) and CaR inhibitor NPS2390 (10 μM) 24 h stimulation. Data are means ± SEM from n=72, *p<0.05 vs control.

It should be noted, that CaR protein and also mRNA levels were significantly higher under ET-1 chronic stimulation (Fig. 18). In the same time PE did not led to CaR protein levels elevation (Fig. 19).

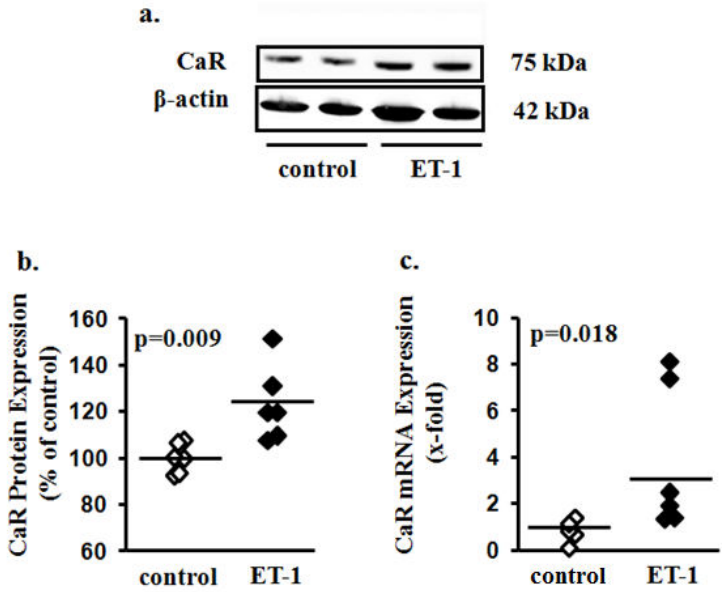


Figure 18. ET-1-dependent CaR upregulation. **a**. Representative immunoblot indicating CaR protein level under ET-1 (100 nM) 24h stimulation. **b.** CaR protein level under ET-1 (100 nM), 24 h stimulation. **c.** CaR mRNA expression under ET-1 (100 nM), 6h stimulation. p<0.05 vs controls indicate significance.

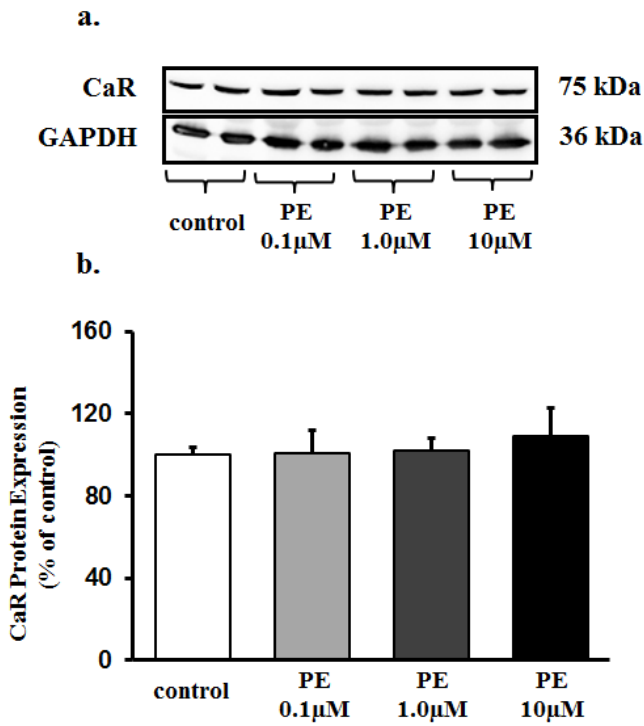


Figure 19. CaR protein expression during PE-induced hypertrophy development. **a.** Representative Western Blot of CaR protein levels under PE concentration-dependent 24 h stimulation **b.** CaR protein expression under PE (0.1 μM, 1 μM and 10 μM) 24 h stimulation. Data are means ± SD from n=2 cell preparations.

To make the effect of CaR inhibition on load free cell shortening under ET-1 more specific, experiments with silenced CaR with siRNA were performed. As it was observed previously, inhibition of the receptors intensified effect of ET-1 (Fig. 20).

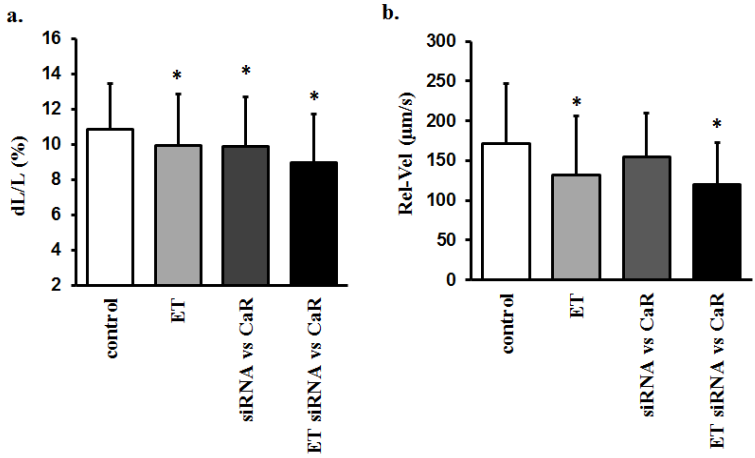


Figure 20. ET-1 effects on cell shortening under downregulated CaR expression. **a.** Load-free cell shortening under ET-1 (100 nM) and siRNA vs CaR (0.05 µM) chronic stimulation. **b.** Relaxation velocity under ET-1 and siRNA vs CaR 24h incubation. Data are means ± SD from n=72. *p<0.05 vs untreated controls.

5.2.3. ET-1-dependent cardiac remodelling

ET-1 as pro-hypertrophic stimuli contributes to ventricular remodelling [Arai et al., 1995; Sakai et al., 1995].

ET-1 *in vitro* increased cell width and volume, whereas cell length was not affected (Fig. 19). CaR inhibitor NPS2390 did not influence ET-1 remodelling effect. Thus, ET-1 action on cardiomyocytes remodelling is CaR-independent.

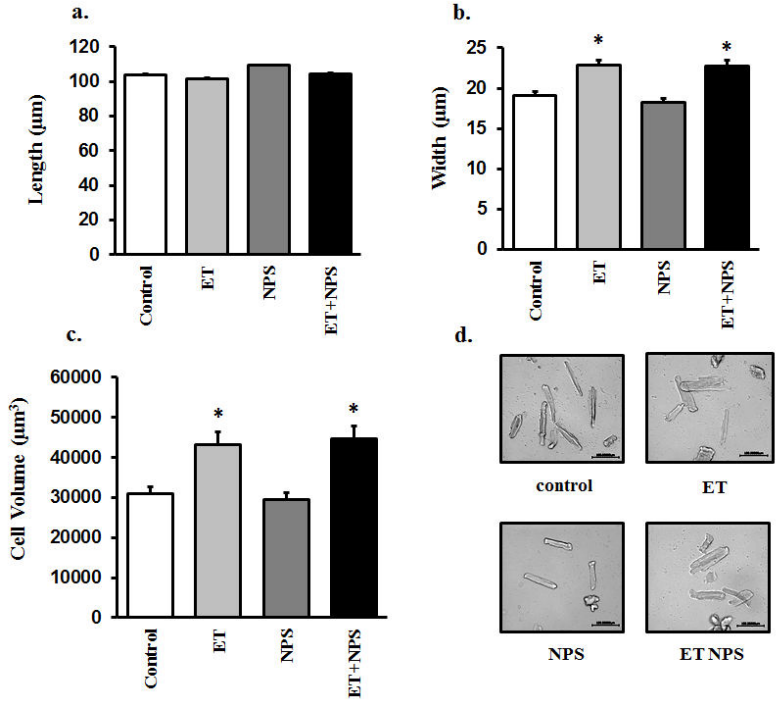


Figure 21. ET-1-induced hypertrophy development *in vitro.* **a.** Cell length under 24 h stimulation with ET-1 (100 nM) and NPS2390 (10 µM) incubation. **b.** Cell width under ET-1 and NPS2390 24 h stimulation. **c.** Cell volume under ET-1 and NPS2390 over-night incubation d. Representative pictures of cardiomyocytes under ET-1 and

NPS2390 stimulation (bar on the pictures equals to 100 µm). Data are means ± SD from n=2 rats, *p<0.05 vs control.

5.2.4. Effects of different types of ET-receptors on cardiomyocytes shortening and CaR expression

As mentioned above, ET-1 action is mediated via ET_A and ET_B receptors [Fareh et al., 1996]. Both of them presented in heart tissue as well as in isolated ventricular cardiomyocytes (Fig. 22). As already indicated there are two splice variants of ET_B receptors in the heart. They differ according to the sensitivity to PD142893. ET_{B1} are PD142893 sensitive and ET_{B2} are PD142893 resistant. As PD142893 is not specific for ET_{B1} it also antagonizes ET_A receptors. Cells were exposed to ET-1 and PD142893 for 24 h in this study. Load-free cell shortening and relaxation velocity were measured. Protein analysis was performed to indicate CaR levels under blocked ET_{B1} and ET_A receptors.

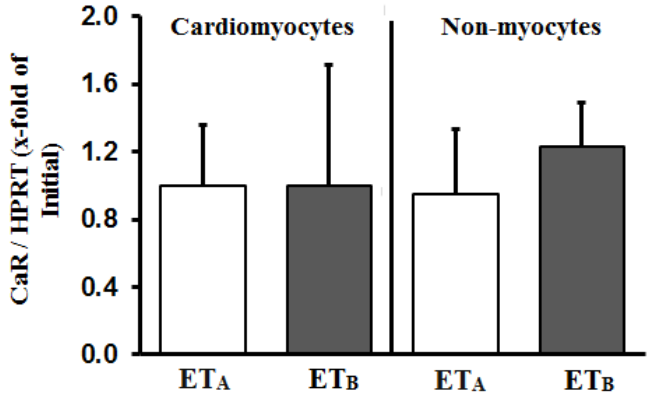


Figure 22. ET-receptors expression in cardiomyocytes and non-myocytes. Data are means ± SD from n=4 rats, normalized to ET_A receptor expression in cardiomyocytes and non-myocytes respectively.

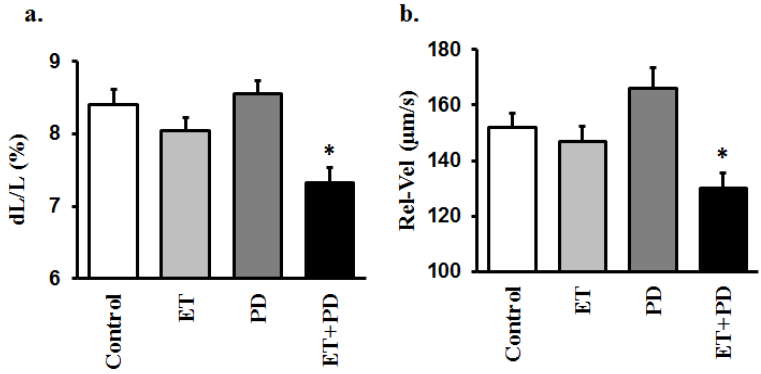


Figure 23. Role of ET_B receptor subtypes in ET-1-dependent modification of cell shortening. **a.** Load-free cell shortening under ET-1

(100 nM) and PD142893 (100 nM) chronic (24 h) stimulation. **b.** Relaxation velocity under ET-1 (100 nM) and PD142893 (100 nM) chronic (24 h) stimulation. Data means ± SD from at least 36-72 cells, *$p<0.05$ vs control values.

Cell shortening was slightly decreased under ET-1, but its effect was much stronger under ET_{B1} and ET_A receptors inhibition (Fig. 23a). Stronger decrease in relaxation velocity was also observed under PD142893 and ET-1 cooperative stimulation comparison to ET-1 alone (Fig 23b).

ET-1 again increased CaR protein levels. This effect was abolished by PD142893 (Fig. 24).

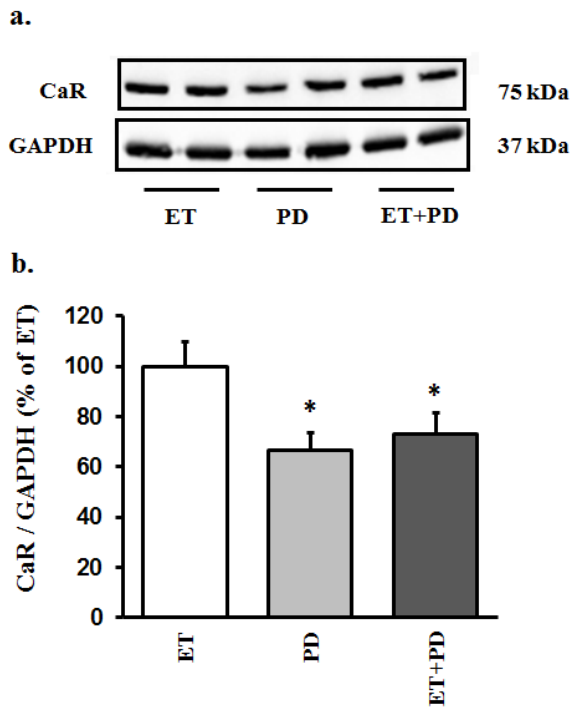


Figure 24. Role of ET_B receptors subtypes in ET-1-dependent up-regulation of CaR **a.** Representative immunoblot; **b.** Quantitative analysis of the expression. Cells were incubated with ET-1 (100 nM) and/or PD142893 (100 nM) for 24 h. Data means ± SD from n=4 cultures and normalized to ET-1 induced values, *$p<0.05$.

To confirm that the negative effect of ET-1 on cell shortening is PD142893-dependent, a second ET_B-receptor-specific inhibitor was used. (Fig. 25). ET-1 together with PD142893 significantly decreased shortening (Fig. 25a) and relaxation velocity (Fig. 25b) of

cardiomyocytes, whereas this effect was blocked by ET_B receptors inhibitor (BQ788).

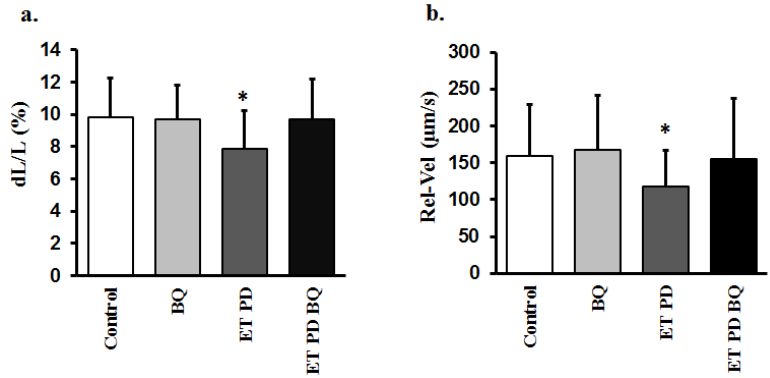


Figure 25. Load-free cell shortening under ETA/ETB receptors inhibition **a.** Load free cell shortening; **b.** Relaxation velocity of cardiomyocytes. Cells were exposed to ET-1 (100 nM), PD142893 (100 nM) and BQ788 (100 nM) for 24. Data are means ± SD from n=72 cells, *p<0.05.

5.2.5. Effect of PD142893 on a hypertrophic remodeling of cardiac myocytes

Finally, we investigated the effect of PD142893 on cardiac hypertrophy. Inhibition of ET_A/ET_{B1} completely attenuated the concentration-dependent increase in cell width and volume (Fig. 23).

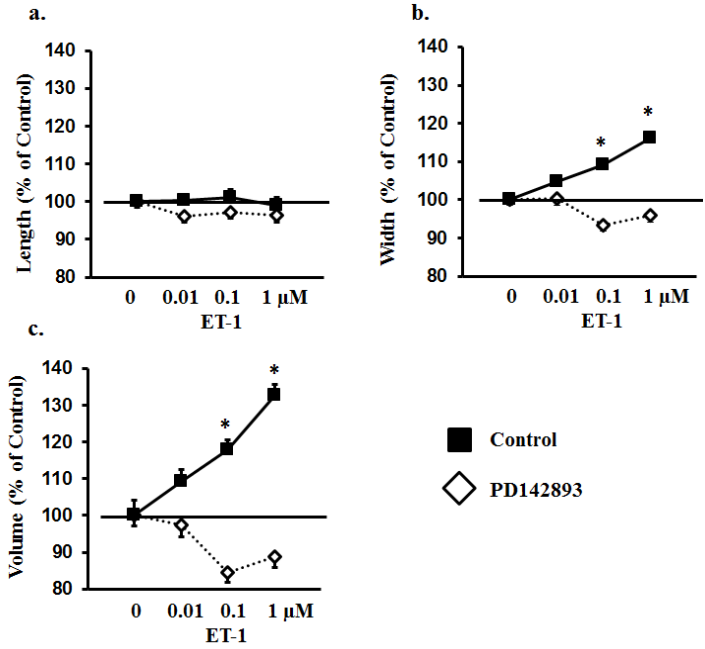


Figure 26. Role of $ET_{A,B1}$ receptor subtypes in ET-1-dependent hypertrophy. **a.-c.** Cells length, width, and volume of cardiomyocytes cultured with ET-1 (100 nM) and PD142893 (100 nM) during 24 h. Data are means from at least 59 cells, *$p<0.05$ vs. untreated controls.

6. Discussion

6.1. CaR involvement in heart contractility

The CaR are presented throughout the entire cardiovascular system: in the cardiac muscle and blood vessels. Its activation leads to different intracellular signaling cascades and various biological reactions. They are supposed to participate in the heart cells contractility and relaxation [Schreckenberg et al., 2015]. Furthermore, they take part in blood pressure modulation [Weston et al., 2005], influence protein expression, and they are involved in I/R injury and apoptosis [Zhen et al., 2016]. The nature of the intracellular pathway, activated by CaR, depends markedly on the cell type in which the receptors are expressed.

In 2003 Wang et al. demonstrated the presence of CaR in the cardiomyocytes that were isolated from Sprague–Dawley rats. Since then further investigations of CaR in the cardiovascular system have been performed. The mRNA of CaR was detected in both - atrial and ventricular cardiomyocytes. In the heart cells the receptors are known to be connected to Gαq proteins leading to the PLC and MAPK pathways activation [Tfelt-Hansen et al., 2005; Tfelt-Hansen et al., 2006].

In our study we confirmed that isolated adult cardiomyocytes of Wistar rats express CaR under physiological conditions and that the receptors contribute to the basal electromechanical coupling.

We were able to detect differences in CaR expression between atrial and ventricular tissue. The nature of this expression differences is not clear yet, but probably it is due to the crucial pumping role of ventricles which is not present in atria.

CaR are mainly activated by Ca^{2+} ions [Hofer et al., 2003]. However, as a crucial molecule in the heart, it also influences cell contractility in CaR-independent way. To distinguish the effect of CaR and Ca^{2+} ions themselves, load-free cell shortening under CaR inhibition and under different extracellular Ca^{2+}-concentration was measured. The highest activity of the receptors was detected under almost physiological Ca^{2+}-concentration (1-1.25 mM).

To study an effect of CaR activation on heart muscle strips and isolated heart muscle cells, positive charged synthetic molecule Gd, petrescine and spermine were used.

Putrescine and spermine are natural polyamines, highly positive-charged molecules, occurring in all living cells. Together with Ca^{2+} ions they belong to the group of natural direct agonists of CaR. Polyamine putrescine is produced from amino acid ornithine under the activity of the ornithine decarboxylase (ODC). The product of ODC, is then converted to spermine under activity of specific aminopropyl-transferase spermine synthase. Polyamine formation is induced by several growth and differentiation stimuli. Interestingly, they are essential for cell proliferation [Oreddsson et al., 2003] and play a role in the development of heart muscle hypertrophy [Caldarera et al., 1971; Bartolome et al., 1980; Pegg et al., 1980]. An increase in polyamine concentration was detected in heart tissue after ascending aortic stenosis [Feldman et al., 1972], stress [Russell et al., 1971], administration of adrenoceptor agonists [Warnica et al., 1975; Cubria et al., 1998; Schlüter et al., 2000] and physical exercise [Caldarera et al., 1974; Retting et al., 1977] – all precursors of cardiac hypertrophy. Increased polyamine levels were

associated with elevated RNA and protein synthesis [Moruzzi et al., 1974, Gibson et al., 1974].

Challenging isolated ventricular cardiomyocytes with polyamines led to an increased contraction force of heart muscle strips. Load free cell shortening was also higher with CaR agonists. At the same time, increased relaxation velocity of cardiac myocytes was observed. Same agonists induced concentration-dependent Ca^{2+}-increase inside the cell. Tfelt-Hansen et al. previously explained increased intracellular Ca^{2+} by IP_3 elevation within the cell, thus suggesting the link of the receptor to PLC/IP_3 pathway [Tfelt-Hansen et al., 2006].

Ca^{2+}-decay was also faster comparison to control values, which corresponds with higher relaxation velocity observed in beating cells under CaR activation. Along with increased IP_3 formation, there is parallel enhanced DAG synthesis (Fig. 27). DAG and Ca^{2+} ions are required for the PKC activation. PKC in turns phosphorylates protein PLB at the positions 10 and 16 [Colyer et al., 1998]. The increased activity of PKC and increased phosphorylated PLB levels were observed in present study. Phosphorylated form of PLB is increasing sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity. Phosphorylated PLB separates from SERCA, and SERCA, in turns, has higher potency of Ca^{2+}-uptake. Enhanced Ca^{2+}-uptake indicates increased relaxation velocity.

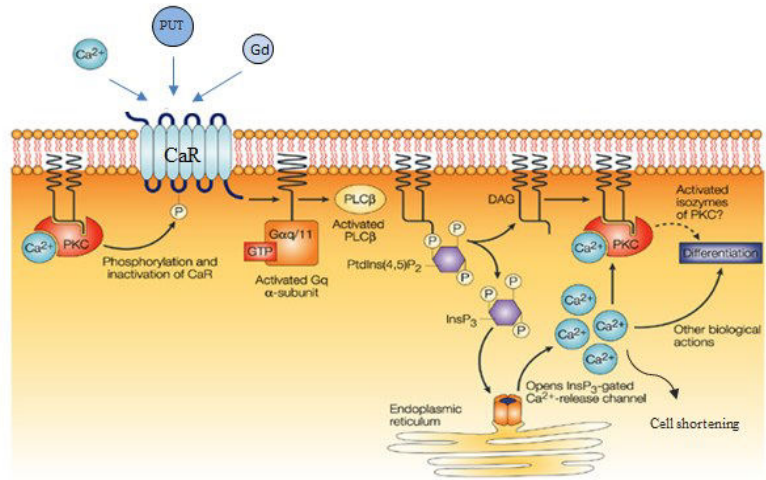


Figure 27. CaR-mediated intracellular signaling pathway in cardiac myocytes [Modified from Sergio et al, 2003].

To determine the role of CaR in the basal cell shortening CaR's non-specific negative allosteric modulator NPS2390 and siRNA vs CaR were applied. They significantly decreased cellular function by approximately 15% in acute and chronic ways. Western Blot confirmed the lack of the receptor protein under siRNA vs CaR and NPS2390 chronic inhibition. NPS2390 also decreased contraction force of isolated heart muscle strips in time-dependent manner.

The CaR belong to the large group of GPCRs and as other receptors from this group require RAMPs for the trafficking, function and appropriate localization [Parameswaran et al., 2006]. These single-transmembrane proteins expressed in many tissues and cells [McLatchie

et al., 1998; Morfis et al., 2003]. Class and individual isoform of these proteins depend on the cell and tissue type where they are shown to be expressed [Parameswaran et al., 2006]. Three types of these proteins are identified: RAMP1, RAMP2 and RAMP3. RAMP1 was isolated from human as a receptor for functioning and trafficking of the calcitonin gene-related peptide (CGRP) in 1998. RAMP2 and RAMP3 were detected later on by database searching [McLatchie et al., 1998]. Although, proteins were shown mostly to mediate activity of class B GPCRs, recently class C was also identified as receptors requiring RAMPs activity.

RAMP1 and RAMP3 association with CaR expressed on the cell surface in COS7 was observed [Bouschet et al., 2005]. Another study performed on HEK293 cells showed alteration in CaR trafficking under downregulated RAMP1 [Bouschet et al., 2005]. RAMPs promote trafficking of the CaR from endoplasmic reticulum to the Golgi apparatus and mediate the terminal glycosylation of the receptor.

Cardiac cells with downregulated RAMP1 had impaired respond to the CaR agonist putrescine in current study. However, immunoblot analysis did not detect any differences in CaR protein expression between control cells and cells with silenced RAMP1. This suggests that RAMP1 deficiency is not responsible for the lack of CaR protein in cardiomyocytes in general, but leads to its absence on the cell surface.

So far we can make intermediate conclusion about the role of the CaR in cardiomyocytes expression and functioning. Our study showed the presence of the receptor in cardiomyocytes derived from Wistar rats and that these receptors significantly contribute to the basal cell shortening. Mechanism of this contribution involves the PLC and the PKC

activation, leading to an increased intracellular Ca^{2+}-release and Ca^{2+}-uptake with subsequent increase in cells shortening and relaxation. It should be noted, that CaR are efficient to increase cellular shortening at nearly physiological Ca^{2+}-concentrations. In addition, for the cell surface expression and proper function CaR require the activity of RAMP1.

6.2 Role of CaR in the heart functioning during hypertrophy development

The presence of the receptor was also shown in blood vessels of different species. The CaR protein was detected in perivascular nerves in rat mesenteric, renal, coronary and vertebral arteries [Bukoski et al., 1997; Wang et al., 1998; Ruat et al., 1995]. For instance, activated vascular endothelial CaR open Ca^{2+}-sensitive potassium channels by inducing subsequent hyperpolarization [Weston et al., 2005]. CaR expressed in perivascular sensory nerves of arteries mediates Ca^{2+}-induced relaxation [Bukoski et al., 1997]. A functional CaR protein has been reported in endothelial cells from rat mesenteric and porcine coronary arteries [Weston et al., 2005]. Other group reported the presence of the CaR in endothelial cells of human aorta where it modulates NO production [Ziegelstein et al., 2006]. These studies indicate the role of the receptor in blood pressure modulation by inducing hyperpolarization and NO synthesis.

They are also supposed to play a role during development of different heart disease. For example, CaR are involved in pulmonary vascular remodeling and pulmonary hypertension [Yamamura et al., 2012; Peng et al., 2014], I/R injury and heart failure development [Yan et al., 2011]. Interestingly, CaR protein levels were increased under Ang-II

stimulation, pointing to the CaR involvement in hypertrophy [Wang et al., 2008]. There is also upcoming evidence of CaR participation in post-ischemic heart recovery and heart failure progression [Gan et al., 2012; Lu et al., 2013]. Thereby, the CaR seem to be an active player on the cell surface of the cardiovascular system also participating in its functioning during heart diseases development and progression.

Hypertension takes a big part among risk factors leading to death later on. High blood pressure affects between 16 and 37 % of the global population [Poulter et al., 2015]. In 2010, hypertension was assessed as 17.8% of premature deaths (9.4 million) and 7% of disability worldwide [Campbell et al., 2015]. Thus, hypertension and developed later on hypertrophy are one of the most important targets of investigation. So far expression of CaR in chronic hypertension was not clearly tested.

As it was indicated above, *in vivo* and *in vitro* hypertrophic models were used to assess the involvement of CaR into the hypertrophy progression. Hypertrophy *in vitro* was evoked by use of the commonly known hypertrophic stimuli - ET-1 and PE [Hinrichs et al., 2011].

An in vivo model is the hypertensive rat model with NO-deficiency. This part of the study was used here as a hypothesis generating part. We hypothesized that chronic NO-deficiency, which also leads to hypertrophy development, increases the cardiac expression of RAMP-1 and CaSR and that ET-1 triggers this effect. NO deficiency is usually associated with endothelial dysfunction in cardiovascular system under stress conditions [Cai et al., 2000]. As a strong vasoconstrictor ET-1 antagonizes the activity of NO in smooth muscle cells and cardiomyocytes. NO reduces ET-1 secretion thus removing ET-1 effect in the heart while rats with developed NO-deficiency show increased

ET-1 release [Brunner et al., 1995]. It should be also noted that NO can reduce polyamine levels, one of the main CaR agonists, and inhibit ODC activity [Bauer et al., 1999] and vice versa, polyamines can inhibit NO synthesis by reducing NOS activity [Hu et al., 1994]. Increased CaR and RAMP1 mRNA levels were observed in rats under NO-deficiency, which confirms correlation between these two proteins.

In vitro ET-1 and PE chronic stimulation led to drop of load free cell shortening and relaxation velocity. NPS2390, used to antagonize the CaR, enhanced the ET-1 effect, but not the effect of PE. Similar results were obtained under silenced CaR and ET-1 stimulation. ET-1 again slightly decreased cell shortening, but its effect was much stronger under downregulated CaR. Following question was addressed: Did chronic ET-1 stimulation and CaR inhibition enhance functional drop together in independent manner or there is an interaction between these receptors pathways?

Protein analysis did not show changes in CaR expression under PE stimulation. However, increased CaR levels were observed under ET-1 (after 24 h), suggesting that CaR can partly compensate loss of cellular function under ET-1-dependent hypertrophy development. Nevertheless, mechanisms of this upregulation remain elusive.

ET-1 effect in the heart is implemented via GPCRs and mediates IP_3-dependent Ca^{2+}-release in acute stimulation. Chronic stimulation shows the opposite effect. The type of ET-receptors, responsible for the cardiac remodeling and the inhibited function of the heart cells, is not known.

Enhanced ET_B receptors mRNA was observed in experimental rat model with NO-deficiency, whereas ET_A and ECE did not show any changes.

Previously mentioned, that ET-1 in long-term exposure leads to cardiac hypertrophy development. Likely, this effect is provided mainly by ET_B receptors type [Lee et al., 2004]. To check which ET receptor type is responsible for the CaR upregulation, different ET-receptor antagonists were used. ET_B receptors are expressed in two different splice variants. The functional importance of the two variants was investigated by pharmacological approaches using PD142893. The inhibitor blocks the activation of ET_A and ET_{B1} receptors, respectively, leaving ET_{B2} receptor signalling intact. Stimulation of cardiomyocytes with PD142893, revealed differences in CaR expression. ET-1 in chronic way increased CaR expression as it was reported in previous experiments, whereas PD142893 abolished this effect. PD142893 potentiated ET-1-dependent loss of the cell function. Specificity of PD142893 was checked by ET_B receptor blocker, which together with PD142893 completely attenuated depressive effect of ET-1. This suggests that likely, receptors remaining unblocked under PD142893 are ET_B. Moreover, increased cell volume under ET-1 was not observed during PD142893 application. Apparently, ET-1 long-term application via ET_A or ET_{B1} induce hypertrophic cardiomyocytes remodelling and CaR upregulation.

Second conclusion indicating the role of CaR in hypertrophy development could be done. It is supposed that ET-1 in chronic stimulation via ET_A or ET_{B1} receptors not only leads to cardiomyocytes remodelling and attenuates their function, but also upregulates CaR expression. CaR upregulation is supposed to compensate loss of contractile function of cardiac myocytes during hypertrophy development. The data are summarized in Fig. 28

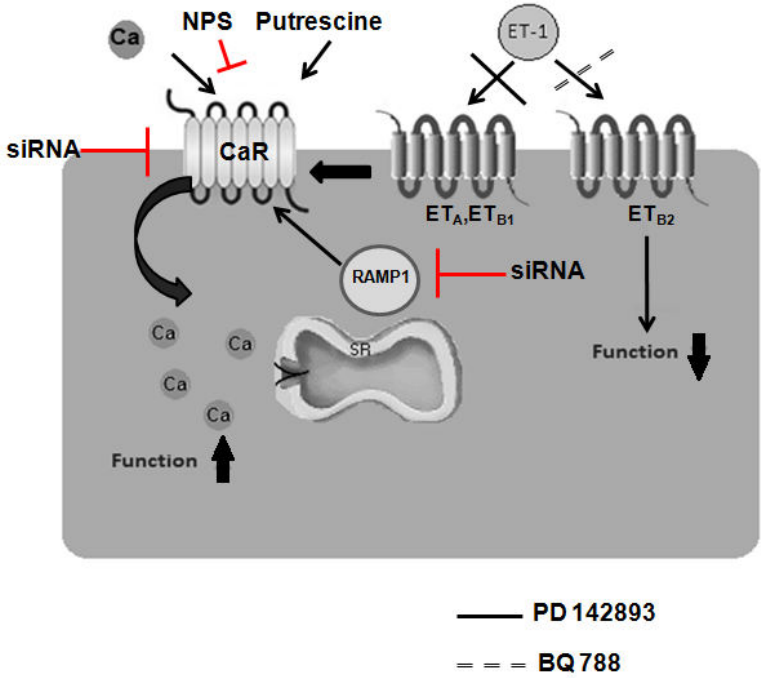


Figure 28. Schematic summary of the data obtained in this study. ET-1 acts on three different populations of ET-receptors (ET_A, ET_B1) and ET_B2. While ET_B2 receptor activation (ET-1 in presence of PD142893) induces a loss of cell function (reduced load-free cell shortening), activation of ET_A and ET_B1 receptors up-regulate CaR that stabilizes cell function. The latter effect requires RAMP-1.

7. Conclusion

In conclusion, our study shows that CaR are involved in physiological Ca^{2+}-handling of adult rat ventricular cardiomyocytes and contribute approximately 15% to basal electromechanical coupling of cardiomyocytes. To be functionally expressed on the cardiomyocytes surface CaR require activity of RAMP1. Furthermore, it revealed that up-regulation of CaR, as it occurs in ET-1-dependent hypertension via ET_A or ET_{B1} receptors stimulation, stabilizes cardiac function. Pharmacologically, selective inhibition of ET_{B1} or ET_A receptors may delay the transition of compensatory hypertrophy to heart failure.

8. References

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9. Summary

Background. Ca^{2+} is commonly known as a crucial molecule in heart tissue. It triggers signal transduction, cellular contractility, and activates enzymes. Moreover, Ca^{2+} is considered to be an important second messenger in cardiovascular tissue. Recently, Ca^{2+} ions were shown to act as a first extracellular messengers via special calcium-sensing receptors (CaR). These receptors are G protein-coupled and are present in many organs and tissues throughout the entire body including the cardiovascular system. They are supposed to activate several signaling cascades in cardiomyocytes. In the past few years, the involvement of CaR in heart diseases was shown in many publications. CaR are involved in ischemia-reperfusion injury, hypertension, hypertrophy and heart failure development. However, underlying mechanisms remained elusive so far. Thus in present study we aimed to investigate on the role of CaR in physiological heart function and during the development of cardiac hypertrophy.

Materials and Methods. Experiments were performed on isolated cardiomyocytes and heart muscle strips. Load-free cell shortening was measured with a special cell-edge detection system. Contraction force of the heart muscle strips was measured using tensometry equipment. Cells and tissue material were exposed to Western Blot and RT-PCR analysis. Ca^{2+}-transients were detected in isolated cardiac myocytes loaded with FURA-2AM by ION Optix system. To determine protein kinase C (PKC) activity under CaR stimulation, special nonradioactive PKC activity assay was used. Hypertrophy was modulated *in vivo* and *in vitro*. As *in vivo* model rats with NO-deficiency were used. Hypertrophy *in vitro* was provoked by adding ET-1 and PE to the cardiomyocytes

culture for 24 h. Polyamines putrescine and spermine and synthetic CaR agonist gadolinium (Gd) were used to activate CaR and NPS2390 and siRNA vs CaR to antagonize the receptor.

Results. CaR in physiological heart function. RT-PCR and Western Blot analysis confirmed the presence of the receptor in adult rat cardiomyocytes. Differences in CaR expression between right and left ventricular and atrial tissues were detected. Cells and heart muscle strips shortening as well as relaxation velocity of cardiomyocytes increased under CaR activation. Inhibition of the receptor lead to a reduced contraction force of the heart strips and decreased load-free cell shortening of cardiomyocytes, and also to a deceleration of the relaxation velocity. Significant effect of CaR inhibition was observed just under physiological Ca^{2+}-concentrations. The effect of CaR agonists and inhibitors on atrial strips was less pronounced. Systolic Ca^{2+} and Ca^{2+}-uptake to the sarcoplasmic reticulum (SR) were enhanced under CaR activation. Putrescine increased the activity of PKC and the levels of phosphorylated phospholamban (PLB). Moreover, inhibition of PKC abolished the effect of CaR activation on load-free cell shortening and relaxation velocity of cardiomyocytes. Effect of CaR was also abolished under downregulated RAMP1.

CaR and hypertrophy. Hypertrophy developed *in vivo* was accompanied by increased mRNA levels of CaR and RAMP1. Interestingly, the mRNA of ET_B receptors was also enhanced in one month L-NAME treated rats, whereas ET_A and ECE showed no differences.

ET-1 and PE chronic stimulation decreased shortening and relaxation velocity of the cells. However, the effect of ET-1 was potentiated by

NPS2390 and siRNA vs CaR. Enhanced CaR protein expression was shown under ET-1 exposure for 24 hours, but not under PE stimulation. Inhibition of ET_{B1} and ET_A receptors cancelled ET-1 remodeling effect and enhanced CaR protein expression.

Conclusion. Cardiomyocytes express CaR under physiological conditions and this contributes to the basal electromechanical coupling of cardiomyocytes under nearly physiological Ca^{2+}concentrations. Activation of the receptor with direct agonists is able to further increase contractility. It is also leading to the enhanced relaxation velocity which is explained by PKC activation, PLB phosphorylation and enhanced SERCA activity. To be functionally expressed on cardiomyocytes' membrane, CaR require the activity of RAMP1.

During the development of pressure induced hypertrophy, ET-1 via ET_A or ET_{B1} receptors is able to upregulate CaR expression. Elevated levels of CaR are supposed to compensate a loss of the heart function during hypertrophy development.

10. Zusammenfassung:

Hintergrund: Ca^{2+} ist ein wichtiges Molekül für die kardiale Funktion. Es triggert Signaltransduktion, Kontraktilität und aktiviert Enzyme. Ca^{2+} kann als wichtiger sekundärer Transmitter betrachtet werden. Allerdings wirkt Ca^{2+} auch als erster Signalbotenstoff durch Bindung an den Calcium-Sensing-Rezeptor (CaR). CaR sind G-Protein gekoppelte Rezeptoren, die sich in vielen Organen des Körpers finden. CaR sind an der Entstehung von Reperfusionsschäden, Hypertension, Hypertrophie und Herzinsuffizienz beteiligt. Die zugrunde liegenden Mechanismen sind weniger klar und Gegenstand dieser Untersuchungen.

Material und Methoden: Funktionelle Untersuchungen wurden an isolierten Kardiomyozyten und Muskelstreifenpräparaten durchgeführt. Die lastfreie Zellverkürzung isolierten Herzmuskelzellen wurde mit Hilfe eines Zellendendetektionssystems bestimmt. Zell- und Gewebeanalysen wurden auch mittels Western Blot und RT-PCR Technik durchgeführt. Calcium-Transienten wurden unter FURA-2AM Beladung der Zellen bestimmt. Proteinkinase C Aktivität wurde mittels nicht-radioaktiven Detektionssystemen (ELISA) bestimmt. Als in vivo-Modell der kardialen Druckbelastung wurden Ratten mit NO-Defizienz untersucht. Zelluläre Hypertrophie wurde durch Zugabe von Phenylephrin und Endothelin ausgelöst. CaR Stimulation erfolgte durch Putrescin und Gadolinium.

Ergebnisse: CaR unter physiologischen Bedingungen: RT-PCR und Western-Blot Analysen bestätigten eine konstitutive Expression des CaR in adulten Rattenkardiomyozyten. Die stärkste Expression fand sich im linken Ventrikel. CaR Aktivierung führte zur Zunahme der Verkürzung und Relaxationsgeschwindigkeit von Kardiomyozyten und

Muskelstreifen. Am stärksten beeinflusste CaR-Stimulation die Zellverkürzung bei nahezu physiologischer Ca^{2+}-Konzentration. Effekte durch Stimulation des CaR waren am Vorhof weniger ausgeprägt. Die Ca^{2+}-Transienten waren unter Stimulation des CaR verbessert. Putrescine aktivierte die Proteinkinase C und bei Hemmung der Proteinkinase C waren die positiv inotropen Wirkungen der CaR-Stimulation unterdrückt.

CaR und Hypertrophie: In dem hier verwendeten Hypertrophie-Modell kam es in vivo zu einer verstärkten Expression von CaR und RAMP1. Interessanterweise nahm auch die Expression des ET_B Rezeptors zu. Langfristig führte eine Exposition der Zellen mit ET-1 zu einer Abnahme der lastfreien Zellverkürzung, aber der Effekt war viel ausgeprägter unter Hemmung des CaR oder nach Herabregulation des Rezeptors. Pharmakologische Experimente zeigen, dass eine Stimulation der ET_A- oder ET_B1-Rezeptoren die negativen Effekte von Endothelin-1 durch Induktion des CaR begrenzen.

Schlussfolgerung: Kardiomyozyten exprimieren konstitutiv einen CaR der zur basalen Zellverkürzung beiträgt. Eine Aktivierung des CaR führt zur Verstärkung der Zellverkürzung, durch Proteinkinase C-abhängige Phosphorylaierung von Phospholamban und nachfolgender Verbesserung der Ca^{2+}-Transienten. Kardiomyozyten benötigen RAMP1 zur Verankerung des Rezeptors an der sarkolemmalen Membran. Bei prohypertropher Stimulation mit Endothelin-1 kann eine Heraufregualtion des CaR zur partiellen Kompensation eines hypertrophie-bedingten Funktionsverlustes beitragen.

11. List of publications:

- **The calcium-sensing receptor contributes to the electromechanical coupling of ventricular cardiomyocytes** / Dyukova E., Schreckenberg R., Schlueter K.-D. // Clin Res Cardiol 2013; 103 (1): V144.
- **Role of calcium-sensing receptors in electromechanical coupling of cardiomyocytes /** Dyukova E.A., Schreckenberg R., Sitdikova G., Schlueter K.-D. // Receptors and intracellular signaling, 2013; 1: 24-28.
- **The calcium-sensing receptor in experimental models of pressure induced cardiac hypertrophy** / Dyukova E., Schreckenberg R., Sitdikova G., Arens C., Schlueter K.-D. // Clin Res Cardiol 2014; 103(1): V523.
- **Mechanisms by which Calcium receptor stimulation modifies electro-mechanical coupling in isolated ventricular cardiomyocytes /** Schreckenberg R., Dyukova E., Sitdikova G., Abdallah Y., Schlueter K.D. // Pflügers Arch; 2015; 467: 379-88.
- **Calcium sensing receptor in the development of adaptive hypertrophy** / Dyukova E., Arens C., Schreckenberg R., Schlueter K.-D // Clin Res Cardiol 2016. Online publication. DOI 10.1007/s00392-016-0967-z.
- **The role of calcium sensing receptor in endothelin-1-dependent effects on adult rat ventricular cardiomyocytes: Possible contribution to adaptive myocardial hypertrophy** / Dyukova E., Schreckenberg R., Arens C., Sitdikova G., Schlüter KD. (Manuscript is under revision)

12. Declaration.

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communication. I have abided by principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Elena Dyukova

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