Calcium-sensing receptor and calcimimetics in the cardiovascular system

Sanela Smajilovic

This review has been accepted as a thesis together with five previously published papers by University of Copenhagen April, 2009 and defended on 26.June 2009

Tutors: Stig Haunso, Jacob Tfelt-Hansen & Majid Sheykhzade

Official opponents: Klaus Ølgaard, Lars Edvinsson & Naibedya Chattopadhyay

Correspondance: Department of Molecular Cardiology, Copenhagen University Hospital, Jagtvej 162, 2100-Copenhagen, Denmark

E-mail: ssmajilovic@yahoo.dk

Dan Med Bull 2010;(6)B4151

PREFACE

This PhD thesis is the result of work performed in the Laboratory of Molecular Cardiology, Department of Cardiology, The Heart centre, Rigshospitalet, in the period 2006-2009. My salary, PhD-fee and an anuum was funded by the Faculty of Health Sciences, University of Copenhagen. The project was supported by generous donations by the Danish Arrhythmia Research Centre. The thesis is based on 2 published reviews and 3 published scientific papers:

The two reviews are

1. Smajilovic S and Tfelt-Hansen J. Calcium as first messenger through calcium-sensing receptor in the cardiovascular system. Cardiovascular Research 75(3):457-67, 2007

2. Smajilovic S and Tfelt-Hansen J. Novel role of the calcium-sensing receptor in blood pressure modulation. Hypertension 52(6):994-1000, 2008

The threre scientific papers are

1. Tfelt-Hansen J, Hansen JL, Smajilovic S, Terwilliger EF, Haunso S, and Sheikh SP. Calcium receptor is functionally expressed in rat neonatal ventricular cardiomyocytes. Am J Physiol Heart Circ Physiol 290: H1165-1171, 2006.

2. Smajilovic S, Hansen JL, Christoffersen TE, Lewin E, Sheikh SP, Terwilliger EF, Brown EM, Haunso S, and Tfelt-Hansen J. Extracellular calcium sensing in rat aortic vascular smooth muscle cells. Biochem Biophys Res Commun 348:1215-1223, 2006.

3. Smajilovic S, Sheykhzade M, Holmegard HN, Haunso S, and Tfelt-Hansen J. Calcimimetic drug AMG 073 induces relaxation on the isolated rat aorta. Vascul Pharmacol. 47(4):222-8, 2007.

The papers are referred to as review 1, review 2, paper 1, paper 2 and paper 3 in the thesis.

LIST OF ABBREVIATIONS

7TM	Seven transmembrane
ANP	Atrial natriuretic peptide
BKCa	Large conductance Ca^{2+}-sensitive K^{+} channels
β-MHC	Beta- myosin heavy chain
$Ca^{2+}i$	Intracellular Ca^{2+}
$Ca^{2+}o$	Extracellular Ca^{2+}
cAMP	Cyclic adenosine monophosphate
CaR	Calcium-sensing receptor
cDNA	Complimentary DNA
ECD	Extracellular domain
ERK	Extracellular signal-regulated kinase
GAPDH	Glyceraldehydephosphate dehydrogenase
GPCR	G protein-coupled receptor
HEK-CaR	CaR-transfected human embryonic kidney
HPRT	Hypoxanthine phosphoribosyl-tranferase
ICD	Intracellular domain
IKCa	Intermediate conductance Ca^{2+}-sensitive K^{+} channels
IP	Inositol phosphate
IP3	Inositol triphosphate
JNK	Jun amino-terminal kinase
MAPK	Mitogen-activated protein kinase
MEK1	MAPK kinase 1
mGlu	Metabotropic glutamate receptor
mRNA	Messenger RNA
NO	Nitric oxide
PI4K	Phosphatidylinositol 4-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PTH	parathyroid hormone
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
TEA	Tetraethylammonium
TMD	Transmembrane domain
VSMC	Vascular smooth muscle cell

INTRODUCTION

INTRODUCTION TO THE CALCIUM-SENSING RECEPTOR

The calcium-sensing receptor (CaR) belongs to the family C of the G protein-coupled receptors (GPCRs), which are also termed seven transmembrane receptors (7TMs) [57]. The 7TMs constitute the largest group of cell surface membrane receptors, and they are one of the most important targets of currently used drugs. The human CaR consist of 1078 amino-acid residues and, like all 7TMs, has three structural domains (see **Figure 1**, Review 1): amino (N)-terminal domain of 612 amino acid residues, which is an unusually large extracellular domain (ECD) characteristic of the family C receptors of the 7TMs; a seven transmembrane domain (TMD) of 250 amino acid residues; and a 216 amino acids long intracellular carboxyl (C) -terminal domain (ICD). The receptor is modified by N-linked glycosylation, which is important for cells-surface expression [47]. The cells-surface CaR is present in a homodimeric configuration, which is crucial for its normal function [4]. The ECD is the main extracellular Ca^{2+} ($Ca^{2+}o$) binding site, but a mutated CaR that lacks the ECD also responds to $Ca^{2+}o$, implying that the TMD also participates in the calcium sensing [48].

Although the main ligand of the CaR is $Ca^{2+}o$, the CaR is a promiscuous receptor with many ligands, which can be divided into type I and type II [23, 57]. Type I are direct agonists, whereas type II are allosteric modulators that change the affinity of the receptor to $Ca^{2+}o$ and other direct agonists. The type I ligands are all polycharged cations, both organic and inorganic (listed in Table 1, Review 1). Furthermore, the CaR has also been shown to be sensitive to changes in ionic strength and pH [44, 45]. Type II agonists compromise two groups: the first group is small pharmacological drugs, termed calcimimetics, and the second group is L-amino acids [13, 23]. The calcimimetics bind to the TMD of the CaR and increase its sensitivity to $Ca^{2+}o$ [37]. The calcimimetic drugs are used in the treatment of uremic secondary hyperparathyroidism [17, 64]. AMG 073 is currently the drug of choice in the clinic due to pharmacokinetic considerations. Drugs that negatively modulate the CaR in an allosteric fashion are termed calcilytics.

The CaR is a low-affinity receptor: $Ca^{2+}o$ produces half-maximal activation of the CaR at about 3.5mM in CaR-transfected human embryonic kidney (HEK-CaR) cells in vitro [46]. However, the Hill coefficient, a measure of how well the receptor responds to small changes in agonists, is 3-4 in HEK-CaR cells. This allows the CaR to detect very small fluctuations in the $Ca^{2+}o$ levels.

Intracellular signalling apparatus of the CaR is very complex (for an overview, see Figure 2, Review 1) and depends markedly on the cell type in which the receptor is expressed. The CaR, like other GPCRs, acts mainly through G-proteins. In most cells, the CaR stimulation elicits phospholipase C (PLC)-mediated inositol triphosphate (IP3) formation with intracellular Ca^{2+} ($Ca^{2+}i$) mobilisation, indicative of Gαq activation [28, 60]. This interaction also induces activation of protein kinase C (PKC), which in turns modulates the activity of the receptor by a negative feedback system [5, 25]. In parallel, the CaR has been shown to activate phosphatidylinositol 4-kinase (PI4K), which is an enzyme that facilitates the first step in inositol lipids biosynthesis [25]. The CaR interacts directly not only with Gαq, but also with pertussis toxin-sensitive Gαi, which results in the inhibition of adenylate cyclase and therefore a reduction in cellular cyclic adenosine monophosphate (cAMP) levels [24].

The CaR has also been linked by several signalling pathways to various mitogen-activated protein kinases (MAPKs) such as MAPK kinase 1 (MEK1), extracellular signal-regulated kinases (ERKs), p38 MAPK, and Jun amino-terminal kinase (JNK), which account for many distal effects of the CaR, such as proliferation, differentiation, regulation of peptide secretion, and ion channel activity [29, 57, 58, 60, 61]. As in the case with many other cell-surface receptors, it is at present poorly understood how the activation of a single receptor type, in this case the CaR, can result in such varied biological endpoints depending on the cellular context in which the receptor is expressed.

The CaR is one of the key players in calcium homeostasis. The two major functions of this receptor are to inhibit parathyroid hormone (PTH) release from the parathyroid glands and to inhibit renal reabsorption of calcium (for a more detailed discussion, refer to the section 2.4 in Review 1). Besides being expressed in the parathyroid glands, kidney and, at lower levels, bone and intestinal cells, all four organs involved in calcium homeostasis, the CaR has also been found to be functionally expressed in tissues not related to calcium homeostasis (for the function of the CaR in these tissues, refer to the section 2.5 in Review 1), including heart and blood vessels [53].

CALCIUM-SENSING RECEPTOR IN THE CARDIOVASCULAR SYSTEM

The expression of a functional CaR has been demonstrated in rat cardiomyocytes [60, 66]. Recently, an immunohistochemical staining of tissue sections from a sheep heart revealed the CaR protein in endocardial endothelium, myocardial microvasculature and cardiac fibroblasts [31]. Although the CaR has been demonstrated in rat cardiomyocytes, it appeared not to be expressed in the cardiomyocytes from sheep. On the other side, we were not able to demonstrate its transcripts in cardiac fibroblasts from rats and humans grown in a cell culture (data not published). These discrepant results might be explained by species variation or by the difference between cell culture systems versus tissue specimens. (For a more detailed discussion on the CaR in the heart, refer to the section 3.1 in Review 1).

In blood vessels, the CaR protein was first reported in the perivascular nerves in rat cerebral, mesenteric, coronary and renal arteries, and was proposed to be involved in $Ca^{2+}o$ – induced relaxation of the precontracted arteries [8, 49, 67]. Later, the CaR has been detected in homogenates of whole vessels from rat subcutaneous small arteries and on the endothelial cells from rat mesenteric and porcine coronary arteries [40, 70]. Stimulating the CaR with an allosteric CaR modulator, Calindol, induced a hyperpolarisation of vascular smooth muscle cells, leading to vasodilation [69, 70]. Moreover, the CaR was shown in immortalised endothelial cells from human aorta, and activation of the receptor induced production of nitric oxide (NO), the most potent vasodilator [72]. In vascular smooth muscle cells (VSMCs), the presence of the CaR is controversial; several groups demonstrated the CaR in VSMCs [2, 35, 52, 71], whereas others reported its absence or suggested the presence of a receptor that is functionally related to, but molecularly distinct from, the CaR [20, 31, 50]. (For a more detailed discussion on the CaR in blood vessels, refer to the section 3.2 in Review 1).

Although it is now clear that the CaR is widely expressed in vascular tissues, the exact expression and function(s) still remain to be elucidated. Several reports indicate that it might be involved in the regulation of myogenic tone, and thereby blood pressure [8, 40, 54, 69, 70]. (For a more detailed discussion on potential roles of the CaR in blood pressure regulation, refer to Review 2). Moreover, growing evidence indicate that the CaR might be involved in vascular calcification [2, 35].

The overall aim of this thesis was:

- To establish whether the CaR is expressed in rat aortic VSMCs and neonatal ventricular cardiomyocytes in primary cell culture systems and to identify the signalling pathways used by the receptor. Furthermore, to investigate functions of Ca^{2+}o and the CaR in these cells.
- To investigate effects of the calcimimetic AMG 073 on the contractility of the rat aorta.

SUMMARY OF RESULTS

CALCIUM-SENSING RECEPTOR AND CALCIMIMETICS IN RAT NEONATAL VENTRICULAR CARDIOMYOCYTES

Paper 1

We established, using Reverse Transcription-Polymerase Chain Reaction (RT-PCR), that CaR messenger RNA (mRNA) is present in an in vitro model of rat neonatal ventricular cardiomyocytes (but not in rat neonatal ventricular fibroblasts; data not published). The presence of the CaR protein was demonstrated by immunocytochemistry using two CaR-specific antibodies. Stimulation with Ca^{2+}o activated the PLC/IP3 pathway in a concentration-dependent manner, assessed by measuring inositol phosphates (IP) accumulation. Addition of the calcimimetic AMG 073 augmented the Ca^{2+}o response, effectively left-shifting the relationship between Ca^{2+}o and IP accumulation. Moreover, infecting the cardiomyocytes with an adeno-associated virus containing the dominant negative CaR (R186Q [3]) significantly inhibited the Ca^{2+}o-induced IP response. These results strongly support the CaR as a mediator of the Ca^{2+}o-induced activation of the PLC/IP3 pathway in the neonatal cardiomyocytes.
Another signalling pathway used by the CaR is MAPK pathway, MEK1/ERK. Stimulation of the neonatal cardiomyocytes with 6mM Ca^{2+}o induced activation of ERK1/2. Moreover, AMG 073 induced ERK1/2 activation in the presence of 0.5mM Ca^{2+}o, and this response occurred more rapidly than with Ca^{2+}o alone.
Since stimulation of the CaR induced activation of ERK1/2, which is an important regulator of the cell cycle, we next investigated a possible role of the CaR in regulating DNA synthesis in the neonatal cardiomyocytes. Ca^{2+}o induced a biphasic response in DNA synthesis, as assessed by [3H]thymidine incorporation. DNA synthesis was upregulated by 3mM Ca^{2+}o, whereas at higher levels of Ca^{2+}o (6-10mM) DNA synthesis was downregulated. AMG 073 reduced DNA synthesis at all Ca^{2+}o concentrations. Furthermore, 3µM AMG 073 was more potent than 0.3µM AMG 073 at 0.5mM Ca^{2+}o. No effect on cell number was observed, as assessed by cell counting.
In conclusion, we demonstrated presence of a functional CaR in the rat neonatal cardiomyocytes, and showed that activation of the receptor regulates DNA synthesis possibly through the ERK1/2 signalling pathway.

CARDIAC HYPERTROPHY

An increase in DNA synthesis is observed in neonatal cardiomyocytes undergoing hypertrophy, perhaps due to partial progression through the cell cycle [6, 34].
Because activation of the CaR in the neonatal cardiomyocytes appeared to regulate DNA synthesis, and no effect on cell number, a measure of cell proliferation, was observed, we investigated a possible role of the CaR in hypertrophy. This was done by a set of experiments studying the effects of calcium and calcimimetic AMG 073 stimulation overnight on mRNA expression of three hypertrophy marker genes, atrial natriuretic peptide (ANP), beta- myosin heavy chain (β-MHC) and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [18], by real-time RT-PCR. Cardiac hypertrophy is usually associated with an increased expression of ANP and β-MHC, and an decrease in expression of SERCA [18].
Increasing levels of Ca^{2+}o from 0.5mM to 2-3mM did not have any significant effect, whereas 6mM Ca^{2+}o decreased gene expression of ANP (Figure 1). AMG 073 decreased gene expression of ANP in the presence of both 0.5mM and 3mM Ca^{2+}o, suggesting that the CaR might be protective against cardiac hypertrophy (Figure 1). No significant changes in the expression of β-MHC and SERCA were observed in response to Ca^{2+}o or AMG 073 (data not shown).

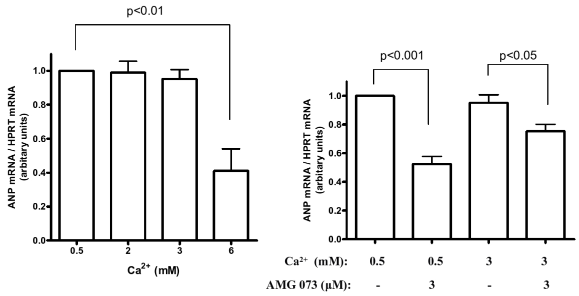

Figure 1

Expression of mRNAs for ANP in rat neonatal ventricular cardiomyocytes
Method: Total RNA was isolated with TriReagent (Molecular Research Centre, Inc, Cincinnati, OH, USA). First-strand cDNA was synthesised from 1µg of total RNA using Omniscript RT kit (Qiagen). Quantitative real-time PCR analyses were performed with the Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) and Quantitect SYBR Green PCR Kit (Qiagen).
The primers were:
ANP, forward: 5'- CCG GTA CCG AAG ATA ACA G-3'
ANP, reverse: 5'- CTC CAG GAG GGT ATT CAC C-3'
HPRT, forward: 5'- CGC AAA GTG GAA AAG CCA AGT-3'
HPRT, reverse: 5'- GCC ACA TCA ACA GGA CTC TTG TAG-3'
The specificity of each set of primers was ensured by 1.4% agarose gel analysis and DNA sequence analysis (GATC Biotech, Konstanz, Germany).
All mRNAs were quantitated in duplicates and the results were normalized to the content of HPRT within the same sample.
All data were analyzed with GraphPad Prism using one-way ANOVA analysis. Data are from ten individual experiments presented as means ±SEM. $P < 0.05$ was considered to represent a statistically significant difference.the results were normalized to the content of HPRT within the same sample.
All data were analyzed with GraphPad Prism using one-way ANOVA analysis. Data are from ten individual experiments presented as means ±SEM. $P < 0.05$ was considered to represent a statistically significant difference

CALCIUM-SENSING RECEPTOR AND CALCIMIMETICS IN RAT AORTA

Paper 2

We established, using RT-PCR, that CaR mRNA is present in rat aortic VSMCs in a cell culture. The presence of the CaR protein was demonstrated by immunocytochemistry. Stimulation with 3mM Ca^{2+}o activated MEK1/ERK pathway and induced MEK1/ERK dependent increase in DNA synthesis, as assessed by

[3H]thymidine incorporation. Neomycin, which is a type I agonist of the CaR, also increased the DNA synthesis. However, $Ca^{2+}o$ induced only a weak increase in IP production, observed merely with very high $Ca^{2+}o$ concentrations (20-30mM), and neomycin did not have any effect in this regard.
Calcium-induced DNA synthesis was attenuated by pre-treatment with NPS 2390, which is an allosteric antagonist of the group I metabotropic glutamate receptors (mGlu1 and mGlu5), shown previously to modulate effects of $Ca^{2+}o$ in other CaR-expressing cells [26, 33]. Two other group I metabotropic glutamate receptor antagonists, AIDA [36] and MPEP [22], either stimulated DNA synthesis (at 0.5mM $Ca^{2+}o$) or had no effect (at 3mM $Ca^{2+}o$). The increase in DNA synthesis was due to cell proliferation, as protein synthesis, protein content and cell number were also increased. Infecting the VSMCs with the adeno-associated virus containing the dominant negative CaR (R186Q [3]) did not attenuate calcium-induced DNA synthesis or high calcium-induced IP accumulation .
In conclusion, we demonstrated that the VSMCs express the CaR and showed that stimulation with $Ca^{2+}o$ and neomycin induces cell proliferation. However, due to conflicting results it could not be established whether the CaR is involved in this effect.

Paper 3
We investigated the effects of the calcimimetic AMG 073 on the contractility of rat aorta by wire myography. Our data showed that AMG 073 attenuates contractile response of the aorta to phenylephrine as well as high (125mM) K^+. Moreover, AMG 073 also induced concentration dependent relaxation in the vessels precontracted with phenylephrine, high K^+ or a non-selective potassium channel blocker, tetraethylammonium (TEA). Other CaR agonists, neomycin and gadolinium, did not have any effect. Inhibition of endothelium function with L-NAME and indomethacin, which are inhibitors of the enzymes NO synthase and cyclooxygenase, respectively, reduced AMG 073 –induced relaxation of the vessels precontracted with phenylephrine, but not with the high K^+. Relaxation to AMG 073 was also observed in the vessels precontracted with BayK 8644, which increases the probability of L-type calcium channels openings.
Although we demonstrated the presence of the CaR in the cultured VSMCs (as described in section 2.2.1), immunohistochemical staining of the aorta with two CaR specific antibodies demonstrated the presence of the CaR protein predominantly in endothelial and adventitial layers. The possible explanation for these discrepant results will be discussed in section 3.2.2.

DISCUSSION AND FUTURE PERSPECTIVES

METHODOLOGICAL CONSIDERATIONS

Cell culture systems

Rat neonatal ventricular cardiomyocytes
Primary culture of rat cardiomyocytes is a widely used experimental model in cardiac research. Cardiomyocytes can be obtained from neonatal rats and adults. Isolation of neonatal cardiomyocytes is less demanding compared to adult cardiomyocytes, which are very sensitive to the concentration of Ca^{2+} in the medium during the whole isolation procedure [12]. A second advantage of neonatal cardiomyocytes is that the phenotype of the cultured neonatal cardiomyocytes is very stable, whereas the phenotype of isolated adult cardiomyocytes is quite different from that of in situ hearts. Furthermore, neonatal cardiomyocytes most likely can grow and divide and the cells beat spontaneously and synchronized when they come into contact. We used neonatal cardiomyocytes isolated from 1 to 3 days old rats.
The most common problems with this type primary cell culture are a small cell yield and contamination with non-cardiomyocytes, such as endothelial cells, fibroblasts or smooth muscle cells. Trypsin was used to dissociate the heart tissue into single cells. Tryspsinization was repeated for short periods of incubation, as this has previously been reported to give a higher proportion of undamaged muscle cells. We discarded the cell suspension after the first two enzyme exposures to remove dead and damaged cells, and non-cardiomyocytes thereby obtaining the higher yields of viable cardiac cells and the quality of the primary culture.
The protocol was previously optimized and routinely used in our laboratory. Busk et al. (from our laboratory) tested the purity of the cultures by immunocytochemistry and found 98% of the cells positive for the cardiomyocyte markers sarcomeric-α-actin and sarcomeric tropomyosin [9]. Only few cells were positive for smooth muscle actin that stains fibroblasts and smooth muscle cells and for the endothelial cell marker von Willebrand Factor. It has been assumed that a minimum of non-cardiomyocytes is necessary in vivo and in vitro for proper functioning of the cardiomyocytes.

RAT AORTIC VASCULAR SMOOTH MUSCLE CELLS
VSMCs have been shown to exist in two phenotypic states, which have been designated contractile and synthetic [42]. Contractile VSMCs have a muscle-like or spindle-shaped appearance and a well-developed contractile apparatus, resulting from a high content of contractile filaments in the cell cytoplasm. The function of these is almost exclusively contraction and the maintenance of vascular tone. VSMCs in the synthetic state have a fibroblast-like appearance, proliferate readily, and synthesize increased levels of various extracellular-matrix components such as fibronectin and collagens. VSMCs in mature, normal blood vessels exhibit the contractile morphology, but injury induces a phenotypic modulation toward the synthetic phenotype, which contributes to intimal hyperplasia, seen in atherosclerotic lesions.
The VSMCs used in our studies were isolated from normal rat aortas. However, the isolated cells undergo a series of spontaneous changes in phenotype over the first days in the culture and assume the synthetic phenotype. Therefore, the VSMCs cultivation techniques provide a reasonable model for examining VSMCs in the synthetic state, but a poor system for studying the contractile phenotype. In addition, VSMCs in the culture undergo rapid and extensive proliferation providing the opportunity for further selection, or even transformation, and thus the appearance of subpopulations that may not have existed in vivo. Furthermore, the VSMCs cultures may be contaminated with other cell types, such as endothelial cells or fibroblasts.
We used a well established and widely used method for isolation of the VSMCs [42]. To obtain a high purity, the tunica adventitia of the aorta was peeled off to minimize fibroblast contamination. The cultures exhibited the “hill-and-valley” appearance characteristic of adult VSMCs. The cells were uniformly positive for smooth muscle α-actin by immunocytochemistry. However, expression of the smooth muscle α-actin was reported in nonmuscle tissues, including fibroblasts [16]. In addition, smooth muscle cells and fibroblasts are very alike in morphology. Therefore, the possible cell line cross-contamination cannot be completely avoided, which is a potential problem common to most primary cell cultures. However, despite the disadvantages described, primary cell cultures are a powerful tool in molecular and cell biology as they

closely mimic the in vivo state and provide a unique opportunity to study expression of proteins and their functions.

DETECTION OF THE CALCIUM-SENSING RECEPTOR

Two approaches, RT-PCR and immunochemical staining, were used to evaluate expression of the CaR.

RT-PCR combines complimentary DNA (cDNA) synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression. Due to the high sensitivity, RT-PCR enables to detect mRNAs from small amounts of RNA, even from cells with a low expression level.

One potential difficulty encountered with RT-PCR is genomic DNA contamination of RNA. Using a good RNA isolation technique minimizes the amount of contaminating genomic DNA in the RNA preparation. Moreover, using intron-spanning primers enable to distinguish amplified cDNA from amplified contaminating genomic DNA, as PCR products derived from cDNA will be shorter than products amplified from contaminating genomic DNA. In addition, control experiments without reverse transcriptase for each RNA template allow determining whether a given fragment is of genomic DNA or cDNA origin: products generated in the absence of reverse transcriptase are of genomic origin.

The major pitfall of RT-PCR is that detection of an mRNA in a particular tissue/cell type does not necessarily imply the expression of a functional protein product. In addition, when RNA is isolated from a particular cell type (e.g. cardiomyocytes or vascular smooth muscle cells) grown in a cell culture, a possible cross-contamination with other cell types might give false-positive signals.

Therefore, we performed another expression analysis on the protein level, immunochemical staining. The technique is widely used for demonstrating both the presence and the subcellular localization of an antigen (protein) in live or fixed cell cultures (immunocytochemistry) or tissues (immunohistochemistry) by use of a specific (primary) antibody, which recognizes the protein in the cell. The primary antibody is then amplified by use of a secondary antibody, which binds to the primary antibody. Three different primary antibodies were used in our studies: monoclonal LRG, polyclonal FF-7 and polyclonal ADI (Alpha Diagnostic), each previously shown to be CaR specific (references: LRG [11], FF-7 [30], ADI [72]). Secondary antibodies were covalently linked to a fluorophore (Alexa Fluors), which is detected in a confocal microscope. As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g. Alexa Flouors). Negative controls omitting the primary antibody are performed to establish the background fluorescence.

THE MYOGRAPH TECHNIQUE

The reactivity of arteries may be investigated using different ex vivo methods. The two approaches most commonly used are the isometric and the isobaric techniques [15]. Isometric preparations are mounted on dual wires or pins, the vascular segments are stretched to a flat shape, and contraction of the arterial segment is isometric, i.e. contraction in which tension increases while length remains constant. In contrast, isobaric preparations are longer, cannulated in both ends and usually stretched in the longitudinal direction to the length of the segment as measured in situ. In this type of preparation, the vessel is allowed to react freely to challenges. In our studies, we used the isometric myograph technique. In vivo vascular reactivity is rather isotonic (vessel length changes) than isometric, and largely dependent on blood flow, the release of humoral factors and vessel innervation, factors which are not present in the in vitro model. Also, in isometric preparations, both the internal and external surfaces are directly accessible to buffers and substances applied to the organ chamber, so that differential application to these surfaces is not possible. Additionally, transluminal pressure gradients and flow-induced shear stress are absent when using isometric preparations.

Conventionally, myograph experiments are performed under non-physiological high oxygen tensions (95% O2) that might influence NO mediated mechanisms and affect the vessel response to vasoactive compounds [41, 63]. Effects of oxygen tension on NO production appear to be specific for the vascular bed or animal species under investigation. Lowering of the oxygen tension to physiological values may more closely approximate the in vivo situation, and should perhaps be considered in future studies.

Smooth muscle cell contractility is dependent on the degree of stretch applied to the vessel, hence the initial stretch or the lumen diameter of the vessel needs to be defined. To ensure standardised conditions for vascular tension development, the vessel segments are normalized. The aim of the normalization procedure is to determine the internal lumen diameter, which the vessel would have if relaxed and under a transmural pressure of 100mmHg, by using a non-linear fitting procedure, assuming a circular lumen. The lumen diameter is obtained from the passive characteristics of the vessel, and the active components of the vessel wall are not included in the determination of the lumen diameter. A further disadvantage of the normalization procedure is that the in vivo transmural pressure at the vessel site is not necessarily 100mmHg.

In isolated vessel experiments, a precontractile agent is required, often in high concentrations, to induce vascular tone from which vasodilation can be measured. In contrast, vascular tone in vivo is maintained by the balance between endogenous vasoconstrictive and dilatory agents, the release of which is controlled by factors such as pressure and flow, the local tissue oxygen and temperature.

However, despite the discrepancies between the in vivo and in vitro situation, the myograph allows the investigation of functional properties in isolated vessels, and is appropriate for pharmacological purposes as it allows repetitive large-scale controlled experiments.

REAL-TIME RT-PCR

Real-time RT-PCR combines simultaneous amplification and detection of cDNA generated from RNA to allow the monitoring of PCR as it progress. Its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. The strength of the technique is its high sensitivity and specificity, which allows amplification and quantification of mRNA from extremely small samples. However, real-time RT-PCR is also a complex assay and all laboratory procedures must be rigorously standardized to obtain reliable and reproducible data [10]. In the present studies all reactions were optimized to avoid mispriming, genomic DNA contamination, reagent impurities, and operator variability.

The amount of cDNA available for amlification of the target and reference (housekeeping) gene in each sample is identical, and

the normalization of data to this reference gene allows for correction of inter-sample variation introduced by the variation in the initial RNA concentrations.
The housekeeping gene or reference gene should be present at a relatively constant level and be universally and constitutively expressed. We used hypoxanthine phosphoribosyl-tranferase (HPRT), responsible for metabolic salvage of nucleotides, as the reference gene in our studies. The comparison of the relative expression of mRNA levels in different treatment groups relies on the assumption that HPRT is expressed at a constant level between the groups. However, it cannot be ruled out that HPRT might be actively regulated by the treatment we applied (calcium and calcimimetics). Therefore, another reference gene, glyceraldehydephosphate dehydrogenase (GAPDH), was also used in some experiments, and similar results were obtained (data not shown).
Contamination of RNA samples with genomic DNA might be difficult to avoid, and therefore primers were designed to span intron-exon junctions, which allows the amplification of cDNA synthesized from mRNA but prevents amplification of genomic DNA. Primer specificity and PCR product size was tested by agarose gel electrophoresis and sequencing of the PCR product.
We used SYBR Green I detection system (Qiagen) based on an imaging system, which via binding of fluorescent dye to the double-stranded DNA, monitors the total amount of DNA produced. SYBR Green I non-selectively adheres to all double-stranded DNA molecules i.e. primer dimers and non-specific products will contribute to the overall emission of fluorescence. This problem was avoided by the use of highly specific primers and careful adjustment of PCR temperature profiles.
Real-time RT-PCR detects steady-state mRNA levels and provides no information on the amount or biological effects of a protein. Although there is a likely relation between the mRNA and protein levels for most genes, a final prediction of the full biological significance of a change in gene expression requires protein analysis such as western blot and immunochemistry followed by functional studies in cell cultures or animals.

RESULTS

CALCIUM-SENSING RECEPTOR AND CALCIMIMETICS IN RAT NEONATAL VENTRICULAR CARDIOMYOCYTES

Paper 1

The first evidence that the CaR is present in heart came in 2003 when Wang et al. demonstrated a functional CaR in rat adult cardiomyocytes [66]. We established that the CaR is also present in rat neonatal cardiomyocytes and showed that it is coupled to the PLC/IP3 and MEK1/ERK pathways [60].
The main ligand of the CaR is Ca^{2+}o, and stimulation with Ca^{2+}o is well documented to induce the release of intracellular Ca^{2+} through activation of the PLC/IP3 pathway in most cell types expressing the CaR. However, it has been demonstrated that Ca^{2+}o may also activate certain other receptors, e.g. some mGlu receptors [32]. Therefore, using Ca^{2+}o as a ligand is thus not sufficient to claim the involvement of the CaR. We used two approaches to verify that the effects observed in the cardiomyocytes were CaR-mediated. First, we infected the cells with the adeno-associated virus containing the dominant negative CaR (R186Q [3]) and compared effects of Ca^{2+}o on IP accumulation with those in cells infected with the control adeno-associated virus expressing β-galactosidase, a protein approximately the same size as the CaR. Expressing the dominant negative CaR produced a downward and rightward shift in the concentration-response curve for Ca^{2+}o- induced IP accumulation in the cardiomyocytes. Similar inhibitory effects of this mutant dominant negative CaR on the response of the wild-type CaR to Ca^{2+}o were also produced in other CaR-expressing cells [3, 59, 61, 62]. We also utilized the calcimimetic AMG 073 to prove CaR's role as a mediator of the effects of Ca^{2+}o. As expected, AMG 073 augmented the effects of Ca^{2+}o on IP accumulation. Furthermore, ERK1/2 activation was more rapid in response to AMG 073 than with Ca^{2+}o alone. However, although Ca^{2+}o induced a biphasic response in DNA synthesis, AMG 073 inhibited DNA synthesis at all levels of Ca^{2+}o. The difference in the effects of the two CaR agonists on the DNA synthesis might be explained by: first, differences in intracellular signalling pathways that they activate (e.g. the duration of ERK1/2 activation); secondly, stimulatory effects of Ca^{2+}o on DNA synthesis might be mediated by other mechanisms than the CaR; or lastly, AMG 073 may also have other effects besides activating the CaR (as discussed further below, section 3.2.1.b).

CARDIAC HYPERTROPHY

Although there is now strong evidence that the CaR is present in the cardiac tissue, the function of the receptor in the heart under physiological and pathophysiological conditions is yet not clear. We investigated whether the CaR is involved in cardiac hypertrophy. Cardiac hypertrophy is associated with, and perhaps in part mediated by, increased expression of several hypertrophic genes, including ANP and β-MHC [18]. Additionally, SERCA tends to be decreased in several hypertrophy models. We demonstrated that both high Ca^{2+}o (6mM) and AMG 073 decreases gene expression of the hypertrophy marker gene ANP, whereas no changes were observed in the expression of two other hypertrophy markers, β-MHC and SERCA.
There are numerous examples of mouse models of cardiac hypertrophy in which classic hypertrophic genes are not expressed as a coherent 'program' [18]. ANP can be increased independent or out of proportion to the other members. The observed decrease in the ANP gene expression combined with downregulation of DNA synthesis suggest that the CaR might be protective against hypertrophy, that is contrary to what has been suggested previously, that the CaR may promote neonatal rat cardiomyocyte apoptosis and Angiotensin II-induced hypertrophy [56, 65]. However, these studies were not very convincing, as they were designed without proper controls and Gd^{3+} was the only agonist used to show the effects of the CaR.
Determining whether stimulation of the CaR during a hypertrophic response protects from an increase in cardiomyocyte size is critical in the understanding of the protective role of the CaR in cardiac hypertrophy, and this would be very interesting to investigate in future studies. In addition, it should be kept in mind that AMG 073 might exert some of its effects through other mechanisms than the CaR. It has previously been suggested that another calcimimetic, NPS R-467, may directly activate a non-specific cation channel in pancreatic β cells [55]. Therefore, using other approaches (e.g. dominant-negative CaR; siRNA) to prove involvement of the CaR in cardiac hypertrophy should also be considered in future studies. Finally, AMG 073 is clinically used in the treatment of uremic secondary hyperparathyroidism [17, 64], and it is therefore interesting to investigate its effects on the heart and identify also if the effects are through other mechanisms than those mediated by the CaR.

CALCIUM-SENSING RECEPTOR AND CALCIMIMETICS IN RAT AORTA

Paper 2

There have been conflicting reports regarding whether the CaR is present in the VSMCs. Wonneberger et al. found CaR mRNA transcripts in the gerbil spiral modiolar artery and demonstrated a biphasic increase in intracellular Ca^{2+} in response to elevations in Ca^{2+}o [71]. In addition, using Ca^{2+}o and other CaR agonists (Gd^{3+}, Ni^{2+}) they induced a biphasic vasoconstriction. Since the increase in intracellular Ca^{2+} was paralleled by the biphasic vasoconstriction, they suggested that the CaR is most likely localized in the VSMCs. However, Farzaneh-Far et al. failed to detect CaR transcripts in the cultured VSMCs from rat aorta, and suggested a presence of a receptor that is functionally related to, but molecularly distinct from, the CaR [20].

We found CaR mRNA in the cultured VSMCs from rat aorta by using RT-PCR [52]. Moreover, the presence of CaR protein was demonstrated by immunocytochemistry. Our data are supported by two recent reports that demonstrated the CaR in human and bovine VSMCs, including aortic VSMCs [2, 35]. Expression of the CaR was markedly reduced in calcified areas of atherosclerotic arteries and in VSMCs cultured to have the mineralized phenotype. Furthermore, it has been demonstrated that incubating VSMCs in the presence of 1.8mM Ca^{2+}o, which is present in a regular culture medium, and 2.5mM Ca^{2+}o for 24 hours decreases the CaR expression compared to that observed in 1.2mM Ca^{2+}o [2]. Interestingly, down-regulation of the CaR expression was observed only in the presence of β-glycerophosphate, which induces VSMCs calcification following long-term treatment. However, the decrease in the CaR expression occurred before any increase in mineralization was detected in the cultures. Thus, level of the CaR expression appears to be regulated by several factors, such as culture conditions and phenotype of the cells, and could therefore account for the inconsistent detection of the CaR expression in the VSMCs in the previous studies. It should also be kept in mind that level of the CaR expression may also vary between species and different arteries.

We also demonstrated that Ca^{2+}o and neomycin induces the VSMCs proliferation, likely through MEK1/ERK pathway. Newly, Molostvov et al. showed a marked increase in human aortic VSMCs proliferation after treatment with neomycin, which seemed to be mediated by MEK1/ERK and PLC/IP3 pathways [35]. The observed increase in cell proliferation was significantly attenuated in cells transfected with CaR siRNA, confirming the direct involvement of the receptor. In contrast, neomycin did not have any effect on the IP accumulation in our study. In addition, Ca^{2+}o had only very weak effect and only at extremely high concentrations (20-30mM), suggesting that the CaR might not be coupled to the PLC/IP3 pathway in the rat VSMCs. Alternatively, this might be due to a low CaR expression under conditions used in this assay; e.g. the cells were grown in 1.8mM Ca^{2+}o for the IP assay, while for DNA/protein synthesis assay and Western blot analysis (for determination of ERK1/2 phosporylation) the cells were starved for several hours in the medium containing 0.5mM Ca^{2+}o, which might have up-regulated the CaR expression.

Infecting the cells with the adeno-associated virus containing the dominant negative CaR did not have any effect on calcium-induced DNA synthesis or high calcium-induced IP accumulation. This might be due to insufficient cell infection or there might be other signalling mechanisms and additional calcium-sensing receptors involved in calcium-induced proliferation of the VSMCs. Interestingly, Wellendorph et al. recently cloned and sequenced GPRC6A, a novel family C GPCR, with a significant homology to the human CaR [68]. It was subsequently suggested that GPRC6A is also activated by calcium (albeit at high concentrations) and calcimimetics [43] .

VSMCs are an important player in the pathogenesis of atherosclerosis, which involves the proliferation of the VSMCs. Atherosclerosis is considered to be an inflammatory disease at least partly [19], and it has been reported that extracellular fluids at sites of inflammation contain high concentration of calcium [27]. Moreover, atherosclerotic plaques may contain osteoclast-like cells, and the levels of Ca^{2+}o under actively resorbing osteoclasts may be 8-40mM [1, 51]. Thus, cells in an atherosclerotic lesion may be exposed to locally high concentrations of Ca^{2+}o. Therefore, our findings that the VSMCs express the CaR and that Ca^{2+}o induces cell proliferation may be of importance in understanding and preventing the disease. Furthermore, VSMCs are important in regulation of vascular tone. It would be therefore interesting to further investigate potential roles of Ca^{2+}o and the CaR in these cells and mechanisms behind.

Paper 3

Calcimimetic AMG 073, also known as Cinacalcet HCl, is used clinically for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease [17, 64]. In addition to its effects on PTH and mineral metabolism, it also appears to have favourable effects on important clinical outcomes, including cardiovascular events [14]. In addition, several reports demonstrated that calcimimetics induce acute hypertension in both uremic and normal rats, which is followed by a marked and sustained hypotensive effects in uremic rats in the presence of parathyroid glands [21, 38, 39]. Although the results indicated that the effects of the calcimimetics on blood pressure depend on the presence of parathyroid glands, other mechanisms, such as direct effects on vessels, may contribute as well.

Using wire myography, we demonstrated that AMG 073 induces relaxation of the precontracted rat aorta by direct effects on the vessel. In the vessels precontracted with phenylephrine, AMG 073-induced relaxation was partially endothelium-dependent, as it was significantly reduced when endothelium function was inhibited. In contrast, relaxation of the vessels precontracted with high K^+, which prevents efflux of K^+ from the cell and induces thereby depolarization, was endothelium-independent. These data suggest that endothelium-dependent relaxation evoked by AMG 073 is depending on hyperpolarizing factor(s), e.g. K^+ channels. Relaxation was also observed in the vessels precontracted with BayK 8644. BayK 8644 increases the probability of L-type calcium channels openings, and it is likely that entry of Ca^{2+}o through L-type calcium-channels plays the dominant role in inducing contraction with BayK 8644.

Taken together, we demonstrated that AMG 073 induces vasorelaxation of the precontracted aorta by both an endothelium-dependent mechanism, which seems to be dependent on hyperpolarising factors, and by an endothelium-independent mechanism, which could involve a direct inhibition of L-type calcium channels. AMG 073 induced relaxation could be, at least partly, mediated by the CaR or/and by a direct action on the ion channels.

Weston et al. demonstrated that the CaR present in the endothelial layer of rat mesenteric and porcine coronary arteries activates intermediate conductance Ca^{2+}-sensitive K^+ channels (IKCa), resulting in K^+-induced hyperpolarization of VSMCs [70]. Although hyperpolarization is usually associated with relaxation, stimulation of the CaR with a specific positive modulator, Calindol, did not have any effect on phenylephrine precontracted mesenteric

arteries. In a novel report, they demonstrated that this was due to phenylephrine-induced increases in K^+, the so-called 'K^+ clouds', emanating from large conductance Ca^{2+}-sensitive K^+ channels (BKCa) present on contracted VSMCs, which make the system less able to respond to further increases in K^+ [69]. Therefore, any further increase in K^+ after the opening of endothelial cell IKCa channels via CaR activation would generate minimal vasorelaxation, which might not be detected. Once the K^+ clouds were inhibited using iberiotoxin, a selective inhibitor of BKCa channels, CaR-induced vasodilation was detected.
In the light of these results, we suggest that AMG 073 might induce endothelium-dependent relaxation of precontracted aorta by activating the CaR in endothelial cells. In favour to our hypothesis, we demonstrated expression of the CaR protein in the endothelial layer of the rat aorta by immunohistochemical staining. Although we demonstrated the presence of CaR in the cultured VSMCs, only very faint staining was observed in the medial layer of the vessel. The discrepancy in the results might be explained by different phenotypes of the VSMCs present in the intact vessel compared to the VSMCs in the culture, as discussed above in section 3.2.2a.
In contrast to Weston et al., we detected relaxation of phenylephrine-precontracted vessel without inhibiting BKCa channels. It might be explained by use of different vessels (aorta versus mesenteric arteries) or different levels of precontraction obtained (e.g. relaxation might be easer to detect in a vessel that is weakly precontracted). Moreover, Weston et al. used pressure myography, while we were using wire myography. Important differences have been reported in the myogenic mechanisms and sensitivity to contractile agonists, when the two methods are applied in the same vessel type [15]. Furthermore, different agonists might differ in potency.
Other CaR agonists, neomycin, gadolinium, and phenylalanine, did not have any effects on the contractility of the aorta. Variable responses to CaR agonists have previously been reported in other systems, including cultured human aortic endothelial cells [7, 72]. Since our experiments were performed without inhibition of BKCa channels, it would be interesting to investigate whether addition of iberiotoxin would enable to induce relaxation with neomycin, gadolinium, and phenylalanine as well as augment endothelium-dependent relaxation evoked by AMG 073.

CONCLUSION

We established the presence of the CaR in neonatal ventricular cardiomycytes from rats and demonstrated that the receptor activates PLC/IP3 and MEK1/ERK signalling pathways. Furthermore, our data suggest that the CaR may have a role in cardiac hypertrophy.
We also established the presence of the CaR in the VSMCs from rat aorta, and showed that $Ca^{2+}o$ up-regulate proliferation of the cells. Further research is required to identify whether the CaR is involved in the calcium-induced VSMCs proliferation. Moreover, we demonstrated that calcimimetic AMG 073 induces relaxation on isolated rat aorta, and this could be, at least partially, mediated by the CaR.
Our data suggest that $Ca^{2+}o$ is a first messenger in normal physiology and patophysiology of the cardiovascular system. Furthermore, the novel clinical use of calcimimetics in the treatment of hyperparathyroidism and a potential use of calcilytics in the treatment of osteoporosis highlights the necessity to understand the role of the CaR in the cardiovascular system. Our results contribute to the emerging picture that the CaR may be of importance in the heart and vascular physiology. Future studies should reveal exact functions and mechanisms by which the CaR may modulate the system.

SUMMARY

Calcium is a crucial signal molecule in the cardiovascular system. Calcium (Ca^{2+}) acts as a second messenger via changes in intracellular Ca^{2+} levels through the actions of calcium channels and pumps. However, it is now well know that calcium may also be an extracellular first messenger through a G-protein-coupled receptor that senses extracellular Ca^{2+} concentration, the calcium-sensing receptor (CaR). The CaR is one of the key players in extracellular calcium homeostasis, but besides being expressed in the major organs involved in calcium homeostasis, the parathyroid gland, kidney and intestine, the CaR has also been found to be functionally expressed in other tissues. Although several studies demonstrated the CaR in heart and blood vessels, exact roles of the receptor in the cardiovascular system still remain to be elucidated. This PhD thesis describes data from our in vitro and ex vivo studies on the expression and function of the CaR in heart and aorta from rats.
We found a functional CaR to be expressed in rat neonatal ventricular cardiomyocytes in cell culture and to downregulate DNA synthesis in these cells, indicating that the CaR might be protective against cardiac hypertrophy. We also demonstrated the presence of the CaR in cultured rat aortic vascular smooth muscle cells and showed a stimulating effect of extracellular calcium on cell proliferation. However, we were not able to demonstrate that extracellular calcium exerted its effects through the CaR. Although we found the CaR to be expressed in cultured vascular smooth muscle cells, immunohistochemical staining of rat aortic tissue sections showed the CaR mainly in the endothelium but not in the medial layer of the vessel. These discrepant results are probably due to difference between cell culture systems versus tissue specimens, demonstrating the importance of using different models in research. We also investigated the effects of a CaR agonist, calcimimetic AMG 073, on contractility of the rat aorta by wire myography, and found it to induce vasodilation of precontracted aorta, an effect that might be, at least partly, mediated by the CaR.
In conclusion, we demonstrated the presence of the CaR in the neonatal ventricular cardiomyocytes and aorta, and showed a vasodilating effect of the calcimimetic AMG 073 on the precontracted aorta. These results support the emerging picture that the CaR may be of importance in the heart physiology and blood pressure regulation.

ACKNOWLEDGEMENTS

I have truly enjoyed my time as a PhD student in the Laboratory of Molecular Cardiology and I have met many people that I would like to thank. A very special thanks goes out to Jacob Tfelt-Hansen for being the best supervisor there is, for our friendship and for all his support. Thanks for sharing your vast knowledge and for keeping my enthusiasm high at all times! Also, I wish to thank Stig Haunsø for his patient mentorship and support. I am deeply grateful to Majid Sheykhzade for teaching me the skills of myography technique and for sharing his huge knowledge of vascular physiology.
I thank Jacob Lerche Hansen and Søren P. Sheikh for their scientific input and collaboration. I also whish to thank Katrine Kastberg for the excellent technical assistance and Karen Astrup for her helpfulness and patience. I am grateful to all the present and

former lab members for their friendship, scientific input and many joyful hours in the laboratory.
I am especially grateful to Edward M. Brown for giving me the opportunity for the six months sabbatical at Brigham and Women's Hospital and Harvard Medical School in Boston.
Finally, I whish to thank my family for the support they provided me through my entire life and in particular, I must acknowledge my life's companion and best friend, Predrag, for his persisting love and encouragement.

REFERENCES

1. Abedin, M., Tintut, Y., Demer, L.L. (2004) Vascular calcification: mechanisms and clinical ramifications. Arterioscler Thromb Vasc Biol, 24, 1161-1170.
2. Alam, M.U., Kirton, J.P., Wilkinson, F.L., Towers, E., Sinha, S., Rouhi, M., Vizard, T.N., Sage, A.P., Martin, D., Ward, D.T., Alexander, M.Y., Riccardi, D., Canfield, A.E. (2009) Calcification is associated with loss of functional calcium-sensing receptor in vascular smooth muscle cells. Cardiovasc Res, 81, 260-268.
3. Bai, M., Pearce, S.H., Kifor, O., Trivedi, S., Stauffer, U.G., Thakker, R.V., Brown, E.M., Steinmann, B. (1997) In vivo and in vitro characterization of neonatal hyperparathyroidism resulting from a de novo, heterozygous mutation in the Ca2+-sensing receptor gene: normal maternal calcium homeostasis as a cause of secondary hyperparathyroidism in familial benign hypocalciuric hypercalcemia. J Clin Invest, 99, 88-96.
4. Bai, M., Trivedi, S., Kifor, O., Quinn, S.J., Brown, E.M. (1999) Intermolecular interactions between dimeric calcium-sensing receptor monomers are important for its normal function. Proc Natl Acad Sci U S A, 96, 2834-2839.
5. Bai, M., Trivedi, S., Lane, C.R., Yang, Y., Quinn, S.J., Brown, E.M. (1998) Protein kinase C phosphorylation of threonine at position 888 in Ca2+o-sensing receptor (CaR) inhibits coupling to Ca2+ store release. J Biol Chem, 273, 21267-21275.
6. Brooks, G., Poolman, R.A., Li, J.M. (1998) Arresting developments in the cardiac myocyte cell cycle: role of cyclin-dependent kinase inhibitors. Cardiovasc Res, 39, 301-311.
7. Bruce, J.I., Yang, X., Ferguson, C.J., Elliott, A.C., Steward, M.C., Case, R.M., Riccardi, D. (1999) Molecular and functional identification of a Ca2+ (polyvalent cation)-sensing receptor in rat pancreas. J Biol Chem, 274, 20561-20568.
8. Bukoski, R.D., Bian, K., Wang, Y., Mupanomunda, M. (1997) Perivascular sensory nerve Ca2+ receptor and Ca2+-induced relaxation of isolated arteries. Hypertension, 30, 1431-1439.
9. Busk, P.K., Hinrichsen, R., Bartkova, J., Hansen, A.H., Christoffersen, T.E., Bartek, J., Haunso, S. (2005) Cyclin D2 induces proliferation of cardiac myocytes and represses hypertrophy. Exp Cell Res, 304, 149-161.
10. Bustin, S.A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol, 29, 23-39.
11. Chattopadhyay, N., Yano, S., Tfelt-Hansen, J., Rooney, P., Kanuparthi, D., Bandyopadhyay, S., Ren, X., Terwilliger, E., Brown, E.M. (2004) Mitogenic action of calcium-sensing receptor on rat calvarial osteoblasts. Endocrinology, 145, 3451-3462.
12. Chlopcikova, S., Psotova, J., Miketova, P. (2001) Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 145, 49-55.
13. Conigrave, A.D., Franks, A.H., Brown, E.M., Quinn, S.J. (2002) L-amino acid sensing by the calcium-sensing receptor: a general mechanism for coupling protein and calcium metabolism? Eur J Clin Nutr, 56, 1072-1080.
14. Cunningham, J., Danese, M., Olson, K., Klassen, P., Chertow, G.M. (2005) Effects of the calcimimetic cinacalcet HCl on cardiovascular disease, fracture, and health-related quality of life in secondary hyperparathyroidism. Kidney Int, 68, 1793-1800.
15. Davis, M.J., Hill, M.A. (1999) Signaling Mechanisms Underlying the Vascular Myogenic Response. PHYSIOLOGICAL REVIEWS, 79, 387-423.
16. Desmouliere, A., Rubbia-Brandt, L., Abdiu, A., Walz, T., Macieira-Coelho, A., Gabbiani, G. (1992) Alpha-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by gamma-interferon. Exp Cell Res, 201, 64-73.
17. Dong, B.J. (2005) Cinacalcet: An oral calcimimetic agent for the management of hyperparathyroidism. Clin Ther, 27, 1725-1751.
18. Dorn, G.W., 2nd, Robbins, J., Sugden, P.H. (2003) Phenotyping hypertrophy: eschew obfuscation. Circ Res, 92, 1171-1175.
19. Dzau, V.J., Braun-Dullaeus, R.C., Sedding, D.G. (2002) Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med, 8, 1249-1256.
20. Farzaneh-Far, A., Proudfoot, D., Weissberg, P.L., Shanahan, C.M. (2000) Matrix gla protein is regulated by a mechanism functionally related to the calcium-sensing receptor. Biochem Biophys Res Commun, 277, 736-740.
21. Fryer, R.M., Segreti, J.A., Widomski, D.L., Franklin, P.H., Banfor, P.N., Koch, K.A., Nakane, M., Wu-Wong, J.R., Cox, B.F., Reinhart, G.A. (2007) Systemic activation of the calcium sensing receptor produces acute effects on vascular tone and circulatory function in uremic and normal rats: focus on central versus peripheral control of vascular tone and blood pressure by cinacalcet. J Pharmacol Exp Ther, 323, 217-226.
22. Gasparini, F., Lingenhohl, K., Stoehr, N., Flor, P.J., Heinrich, M., Vranesic, I., Biollaz, M., Allgeier, H., Heckendorn, R., Urwyler, S., Varney, M.A., Johnson, E.C., Hess, S.D., Rao, S.P., Sacaan, A.I., Santori, E.M., Velicelebi, G., Kuhn, R. (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology, 38, 1493-1503.
23. Hofer, A.M., Brown, E.M. (2003) Extracellular calcium sensing and signalling. Nat Rev Mol Cell Biol, 4, 530-538.
24. Holstein, D.M., Berg, K.A., Leeb-Lundberg, L.M., Olson, M.S., Saunders, C. (2004) Calcium-sensing receptor-mediated ERK1/2 activation requires Galphai2 coupling and dynamin-independent receptor internalization. J Biol Chem, 279, 10060-10069.
25. Huang, C., Handlogten, M.E., Miller, R.T. (2002) Parallel activation of phosphatidylinositol 4-kinase and phospholipase C by the extracellular calcium-sensing receptor. J Biol Chem, 277, 20293-20300.
26. Jung, S.Y., Kwak, J.O., Kim, H.W., Kim, D.S., Ryu, S.D., Ko, C.B., Cha, S.H. (2005) Calcium sensing receptor forms complex with and is up-regulated by caveolin-1 in cultured human osteosarcoma (Saos-2) cells. Exp Mol Med, 37, 91-100.
27. Kaslick, R.S., Chasens, A.I., Mandel, I.D., Weinstein, D., Waldman, R., Pluhar, T., Lazzara, R. (1970) Quantitative analysis of sodium, potassium and calcium in gingival fluid

from gingiva in varying degrees of inflammation. J Periodontol, 41, 93-97.
28. Kifor, O., Diaz, R., Butters, R., Brown, E.M. (1997) The Ca2+-sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells. J Bone Miner Res, 12, 715-725.
29. Kifor, O., MacLeod, R.J., Diaz, R., Bai, M., Yamaguchi, T., Yao, T., Kifor, I., Brown, E.M. (2001) Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK293 cells. Am J Physiol Renal Physiol, 280, F291-302.
30. Kifor, O., Moore, F.D., Jr., Wang, P., Goldstein, M., Vassilev, P., Kifor, I., Hebert, S.C., Brown, E.M. (1996) Reduced immunostaining for the extracellular Ca2+-sensing receptor in primary and uremic secondary hyperparathyroidism. J Clin Endocrinol Metab, 81, 1598-1606.
31. Klein, G.L., Enkhbaatar, P., Traber, D.L., Buja, L.M., Jonkam, C.C., Poindexter, B.J., Bick, R.J. (2008) Cardiovascular distribution of the calcium sensing receptor before and after burns. Burns, 34, 370-375.
32. Kubo, Y., Miyashita, T., Murata, Y. (1998) Structural basis for a Ca2+-sensing function of the metabotropic glutamate receptors. Science, 279, 1722-1725.
33. Kwak, J.O., Kwak, J., Kim, H.W., Oh, K.J., Kim, Y.T., Jung, S.M., Cha, S.H. (2005) The extracellular calcium sensing receptor is expressed in mouse mesangial cells and modulates cell proliferation. Exp Mol Med, 37, 457-465.
34. Matturri, L., Milei, J., Grana, D.R., Lavezzi, A.M. (2002) Characterization of myocardial hypertrophy by DNA content, PCNA expression and apoptotic index. Int J Cardiol, 82, 33-39.
35. Molostvov, G., Fletcher, S., Bland, R., Zehnder, D. (2008) Extracellular calcium-sensing receptor mediated signalling is involved in human vascular smooth muscle cell proliferation and apoptosis. Cell Physiol Biochem, 22, 413-422.
36. Moroni, F., Lombardi, G., Thomsen, C., Leonardi, P., Attucci, S., Peruginelli, F., Torregrossa, S.A., Pellegrini-Giampietro, D.E., Luneia, R., Pellicciari, R. (1997) Pharmacological characterization of 1-aminoindan-1,5-dicarboxylic acid, a potent mGluR1 antagonist. J Pharmacol Exp Ther, 281, 721-729.
37. Nemeth, E.F., Steffey, M.E., Hammerland, L.G., Hung, B.C., Van Wagenen, B.C., DelMar, E.G., Balandrin, M.F. (1998) Calcimimetics with potent and selective activity on the parathyroid calcium receptor. Proc Natl Acad Sci U S A, 95, 4040-4045.
38. Odenwald, T., Nakagawa, K., Hadtstein, C., Roesch, F., Gohlke, P., Ritz, E., Schaefer, F., Schmitt, C.P. (2006) Acute blood pressure effects and chronic hypotensive action of calcimimetics in uremic rats. J Am Soc Nephrol, 17, 655-662.
39. Ogata, H., Ritz, E., Odoni, G., Amann, K., Orth, S.R. (2003) Beneficial effects of calcimimetics on progression of renal failure and cardiovascular risk factors. J Am Soc Nephrol, 14, 959-967.
40. Ohanian, J., Gatfield, K.M., Ward, D.T., Ohanian, V. (2005) Evidence for a functional calcium-sensing receptor that modulates myogenic tone in rat subcutaneous small arteries. Am J Physiol Heart Circ Physiol, 288, H1756-1762.
41. Pasgaard, T., Stankevicius, E., Jorgensen, M.M., Ostergaard, L., Simonsen, U., Frobert, O. (2007) Hyperoxia reduces basal release of nitric oxide and contracts porcine coronary arteries. Acta Physiol (Oxf), 191, 285-296.
42. Pauly, R.R., Bilato, C., Cheng, L., Monticone, R., Crow, M.T. (1997) Vascular smooth muscle cell cultures. Methods Cell Biol, 52, 133-154.
43. Pi, M., Faber, P., Ekema, G., Jackson, P.D., Ting, A., Wang, N., Fontilla-Poole, M., Mays, R.W., Brunden, K.R., Harrington, J.J., Quarles, L.D. (2005) Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J Biol Chem, 280, 40201-40209.
44. Quinn, S.J., Bai, M., Brown, E.M. (2004) pH sensing by the calcium receptor. J Biol Chem, 279, 37241-37249.
45 Quinn, S.J., Kifor, O., Trivedi, S., Diaz, R., Vassilev, P., Brown, E. (1998) Sodium and ionic strength sensing by the calcium receptor. J Biol Chem, 273, 19579-19586.
46. Quinn, S.J., Ye, C.P., Diaz, R., Kifor, O., Bai, M., Vassilev, P., Brown, E. (1997) The Ca2+-sensing receptor: a target for polyamines. Am J Physiol, 273, C1315-1323.
47. Ray, K., Clapp, P., Goldsmith, P.K., Spiegel, A.M. (1998) Identification of the sites of N-linked glycosylation on the human calcium receptor and assessment of their role in cell surface expression and signal transduction. J Biol Chem, 273, 34558-34567.
48. Ray, K., Northup, J. (2002) Evidence for distinct cation and calcimimetic compound (NPS 568) recognition domains in the transmembrane regions of the human Ca2+ receptor. J Biol Chem, 277, 18908-18913.
49. Ruat, M., Molliver, M.E., Snowman, A.M., Snyder, S.H. (1995) Calcium sensing receptor: molecular cloning in rat and localization to nerve terminals. Proc Natl Acad Sci U S A, 92, 3161-3165.
50. Shalhoub, V., Shatzen, E., Henley, C., Boedigheimer, M., McNinch, J., Manoukian, R., Damore, M., Fitzpatrick, D., Haas, K., Twomey, B., Kiaei, P., Ward, S., Lacey, D.L., Martin, D. (2006) Calcification inhibitors and Wnt signaling proteins are implicated in bovine artery smooth muscle cell calcification in the presence of phosphate and vitamin D sterols. Calcif Tissue Int, 79, 431-442.
51. Silver, I.A., Murrills, R.J., Etherington, D.J. (1988) Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp Cell Res, 175, 266-276.
52. Smajilovic, S., Hansen, J.L., Christoffersen, T.E., Lewin, E., Sheikh, S.P., Terwilliger, E.F., Brown, E.M., Haunso, S., Tfelt-Hansen, J. (2006) Extracellular calcium sensing in rat aortic vascular smooth muscle cells. Biochem Biophys Res Commun, 348, 1215-1223.
53. Smajilovic, S., Tfelt-Hansen, J. (2007) Calcium acts as a first messenger through the calcium-sensing receptor in the cardiovascular system. Cardiovasc Res, 75, 457-467.
54. Smajilovic, S., Tfelt-Hansen, J. (2008) Novel role of the calcium-sensing receptor in blood pressure modulation. Hypertension, 52, 994-1000.
55. Straub, S.G., Kornreich, B., Oswald, R.E., Nemeth, E.F., Sharp, G.W. (2000) The calcimimetic R-467 potentiates insulin secretion in pancreatic beta cells by activation of a nonspecific cation channel. J Biol Chem, 275, 18777-18784.
56. Sun, Y.H., Liu, M.N., Li, H., Shi, S., Zhao, Y.J., Wang, R., Xu, C.Q. (2006) Calcium-sensing receptor induces rat neonatal ventricular cardiomyocyte apoptosis. Biochem Biophys Res Commun, 350, 942-948.
57. Tfelt-Hansen, J., Brown, E.M. (2005) The calcium-sensing receptor in normal physiology and pathophysiology: a review. Crit Rev Clin Lab Sci, 42, 35-70.

58. Tfelt-Hansen, J., Chattopadhyay, N., Yano, S., Kanuparthi, D., Rooney, P., Schwarz, P., Brown, E.M. (2004) Calcium-sensing receptor induces proliferation through p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase but not extracellularly regulated kinase in a model of humoral hypercalcemia of malignancy. Endocrinology, 145, 1211-1217.

59. Tfelt-Hansen, J., Ferreira, A., Yano, S., Kanuparthi, D., Romero, J.R., Brown, E.M., Chattopadhyay, N. (2005) Calcium-sensing receptor activation induces nitric oxide production in H-500 Leydig cancer cells. Am J Physiol Endocrinol Metab, 288, E1206-1213.
60. Tfelt-Hansen, J., Hansen, J.L., Smajilovic, S., Terwilliger, E.F., Haunso, S., Sheikh, S.P. (2006) Calcium receptor is functionally expressed in rat neonatal ventricular cardiomyocytes. Am J Physiol Heart Circ Physiol, 290, H1165-1171.
61. Tfelt-Hansen, J., MacLeod, R.J., Chattopadhyay, N., Yano, S., Quinn, S., Ren, X., Terwilliger, E.F., Schwarz, P., Brown, E.M. (2003) Calcium-sensing receptor stimulates PTHrP release by pathways dependent on PKC, p38 MAPK, JNK, and ERK1/2 in H-500 cells. Am J Physiol Endocrinol Metab, 285, E329-337.
62. Tfelt-Hansen, J., Schwarz, P., Terwilliger, E.F., Brown, E.M., Chattopadhyay, N. (2003) Calcium-sensing receptor induces messenger ribonucleic acid of human securin, pituitary tumor transforming gene, in rat testicular cancer. Endocrinology, 144, 5188-5193.
63. Thom, S.R., Fisher, D., Zhang, J., Bhopale, V.M., Ohnishi, S.T., Kotake, Y., Ohnishi, T., Buerk, D.G. (2003) Stimulation of perivascular nitric oxide synthesis by oxygen. Am J Physiol Heart Circ Physiol, 284, H1230-1239.
64. Urena, P. (2003) Use of calcimimetics in uremic patients with secondary hyperparathyroidism: review. Artif Organs, 27, 759-764.
65. Wang, L.N., Wang, C., Lin, Y., Xi, Y.H., Zhang, W.H., Zhao, Y.J., Li, H.Z., Tian, Y., Lv, Y.J., Yang, B.F., Xu, C.Q. (2008) Involvement of calcium-sensing receptor in cardiac hypertrophy-induced by angiotensinII through calcineurin pathway in cultured neonatal rat cardiomyocytes. Biochem Biophys Res Commun, 369, 584-589.
66. Wang, R., Xu, C., Zhao, W., Zhang, J., Cao, K., Yang, B., Wu, L. (2003) Calcium and polyamine regulated calcium-sensing receptors in cardiac tissues. Eur J Biochem, 270, 2680-2688.
67. Wang, Y., Bukoski, R.D. (1998) Distribution of the perivascular nerve Ca2+ receptor in rat arteries. Br J Pharmacol, 125, 1397-1404.
68. Wellendorph, P., Brauner-Osborne, H. (2004) Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. Gene, 335, 37-46.
69. Weston, A.H., Absi, M., Harno, E., Geraghty, A.R., Ward, D.T., Ruat, M., Dodd, R.H., Dauban, P., Edwards, G. (2008) The expression and function of Ca(2+)-sensing receptors in rat mesenteric artery; comparative studies using a model of type II diabetes. Br J Pharmacol, 154, 652-662.
70. Weston, A.H., Absi, M., Ward, D.T., Ohanian, J., Dodd, R.H., Dauban, P., Petrel, C., Ruat, M., Edwards, G. (2005) Evidence in favor of a calcium-sensing receptor in arterial endothelial cells: studies with calindol and Calhex 231. Circ Res, 97, 391-398.
71. Wonneberger, K., Scofield, M.A., Wangemann, P. (2000) Evidence for a calcium-sensing receptor in the vascular smooth muscle cells of the spiral modiolar artery. J Membr Biol, 175, 203-212.
72. Ziegelstein, R.C., Xiong, Y., He, C., Hu, Q. (2006) Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells. Biochem Biophys Res Commun, 342, 153-163.