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CELL MODEL SYSTEMS IN CHARACTERIZING α_2-ADRENOCEPTORS AS DRUG TARGETS

Susann Björk

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“Everything comes in time to him who knows how to wait”

Leo Tolstoy

ABSTRACT

Susann Björk

Cell model systems in characterizing α_2-adrenoceptors as drug targets

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The three α_2-adrenoceptor (α_2-AR) subtypes belong to the G protein-coupled receptor superfamily and represent potential drug targets. These receptors have many vital physiological functions, but their actions are complex and often oppose each other. Current research is therefore driven towards discovering drugs that selectively interact with a specific subtype. Cell model systems can be used to evaluate a chemical compound's activity in complex biological systems. The aim of this thesis was to optimize and validate cell-based model systems and assays to investigate α_2-ARs as drug targets.

The use of immortalized cell lines as model systems is firmly established but poses several problems, since the protein of interest is expressed in a foreign environment, and thus essential components of receptor regulation or signaling cascades might be missing. Careful cell model validation is thus required; this was exemplified by three different approaches. In cells heterologously expressing α_{2A}-ARs, it was noted that the transfection technique affected the test outcome; false negative adenylyl cyclase test results were produced unless a cell population expressing receptors in a homogenous fashion was used. Recombinant α_{2C}-ARs in non-neuronal cells were retained inside the cells, and not expressed in the cell membrane, complicating investigation of this receptor subtype. Receptor expression enhancing proteins (REEPs) were found to be neuronal-specific adapter proteins that regulate the processing of the α_{2C}-AR, resulting in an increased level of total receptor expression. Current trends call for the use of primary cells endogenously expressing the receptor of interest; therefore, primary human vascular smooth muscle cells (SMC) expressing α_2-ARs were tested in a functional assay monitoring contractility with a myosin light chain phosphorylation assay. However, these cells were not compatible with this assay due to the loss of differentiation. A rat aortic SMC cell line transfected to express the human α_{2B}-AR was adapted for the assay, and it was found that the α_2-AR agonist, dexmedetomidine, evoked myosin light chain phosphorylation in this model.

Keywords: α_2-adrenoceptor, cell model, intracellular signaling, drug discovery

TIIVISTELMÄ

Susann Björk
Solumallien käyttö α_2-adrenergisten reseptorien karakterisoinnissa lääkekohteina

Farmakologia, lääkekehitys ja lääkehoito, Biolääketieteen laitos, Turun biolääketieteellinen tohtorikoulu ja lääkekehityksen tohtoriohjelma, Turun yliopisto sekä Department of Molecular and Cellular Physiology, Stanford University, CA, USA.

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α_2-adrenergisten reseptorien (α_2-AR) kolme alatyyppiä ovat G-proteiinikytkentäisiä reseptoreja ja mahdollisia lääkevaikutusten kohdemolekyylejä. Ne välittävät monia tärkeitä elimistön säätelytehtäviä, mutta niiden toiminnot ovat monimutkaisia ja usein vastakkaisia. Siksi yritetään kehittää uusia lääkeaineita, jotka vaikuttaisivat kohdennetusti vain yhteen alatyyppiin. Soluperustaisten koemallien avulla on mahdollista arvioida kemiallisen yhdisteen vaikutuksia monimutkaisissa biologisissa järjestelmissä. Tämän väitöstutkimuksen tavoitteena oli kehittää ja validoida soluperustaisia koemalleja ja mittausmenetelmiä α_2-AR:ien ja niihin vaikuttavien lääkeaineiden tutkimiseksi.

Jatkuvasti jakautuvien solulinjojen käyttö koemalleina on yleistä, mutta siihen liittyy ongelmia, sillä tutkimuksen kohteena olevaa proteiinia ilmennetään sille vieraassa ympäristössä. Isäntäsoluista saattaa puuttua reseptorin viestinvälityksen tai säätelyn kannalta olennaisia tekijöitä. Tämän vuoksi tarvitaan koemallien huolellista validointia. Väitöstutkimuksessa esitetään tästä kolme esimerkkiä. Kun ihmisen α_{2A}-AR:eja ilmennettiin solulinjassa, todettiin, että käytetty geeninsiirtotekniikka vaikutti koemallin avulla saataviin tuloksiin. Jos reseptorien ilmentyminen soluissa oli epätasaista, tuotti adenylaattisyklaasin estoon perustuva mittausmenetelmä väärän negatiivisen mittaustuloksen. Kun α_{2C}-AR:eja tuotettiin muissa kuin hermostoperäisissä soluissa, eivät reseptorit päätyneet solukalvolle, vaan jäivät solun sisään, mikä vaikeutti tämän reseptorialatyypin tutkimista. Reseptorien ilmentymistä lisäävien REEP proteiinien todettiin olevan hermosoluille tyypillisiä säätelijäproteiineja, jotka tehostivat α_{2C}-AR:ien soluliikennettä ja lisäsivät niiden ilmentymistä. Nykyisin pyritään reseptorien tutkimuksessa käyttämään primaarisoluja, jotka luontaisesti ilmentävät tutkittavaa reseptoria. Tämän mukaisesti selvitettiin mahdollisuutta käyttää ihmisen kudoksista eristettyjä verisuonten sileälihassoluja sen tutkimiseksi, miten α_2-AR:t säätelevät verisuonten sileälihassolujen supistustoimintaa. Koemalli perustui solujen supistuksen kannalta keskeisen biokemiallisen ilmiön, myosiinin kevyen ketjun fosforylaation mittaamiseen. Osoittautui, että ihmisen suonista eristetyt sileälihassolut eivät soveltuneet tähän käyttöön, sillä ne menettivät erilaistuneet ominaisuutensa niitä viljeltäessä. Sen sijaan rotan aortasta peräisin, α_{2B}-AR:eja ilmentävän sileälihassolulinjan todettiin soveltuvan koemalliksi. Näissä soluissa α_2-AR:eja aktivoiva lääkeaine deksmedetomidiini aiheutti odotetun vasteen, myosiinin kevyen ketjun fosforylaation.

Avainsanat: α_2-adrenerginen reseptori, lääkeaine, solumalli, soluviestintä

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ABBREVIATIONS

AC	Adenylyl cyclase
AR	Adrenoceptor
$[Ca^{2+}]_i$	Intracellular calcium
CHO	Chinese hamster ovary
CNS	Central nervous system
DAG	Diacylglycerol
EC	Endothelial cell
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
G protein	Guanosine nucleotide-binding protein
GPCR	G protein-coupled receptor
GTPase	Guanosine triphosphatase
HA	Human influenza hemagglutinin
HBSS	Hank's balanced salt solution
HEL	Human erythroleukemia
hMSC	Human mesenchymal stem cell
IP_3	Inositol 1,4,5-trisphosphate
iPSC	Induced pluripotent stem cell
HCS	High-content screening
HSP	Hereditary spastic paraplegia
HTS	High-throughput screening
NA	Noradrenaline
NO	Nitric oxide
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
PFA	Paraformaldehyde

PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PTx	Pertussis toxin
RAMP	Receptor activity modifying protein
REEP	Receptor expression enhancing protein
RGS	Regulator of G protein signaling
RTP	Receptor transporting protein
SGN	Sympathetic ganglion neuron
SMC	Smooth muscle cell
SR	Sarcoplasmic reticulum
SVSMC	Saphenous vein smooth muscle cell
TM	Transmembrane
VSMC	Vascular smooth muscle cell

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–IV. Additionally, unpublished data are presented.

I **Björk S**, Vainio M and Scheinin M (2005). Uneven cellular expression of recombinant α_{2A}-adrenoceptors in transfected CHO cells results in loss of response in adenylyl cyclase inhibition. *Biochim Biophys Acta.* 1744 (1): 38–46.

II **Björk S**[*], Hurt CM[*], Ho V and Angelotti T (2013). REEPs are membrane shaping adapter proteins that modulate specific G protein-coupled receptor trafficking by affecting ER cargo capacity. *PLoS One.* 8(10): e76366.

III Hurt CM[*], **Björk S**[*], Ho V, Gilsbach R, Hein L and Angelotti T (2014). REEP1 and REEP2 proteins are preferentially expressed in neuronal and neuronal-like exocytotic tissues. *Brain Res*. 1545: 12–22.

IV **Björk S**[*], Huhtinen A[*], Vuorenpää A and Scheinin M. Quantitative determination of α_{2B}-adrenoceptor-evoked myosin light chain phosphorylation in vascular smooth muscle cells. *Manuscript submitted for publication*.

[*] authors contributed equally

1 INTRODUCTION

G protein-coupled receptors (GPCRs) constitute a large and important protein family in our bodies, e.g. as acknowledged by the award of the 2012 Nobel Prize in Chemistry. These receptors have important roles as gatekeepers and molecular messengers within cells and have crucial roles in all major physiological systems, such as the sensory, cardiovascular and endocrine systems as well as in brain function. GPCRs function by transmitting messages from neurotransmitters, hormones or other extracellular chemical ligands across the cell membrane, allowing various types of cells in our body to sense their chemical environment and to communicate with each other. Activation of a GPCR results in dynamic biochemical changes within the cell, involving complex signaling pathways that regulate many important cellular functions. Many widely used drugs bind to GPCRs and either activate or block receptor activation, in fact according to some recent estimates, almost half of our current drugs directly or indirectly target GPCRs; these receptors are therefore vital targets for drug discovery and development (Audet and Bouvier, 2012; Kobilka, 2013).

α_2-adrenoceptors (α_2-ARs) belong to the family of GPCRs and are found throughout the human body. Three mammalian α_2-AR subtypes have been identified by molecular cloning. They regulate a wide variety of functions, such as sympathetic tone including blood pressure, glucose and lipid metabolism, as well as platelet aggregation, neurotransmitter release, pain, alertness and cognitive processes. Therefore, potentially these receptors could be involved in the therapy or pathogenesis of many serious human diseases, such as hypertension, coronary heart disease, obesity, diabetes, chronic pain states as well as neuropsychiatric disorders. The functions mediated by the different α_2-AR subtypes are complex and incompletely understood, but it is evident that novel drugs targeting single receptor subtypes could perhaps achieve more specific drug responses. However, no subtype-selective α_2-AR ligands have so far been developed to therapeutic drugs (Ruffolo and Hieble, 1994; Perez, 2006; Gilsbach and Hein, 2012).

Validated cell-based model systems are needed if one wishes to investigate these receptors and their signaling properties, as well as to screen potential (subtype-selective) therapeutic drug candidates or unknown compounds in the search for desired effects. Those kinds of cell systems have conventionally involved the use of established cell lines, transfected to express the receptor under study, but current research is now directed towards using primary cells endogenously expressing receptors in their natural environment, or inducible stem cells. In these cell models, multiple biochemical and morphological cellular outputs to physical, chemical or biological stimulation are measured when screening drugs during the initial phases of drug discovery (Horrocks et al., 2003; Kenakin, 2009; Macarron et al., 2011; Takahashi and Yamanaka, 2013). The main focus of this thesis is on the development and validation of different cell-based models for investigating the subtypes of α_2-ARs. Novel and more detailed knowledge of receptor subtype functions could be anticipated to lead to improved drug therapy.

2 REVIEW OF THE LITERATURE

2.1 α_2-Adrenoceptors, members of the G protein-coupled receptor superfamily

2.1.1 A historical perspective of GPCRs

Cells constitute the living material in the human body through which all vital functions are executed. Mammalian cells are surrounded by a cell membrane which is essentially constituted of a lipid bilayer. To ensure that important information can be conveyed across this membrane, there has to be some kind of molecular framework in order that cells can communicate with each other, to sense their surroundings and to react to its changes. GPCRs constitute an important part of this molecular framework and act as transducers of signals from the outside (consisting of chemical signals such as hormones, neurotransmitters, ions, odorants and taste molecules, and photons in the retina) to the inside of the cell membrane where they activate G proteins that convey the signals further. GPCRs have been the focus of intensive research for over a century, with important milestones such as the awarding of the Nobel Prize in Physiology and Medicine (1994) to Alfred G. Gilman and Martin Rodbell for the discovery of G proteins and their role in cellular signal transduction and the recent Nobel Prize in Chemistry (2012) to Brian K. Kobilka and Robert J. Lefkowitz for answering several key questions related to GPCR structure and function (Audet and Bouvier, 2012; Snogerup Linse, 2012; Kobilka, 2013).

The unraveling and understanding of GPCR structure and function have been a long and fascinating epic, which can only be very briefly summarized here. More detailed accounts have been given elsewhere (e.g. Baldwin, 1994; Lefkowitz, 2000; Pierce et al., 2002; Lefkowitz, 2003; Perez, 2006; Giraldo and Pin, 2011; Kobilka, 2013). Already at the very beginning of the 20th century, J. N. Langley and H. H. Dale postulated that reactive cells must have a "receptive substance" on their surface, as they studied the effects of cholinergic and adrenergic agonists and antagonists on different target organs (Langley, 1901; Dale, 1906). This was followed by a period (1920–70) of evolution of the classical receptor theory, with researchers like Clark, Ariens, Stephenson, Black and Furchgott laying the foundations (Lefkowitz, 2003; Snogerup Linse, 2012). The first definition of the term "receptor" was made by Furchgott to "indicate the postulated specific molecular sites or structures in (or on) an effector cell with which molecules of a specific agonist must react in order to elicit the characteristic response of the cell to the agonist" (Furchgott, 1964). Raymond Ahlquist investigated the properties of catecholamines in different organs and in 1948 he was the first to propose a pharmacological separation of "adrenotropic" receptors into α- and β-adrenoceptors. He suggested that epinephrine (adrenaline) was the endogenous mediator in the adrenergic system and that whether the agonist response was inhibitory or excitatory solely depended on the type of receptor it activated (Ahlquist, 1948). A

separation of pre- (α_2) and postjunctional (α_1) α-adrenoceptors was proposed by Langer in 1974 (Langer, 1974), but this was revised in 1977 when it became evident that anatomical location alone was not sufficient to classify these receptor types. Instead, a classification based on the functions mediated by the α-adrenoceptors was proposed (Berthelsen and Pettinger, 1977). This classification into an inhibitory and an excitatory category was once again refuted as antagonists of both receptor subtypes were able to inhibit noradrenaline-induced vasoconstriction (Drew and Whiting, 1979). It was therefore concluded that neither anatomical nor functional classifications could be reliably used in the classification of adrenoceptor subtypes, and instead a pharmacological classification depending on their reactivity to agonists and antagonists in different tissues was postulated (Bylund, 1985; Bylund et al., 1994; Ruffolo and Hieble, 1994). Shortly thereafter, a pharmacological classification of the receptors evolved, and with the advances of radioligand binding as a tool to study receptor characteristics, combined with the revolutionary technique of molecular cloning, it was finally appreciated that GPRCs constitute a large superfamily of receptors with structural and functional similarities. This became evident with the cloning of the mammalian β_2-adrenoceptor gene, as surprisingly it was found that the corresponding gene was intronless and significantly homologous to the gene encoding bovine rhodopsin (Dixon et al., 1986). Subsequently, evidence was put forward for nine different subtypes of adrenoceptors, with much of the pioneering work on their biochemical and structural characterization being done in Dr. Lefkowitz's laboratory in Duke University (Benovic, 2012). With the revelation of the β_2-adrenoceptor existing as a distinct protein entity coupling high-affinity agonist binding to the activation of heterotrimeric G proteins, the concept of a ternary complex was introduced in 1980 by the same group (De Lean et al., 1980). Very recently, 30 years later, the ternary complex was beautifully visualized by Dr. Kobilka's research group in Stanford University, as the high resolution three-dimensional crystal structure of the β_2-adrenoceptor in complex with its agonist and a G_s protein molecule was reported (Rasmussen et al., 2011).

Today, it is known that approximately 720–800 genes in the human genome encode GPCRs, also termed 7TM (transmembrane) receptors since their polypeptide chain passes seven times across the lipid bilayer of the cell membrane. Members of this family include the adrenoceptors, histamine and dopamine receptors, as well as olfactory and chemosensory receptors. Together they represent the largest family of proteins involved in signal transduction across the plasma membrane (Siehler, 2008; Audet and Bouvier, 2012; Venkatakrishnan et al., 2013). They were classically grouped into class A (rhodopsin-like), B (secretin-like) and C (metabotrophic glutamate-like), where the α_2-adrenoceptors belong to the A class (Fredriksson et al., 2003; Lagerstrom and Schioth, 2008; Audet and Bouvier, 2012; Venkatakrishnan et al., 2013). This classification has been expanded to include Frizzled (and the closely related Smoothened receptors (Schulte, 2010) and adhesion receptors (Bjarnadottir et al., 2007).

2.1.2 Subtypes, structure and pharmacology

The adrenoceptor family is divided into α_1-, α_2- and β-adrenoceptors, each of which has three subtypes in human and other mammals (Vassilatis et al., 2003). Initially, however, α_2-ARs were thought to have four subtypes, since the rodent (rat and mouse) α_{2A} subtype was classified as the α_{2D} subtype, due to some differences in its pharmacological properties in comparison to the human α_{2A}-AR (Lanier et al., 1991; Simonneaux et al., 1991), but it was soon proposed (and later confirmed) that these were species variants of the same receptor subtype (Link et al., 1992; Bylund et al., 1994). The genes that encode each of the three α_2-AR subtypes were cloned, as part of the early GPCR studies in Dr. Lefkowitz's laboratory, and the receptor subtypes were named α_2-C10 (Kobilka et al., 1987), α_2-C2 (Lomasney et al., 1990) and α_2-C4 (Regan et al., 1988) based on their locations on human chromosomes 10, 2 and 4, respectively. Their gene products correspond to the pharmacological α_2-AR subtypes α_{2A}, α_{2B} and α_{2C}, respectively, in humans and other mammalians. Non-mammalian vertebrate animals may have different complements of adrenoceptor subtypes in their genomes, e.g. five α_2-AR subtype genes have been identified in the zebrafish genome (Ruuskanen et al., 2004). Bacteria, plants and invertebrate animals do not have catecholamine-binding adrenoceptors, but they have evolutionarily related receptors for other monoamines that structurally resemble noradrenaline. For example, insects and crustaceans have a GPCR subfamily that is activated by octopamine and tyramine, and an α-adrenoceptor-like octapamine receptor that recognizes the α_2-AR agonist medetomidine (Evans and Maqueira, 2005; Lind et al., 2010) as well as a recently identified octopamine autoreceptor that was shown to exert adrenoceptor-like inhibitory actions on cAMP production (Koon and Budnik, 2012).

As the first adrenoceptor genes were cloned, an interest in understanding their structure was kindled, and already in the beginning of the 1990's Kobilka and co-workers started a major project to crystallize the β_2-adrenoceptor. The first glimpses of GPCR structure came from a low resolution model of bacterio-rhodopsin (Henderson and Unwin, 1975; Henderson et al., 1990), but the first true GPCR model was an electron microscopy (7 Å) structure of bovine rhodopsin (Henderson and Unwin, 1975; Schertler et al., 1993), which allowed for homology model design to help delineate GPCR structures (Henderson et al., 1990; Baldwin et al., 1997). The first crystal-based structure of a GPCR was solved by Palczewski et al., 2000, who published the X-ray structure of bovine rhodopsin at 2.8 Å resolution (Palczewski et al., 2000), providing researchers with important detailed information on the structure of a GPCR in the inactive state, as well as its side-chain amino acid conformation, N- and C-terminal domains and the positions of its extra- and intracellular loops. Nonetheless, structural analysis of GPCRs was still hindered by their inherent structural flexibility and instability in detergent solutions. Therefore it took another seven years before the next GPCR structure was resolved by Dr. Kobilka's research group. In 2007, a 3.4/3.7 Å resolution structure of the β_2-adrenoceptor bound to an inverse agonist was published and this yielded insights into the transmembrane domains and intracellular portions of the

receptor (Rasmussen et al., 2007). The key to success was the use of a Fab antibody fragment to stabilize the otherwise very flexible third intracellular loop of the receptor, or alternatively the replacement of this loop with a T4 lysozyme domain to help provide extended crystal contacts (Cherezov et al., 2007; Rosenbaum et al., 2007). This trio of papers marked an important milestone in structural biology.

Finally in 2011, Rasmussen, Sunahara, Kobilka and co-workers discovered a detergent for stabilizing the receptor with its G protein, hence providing a lipid scaffold that allowed the receptor to be supported, along with an antibody that could hold it together. This crystallization project culminated in a 3.2 Å resolution picture of the agonist-occupied receptor frozen at the exact instant as it activates the G_s protein (in complex with it or with a nanobody) (Rasmussen et al., 2011; Rasmussen et al., 2011). Recently, the conformation of the active β_2-AR bound to its endogenous neurotransmitter was reported (Ring et al., 2013). This has led to an explosion in the field, and numerous structures of other GPCRs have emerged in the recent few years (Audet and Bouvier, 2012; Benovic, 2012), although the α_2-AR structures still remain unresolved.

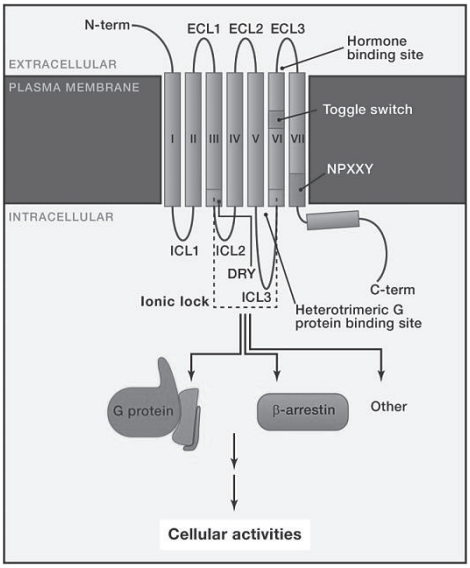

Figure 1. Schematic representation of a typical GPCR. GPCRs have an N-terminus on the extracellular side, followed by seven α-helical membrane-spanning segments separated by extracellular (ECL) and intracellular (ICL) loops that terminate in an intracellular C-terminus. Motifs (DRY, toggle switch and NPXXY) that mostly are conserved among class A GPCRs are outlined, as well as the G protein binding site (Audet and Bouvier, 2012).

Generally, it can be said that α_2-ARs and other members of class A GPCRs contain an extracellular region, consisting of an N-terminal domain and three extracellular loops that act as a vestibule, directing the way that the ligands access the receptor-binding pocket, as outlined in Fig. 1. Seven transmembrane (TM) spanning α-helices form the structural core of the receptor, embedded in the lipid bilayer. This is the location where ligands bind and transduce the information to the inside of the cell by inducing or stabilizing conformational changes in the α-helices. Each receptor has a binding site

that is specifically adapted to the nature of its ligands. The ligands penetrate to different depths within this pocket, whose conformation in turn is defined by the conformational changes in the overall receptor architecture. In particular, TM3 seems to be very important for GPCR structure and function, and it has a crucial role as a structural and functional hub. Finally, the intracellular region, containing the C-terminus and the three intracellular loops, regulates the interface with cytosolic signaling proteins (Audet and Bouvier, 2012; Venkatakrishnan et al., 2013).

So far, no high-resolution structure has been resolved for the α_2-ARs, and current α_2-AR models are therefore based on modeling from existing related structures of other class A GPCRs, mainly the β-adrenoceptors. The α_2-ARs are polypeptides that in humans consist of 450 (α_{2A} and α_{2B}) or 461 (α_{2C}) amino acids. Similar to the other GPCR subfamilies, the different subtypes of the α_2-ARs show high conservation, having 45% overall amino acid identity between the subtypes, and approximately 75% within the conserved TM regions (Bylund et al., 1992). The ligand binding pocket is formed by TM2–7, and in these, 29 of the 33 amino acids lining the binding pocket are identical. Due to this high structural similarity, the ligand binding properties of the three receptor subtypes are rather similar (Ruuskanen et al., 2005; Xhaard et al., 2005). However, TM1 has been shown to play an important role in the subtype-selective binding of some α_2-AR antagonists (Laurila et al., 2011), and the second extracellular loop forms a lid over the binding cavity, and is involved in determining the species differences of antagonist binding profiles in human and mouse α_{2A}-ARs (Laurila et al., 2007). The α_2-ARs have exceptionally long third intracellular loops (about 150 amino acids), which is 2–3 times the length of the corresponding structures found in the other adrenoceptors, and thus constitute the most pronounced structural difference (aside from variations in the N- and C-terminal domains) between the adrenoceptor family members (Ruffolo and Hieble, 1994).

The first models of GPCR activation described the situation with an agonist, the *activated* receptor and the G protein in a so-called ternary complex (De Lean et al., 1980). As it was conceived that receptors were able to activate G proteins in the absence of ligands (constitutive activation) and to take into account the differential properties of drugs (full, partial and inverse agonists as well as neutral antagonists) on signaling, this model was extended (Samama et al., 1993; Chidiac et al., 1994), and it was proposed that receptors existed in an equilibrium of two functionally distinct states: the inactive and the active state. When the receptor was not occupied by an agonist, there was a certain degree of basal activity of the receptor that was proportional to the current balance of the equilibrium of the two states. The balance will be shifted when an agonist binds and the efficacy of a ligand therefore thought to be a reflection of its ability to alter the equilibrium between these two states (Gether and Kobilka, 1998). Today, GPCRs are considered as molecular regulators, able to exist in a continuum of conformations with relatively closely spaced energies. Specific ligands can stabilize the receptor in a particular conformation, thereby also allowing the receptor to interact with a specific effector, resulting in the activation of a distinct signaling pathway. The receptor can be active in more ways than one; it can

simultaneously appear to be inactive with respect to a certain signaling pathway but exhibit functional activity in terms of another. In this model, agonist binding increases the likelihood of a receptor assuming an active-state conformation, but it is only G protein binding that can fully stabilize the active state (Rosenbaum et al., 2009). In addition, agonists do not always activate receptors through stabilization of the same active state but rather can stabilize unique active states to create a signal that can then be "biased" towards a certain signaling pathway (biased agonism) (Kenakin and Christopoulos, 2013). The recent description of the crystal structure of the agonist-occupied β_2-AR in complex with its G protein represented the first high resolution view of the active ternary complex (Rasmussen et al., 2011), revealing how small structural changes around the binding pocket could be amplified into very large structural changes in the G protein. The resolved structure has been very valuable for the understanding of the dynamic nature of receptor activation.

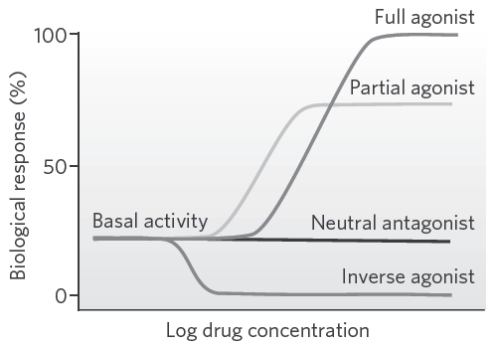

Figure 2. Classification of ligand efficacy for GPCRs. Many GPCRs exhibit basal, agonist-independent activity. An inverse agonist will inhibit this activity, whereas a neutral antagonist will have no effect. Full and partial agonists stimulate biological responses above the basal activity (Rosenbaum et al., 2009).

Efficacy, i.e. the effect that a ligand exerts on the structure, conformation and thus on the biological response of a receptor, is used to group ligands into four different classes, as shown in Fig. 2: 1) full agonists that achieve maximal receptor stimulation, 2) partial agonists that never completely activate the receptor, 3) neutral antagonists that in themselves do not activate receptor signaling, but prevent other ligands (both agonists and inverse agonists) from binding to the receptor and 4) inverse agonists that reduce the level of basal or constitutive activity below that of the unbound receptor (Rosenbaum et al., 2009). However, agonists can also behave as positive (in normal systems) and inverse agonists (in constitutively active systems) on the same receptor (protean agonists), and behave as ligand-selective agonists, i.e. differ in the stimulus pattern they produce in physiological systems (Kenakin, 2001).

All α_2-ARs recognize the two catecholamines, noradrenaline and adrenaline, as their physiological ligands with rather similar affinities. Dexmedetomidine and clonidine are synthetic agonists that display affinity for all three subtypes, although dexmedetomidine is a full agonist only at the α_{2B}-subtype. Brimonidine (UK 14,304) is a full agonist of the α_{2A}-AR but only a partial agonist of the α_{2B}- and α_{2C}-ARs

(Peltonen et al., 1998). The α_2-antagonists, JP-1302 and ORM-12741, have been reported to be highly selective for α_{2C}-ARs (Sallinen et al., 2007; Herrick et al., 2012). Some relatively subtype-selective antagonists have also been developed; for example, oxymetazoline, guanfazine, BRL44408 and BRL 48962 are selective for α_{2A}-ARs, whereas prazosin, spiperone, spiroxatrine and ARC-239 have relatively high affinity for the α_{2B}- and α_{2C}-AR subtypes, but low affinity for α_{2A}-ARs. MK912 exhibits some selectivity for α_{2C}-ARs, but atipamezole, rauwolscine, RS-79948-197, RX821002, yohimbine and idazoxan bind almost equally well to all human α_2-AR subtypes (Bylund et al., 1988; Lomasney et al., 1991; Bylund et al., 1992; Jasper et al., 1998; Peltonen et al., 1998; Peltonen et al., 2003; Ruuskanen et al., 2005; Sallinen et al., 2007; Laurila et al., 2011). The lack of good tools for the investigation of the subtype specific effects of the α_2-ARs has hampered research, and even today no truly subtype-selective compounds have progressed all the way to the clinic.

2.1.3 Coupling to G proteins

GPCRs transduce a wide range of extracellular signals across the plasma membrane of the cell into discrete intracellular messages capable of regulating numerous and diverse cell functions. These chemical signals, which may represent drugs, chemokines, neuromodulators as well as autocrine and paracrine factors, act as molecular ligands for receptors, and upon ligand binding, stabilize the receptor in an active conformation with increased affinity for their cognate G proteins (Gilman, 1987; Neves et al., 2002). G proteins function as intermediates in transmembrane signaling and transduce the signals by activating intracellular signaling pathways, which in turn interact with one another to create a network that regulates effector proteins, i.e. ion channels, transporters and metabolic enzymes that control a wide spectrum of cellular processes, e.g. gene transcription, motility, contraction, secretion and cell proliferation (Hepler and Gilman, 1992; Neves et al., 2002; Wettschureck and Offermanns, 2005).

G proteins are attached to the inside of the plasma membrane and are heterotrimers consisting of an α (39–46 kDa), β (37 kDa) and γ (8 kDa) subunit. The α subunits define the different subclasses of G proteins, whereas the β and γ subunits are commonly shared. The α subunits possess a single high affinity binding site for guanine nucleotides (GDP or GTP). When an activated receptor interacts with a heterotrimeric G protein, it induces a major rigid body rotation of 130° in the G protein (Chung et al., 2011; Rasmussen et al., 2011), which permits the release of the GDP normally bound in the α subunit, allowing free intracellular GTP to bind to this site. The βγ dimer consequently dissociates from the complex, and both subunits (α and βγ) are capable of activating downstream effector molecules independently or synergistically, with their activation persisting as long as GTP is bound to the α subunit and α and βγ remain separated. A single receptor can activate several G proteins and multiple intracellular effectors, which amplifies the signal. The effectors in turn generate second messengers that regulate a wide range of cellular processes, resulting in further signal amplification and achieving the distinctive pattern inherent in

the intricate network created by G protein-coupled interactions. The α subunit has an intrinsic GTPase enzyme activity, i.e. the bound GTP is hydrolyzed to GDP and an inorganic phosphate residue (P_i), which then leads to deactivation of G protein signaling (Ross and Gilman, 1977; Kwok-Keung Fung and Stryer, 1980; Pedersen and Ross, 1982; Gilman, 1987; Hamm, 2001). This activation/inactivation process is termed the G protein cycle and it is illustrated in Fig. 3. The established view is that GPCR signaling takes place only at the plasma membrane interface, but some recent results have refuted this proposition. It has been shown that upon G_s protein activation by β-ARs, the agonist-occupied receptors can be internalized and elicit a canonical second phase of signaling in the endosomes, which at least in the case for the β-AR, contributed to the overall cellular cyclic AMP response occurring within several minutes after agonist application (Irannejad et al., 2013).

Certain bacterial toxins act by causing covalent chemical changes in mammalian G proteins. Some α subunits contain specific amino acid sequences that can be covalently modified by bacterial toxins, e.g. cholera toxin stabilizes the α_s subunit in its active state by ADP-ribosylating it (Cassel and Pfeuffer, 1978), and pertussis toxin (PTx) prevents the receptor-mediated activation of G_i proteins, locking both the α and βγ subunits into their inactive states (Bokoch et al., 1983; Hepler and Gilman, 1992). These toxins have proved to be useful tools in the investigation of G protein-mediated signaling.

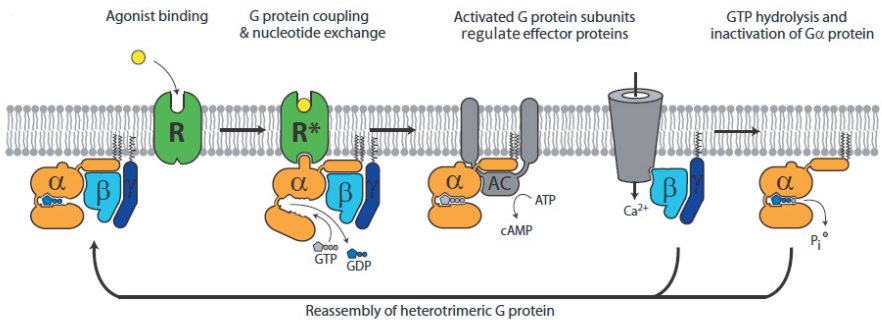

Figure 3. The G protein cycle. R; receptor, AC; adenylyl cyclase. Adapted from (Rasmussen et al., 2011).

There are four main classes of mammalian Gα proteins, classified according to sequence similarity, i.e. G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$, which all show distinct expression patterns and couple to distinct second messenger systems capable of regulating cellular functions. The extensive networks of G protein-activated effector molecules have been reviewed in detail by others (Hepler and Gilman, 1992; Neves et al., 2002; Wettschureck and Offermanns, 2005) and are only briefly summarized here. The specific G protein pathways involved in α_2-AR signaling are presented in the next section.

G_s proteins (G_s and G_{olf}) activate adenylyl cyclase (AC) enzymes in the plasma membrane (Rodbell et al., 1971; Ross and Gilman, 1977; Gilman, 1990; Tang et al., 1991; Tang et al., 1991; Sunahara et al., 1996). AC isoforms convert ATP to the cyclic monophosphorylated form of the nucleotide cAMP. cAMP is an intracellular signaling molecule that regulates a wide range of cellular functions, such as cardiac myocyte contraction, smooth muscle relaxation, insulin secretion, and neurotransmitter release, often by activating protein kinase A (PKA). $G\alpha_s$ serves important functions not only in regulating L-type Ca^{2+} channels in both skeletal muscle and cardiac myocytes (Mattera et al., 1989; Weiss et al., 2013) but also cardiac Na^+ channels (Schubert et al., 1989; Savio-Galimberti et al., 2012). In addition, new evidence has emerged that canonical G_s-dependent signaling can arise from internalized ligand-activated GPCRs in endosomes, which might result in novel downstream cellular responses (Calebiro et al., 2009; Irannejad et al., 2013). Furthermore, cAMP may directly activate Epac (exchange protein directly activated by cAMP), which in turn functions as a guanine-nucleotide exchange factor for the Ras family monomeric G proteins Rap1 and 2, thereby regulating the cell's actin cytoskeleton, motility and control of cell adhesion and cell-cell junction formation as well as secretory granule dynamics (Gloerich and Bos, 2010). Epac has also been shown to connect to phospholipase Cε (PLCε) specifically through the activation of Rap2 resulting in the subsequent release of calcium from intracellular stores in HEK293 and neuroblastoma cell lines (Schmidt et al., 2001).

Most $G_{i/o}$ proteins (G_{i1-3}, G_o, G_z, G_{gust}, G_{t-r} and G_{t-c}) are sensitive to PTx (except G_z) (Fong et al., 1988). It was recognized some time ago that $G\alpha_i$ activation led to inhibition of AC activity and thus to reduced generation of cAMP (Hildebrandt et al., 1983; Sunahara et al., 1996). The Gα isoforms gustaducin and transducin have established roles in sensory functions (Wettschureck and Offermanns, 2005). G_i-mediated signaling has been implicated in vascular smooth muscle cells (SMC), but seems to differ between vessel types, e.g. being present in the rat tail artery where G_q-derived (α_1-AR) calcium mobilization and contraction is followed by a G_i-derived amplification of the intracellular calcium sensitivity of the noradrenaline-induced tension (Petitcolin et al., 2001). $G_{i/o}$, apart from inhibiting AC, can converge signals from many $G_{i/o}$-coupled receptors to signal transducer and activator of transcription (STAT) 3 and 5 e.g. to increase neurite survival, outgrowth and differentiation (Strittmatter et al., 1994; Bromberg et al., 2008; Georganta et al., 2013). $G\alpha_o$ proteins mediate inhibition of AC (Watts et al., 1998; Chamero et al., 2011) and have been implicated in inhibition of synaptic plasticity and behaviour in invertebrate octopaminergic signaling (Koon and Budnik, 2012).

G_q proteins (G_q, G_{11}, G_{14} and $G_{15/16}$) mainly couple to phospholipase C (PLC) and serve an important role in the regulation of vascular tone. Activated PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC), and IP_3 accumulation results in the release of Ca^{2+} from intracellular stores, i.e. from the endoplasmic (ER) or sarcoplasmic (SR) reticulum (Taylor et al., 1990; Smrcka et al.,

1991; Offermanns and Simon, 1995; Exton, 1996). Increased Ca^{2+} concentration within the lumen of the Ca^{2+}-storing organelles subsequently activates plasma membrane Ca^{2+} channels at the cell surface to induce influx of extracellular Ca^{2+}. In addition, $G\alpha_{q/11}$ can activate the monomeric G protein RhoA to regulate calcium-independent SMC contraction (Somlyo and Somlyo, 2003).

The fourth class of G proteins, $G_{12/13}$, was discovered in 1991 (Strathmann and Simon, 1991) and their pathways remain the least studied (Suzuki et al., 2009). They have an important role in regulating the actin cytoskeleton and in modulating its contractility by increasing the activity through guanine nucleotide exchange factors (Buhl et al., 1995; Kozasa et al., 1998). G_{12} has been reported to directly interact with Ras GTPase-activating protein Gap1m and Bruton's tyrosine kinase (Jiang et al., 1998). G_{13} binds and stimulates the non-receptor tyrosine kinase PYK2 to activate serum-response element-dependent gene expression (Shi et al., 2000). G_{13} also plays a crucial role in the development of the vascular system, since its embryonic deficiency is lethal (Offermanns et al., 1997), and is an essential mediator of platelet activation in hemostasis and thrombosis (Moers et al., 2003). Finally, dual regulation of vascular smooth muscle tone by vasoconstrictors is achieved by dual coupling to the G_q-activated pathway as well as to the $G_{12/13}$-activated Rho/Rho-kinase pathway (Gohla et al., 2000; Momotani and Somlyo, 2012).

The Gβγ subunit was classically perceived as the more passive of the G protein subunits, but this paradigm changed fundamentally when βγ subunits were shown to couple to K^+ channels in heart tissue (Logothetis et al., 1987). Even though the effects mediated by the Gβγ subunits have not been fully elucidated and there is no satisfactory mechanistic explanation to date for the selectivity for the different Gβγ subunits, new pathways are constantly emerging and Gβγ subunits are considered to hold great potential as therapeutic targets (Smrcka, 2008). The α subunit masks effector binding surfaces on the Gβγ subunit which are exposed as the heterotrimer dissociates. The conformation of free Gβγ subunits was initially thought to remain unchanged (Sondek et al., 1996), but newer structural results have revealed that Gβγ subunits can exist in a range of conformations that can be exploited during molecular recognition by diverse binding partners (Smrcka et al., 2010). The major coupling pathway for Gβγ subunit activation is through $G_{i/o}$ proteins (Smrcka, 2008) and this has several effects including the inhibition of AC activity (types 1, 3, 5 and 7) (Tang et al., 1991; Taussig et al., 1994; Diel et al., 2006). However, βγ can also stimulate AC activity (types 2, 4, and 7) (Tang and Gilman, 1991; Federman et al., 1992; Taussig et al., 1994), but the stimulatory effect of Gβγ subunits requires that AC is primed by α_s subunits. This demonstrates how the G protein subunits can both activate and antagonize each other's effects (Clapham and Neer, 1993). Gβγ subunits have further been reported to couple to inhibition of T-, N-, P/Q- and R-type voltage-dependent Ca^{2+} channels (Herlitze et al., 1996; Delmas et al., 1999; Wettschureck and Offermanns, 2005), to activation of phosphoinositide 3-kinase (PI3K) (Stephens et al., 1994) and to stimulation of PLCβ, which was suggested already in (Taylor et al., 1990)

and subsequently confirmed (Camps et al., 1992; Smrcka and Sternweis, 1993; Lyon and Tesmer, 2013). Gβγ subunits may also modulate receptor function by regulating receptor-specific protein kinases (GRK2 and 3) that blunt receptor activity (Pitcher et al., 1992) and have been shown to mediate Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway and thereby regulate multiple cellular proliferation pathways (Crespo et al., 1994; Sachdev et al., 2007).

G protein activity can further be modified by RGS (regulators of G protein signaling) molecules (Dohlman and Thorner, 1997). These proteins accelerate the termination of the G protein cycle by activating the intrinsic GTPase-activity of the α subunit, thereby reducing the amplitude and duration of signaling but are also able to inhibit Gα subunit activation of effector proteins by serving as competitive binders (Ross and Wilkie, 2000; Hollinger and Hepler, 2002; Kach et al., 2012). Emerging evidence proposes that these proteins can both modulate and integrate G protein signaling differentially depending on the cell type and context, providing an efficient means of regulating GPCR activity and they are thus appealing targets for drug discovery (Sjogren et al., 2010; Storaska et al., 2013). Finally, G protein-coupled receptor kinases and β-arrestins serve as universal regulators of GPCRs with a prominent role in mediating receptor desensitization mechanisms and the attenuation of signaling (Shenoy and Lefkowitz, 2011).

2.1.4 Signal transduction of α_2-adrenoceptors

2.1.4.1 In recombinant cells

The signal transduction pathways modulated by α_2-ARs have been explored since the 1970's, i.e. long before the receptor genes were cloned in the 1990's, but are still not completely understood. There are numerous reports of α_2-AR coupling to a multitude of effectors mostly depending on the receptor subtype or on the cell type or in the tissue in which they are expressed. This reflects the complexity of these receptors but perhaps also provides the foundation for these receptors' rather astonishing capability to accomplish such a wide range of physiological functions in the human body.

Most of the early studies on human receptor subtypes were conducted on cell lines expressing recombinant α_2-ARs. All subtypes of α_2-ARs were reported to couple to PTx-sensitive $G_{i/o}$ proteins with the subsequent inhibition of AC and reduced intracellular cAMP (McKernan et al., 1987; Cotecchia et al., 1990; Eason et al., 1992; Jansson et al., 1994a; Dorn et al., 1997). It was also noted that high concentrations of agonists could evoke coupling of these receptors to G_s proteins (especially in PTx-treated cells), leading to stimulation of AC. This was first reported as most prevalent for the α_{2A}-AR subtype (Eason et al., 1992), but subsequent studies reported very marked stimulation of AC also for the α_{2B} subtype (Jansson et al., 1994a; Näsman et al., 1997; Pohjanoksa et al., 1997). The type of cell hosting the investigated receptor

evidently exerts important influences on the output; more specifically, the selection of a particular signal transduction pathway is affected by the different isoforms of G_i proteins (Gerhardt and Neubig, 1991; Albarran-Juarez et al., 2009), AC isoforms and other components of the signaling pathways expressed in different cell types (Duzic and Lanier, 1992; Kukkonen et al., 1998).

Coupling to additional signaling pathways has also been demonstrated for the α_2-ARs, including regulation of Na^+/H^+-exchange (Pihlavisto and Scheinin, 1999), arachidonic acid mobilization, presumably via phospholipase A_2 (Jones et al., 1991), and PTx-sensitive coupling of the α_{2A} subtype to phospholipase D (MacNulty et al., 1992). Stimulation of PLC activity in fibroblasts was reported for the α_{2A} (Chabre et al., 1994) and the α_{2C} subtype (Cotecchia et al., 1990). This PLC coupling was postulated to be mediated by Gβγ subunits and was followed by an IP_3-induced increase in intracellular calcium ($[Ca^{2+}]_i$) levels (Dorn et al., 1997).

Pertaining to their roles in neurons, α_2-ARs are actively engaged in the modulation of ion channel activity. PTx-sensitive coupling to K^+ channels by α_{2A} has been reported in neuronal-like cells (Surprenant et al., 1992). Inhibition of L-type Ca^{2+} channels was reported in neuronal cell lines transfected to express the α_{2A}- (Surprenant et al., 1992) and α_{2B}-AR subtypes (Soini et al., 1998). However, Soini and colleagues reported dual modulation of the channel, as the α_{2A}- and α_{2B}-ARs also coupled to stimulation of Ca^{2+} currents in a PTx-insensitive manner, in addition to the PTx-sensitive inhibition of the same channels (Soini et al., 1998). It was speculated that stimulation of Ca^{2+} currents might be mediated by βγ subunits released from G_i proteins, as suggested by (Herlitze et al., 1996). Increases in intracellular Ca^{2+} were reported for the α_{2B}-AR in Sf9 insects cells (Holmberg et al., 1998).

α_2-AR activated MAPK pathways play a prominent role in cell growth and proliferation. Mitogenic responses to α_2-AR agonists were first reported by Alblas et al., (1993), who showed that α_{2A}-AR activation in transfected fibroblasts resulted in rapid activation of Ras in a PTx-sensitive manner, followed by rapid phosphorylation of p42 MAPK (Alblas et al., 1993). This was corroborated by a second study showing α_2-agonist-induced activation of the p42 and p44 isoforms of the MAP kinase (Anderson and Milligan, 1994). Alblas et al. already speculated that the G_i-mediated response would be mediated by Gβγ subunits, and these initial speculations were confirmed (Koch et al., 1994). PI3K was shown to be the intermediate in the G_i-derived Gβγ-mediated MAPK activation of $p21^{ras}$ (Hawes et al., 1996). All of the above studies were performed on fibroblast cells stably expressing the α_{2A} subtype and together provide evidence for α_2-mediated activation of the MAPK-induced phosphorylation and activation of transcription factors involved in cell growth and proliferation. Activation of the MAPK cascade and enhanced cell proliferation has also been shown upon activation of α_{2B}-ARs (Schramm and Limbird, 1999; Cussac et al., 2002; Huhtinen and Scheinin, 2008) and other α_2-ARs (Seuwen et al., 1990; Flordellis et al., 1995; Karkoulias et al., 2006).

2.1.4.2 In native cells

The first insights on endogenously expressed α_2-AR-mediated signaling came from studies on isolated neurons where α_2-agonists induced activation of membrane K^+ conductance through elevated $[Ca^{2+}]_i$ levels (Morita and North, 1981). Increases in the $[Ca^{2+}]_i$ level were also reported for endogenously expressed α_{2B}-ARs in neuroblastoma x rat glioma cells (Holmberg et al., 1998) and inhibition of L-type Ca^{2+} channels was reported in dorsal root ganglion and rat sympathetic neurons (Holz et al., 1986; Delmas et al., 1999). Studies on human erythroleukemia (HEL) cells, that endogenously express the α_{2A}-AR, reported receptor-evoked increases in $[Ca^{2+}]_i$ (Michel et al., 1989; Musgrave and Seifert, 1995; Jansson et al., 1998; Kukkonen et al., 2001). The calcium mobilization was connected to increases in IP_3 levels (Michel et al., 1989) and postulated to be mediated by Gβγ subunit activation of PLC (Dorn et al., 1997).

Furthermore, α_{2A}-ARs were able to mediate inhibition of AC activity in HEL cells (McKernan et al., 1987; Jansson et al., 1998) and stimulation of α_2-ARs in HT29 human colonic adenocarcinoma cells resulted in increased in forskolin-stimulated cAMP production (Jones et al., 1987). Regulation of Na^+/H^+-exchange has been demonstrated in opossum kidney and neuroblastoma x rat glioma cells (Isom et al., 1987; Clarke et al., 1990; Pihlavisto and Scheinin, 1999) and activation of the 42 and 44 kDa ERK MAP kinases have been reported in opossum kidney cells that endogenously express α_{2C}-ARs (Kribben et al., 1997).

There are numerous published reports on the roles of α_2-ARs in the regulation of vascular smooth muscle function, but so far the signaling pathways involved have not been fully elucidated, and the results have varied depending on the anatomical location and diameter of the investigated vessels. Studies on smooth muscle cells from both arterioles and veins have shown that α_2-ARs mobilize intracellular Ca^{2+} (Aburto et al., 1993; Chotani et al., 2004) and a PTx-sensitive, G_i-mediated activation of L-type Ca^{2+} channels has been postulated, with the possible involvement of PKC (Lepretre and Mironneau, 1994; Mironneau and Macrez-Lepretre, 1995; Hughes et al., 1996). Experiments using isometric tension measurements on whole or endothelium-denuded blood vessels have confirmed the involvement of G_i-mediated activation of L-type Ca^{2+} channels in the α_2-AR mediated contraction of smooth muscle (Parkinson and Hughes, 1995; Roberts, 2001). A recent study highlighted the possibly important role of PKC in dexmedetomidine-induced blood vessel contraction of healthy human subjects, since a reduced drug response was associated with a polymorphism in the gene encoding PKCβ (Posti et al., 2013). Other studies have postulated that the mechanism of L-type Ca^{2+} channel activation may involve interactions with the MAPK pathway. More specifically, Src kinase activation of PI3K is followed by Ca^{2+} channel activation which in turn leads to transactivation of EGF receptors and activation of the MAPK cascade that eventually results in vasoconstriction (Roberts, 2001; Roberts, 2003). Relating to this, the Src family of tyrosine kinases has been shown to have an important role in angiotensin II-mediated Ca^{2+} signaling and vascular contraction

(Touyz et al., 2001), and PI3K has been shown to be involved in the activation of L-type Ca^{2+} channel opening (Seki et al., 1999), and appears to be an early intermediate in the G_i-derived Gβγ-mediated MAPK signaling pathway (Koch et al., 1994; Hawes et al., 1996). Finally, inhibition of ATP-sensitive K^+ channels has also been documented in rat vascular smooth muscle cells and arteries (Proks and Ashcroft, 1997; Tan et al., 2007; Kawahito et al., 2011; Rummery and Brock, 2011).

2.1.5 Cellular localization and intracellular processing

The subcellular localization of a GPCR is an important factor in defining the specialized functions of homologous receptors (Xiang and Kobilka, 2003). Although most GPCRs are found on the cell surface, it has been shown for some receptors that a significant proportion of the receptors are retained in intracellular compartments. The ER constitutes a vital quality control system of cells that together with a conglomeration of folding factors, escort proteins, retention factors, enzymes, and members of major molecular chaperone families ensures the correct folding and maturation of proteins, marks them for degradation or promotes their entry into the secretory pathway (Fig. 4) (Ellgaard and Helenius, 2003; Dunham and Hall, 2009; Ulloa-Aguirre et al., 2013).

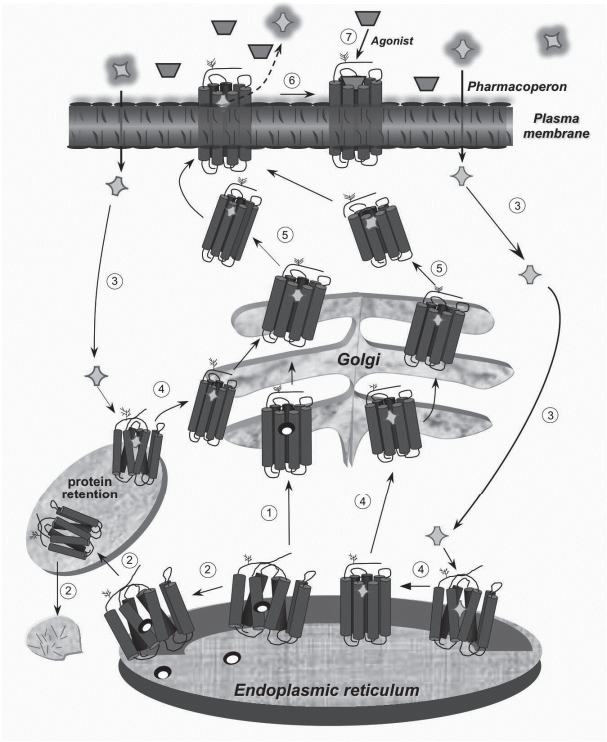

Figure 4. Regulation of GPCR trafficking in the cell. The endoplasmic reticulum (ER) regulates the correct folding of proteins through e.g. chaperones (ovals). Newly synthesized proteins are either 1) translocated to the Golgi or 2) retained in the ER and targeted for degradation. Pharmacological chaperones, pharmacoperones (rhomboids), 3) diffuse into the cell and can correct misfolded proteins which 4) promotes their export to the Golgi. 5) Mature and processed (e.g. glycosylation in the Golgi) proteins are then delivered to the plasma membrane, where a bound pharmacoperone can dissociate from the protein, allowing the receptor to bind to its agonist (Ulloa-Aguirre et al., 2013).

Protein processing in the ER therefore constitutes a potential post-translational mechanism for the control of cell membrane expression of GPCRs (Angelotti et al., 2010) but together with the Golgi also constitute a bottleneck in the production of secreted proteins, as only correctly folded proteins pass the barrier. Because of this stringent quality control mechanism, mutations that result in protein misfolding frequently lead to their retention in the ER (Ulloa-Aguirre and Michael Conn, 2011; Ulloa-Aguirre et al., 2013). Proteins may also be retained in the ER due to lack of accessory proteins in the host cells in which they are expressed.

The α_2-ARs share high structural and functional similarity, but they still have different cellular targeting characteristics and tissue expression. Clear differences in the temporal and spatial trafficking characteristics of endogenously expressed α_{2A}- and α_{2C}-ARs in cultured sympathetic ganglion neurons (SGN) from superior cervical ganglia of newborn mice were demonstrated by Brum et al. (2006). At early stages of culture, α_{2A}-ARs were localized predominantly to somatodendritic regions, but as the culture matured (from day 8 forward), spreading to axonal sites was observed. α_{2C}-ARs, however, were at the early stages predominantly found in the small intracellular vesicular compartments in the cell body and at day 8 still revealed limited plasma membrane expression. It was not until day 16 of culture that specific expression of α_{2C}-ARs at axonal sites became evident. Furthermore, at this late stage, α_{2A}-ARs were localized diffusely in the plasma membrane, whereas α_{2C}-ARs were found predominantly at synaptic sites. Therefore, α_2-ARs traffic differently and seem to serve different roles in SGNs as they mature (Brum et al., 2006). Previous research has also revealed distinctly different trafficking characteristics for the α_2-AR subtypes in transfected cell lines. In a variety of non-neuronal (e.g. NRK, Rat1) cell lines, α_{2C}-ARs were largely retained in the ER, whereas α_{2A}-ARs were targeted primarily to the plasma membrane (von Zastrow et al., 1993; Daunt et al., 1997; Olli-Lähdesmäki et al., 1999; Hurt et al., 2000). In contrast, in neuronal cell lines (e.g. PC12), both subtypes were expressed on the surface of the cell. Hurt et al., (2000) showed that the combined intracellular and plasma membrane fractions of NRK cells and PC12 cells are comparable in terms of receptor expression and function, and therefore postulated that the intracellular α_{2C}-ARs expressed in NRK cells are probably not misfolded but instead processed in the *cis*/medial Golgi and actively retrieved from the early Golgi regions to the ER where they are retained, perhaps due to a lack of some accessory proteins that PC12 cells seem to possess (Hurt et al., 2000). Hence, the nature of the cell plays a significant part in determining how receptors are expressed on the cell surface.

Appropriate receptor localization on the cell surface is also important for extracellular receptor ligands to bind to the receptor and consequently to activate its signal transduction machinery. Most studies on GPCRs in intact cells, aiming to identify novel compounds that act as agonists, antagonists or allosteric modulators on a receptor, have relied on recombinant protein expression in heterologous cells (Dunham and Hall, 2009). However, these experiments assumes that the receptor is localized on

the cell surface. Intracellular retention has been reported for several types of GPCRs, and most often render the receptor non-functional, in contrast to the functional intracellularly retained α_{2C}-ARs (Hurt et al., 2000). From the point of view of drug discovery, it is important to understand the nature of these proteins, whether the intracellular retention is a consequence of expression in a new host cell lacking some component(s) normally needed for native protein expression, or whether the retention represents a physiological mechanism.

There are several approaches which can be applied to artificially enhance the trafficking of particular GPCRs to the plasma membrane, one being the addition of sequences to the receptor. This was first attempted by the addition of an artificial signal sequence to the N-terminus of the β_2-AR and later to the cannabinoid CB1 receptor (Dunham and Hall, 2009). By utilizing a chimeric α_{2A}- / α_{2C}-AR strategy, Angelotti et al., (2010) identified an ER retention signal in the N-terminal region of the α_{2C}-AR that in part was responsible for its subtype-specific trafficking. Removal or disruption of the ER retention signal dramatically increased plasma membrane expression and decreased ER retention. Conversely, transplantation of this signal into the α_{2A}-AR reduced its plasma membrane expression and increased its ER retention (Angelotti et al., 2010; Jahnsen and Uhlen, 2012).

Molecular chaperones are ER-resident proteins that bind to and stabilize unstable conformers of nascent polypeptides to assist in their folding or assembly to ensure efficient ER export, preventing aggregation or incorrect interactions between misfolded proteins and other molecules. Treatment with pharmacological chaperones, small compounds that may rescue misfolded proteins from being trapped in the ER and ensure their proper transport to the final destination in the cell, may therefore represent an intervention in some diseases associated to protein misfolding (Fig. 4) (Ulloa-Aguirre and Conn, 2011; Babcock and Li, 2013; Ulloa-Aguirre et al., 2013). For example, mutations in the V_2 vasopressin receptor have been associated with vasopressin-insensitive diabetes insipidus. These mutant receptors are not properly folded and are therefore intracellularly retained, but their cell-surface delivery can be rescued by vasopressin antagonists that bind and stabilize the misfolded receptors (Morello et al., 2000). Other examples of intracellular ligand receptor targets and therapeutic applications have been thoroughly reviewed (Babcock and Li, 2013).

Some GPCRs require accessory proteins in order to reach their physiological localization, e.g. NinaA for *Drosophila* rhodopsin, RAMPs for the calcitonin receptor-like receptor, ODR-4 for *C. elegans* chemosensory receptors, Drip78 for the D_1 dopamine receptor and MRAP for the MC_2 melanocortin receptor (Tan et al., 2004; Cooray et al., 2009). The existence of *r*eceptor *t*ransporting *p*roteins (RTP1–4) and *r*eceptor *e*xpression *e*nhancing *p*roteins (REEP1–6) were reported by Saito et al., (2004). They showed that co-expression of RTP1 and 2 with odorant receptors promoted functional cell surface expression, which could not be detected with expression of these receptors alone in heterologous cell systems (Saito et al., 2004). Since then a number of reports have been published on different RTP or REEP family

members involved in trafficking or expression of other GPCRs. RTP3 and RTP4 were recognized to act as co-factors for functional expression of some bitter taste receptors (human TAS2R in HEK293T cells) (Behrens et al., 2006). RTP4 was shown to protect μ and δ opioid receptors from ubiquitination and degradation and consequently increase the level of cell surface heterodimers (Decaillot et al., 2008). REEPs belong to the Yip protein family (Ypt interacting protein), and their family members can interact directly with SNAREs, Rab GTPases and other ER/Golgi vesicle proteins to regulate intracellular trafficking and targeting of cargo proteins within yeast cells and neurons (Calero et al., 2001). These accessory proteins may function as molecular chaperones promoting the correct folding of odorant receptors in the ER, facilitate transport of odorant receptors to the cell surface or even be associated as co-receptors with the receptors during ligand binding (Cooray et al., 2009). Genetic knock-out of some of these accessory proteins has shown that they are critical for receptor regulation in native tissues in the mouse (Dunham and Hall, 2009). Physiological significance of the REEP proteins was furthermore implied as mutations in the REEP1 protein was linked to a neurodegenerative disease, namely hereditary spastic paraplegia (HSP). Up to sixty percent of North American HSP cases were reported to be due to mutations in M1-spastin, atlastin-1, strumpellin or REEP1 (Park et al., 2010; Blackstone et al., 2011). Together these proteins constitute a complex of important determinants of curved ER tubule formation, elongation, and microtubule network interactions and regulate polarized membrane and protein trafficking along microtubules to distant sites within the cells of the central nervous system (CNS). Consequently, disruption of these proteins particularly will lead to defects in ER organization within the cell and thus axonal degeneration of corticospinal neurons which is the basis of HSP pathogenesis (Park and Blackstone, 2010; Park et al., 2010; Blackstone et al., 2011), highlighting the important role of the ER in protein processing.

Glycosylation is a critical step in glycoprotein folding and subsequent quality control (Perez, 2006). The N-terminus is the proposed site of asparagine-linked glycosylation in most GPCRs, and the α_{2A}- and α_{2C}-ARs contain two consensus sites for covalent attachment of carbohydrates, whereas the α_{2B} subtype does not appear to be glycosylated (Regan et al., 1988; Lomasney et al., 1991).

As noted above, the life-cycle of a GPCR is dynamic. Plasma membrane localization of GPCRs is balanced between transport to and from the ER (Angelotti et al., 2010). Receptors are processed in the ER, further translocated through the Golgi to its final destination, most often in the plasma membrane. Alternatively, unfolded or misfolded receptors are either retained or marked for transport to lysosomes for degradation. In the plasma membrane, the receptor is not a rigid entity, but assumes a continuum of different conformations. Ligand binding to the receptor includes chemical interactions between the ligand and the receptor that stabilizes the receptor in a conformation or ensemble of conformations that interacts with cytoplasmic signaling and regulatory proteins (Kobilka, 2013). Furthermore, upon prolonged ligand binding, receptors can be desensitized, meaning that the response to an agonists wanes over time, which acts as a feedback mechanism for the receptor to prevent acute and chronic overstimulation

(Ferguson, 2001). The desensitization process involves phosphorylation of the receptor, capture of the GPCR in cytoplasmic vesicles that have been pinched off from the plasma membrane and finally arrestin-mediated internalization into endosomes (endocytosis). Endocytosis has many effects on signal transduction and conversely, receptor signaling regulates the endocytotic machinery. Internalized receptors may be either recycled to the plasma membrane or degraded (Sorkin and von Zastrow, 2009; Irannejad et al., 2013).

2.1.6 Tissue distribution

α_{2A}- and α_{2C}-ARs have widespread distributions in the mouse and rat CNS, with α_{2A}-ARs located in the brain stem, olfactory system, cerebral cortex, septum, basal ganglia, midbrain, cerebellum, pons, hypothalamus and medulla and α_{2C}-ARs located especially in the basal ganglia and the olfactory system, but at lower densities also in the cerebral cortex, hippocampal formation, amygdala, thalamus, hypothalamus, midbrain, pons and medulla. The α_{2B} subtype, on the other hand, appears to be present only in very discrete areas of the mouse brain and in the rat thalamus (Nicholas et al., 1993; Scheinin et al., 1994; Nicholas et al., 1996; Rosin et al., 1996; Wang et al., 1996; Shi et al., 1999). In the rat brain, expression of α_{2A} and α_{2C} are somewhat regionally distinct and related to their different physiological roles. The α_{2A}-AR is the only subtype prominently expressed in the locus coeruleus, an important noradrenergic brain stem center. In contrast, in the basal ganglia, which is a main site of dopaminergic neurotransmission involved in motor control, α_{2C}-ARs are abundant and α_{2A}-expression is low (Sallinen et al., 1998; Fagerholm et al., 2008).

In peripheral tissues, α_2-ARs are differentially distributed e.g. in blood vessels, blood platelets, spleen, lung, heart, liver, kidney, pancreatic islets, adrenal gland, skeletal muscle, adipose tissue, placenta, salivary glands and in the gastrointestinal tract. In contrast to many other tissues where expression of only a single subtype can be detected, blood vessels appear to contain multiple subtypes of both α_1- and α_2-ARs. However, the receptor subtypes are often distributed heterogeneously among different blood vessels, with differences also between animal species, which has made it hard to draw clear conclusions on their specific roles in the regulation blood vessel tone (Ping and Faber, 1993). In addition to α-ARs, vascular smooth muscle cells also possess β_2-ARs, which means that the endogenous agonist adrenaline activates several types of receptors, and that the net response depends on the relative importance of each receptor subtype population. As a general statement it can be stated that α_2-ARs are more prominent in veins and small arteries/arterioles but are not abundant in large arteries like the aorta (where α_1-ARs are more prominent) (Guimaraes and Moura, 2001; Chotani et al., 2004). This was exemplified in the human saphenous vein (contractions by α_{2C}-ARs) and internal mammary artery (contraction by α_1-ARs) (Docherty and Hyland, 1985; Gavin et al., 1997; Rizzo et al., 2001; Giessler et al., 2002). α_{2A}-ARs seem to be expressed in arteries with larger diameters (Faber et al., 2001) compared to the other subtypes. α_{2B} is more often found in smaller arteries and veins and α_{2C} is

mostly found in veins (Ping and Faber, 1993; Gavin et al., 1997; Gornemann et al., 2007). The contribution of α_1- vs. α_2-mediated vasoconstriction also changes along the length of the vessel, e.g. in the arteries of the limbs, the participation of α_2-ARs in the noradrenaline-induced α-adrenoceptor-mediated vasoconstriction is more pronounced in the distal parts as compared to the proximal parts of the vessel (Guimaraes and Moura, 2001). Furthermore, the three layers of the blood vessel wall might contain different α_2-AR subtypes. α_{2A}-ARs, as well as some α_{2C}-ARs are most commonly expressed in the endothelial cell layer (Angus et al., 1986; Bockman et al., 1996). The smooth muscle cell layer again was found to host all three subtypes whereas the adventitia (connective tissue layer surrounding the vessel) mainly express α_{2A}-ARs (Faber et al., 2001). At the prejunctional level, α_2-ARs have been found in *in vitro* experiments in all vascular tissues (arteries and veins), where they mediate modulation of noradrenaline release (Guimaraes and Moura, 2001).

2.1.7 Physiological functions and therapeutic applications

Adrenoceptors form the interface between the sympathetic nervous system and the cardiovascular system as well as many endocrine and parenchymal tissues. α_2-ARs mediate the pre- and postsynaptic inhibitory actions of the catecholamines adrenaline and noradrenaline in the central and peripheral nervous system, and consequently, α_2-ARs regulate a wide variety of functions, such as sympathetic tone and thus blood pressure, glucose and lipid metabolism, as well as platelet aggregation, neurotransmitter release, pain, alertness and cognitive functions. Recently, important insights from transgenic animals have broadened our views and made it possible to distinguish between α_2-AR functions mediated by the classical presynaptic α_2-AR feedback "autoreceptors" in noradrenergic neurons and α_2-AR functions mediated by "heteroreceptors" in non-adrenergic neurons and cells. It has become evident that most of the pharmacological effects of α_2-AR agonists such as analgesia, hypothermia, sedation, anesthetic-sparing and modulation of working memory are in fact mediated by α_2-ARs located on non-adrenergic cells (see Fig. 5). These findings extend the current view of the synaptic localization and function of α_2-ARs in the nervous system and provide important new avenues for future drug development (Gilsbach et al., 2011; Gilsbach and Hein, 2012). The elucidation of the physiological roles of α_2-ARs has been hampered by the lack of subtype-selective compounds and the site of tissue expression of a certain subtype has not always been possible to be linked to specific physiological functions. Genetic inactivation (knock-out) of the three subtypes of α_2-ARs in mice has instead provided useful information and helped in the elucidation of the specific roles of each receptor subtype (Philipp et al., 2002a; Gyires et al., 2009; Gilsbach and Hein, 2012). Today, some non-subtype selective α_2-AR agonists such as clonidine, dexmedetomidine and brimonidine are being used in the clinic to treat patients with hypertension, glaucoma, attention-deficit disorder, postoperative pain and shivering and as anesthetic adjuncts, but because of their serious side-effects, their use is restricted. However, the lack of truly subtype-selective agonists has seriously limited

the understanding of the characteristic structural features of α_2-AR agonists, the design and development of subtype-selective compounds, and, thereby, the more precise evaluation of the *in vivo* pharmacological roles of the α_2-AR subtypes (Balogh et al., 2009).

Feedback control of neurotransmitter release. All α_2-AR subtypes serve as presynaptic autoreceptors regulating feedback inhibition of noradrenaline release in sympathetic nerve endings. Noradrenergic neurons of the brain and spinal cord express α_{2A}- and α_{2C}-ARs as autoreceptors, whereas postganglionic sympathetic neurons additionally express α_{2B}-ARs (Hein et al., 1999; Trendelenburg et al., 2003). α_{2A}-AR is the principal presynaptic subtype regulating the release of noradrenaline from central and peripheral sympathetic nerves. It remains unclear why all of the subtypes have been conserved during the course of evolution to act as autoreceptors in the sympathetic nervous system, especially since these receptor subtypes share such a high degree of structural and functional similarity. However, in the sympathetic neurons innervating the mouse atria, α_{2A}-ARs were shown to inhibit noradrenaline release at high action potential frequencies in nerve endings, whereas α_{2C}-ARs responded to lower frequencies (Hein et al., 1999).

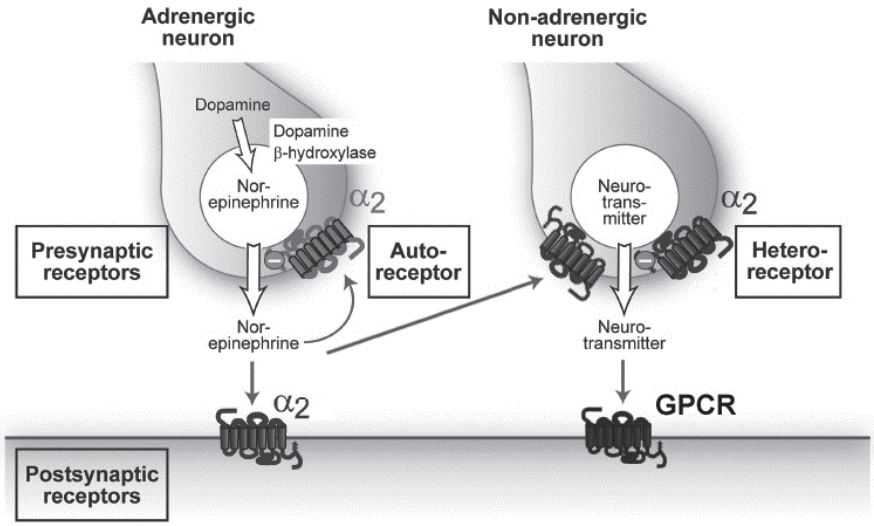

Figure 5. The concept of pre- versus postsynaptic α_2-adrenoceptors. The catecholamine noradrenaline (NA) is synthesized from dopamine in noradrenergic neurons. α_2-ARs regulate the exocytosis of NA by presynaptic "autoreceptors" that inhibit further release of NA from noradrenergic neurons or the release of other neurotransmitters from non-adrenergic neurons ("heteroreceptor" function). Released NA mediates its effects on post-synaptic α_2-ARs, which in turn regulate a variety of functions. Modified from (Gilsbach et al., 2011).

The receptor subtypes may therefore share overlapping but still unique physiological roles, which are influenced also by temporal and spatial differences in the localization of the receptors. The physiological significance of α_{2C}-ARs in mediating feedback inhibition was apparent in mice with single or double knock-out of α_{2A}- and α_{2C}-ARs, and by the discovery that a polymorphic variant of the C subtype could impair effector coupling and represents a pharmacogenetic locus which may be a basis for variation between individuals in the cardiovascular or CNS functions of this receptor subtype (Hein et al., 1999; Small et al., 2000; Brede et al., 2002; Chotani and Flavahan, 2011). In addition, α_2-ARs act as heteroreceptors in non-adrenergic neurons, regulating the release of other neurotransmitters in the central and peripheral nervous systems (Brede et al., 2004; Gilsbach and Hein, 2012). In the basal ganglia of the brain, α_{2A}- and α_{2C}-ARs inhibit the release of dopamine (Bucheler et al., 2002) and in the hippocampus and cerebral cortex they control the release of serotonin (Scheibner et al., 2001). α_2-Agonist-induced reductions in the release and metabolism of noradrenaline, dopamine and serotonin are mainly mediated by the α_{2A}-AR, with some involvement of the C subtype (Sallinen et al., 1997). Both α_{2A} and α_{2C} inhibit the release of noradrenaline in the adrenal medulla, although it has been claimed that the α_{2C}-AR is the dominant subtype in mediating inhibition of adrenaline secretion and autocrine feedback regulation of adrenaline secretion in the adrenal gland and thus it controls circulating adrenaline levels (Brede et al., 2003; Moura et al., 2006).

Cardiovascular effects. As can be concluded from the heterogeneous tissue distribution of vascular α_2-ARs, the cardiovascular effects of α_2-ARs are complex and mediated by several factors; central modulation of sympathetic output, regulation of myocardial contractility as well as direct effects on smooth muscle and endothelial cells in the blood vessel wall (see Fig. 6). Activation of central α_{2A}-ARs in the brain induces a reduction in sympathetic outflow and an augmentation of parasympathetic outflow, which leads to a reduction in arterial blood pressure and bradycardia. The hypotensive effect is in part mediated by presynaptic α_{2A}-autoreceptor feedback regulation but mostly by α_{2A}-heteroreceptors (Gilsbach and Hein, 2012). Studies with a mutant (α_{2A}-D79N) receptor subtype in mice, a mutation that was shown to selectively uncouple the receptor from some of its signaling pathways (Surprenant et al., 1992) revealed loss of α_{2A}-AR coupling to K^+ channels and Ca^{2+} channels *in vivo*, and a blunted bradycardic response (Macmillan et al., 1996; Lakhlani et al., 1997; Altman et al., 1999). The bradycardic affect is partly mediated by α_{2A}-ARs and partly by baroreflex mechanisms. Postsynaptic vascular endothelial α_{2A}-ARs further enhance the hypotensive effect, by nitric oxide-dependent vasorelaxation (Bockman et al., 1993; Figueroa et al., 2001; Wong et al., 2010), but α_2-ARs located in the smooth muscle layer of the blood vessel wall counteract the vasodilatory response by inducing vascular contraction, in part through the actions of prostanoids (Wong et al., 2010). *In vivo* and *in vitro* studies have revealed the great diversity in the postsynaptic contractile response to α_2-AR activation, depending on the animal species, vessel type and diameter as well as on its anatomical location (Hughes et al., 1988; Nielsen et al., 1989; Nielsen et al., 1990; Nielsen et al., 1991; Chotani et al., 2004). As a consequence

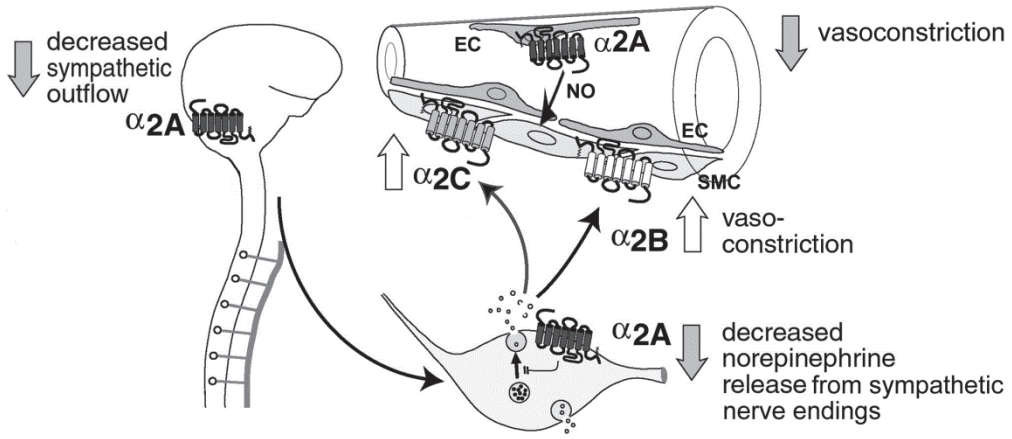

Figure 6. α_2-adrenoceptor-mediated regulation of blood pressure. Presynaptic α_{2A}-ARs in the brain inhibit sympathetic outflow and inhibit noradrenaline release from sympathetic nerves, leading to a decrease in blood pressure. Peripheral α_{2A}-ARs on endothelial cells (EC) in the blood vessel wall further enhance the hypotensive effect by regulating the release of nitric oxide (NO), which exerts its relaxing effect on nearby smooth muscle cells (SMC). This effect is counteracted by peripheral α_{2B}-ARs on SMCs that mediate vasoconstriction. Silent α_{2C}-ARs in SMCs are activated upon exposure to cold and can mediate vasoconstriction. (Modified from Philipp et al., 2002a and Wong et al., 2010).

of the contrasting central and postsynaptic hemodynamic responses to α_2-AR activation, it has been shown that when administered intravenously, an α_2-AR agonist produces a biphasic response in humans and other mammals (rat, cats and dogs) (Savola, 1989; Bloor et al., 1992a; Bloor et al., 1992b; Flacke et al., 1993). An initial, short-lived vasoconstriction, due to activation of peripheral arterial α_2-ARs (and α_1-ARs because of incomplete specificity of the drugs), is followed by a gradual decline in arterial blood pressure. Studies on transgenic mice have postulated that the biphasic response is mediated by different α_2-AR subtypes, with the B subtype probably playing the most important role in the initial vasoconstriction (Link et al., 1996; Talke et al., 2003) and the A subtype in the brain mediating the reduction in blood pressure (Macmillan et al., 1996; Altman et al., 1999; Ebert et al., 2000).

Therefore, central α_{2A}-ARs are currently recognized as targets of antihypertensive agents (Aantaa and Jalonen, 2006); in fact the use of clonidine as a clinical anti-hypertensive agent was established already in the 1960's (Seedat et al., 1969; Khan et al., 1970), but due to its prominent sedative and bradycardic side effects today its use is quite limited. Vascular α_2-ARs have not so far been exploited in the search for antihypertensive drug therapy. A peripherally acting subtype-nonselective α_2-AR antagonist was shown to counteract the vasoconstriction evoked by the potent α_2-agonist, dexmedetomidine (Honkavaara et al., 2012). However, chronic subtype-nonselective α_2-AR blockade is likely to lead to adverse cardiovascular effects, which accentuates the need for novel antagonists of the α_{2B}-AR subtype. An α_{2A}-AR-selective

agonist without any α_{2B}-AR activity counteracting the response might be a more potent hypotensive drug than clonidine. However, the principal side effects of clonidine are attributable to its sedative property, which originates from the same subtype (α_{2A}) and therefore subtype-selective agonism would not seem to offer any clear advantages over clonidine (MacDonald et al., 1997), even though it has been speculated that partial agonism at the α_{2A}-AR might selectively achieve hypotensive actions without sedation (Tan et al., 2002). This provides a good example of the complexity of α_2-AR drug development, with the fields of therapeutic application and unwanted side effect overlapping. With respect to the possibility of using a peripherally acting α_{2A}-AR-selective agonist for treatment of hypertension, in order to avoid sedation and other CNS-mediated adverse effects, the prospects are not promising. No such agent has been tested in clinical practice, but it appears that the hypotensive and sedative drug effects cannot be separated. In a study comparing healthy and tetraplegic subjects, blood pressure was not reduced by clonidine in the subjects with spinal cord transection, indicating that the reduction in blood pressure and cardiac output in normal subjects after clonidine administration was due to clonidine's central sympatholytic action (Reid et al., 1977; Kooner et al., 1988).

Feedback inhibition of noradrenaline release from sympathetic nerve endings by α_{2A}-autoreceptors serves a protective role in the heart under normal resting conditions (Gilsbach and Hein, 2012). As compared to wild-type mice, α_{2A} knockout mice had higher systemic blood pressure and heart rates that correlated with increased noradrenaline release from cardiac sympathetic nerves. Mice with deletion of both α_{2A}- and α_{2C}-ARs also showed elevated plasma noradrenaline concentrations and had an increased risk for suffering cardiac hypertrophy and fibrosis, heart failure and mortality in comparison with wild-type or α_{2B}-deficient mice (Hein et al., 1999; Brede et al., 2002). Clinical studies have evaluated whether inhibition of catecholamine release by presynaptic α_2-ARs could be targeted in chronic heart disease (Swedberg et al., 2002), but the results have been inconclusive (Gilsbach and Hein, 2012).

The expression of α_{2B}-ARs is very limited in the brain; instead these are abundant in vascular smooth muscle, which highlights this receptor subtype's possible role in cardiovascular regulation. The α_{2B}-AR evokes peripheral vasoconstriction in response to α_2-agonists and is required for the development of salt-sensitive hypertension (Makaritsis et al., 1999).

α_{2C}-ARs were originally seen as non-vascular receptors. However, vascular α_{2C}-AR expression was first detected in the study of Richman and Regan, (1998), and later, several studies have found expression of α_{2C}-ARs on SMCs, but their contribution to vascular contraction remains unresolved. Several studies have indicated that α_{2C}-ARs do not normally contribute to vasoconstriction, but are rapidly activated only in response to cold-induced vasoconstriction. Cold-induced cutaneous vasoconstriction plays a protective physiological role to reduce body's heat loss and to ensure the maintenance of normal body temperature. Hence, inhibition of α_{2C}-AR activity has

been speculated to be useful in the therapy of vasospastic episodes in Raynaud's disease, and indeed blockade of α_2-ARs has in some studies abolished the vasospasm of patients with this disease (Chotani et al., 2000; Jeyaraj et al., 2001; Chotani and Flavahan, 2011). A recent clinical trial of this concept with the experimental α_{2C}-AR antagonist, ORM-12741, was, however, entirely negative and failed to provide proof of this concept (Herrick et al., 2012). Since α_2-AR responsiveness is increased when pulmonary vascular tone is elevated, α_{2C}-AR antagonists may prove beneficial in related diseases such as pulmonary hypertension or congestive heart failure (Gornemann et al., 2007).

Other vascular effects of α_2-AR activation have also been investigated. For instance, activation of endothelial α_{2A}-ARs stimulated the release of nitric oxide, and this was able to attenuate postjunctional vascular α_1-AR-induced vasoconstriction, therefore producing vascular relaxation (Vanhoutte and Miller, 1989; Figueroa et al., 2001; Guimaraes and Moura, 2001; Snapir et al., 2009). Dexmedetomidine at low concentrations, induces endothelium-dependent relaxation of rat mesenteric arteries, followed by a contraction at higher doses. It was suggested that the relaxing effect was predominantly mediated by α_{2A}-ARs and to a lesser extent by α_{2B}-ARs (Wong et al., 2010). Activation of α_2-ARs on microvessels in the heart resulted in vessel constriction that reduced coronary blood flow. This constrictive activity of α_2-ARs was augmented in patients with atherosclerosis and could lead to myocardial ischemia; therefore, an α_2-AR antagonist might have therapeutic value in patients with atherosclerotic coronary arteries (Baumgart et al., 1999). Elevated noradrenaline release in the heart (activated by blockade of presynaptic α_{2A}-ARs with yohimbine) has improved cardiac function in lipopolysaccharide-challenged mice (a model of septic shock) which may be a novel therapeutic endpoint for treatment of myocardial dysfunction in septic patients (Wang et al., 2013). α_{2C}-ARs regulate cerebral blood flow through constriction of the carotid artery, and a beneficial role of α_{2C}-AR agonists has therefore been proposed in acute migraine therapy (Willems et al., 2003; Villamil-Hernandez et al., 2013).

Endocrine regulation. The α_{2A}-AR has been identified as an important regulator of blood glucose homeostasis. In the resting state, insulin secreted by pancreatic β-cells maintains normal blood glucose levels. Upon sympatho-adrenal activation, neuronally released noradrenaline together with blood-borne catecholamines bind to α_2-ARs on the β-cells, resulting in reduced secretion of insulin and increased blood glucose levels (Fagerholm et al., 2011). Studies on α_2-AR knock-out mice *in vivo* have revealed a key role for the A subtype in the inhibition of insulin secretion and thus in glucose regulation (Fagerholm et al., 2004; Savontaus et al., 2008). A recent study corroborated this finding, and showed that only postsynaptic α_{2A}-ARs (not autoreceptors) in β-cells inhibited insulin secretion, and that deletion of the α_{2C} subtype led to increased adrenaline secretion and had the potential to increase blood glucose levels via enhanced glycogenolysis (Ruohonen et al., 2012). Heightened sympathetic activity and increased α_{2A}-AR signaling in the pancreas or alternatively α_{2A} overexpression were

associated with the metabolic syndrome and impaired insulin secretion, and were thus risk factors for type 2 diabetes (Rosengren et al., 2010). Regulation of blood glucose levels and insulin secretion with an α_2-AR antagonist might therefore represent a therapeutic opportunity, but there might possibly be adverse effects due to the enhanced sympathetic activity. It has also been postulated that α_2-AR antagonists might worsen sulphonylurea-induced hypoglycemia in type 2 diabetes (Fagerholm et al., 2011).

Fetal development. Mice with deletion of all three α_2-AR subtype genes die during mid-gestation; the developmental defect is probably largely due to deletion of the α_{2B}-AR gene. Hence, α_2-ARs are crucial for lung (Haubold et al., 2010) and placental vascular development to ensure oxygen and nutrient transport to the embryo (Philipp et al., 2002b). In addition, mice with a homozygous deletion genotype of α_{2B}-AR do not breed well (Link et al., 1996), further supporting a role for α_{2B} in development and reproduction.

Pain, sedation, analgesia and behavior. α_2-ARs are critically involved in the regulation of pain and sedation. Furthermore, spinal α_2-ARs are considered to be major players in mediating noradrenergic suppression of pain signals (Pertovaara, 2013). The α_{2A}-AR is the predominant subtype involved in the mediation of acute antinociception and sedation evoked by dexmedetomidine in mice, since α_{2A} knockout mice failed to show any antinociceptive response to dexmedetomidine, wheras no alterations in the sedative response to dexmedetomidine were observed in mice lacking α_{2B}- and α_{2C}-ARs (Hunter et al., 1997). However, moxonidine evoked spinal antinociception that was at least partially dependent on the α_{2C} subtype (Fairbanks et al., 2002), but elucidating the role of spinal α_{2C}-ARs in pain modulation has remained elusive (Pertovaara, 2013). α_2-AR agonists are used as sedative agents in intensive care to lower the need for inhaled anesthetics, and because of their stabilizing effect on hemodynamics including attenuated blood pressure and heart rate responses to stress (Aantaa and Jalonen, 2006; Grewal, 2011). In addition, spinal α_2-ARs induce analgesia by modulating pain pathways. The type of sedation induced by α_2-AR agonists is unique, patients are sedated but can be aroused easily but quickly fall back into sedation again (Gilsbach and Hein, 2012). Dexmedetomidine provides similar sedation in intensive care as can be obtained with midazolam and propofol, but its cardiovascular side-effects, particularly bradycardia, limit its widespread use (MacDonald et al., 1997; Ahmed and Murugan, 2013). When the effects of increasing plasma concentrations of dexmedetomidine in humans were investigated, it was found that low doses of dexmedetomidine were useful for sedative purposes and mild analgesia, leaving cardiovascular functions nearly intact. As the dose was increased, heart rate was lowered, but blood pressure exhibited a biphasic response (low, then high). Higher concentrations of dexmedetomidine resulted in further sedation and analgesia, but cardiovascular side effects limited its usefulness (Ebert et al., 2000; Snapir et al., 2006). The use of dexmedetomidine (Precedex®, Dexdor®) for intensive care unit sedation has been approved in the USA, Japan and all 28 EU countries (see

www.orion.fi). In addition to dexmedetomidine, also racemic medetomidine and two other α_2-AR agonists, detomidine and xylazine, are employed in veterinary medicine as tranquilizers, sedative-anesthetics and analgesics (England and Clarke, 1996). Nonetheless, it has been argued that α_2-AR agonists might have so far unexploited therapeutic usefulness in the treatment of pain (Pertovaara, 2013).

α_{2A}- and α_{2C}-ARs play important roles in behavioral responses, such as in the startle reflex, in stress responses and in the regulation of locomotor activity, body temperature and dopamine metabolism (Sallinen et al., 1997; Lahdesmaki et al., 2003), as well as in reducing hyper-reactivity and impulsivity (Sallinen et al., 1998). Therefore, activation of these receptors might be beneficial in the treatment of disorders associated with enhanced startle responses and various neuropsychiatric disorders such as schizophrenia, post-traumatic stress disorder and drug withdrawal (Scheinin et al., 2001; Philipp et al., 2002a). Due to the functional interactions between the monoamines noradrenaline, serotonin and dopamine and α_{2C}-ARs, this subtype may have a modulatory role in psychomotor functions and possibly also in disorders such as depression, schizophrenia, and Alzheimer's and Parkinson's diseases (Sallinen et al., 1998; Sallinen et al., 2007; Jantschak et al., 2013; Muguruza et al., 2013). Consequently, piribedil, a combined partial dopamine receptor agonist and α_2-AR antagonist is claimed to hold great potential in the treatment of Parkinson's disease (Millan, 2010), and a combined serotonin receptor inverse agonist and α_2-AR blocker was speculated to be a promising novel antidepressant drug due to its beneficial influence on mood, cognition, sleep and sexual function (Millan et al., 2012).

Due to the presence of α_{2A}-ARs in the prefrontal cortex, α_2-ARs enhance working memory (Wang et al., 2007) and α_2-AR agonists (clonidine, guanfacine) have been beneficial in the treatment of attention deficit hyperactivity disorder (Levy, 2008; Sallee et al., 2013). According to www.centerwatch.com, clonidine (Kapvay®) is currently approved by regulatory authorities (FDA) for this purpose.

Other functions. α_{2A}-ARs regulate the seizure threshold and mediate antiepileptogenic effects in mice (Janumpalli et al., 1998). The α_2-AR agonists apraclonidine and brimonidine have been used for their intraocular-pressure lowering properties to treat glaucoma (Serle, 1994). α_2-ARs also decrease lipolysis in adipose tissue and α_2-AR antagonists might have therapeutic potential to combat obesity (Lafontan et al., 1992). In the kidney, these receptors modulate water clearance in both proximal and distal tubules as well as sodium excretion (Philipp et al., 2002a). All three subtypes of α_2-ARs have been detected in the gastric mucosa of rats, where they inhibit gastric acid secretion, gastric motility and provide protection for the gastric mucosa (Gyires et al., 2009). Finally, recently developed novel small molecule inhibitors of $G\beta\gamma$ subunits (or actually of the $G\beta\gamma$ effector interaction) have also been speculated to represent a therapeutic intervention point for α_2-ARs (Smrcka et al., 2008).

Receptor gene polymorphisms are relatively common in humans, but have only rarely been associated with diseases or marked inter-individual differences in responses to a drug. A genetic variant of the α_{2A}-AR, associated with impaired insulin secretion and increased risk for type 2 diabetes, exhibited a more prominent decrease in plasma insulin concentrations upon dexmedetomidine administration (Ghimire et al., 2013). A variant form of the α_{2B}-AR with a three amino acid deletion in the third intracellular loop (Δ301–303), which in transfected fibroblasts showed impaired agonist-promoted receptor desensitization, was associated with an elevated risk for myocardial infarction, sudden cardiac death and reduced metabolic rate (Heinonen et al., 1999; Small et al., 2001; Snapir et al., 2001; Snapir et al., 2003). A deletion variant with loss of four amino acids from the third intracellular loop of the α_{2C}-AR (Δ322–325) was cloned and expressed in CHO cells, and this mutation led to significantly diminished AC signaling, stimulation of IP_3 accumulation and activation of the MAPK pathway (Small et al., 2000). Furthermore, the deletion form was associated with an increased risk of worsening of congestive heart failure in a clinical association study (Small et al., 2000; Brede et al., 2002; Small et al., 2002).

2.2 Cell models in drug discovery and development

It usually takes 12–24 years to invent and to develop a new drug since it is a process that involves multiple steps, as outlined in Fig. 7. The process is both risky and costly, since at any phase a drug candidate may be deemed unsuitable for further development. Experience has shown that approximately only one out of 15–25 drug candidates survives the rigorous safety and efficacy testing in animals and humans that is required for it to become a marketed product (Lombardino and Lowe, 2004; Rubin, 2008), and the average cost of bringing a drug to the market is estimated to be over 1.5 billion US dollars (Macarron et al., 2011).

Nowadays, it is common that computational chemists design and select novel chemicals for further screening (Lombardino and Lowe, 2004), and these potentially bioactive molecules are further validated in cell models and cell-based assays (Kenakin, 2009). This process usually yields “hits” with desired biological activity *in vitro*; these compounds are consequently further tested in animal studies for activity *in vivo*, resulting in one lead compound that is chosen as the drug development candidate. This compound undergoes toxicological testing in animals. After receiving appropriate approval by a governmental agency, the clinical lead compound can be tested in clinical Phases I (human safety and tolerability), II (safety and preliminary efficacy assessment and dose range in patients) and III (large-scale clinical trials in many patients). The final approval of a drug candidate for marketing and clinical use is conducted under the supervision of a national or multi-national regulatory agency (e.g. the FDA in the U.S., the EMA in the EU and Fimea in Finland). This enables the use of the drug by physicians and their patients for the treatment of the disease for which it was designed (Lombardino and Lowe, 2004; Bowes et al., 2012). The process is outlined in Fig. 7.

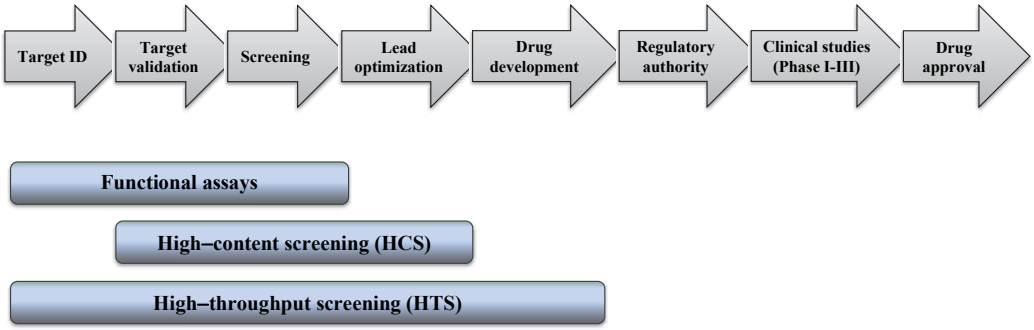

Figure 7. The drug discovery and development pipeline. The use of functional assays as well as HTS/HCS screening is outlined to reveal a perspective of how different phases of drug discovery utilize these various screens. (Modified from Bowes et al., 2012 and Cooper, 2002).

2.2.1 Cell-based assays as tools to study signaling pathways and to characterize drug candidates

Pharmacological studies of ligand properties were previously mostly carried out on isolated tissues and whole animals, which allowed for studies in real time. Unfortunately, these types of assays are very tedious if one has to screen a large library of compounds. Moreover, it is difficult to obtain human tissues and most studies are therefore performed on animal tissues. As drug effects often vary between species, this influence must be taken into consideration. Transfected cell lines expressing the human form of a recombinant protein have largely circumvented this problem and pharmacological screening assays during the last two decades have mostly involved ligand binding assays (in which drug candidates compete with a receptor-specific, high-affinity radiolabeled ligand for binding to the target receptor) as rapid initial screening tools of receptor affinity. To complement such ligand binding assays, other cell-based assays have been developed which attempt to measure the consequences of receptor activation, e.g. by monitoring second messenger production (cAMP, IP_3 etc.), Ca^{2+} fluxes or G protein activation (e.g. GTPγS binding assays). If they are to be useful, such assays should confer advantages such as relative ease of application, robustness of response and low cost, but their information content is limited because the host cell may lack the components that are required for characteristic therapeutic phenotypes or signal transduction pathways (Kenakin, 2009; Allen and Roth, 2011).

High-throughput screening (HTS) technology represents an integral part of the pharmaceutical research and biotechnology industry. HTS refers to systems where large numbers of compounds (up to tens of thousands of compounds daily) are screened to identify biologically active hits or leads for further evaluation in the drug discovery and development pipeline. HTS systems can be subdivided into biochemical and cell-based assays, with the latter representing approximately half of all high-

throughput screens currently being performed (An and Tolliday, 2010). Cell-based assays are considered more physiologically relevant models as compared to biochemical screens since not only is the reaction with the molecular target itself taken into account, but also the entity that the cell provides, therefore allowing an assessment of a compound's membrane permeability and cytotoxicity, which are critical for the eventual clinical application. Cell models in HTS also have an established role in drug development for testing metabolism, toxicity and transport (Horrocks et al., 2003; An and Tolliday, 2010; Macarron et al., 2011; Xia and Wong, 2012).

Cell-based assays designed to monitor second messenger signaling pathways are usually limited to detecting agonist efficacy that results from one particular mechanism. Furthermore, the signals detected are averages of the population of all of the cells in the system, i.e. they do not take into consideration the heterogeneity of cellular responses (Kenakin, 2009; Xia and Wong, 2012). Additionally, GPCRs do not simply function as "on-off" switches but rather have the ability to process ligand-evoked signals in different ways, i.e. they may produce partial activation at one system and full agonism at some other cellular signaling pathway. Therefore, as previously noted, one agonist may be biased towards activation of one cellular signaling pathway over others, by stabilizing a unique GPCR conformation linked to a specific intracellular pathway. For example, an old antidepressant drug, desipramine, was classically characterized as an inhibitor of noradrenaline reuptake, but later studies have recognized it as an arrestin-biased α_{2A}-AR agonist, and prolonged exposure to desipramine *in vitro* had triggered arrestin-mediated internalization and downregulation of the α_{2A}-AR with a concurrent reduction in endogenous agonist signaling through that receptor (Cottingham et al., 2011). In addition, in HEL 92.1.7 cells endogenously expressing α_{2A}-ARs, pathway-selective ligand specificity was observed for catecholamines and some other α_2-AR agonists (Kukkonen et al., 2001). In order to optimally predict the therapeutic potential of such biased agonists, their varied effects on the alternative signaling pathways need to be determined, with sequential screening of molecules in different assays. Although screening of biased agonist efficacy might appear complex, it does allow for more accurate predictions of an agonist's therapeutic value (Kenakin, 2009; Kenakin and Christopoulos, 2013).

This highlights the importance of functional assays that can monitor cellular responses in a broader context, where the full complement of signaling reactants, the complete regulatory network and feedback control mechanisms of the cell are present (Silverman and Loscalzo, 2013). It can be postulated that functional assays with primary cell systems would provide a better chance to discover new therapeutic drugs than standard assays with recombinant cell models. These cells can be utilized in systems where the complexity of disease processes would be mimicked by incorporating multiple cell types and by allowing activation of multiple pathways together. These systems could therefore be used to scan, in a predictive manner, a wide range of physiological and disease states more rapidly and cost-efficiently than classical *in vivo* studies, and this would also favour the discovery of unexpected therapeutic properties. However, it is important to remember that *in vitro* models are never as representative as whole animal

studies even if multiple cell types are present in the model (Butcher, 2005; Kenakin, 2009). Today, multiple biochemical and morphological cellular outputs to physical, chemical or biological stimulation from the environment can be measured simultaneously with online high-content assays in primary cells. These assays represent another level of detection of real-time changes (from small molecules or genetic manipulations) on the local ionic environment, proliferation rate, cell number, morphology, volume and adherence, up- or down-regulation of metabolic activity of cells as well as cell-to-cell contacts (Wolf et al., 2013) (Fig 8). The technique of combining HTS with microscopy is termed high-content screening (HCS) and is particularly well-suited for stem cell research and drug discovery, since examining cells at a single cell level allows for detection of rare phenotypes in heterogeneous cultures (Xia and Wong, 2012). HCS is not always superior to other methods, instead, choosing which screen to select depends on the balance between the readout type, the sensitivity and the statistical quality, the throughput as well as on time and cost constraints.

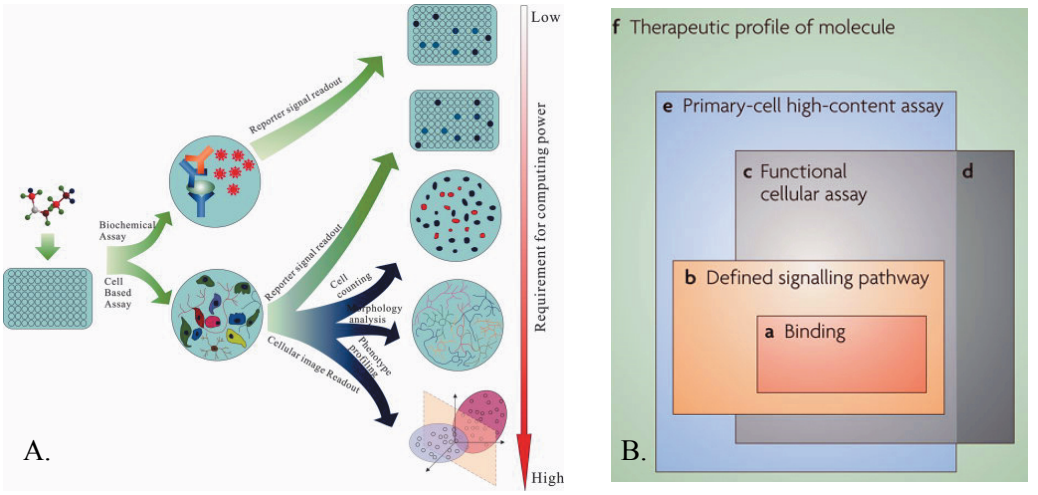

Figure 8. The use of cell-based and biochemical assays in drug discovery and the various detection ranges for different pharmacological assays. A. An overview of data output gained from biochemical and cell-based HTS-assays (green). By combining HTS-assays with cellular image assays (blue) multiple readouts of a compound's effects can be evaluated. B. Receptor binding studies (a) are the most limited pharmacological assays, allowing only for detection of a ligand's ability to bind to a receptor. At the next level, signaling pathways are integrated and detection of second messenger generation is accomplished with either biochemical or cell-based assays (b). In functional cell-based assays (c) a wider range of cellular behavior is assessed, including the receptor's interaction with other partners, e.g. β-arrestins, in addition to G proteins, this for example allows for detection of internalization of receptors without the concomitant activation of a second messenger (d). With HCS assays (e), cellular signaling pathways are investigated while simultaneously detecting changes in cellular function, morphology etc. over time. Finally the therapeutic profile of a molecule can be investigated in a tissue subjected to some disease pathology (f). (Images from Kenakin, 2009; Xia and Wong, 2012).

Finally, assays can be designed to investigate the drug target in a therapeutically relevant tissue which is expressing the symptoms of some disease. This represents an important missing link in a system in which drugs are optimized in test assays from healthy tissue (Butcher, 2005; Kenakin, 2009). Moreover, accessing technology to generate induced pluripotent stem cell (iPSC) lines from patients affected with specific diseases may provide a long-term supply of these cells for further studies in a relevant pathological setting and thus enable the screening of drugs to correct some disease phenotype (Rubin and Haston, 2011). Studying these cells may also aid in understanding the actual diseases and improve their diagnosis as well as allowing patients to enter drug development processes through preclinical trials much earlier than at present (Ebert and Svendsen, 2010; Bellin et al., 2012; Takahashi and Yamanaka, 2013). There is a detailed review of the diseases that have been modeled with human iPSC (Robinton and Daley, 2012). It is hoped that by combining physiologically relevant iPSC disease models with experimental platforms, the drug discovery industry will be able to generate high quality drug targets and HCS screening assays. One could argue that access to better drug targets, more relevant safety profiles (resulting from toxicity tests in e.g. iPSC-derived liver cells) and a more relevant choice of patient population for testing of drug candidates, the cost of drug discovery could be considerably reduced from its current level (Rubin and Haston, 2011).

Many clinically efficacious drugs exhibit so-called polypharmacology at GPCRs, i.e. they are able to bind to several GPCR targets. This further complicates assay design but also presents new opportunities for the discovery of better drugs since it incorporates the concept of network pharmacology. Here the role of a drug's action on receptor networks in a biological system is investigated in order to understand complex diseases. Information thus gained can be used to inform and guide drug development (Allen and Roth, 2011; Peters, 2013; Silverman and Loscalzo, 2013). Furthermore, the drug industry now employs *in vitro* pharmacological profiling to screen compounds against a broad range of targets that are distinct from the intended therapeutic target in order to identify undesirable off-target activity that may hinder the development of candidate leads (Bowes et al., 2012).

Setting up a new functional assay for drug discovery requires rigorous validation and optimization of the protocol. These steps include titration of cell density, titration of assay reagent(s), determination of optimal concentrations of modulator(s) and incubation times with compounds as well as the identification of the correct positive controls and vehicle controls for normalization (Kenakin, 2009; An and Tolliday, 2010). If one wishes to evaluate α_2-ARs, their signaling properties as well as potential therapeutic drugs against these receptors, one must have well validated cell model systems. A summary of the most common readouts in functional assays developed and used with α_2-ARs are presented in Table 1.

Table 1. Reported readouts of α_2-AR activation in immortalized cell lines or cell lines endogenously expressing α_2-AR subtypes. Reported as first observation per cell line in the given reference.

Cell-based assay readouts	Cell lines	References
cAMP	Neuroblastoma x rat glioma cells (NG108-15)	Kurose et al., 1983
	Human erythroleukemia (HEL)	McKernan et al., 1987
	Human colonic adenocarcinoma cells (HT29)	Jones et., 1987
	Opossum kidney (OK)	Murphy et al., 1988
	Chinese hamster ovary (CHO)	Fraser et al., 1989
	Chinese hamster lung fibroblasts (strain PS120)	Cotecchia et al., 1990
	Shionogi 115 mouse mammary tumour (S115)	Marjamäki et al., 1992
	Rat fibroblast (Rat1)	MacNulty et al., 1992
	NIH-3T3 fibroblasts	Duzic et al., 1992
	DDT1 MF-2 smooth muscle cells	Duzic et al., 1992
	Rat adrenal pheochromocytoma (PC12)	Duzic et al., 1992
	Human choriocarcinoma (JEG-3) cells	Pepperl & Regan, 1993
	Human embryonic kidney (HEK293)	Chabre et al., 1994
	Sf9 insect cells	Jansson et al., 1995
	Mouse distal tubule cells (DCT)	Gesek et al., 1996
Polyphosphoinositide formation	Human erythroleukemia (HEL)	Michel et al., 1989
	Chinese hamster lung fibroblasts (strain PS120)	Cotecchia et al., 1990
	Monkey kidney fibroblast (COS-7)	Cotecchia et al., 1990
	Human embryonic kidney (HEK293)	Conclin et al., 1992
	Mouse distal tubule cells (DCT)	Gesek et al., 1996
Voltage-dependent Ca^{2+} currents	Mouse pituitary tumor cell (AtT20)	Surprenant et al., 1992
	Human erythroleukemia (HEL)	Musgrave & Seifert, 1995
Intracellular calcium	Human erythroleukemia (HEL)	Michel et al., 1989
	Chinese hamster ovary (CHO)	Dorn et al., 1997
	Monkey kidney fibroblast (COS-7)	Dorn et al., 1997
	Human aortic vascular SMC	Chotani et al., 2004
	Neuroblastoma x rat glioma cells (NG108-15)	Holmberg et al., 1998
	Sf9 insect cells	Holmberg et al., 1998
Potassium currents	Mouse pituitary tumor cell (AtT20)	Surprenant et al., 1992
	Rat adrenal pheochromocytoma (PC12)	Soini et al., 1998
GTPγS activation	Human embryonic kidney (HEK293)	Jasper et al., 1998
	Chinese hamster ovary (CHO)	Peltonen et al., 1998
	Rat aortic smooth muscle (A7r5)	Huhtinen et al., 2008

Cell-based assay readouts	Cell lines	References
MAPK activation	Rat fibroblast (Rat1)	Alblas et al., 1993
	Monkey kidney fibroblast (COS-7)	Koch et al., 1994
	Chinese hamster ovary (CHO)	Flordellis et al., 1995
	Opossum kidney (OK)	Kribben et al., 1997
	Human embryonic kidney (HEK293)	Schramm et al., 1999
	Pig renal tubular (LLC-PK1)	Cussac et al., 2002
	Rat adrenal pheochromocytoma (PC12)	Karkoulias et al., 2006
Phospholipase D	Rat fibroblast (Rat1)	MacNulty et al., 1992
Phospholipase A2	Chinese hamster ovary (CHO)	Jones et al., 1991
Na^+/H^+ exchange	Neuroblastoma x rat glioma cells (NG108-15)	Isom et al., 1987
	Opossum kidney (OK)	Clarke et al., 1990
	Chinese hamster ovary (CHO)	Pihlavisto et al., 1999

2.2.2 Recombinant expression of GPCRs in mammalian cell lines

The two principal advantages of using cultured cells (as compared to *in vivo* studies where genetic diversity and cell-cell interactions dominate) are the possibility to better control experimental variables in a single population of identical cells and to provide a more reliable, controllable and consistent source of living material needed for molecular and biochemical studies (Owens, 1995). The most commonly employed cell types in cell-based drug discovery models can be divided into two major categories; namely cell lines, defined as permanently established immortalized cells that can grow indefinitely in culture; and primary cells that may be highly differentiated and are cultured directly from a donor subject (Gasparri, 2009). The typical simple cell models commonly used in drug discovery are based on heterologous expression of a recombinant protein transfected into an immortalized cell line, which allows for rapid pharmacological analysis of drug candidates against a defined target, e.g. a receptor subtype, as well as a functional analysis of receptor coupling mechanisms in a controlled well-defined cellular environment (Horrocks et al., 2003; Kenakin, 2009). Mammalian cells provide suitable environments for the expression of specific GPCRs permitting investigation of their functions, since many of the components necessary for the interaction between GPCRs and their G proteins and effector coupling systems are all present. Ideally, however, drug screening should be performed with target cells from the tissue of interest to ensure that the results will be faithfully transferred to subsequent applications *in vivo*. Nonetheless, this is often impossible and thus immortalized cell lines continue to have an established role in HTS and HCS, since they have the advantages of being inexpensive, easily grown and amplified in virtually

unlimited quantities, and can be easily modified with gene technology (Xia and Wong, 2012).

Careful selection of the expression system is necessary when setting up assays with transformed cells. The most commonly used transformed cell lines in cell-based HTS and HCS are cells of human origin such as HeLa or HEK293 cells and Chinese hamster ovary (CHO) cells (Gasparri, 2009). Screening of receptor binding properties of different α_2-AR ligands in the pre-cloning days took advantage of cell lines that endogenously express a single α_2-AR subtype; these included the megakaryocyte-derived erythroleukemia cell line HEL and the colon adenocarcinoma cell line HT29 for α_{2A}, the neuroblastoma hybrid cell line NG108-15 for α_{2B} (Bylund et al., 1988), and an opossum kidney OK epithelial cell line (Murphy and Bylund, 1988; Blaxall et al., 1994) or the hepatoma cell line HepG2 for α_{2C} (Schaak et al., 1997).

Nevertheless, there are many drawbacks and limitations associated with transformed cell lines; especially the immortalization of a cell line (introduction of a mutation to make the cells undergo infinite cell division) to produce expanded cell lines inevitably introduces changes into the cells' behavior. Even if the target receptor is of human origin, the host cell line is commonly derived from another species and therefore care should always be taken in the biological interpretation of the observed signaling pathways irrespective of whether they are activated or inhibited, as they only provide indications of what might happen in the native tissue (Horrocks et al., 2003; Kenakin, 2009; Ebert and Svendsen, 2010). Several potential problems are related to the expression of proteins in a foreign environment. For example, this is the case for odorant receptors, taste receptors and α_{2C}-ARs, which when expressed in heterologous cell systems do not usually reach the plasma membrane but are retained in the endoplasmic reticulum (Hurt et al., 2000; Saito et al., 2004; Behrens et al., 2006). This may be caused by the lack of a transport/chaperone protein in that particular cell type, which normally would be present in the cell population where the receptor is endogenously expressed. The high rate of protein synthesis in transient expression systems may lead to incomplete and heterogeneous post-translational modifications and might also result in receptor promiscuity, i.e. coupling of a receptor to multiple G protein isoforms, as was also shown for the α_2-ARs (Eason et al., 1992; Pohjanoksa et al., 1997). Therefore, care must be taken in the interpretation of the results, as they may not necessary reflect the *in vivo* situation in the fully differentiated cell in its normal environment (Owens, 1995).

2.2.3 Endogenous expression of GPCRs in primary cells

Cultured mammalian cell lines might serve as good preliminary cell models for screening of drugs in the early stages of drug development, but the closer a cultured cell model system matches to the real human target tissue, the more likely it is that the results obtained in such systems will be predictive of efficacy and safety *in vivo* (Horrocks et al., 2003; Kenakin, 2009). Primary cells are mostly employed as freshly

isolated cells from individuals (human or animal sources) from the tissue of interest, but some ready-made preparations are also commercially available. The clear advantage of primary cells is that they much more genuinely represent the *in vivo* physiological state of differentiated cell types. However, primary cells also have many drawbacks: they only survive in culture for a limited duration of time or number of cell divisions and cannot be passaged indefinitely, they may quite rapidly lose their differentiated phenotype in culture and there is poor availability of human tissue samples. Between-subject genetic variations in different isolated populations may exist, resulting in cell heterogeneity between patients and this can affect the results. Furthermore, primary cells are not easily transfected and there are limitations to experimental approaches, e.g. tagging of an endogenously expressed receptor with an epitope that is conveniently recognized by an antibody is not possible (Gasparri, 2009; Gresch and Altrogge, 2012). The limitations associated with immortalized cell lines and primary cells can partly be avoided by the use of stem cells. The Nobel Prize in Physiology or Medicine 2012 was awarded to Sir John B. Gurdon and Dr. Shinya Yamanaka for the discovery that mature mouse and human somatic cells could be genetically reprogrammed (by the addition of four powerful genes) to become pluripotent, i.e. induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Takahashi and Yamanaka, 2013). These cells adopt similar characteristics to embryonic stem cells and they are therefore in principle able to be differentiated to any human cell type (e.g. neurons, blood cells and cardiac myocytes) in limitless supplies, including disease-relevant cell types, providing a new paradigm for drug screening. This allows for the generation of physiologically relevant cellular models that are tailored to the disease that the drug candidate is intended to treat, and iPSCs also constitute an ideal source of cells for estimating the toxicity of drug candidates. Furthermore, patient-specific iPSC lines can be established, representing a unique cell source for personalized drug discovery and disease modeling (Ebert and Svendsen, 2010; Bellin et al., 2012; Xia and Wong, 2012; Takahashi and Yamanaka, 2013). The advantages vs. disadvantages of different cell models are summarized in Table 2.

Table 2. Advantages and disadvantages of human cells used in drug research. Modified from (Ebert and Svendsen, 2010).

Cell types	Advantages	Disadvantages
Immortalized cell lines	Low cost of growth and maintenance Homogeneous cell population	Lack important aspects of native function Not representative of all cell types
Primary cells	Fully differentiated cell types Close resemblance of native function	Poor availability of human tissue and other cell types Require fresh preparation Questionable reproducibility
Stem cells	Readily available source of all cell types High quality Fully differentiated cell types Close resemblance of native function	Expensive growth and maintenance Time-scale of obtaining fully differentiated cell types Difficulty to produce pure populations Difficulty to induce into a specific cell type

If one considers the investigation of α_2-ARs as cardiovascular drug targets, then a physiologically relevant cell model system would be primary cultures of fully differentiated cells, derived from the relevant human tissue and expressing the target protein of interest endogenously at physiological levels, e.g. α_2-ARs expressed in human vascular smooth muscle cells (SMC). These kinds of cells have been used as a model for a variety of pharmacological and physiological studies directed towards understanding the processes involved in vascular diseases and hypertension (Chamley-Campbell et al., 1979; Truskey, 2010; Proudfoot and Shanahan, 2012).

SMCs are found in the middle layer (media) of the blood vessel wall, where they form circular layers. On the outside, they are surrounded by connective tissue and fibroblasts (adventitia), and on the inside, of a continuous single flattened sheet lining of endothelial cells (intima) that forms a barrier to the circulating blood. The different layers are separated by basement membranes composed of extracellular matrix proteins. SMCs *in vivo* exist in a quiescent differentiated state and express markers for contractile proteins (α-smooth muscle actin, calponin, myosin heavy chain, SM22α, desmin, h-caldesmon, metavinculin and smoothelin). After injury to the vascular wall or in diseases such as hypertension and atherosclerosis, SMCs can start to proliferate and assume a de-differentiated synthetic state, with reduced expression of contractile markers and increased migratory and secretory abilities. As SMCs are placed in culture, they gradually adopt a similar de-differentiated phenotype (Chamley-Campbell et al., 1979; Shanahan et al., 1993; Owens, 1995; Proudfoot and Shanahan, 2012).

SMCs mediate regulation of blood pressure and blood flow through coordinated vasoconstriction and vasodilation. They contain thick and thin filaments of myosin and actin that are organized in a way well suited for their role in maintaining tonic

contractions and reducing lumen diameter. Contraction of SMCs can be initiated through mechanical, electrical and chemical stimuli. Drugs, neurotransmitters or hormones, originating from sympathetic nerves, endothelial cells or from the bloodstream, bind to receptors in the SMC membrane and activate different signal transduction pathways, many of which converge to increase the levels of intracellular Ca^{2+}. The free Ca^{2+} in the cell binds to calmodulin, and the Ca^{2+}-calmodulin complex activates myosin light chain kinase (MLCK), which in turn phosphorylates the 20 kDa regulatory proteins of myosin light chains (MLC). This phosphorylation in combination with ATP hydrolysis leads to cross-bridge formation between myosin heads and the actin filaments, and hence smooth muscle contraction. The intracellular signaling events involved in the contraction process vary depending on which GPCR is activated, but many of these routes involve activation of the PLC pathway to increase intracellular Ca^{2+} (Somlyo and Somlyo, 1968; Small and Sobieszek, 1977; Chamley-Campbell et al., 1979; Kamm and Stull, 1985).

3 AIMS OF THE STUDY

The α_2-ARs constitute important targets for drug discovery and development as they are critically involved in the regulation of cardiovascular, endocrine and sensori/motor functions of the body. They regulate a complex and sometimes mutually opposing network of functions by mediating responses to the two endogenous catecholamines, noradrenaline and adrenaline. Current research is thus driven towards developing drugs that selectively act on specific subtypes of the α_2-ARs in order to avoid adverse effects. Different types of cell model systems are employed in the early phases of the drug discovery and development process for initial evaluation of new chemical compounds and their efficacy in complex biological systems. Several different types of cell models can be employed depending on the investigated target and function; each system has its own characteristic benefits and drawbacks. The development and validation of cell-based models and functional assays to investigate α_2-ARs as drug targets were the main focus of this thesis. It is anticipated that improved detailed knowledge of receptor subtype functions can lead ultimately to improved drug therapy.

The studies presented in this thesis had the following aims:

1. To validate and optimize a functional assay in order to investigate α_2-AR-mediated inhibition of AC activity and to compare previously generated mutated α_{2A}-AR constructs for the effects of the receptor mutations on second messenger signaling (agonist-dependent inhibition of AC activity).

2. To create cell models for investigation of the trafficking and localization properties of the α_{2A}- and α_{2C}-AR subtypes in order to understand why these receptor subtypes are differentially targeted in neuronal and non-neuronal cells, as well as to in detail examine how membrane accessory proteins (RTPs and REEPs) can affect the expression and trafficking of these receptor subtypes.

3. To set up a functional assay to screen α_2-AR agonists and antagonists for their capacity to evoke or inhibit vascular smooth muscle cell contraction, and to validate the use of primary human vascular smooth muscle cells endogenously expressing α_2-ARs in the developed MLC phosphorylation assay.

4 MATERIALS AND METHODS

4.1 Construction of expression vectors and transfections

In Study I, an episomally kept expression vector, pREP4 (InVitrogen, NV Leek, Holland), containing the human α_{2A}-AR cDNA (originally a gift from Dr. R.J. Lefkowitz, Duke University) was employed.The expression vector also contains a gene for hygromycin B resistance (Marjamäki et al., 1998). A stable cell population expressing human α_{2A}-ARs was previously produced with the same insert placed in the expression vector pMAMneo (Clontech, Palo Alto, CA, USA), containing a neomycin resistance gene (Pohjanoksa et al., 1997). The human influenza hemagglutinin (HA) epitope was placed in front of the α_{2A}-AR coding sequence. It has previously been shown that this epitope does not affect ligand binding of murine α_2-ARs (Daunt et al., 1997), or the functional coupling of murine α_{2C}-ARs (Hurt et al., 2000).

Most of the original RTP and REEP constructs in the pCI vector were kind gifts from Dr. H. Matsunami, (Duke University). Mouse REEP1 and REEP2 cDNAs were transferred to the pcDNA3.1+ vector (Invitrogen). REEP6 was cloned from mouse embryo cDNA (Ambion, Austin, TX, USA) with PCR, using the Platinum Taq High Fidelity DNA polymerase (Invitrogen), and ligated into the pcDNA3.1+ vector. REEP1, REEP2, and REEP6 were epitope-tagged on their extreme carboxyl terminus with the Flag epitope (DYKDDDDKA), using the QuickChange® Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All α_2-AR constructs used in Studies II and III had been made previously and contained an extracellular HA epitope tag at the amino terminus (Daunt et al., 1997; Hurt et al., 2000). Transient expression of HA-tagged α_2-ARs and Flag-tagged REEPs was achieved using the Effectene (Qiagen, Valencia, CA, USA) transfection reagent. The empty pcDNA3.1 plasmid was used for control transfections.

Study IV utilized the A7r5 rat aortic smooth muscle cell line transfected to express the human α_{2B}-AR subtype, as described earlier (Huhtinen and Scheinin, 2008).

4.2 Cell culture

4.2.1 Cell lines

In Study I, adherent Chinese hamster ovary (CHO) cells (American Type Culture Collection, ATCC, Rockville, MD, USA) were cultured in α-MEM (MEM Alpha Medium, Gibco BRL, U.K.) supplemented with 26 mM $NaHCO_3$, 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich) and 10% heat-inactivated fetal calf serum (FCS, Bioclear, Australia), referred to as growth medium. The semistable cell population was maintained in growth medium supplemented with Hygromycin B® (200

μg/ml, Roche, Pentzberg, Germany) and the stable cell population was maintained in growth medium supplemented with Geneticin® (200 μg/ml, Sigma-Aldrich Corporation, St. Louis, MO, USA). All cell cultures were grown at 37 °C in 5% CO_2. CHO cells (90% confluent) were harvested for experiments into phosphate-buffered saline (PBS) with normal harvesting methods. In the ligand binding studies, cell pellets were stored frozen prior to analysis. Prior to the measurement of cAMP production, cells were counted after harvesting and plated on 96-well plates (~23000 cells/well; for PTx treatment ~35000 cells/well).

In Studies II and III, immortalized cell lines including NRK, Rat1, HEK293 and AtT20 (ATTC) were cultured at 37° C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gemini Bio-Products, Inc., Calabasa, CA, USA) and gentamicin (25 μg/ml; Roche Molecular Biochemicals, Indianapolis, IN, USA). Pheochromocytoma-derived PC12 cells were cultured at 37 °C in 10% CO_2 in DMEM supplemented with 10% horse serum (EDS, Hyclone) and 5% fetal bovine serum (BCS, Hyclone, USA). Cells were harvested for experiments into PBS with trypsin.

In Study IV, A7r5 cells (ATCC) were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml geneticin (Sigma-Aldrich). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 3 days and the cells were passaged approximately once a week by dissociation with a PBS solution containing 0.025% trypsin and 0.1% EDTA.

4.2.2 Generation of monoclonal cell lines

Monoclonal (stable) cell populations were produced from the semistable cell population. Diluted cell suspensions were plated on Petri dishes. Cells were kept in growth medium supplemented with Hygromycin B® (200 μg/ml). Single cell clones were marked after cell attachment, and clonal cell colonies originating from a single cell were harvested 4 days later by detachment with trypsin and transfer of the colonies to a 6-well plate. Alternatively, the diluted cell suspension was divided on a 96-well plate with approximately one cell per well. Wells containing only a single cell clone were subsequently expanded. Established monoclonal cell populations were maintained in growth medium supplemented with Hygromycin B® (200 μg/ml).

4.2.3 Primary human vascular smooth muscle cells

Primary cultures of SMCs were derived from human saphenous (SVSMC) vein grafts from patients undergoing coronary bypass surgery in Turku University Hospital. The collection and use of human tissue samples was based on informed consent by the tissue donors. The study protocol was approved by the Ethics Committee of South-

West Finland Hospital District. The veins were collected in cold Hank's balanced salt solution (HBSS) supplemented with 5% FCS (PAA Laboratories) and processed as soon as possible. The explant method was used as the isolation technique (Campbell and Campbell, 1993) since it has been reported to be the most reliable way to obtain vascular SMCs (Proudfoot and Shanahan, 2012). The vessel was washed several times in HBSS, cleaned of fat and connective tissue and cut longitudinally. The endothelial lining was carefully scraped off with a scalpel, taking care not to damage the sub-endothelial layer, and the exposed media layer was cleaned with sterile gauze soaked in HBSS to remove all remaining endothelial cells. The media was washed several times with HBSS. Then the inner two-thirds of the media layer were removed from the adventitia by forceps in one or several horizontal strips, and cut into 1–2 mm^2 pieces with a razor blade. The explant pieces were transferred with the luminal side (intima) face down onto a plastic cell culture dish (35 mm) that had been pre-wetted with culture medium prior to placing in an incubator. A delicate balance was maintained between keeping the explant pieces moist and not adding too much liquid (so that the explants floated off). This was achieved by adding droplets of liquid carefully at 6–12 h intervals during the first 48–72 h. After this, 1 ml of cell culture medium could be added to cover the whole plate. The cell culture medium was composed of Medium 231 (Gibco) supplemented with 10% FCS, 2 ng/ml human basic fibroblast growth factor (Gibco), 0.5 ng/ml human epidermal growth factor (Gibco), and 5 μg/ml insulin (Sigma-Aldrich), as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). The culture medium was changed every 3 days by removing 2/3 of it and replacing it with fresh medium, again very carefully to avoid detachment of the explants. In approximately two weeks, the cells started to migrate out of the explant pieces and were grown for several weeks before passage with 0.05% trypsin-0.2% EDTA in PBS (10 min) and centrifugation at 1000 rpm for 7 min. It was crucial for cell passaging that cells were plated out relatively densely (covering about 40% of the culture dish) as this encouraged their growth. The cells could not be passaged more than 10 times; thereafter they declined into senescence (Proudfoot and Shanahan, 2012). In the differentiation studies, cells were grown until confluent and were then kept in a serum-free differentiation medium containing M199 medium supplemented with porcine heparin (400 μg/ml) to arrest cell proliferation, SITE (a mixture of bovine insulin, human transferrin, sodium selenite and ethanolamine, used to maintain SMC quiescence without nutritional deprivation), and penicillin-streptomycin (all from Sigma-Aldrich) for 6 days (Desmouliere et al., 1991).

4.3 RNA extraction, RT-PCR and quantitative real-time RT-PCR

Isolation of mRNA and cDNA synthesis (Study II, III) were done with a Cells-to-cDNA™ kit (Ambion), separately for each cell line, using ~100 000 cells. The standard protocol of the kit was followed, except that the DNase I reaction was prolonged to 60 min at 37 °C to ensure complete DNA digestion. Reverse transcription was conducted with a two-step protocol using random decamers. Samples were preheated at 70 °C for 3 min before addition of enzymes, then incubated at 42 °C for 45 min and inactivated

at 94 °C for 10 min. Primers specific for the mouse or rat sequences of RTP1–4 and REEP1–6 were designed using DNAStar software (Madison, WI, USA) and synthesized based on known GenBank sequences. PCR amplifying conditions were optimized separately for each rat and mouse RTP1–4 and REEP1–6 gene, using either commercial mouse embryo cDNA (Ambion), mouse brain or rat brain cDNA (Maximbio, CA, USA). In order to check the integrity of the cell-derived samples, expression of GAPDH was assessed. The annealing temperature, as well as other conditions, were separately optimized for each primer pair, as indicated in Table 4. The final PCR protocol consisted of 30–45 cycles of denaturation (30 sec, 94 °C), annealing (30 sec), and extension (72 °C, 1 min), preceded by a 3 min denaturation at 94 °C, and followed by a 3 min final extension at 72 °C. PCR products were analyzed with standard gel electrophoresis methods and confirmed by either restriction enzyme digestion or sequencing.

Isolation of mRNA from primary human smooth muscle cells (Study IV) was done with the RNeasy Mini Kit (Qiagen), and sample concentrations were determined by UV absorbance. Only RNA of high quality was used (OD_{260} / OD_{280} ratio of approximately 2.0). A total of 1 μg of RNA/reaction was converted to cDNA using the DyNAmo™ cDNA Synthesis Kit (Finnzymes Oy, Vantaa, Finland), including controls with no reverse transcriptase enzyme included.

Quantitative real-time reverse transcription (RT)-PCR was performed using the KAPA SYBR® FAST qPCR kit (Master mix, ABI Prism™, Wilmington, MA, USA), using 20 ng of cDNA template, and incorporating all necessary controls to check for purity. Primers against the ribosomal S29 protein (commonly used as a housekeeping gene) were always added in each run for use as a positive control. The temperature program was as follows (Table 3):

Table 3. Quantitative PCR-conditions for assessment of α_2-adrenoceptor expression and SMC specific markers in human primary vascular SMC.

Step	Temperature	Duration	Cycles
Enzyme activation	95 °C	90 sec	hold
Denaturation	95 °C	3 sec	40
Annealing/extension	60 °C	30 sec	
Dissociation	95 °C	15 sec	
	60 °C	60 sec	
	95 °C	15 sec	
	60 °C	15 sec	

Table 4. Mouse and rat RTP1−4 and REEP1−6 primer sequences and optimized final PCR conditions.

Primer name	Sequence (forward primer)	Sequence (reverse primer)	Annealing T (°C)	# of cycles	$MgSO_4$ (mM)	Product size (bp)
GAPDH	GTCATCATCTCCGCCCCTTCT	TGCCTGCTTCACCACCTTCTT	60	30	2	441
mRTP1	CCCTGCCTTACACTTACCCTCT	GCTCTCGCAAACTGGTGATGA	62,7	40	3	458
mRTP2	TCCACCAGCCTGACCACTT	GCCACCATCCTCATCGTAAC	62,7	40−45	3	402
mRTP3	TCAAATGCCTTCTCCCACTC	GAAAGGCCAAGCAACACAGT	62,7	40	3	413
mRTP4	AGACAGTGCTTGGCAGGTTC	GGCACATCTTCCATGAGAGT	60,3	40	3	362
mREEP1	TATTTGGCACCCTTTATCCTG	CATCTTGGGCTGGCTGTGTTT	60,1	40	1	524
mREEP2	TAGTCTTCGCCTTCTTCACCACA	GACTTAGGGCAGGCTCATCTC	60,1	40	1	459
mREEP3	ACGTTCGCTGGATGATGTATTG	CCCTCCGCTTCTTCGTCTGTCT	62,1	35	3	514
mREEP4	CAAGGCCGTGAAGAGCAAGAA	CAGGGGTGTCAGGGATAGAG	62,2	40	1	419
mREEP5	TTTGTATCTGGTGTTCGGTT	TGCAGTCTCTTTGGCTTTGTCCTT	60,6	40	3	364
mREEP6	AAGCAAGGACCGGTGTAGAGAA	GTTGATGCTGGGGTGACGAGAG	62,2	40	1	490
rRTP1	TGACGAAGACATGTGTAAGAGTG	CGGAAGGAGAATTGCAGGTA	62,1	40	3	681
rRTP2	TCCACCAGCCTGACCACTT	CATGCGCACTGAGCCAAC	62,1	40	4	264
rRTP3	AAGCCAGGATGGACGCAA	TGCTGGTGGTTTTGAGGC	59,1	40	1	411
rRTP4	GCTGTTCTCCGATGACTTCAG	TATCCAAGTGCAGGGTCCAC	60,1	40	2	95
rREEP1	TATTTGGCACCCTTTATCCTG	CATCTTGGGCTGGCTGTGTTT	60,1	40	3	524
rREEP2	TAGTCTTCGCCTTCTTCACCACA	GACTTAGGGCAGGCTCATCTC	60,1	40	1	459
rREEP3	ACGTTCGCTGGATGATGTATTG	CCCTCCGCTTCTTCGTCTGTCT	60,1	40	3	514
rREEP4	CAAGGCCGTGAAGAGCAAGAA	CAGGGGTGTCAGGGATAGAG	62,1	40	2	419
rREEP5	TTTGTATCTGGTGTTCGGTT	TGCAGTCTCTTTGGCTTTGTCCTT	60,1	40	3	364
rREEP6	AAGCAAGGACCGGTGTAGAGAA	GTTGATGCTGGGGTGACGAGAG	62,1	40	2	490

4.4 Receptor density determinations

All radioligand binding experiments were performed on CHO cell homogenates in 50 mM K^+ phosphate buffer. Receptor densities were determined with saturation binding assays, using the α_2-AR antagonist radioligand [^{3}H]RX821002 ((1,4-[6,7(n)-^{3}H]benzodioxan-2-methoxy-2yl)-2-imidazoline hydrochloride, 54–60 Ci/mmol, 0.0625–8 nM) (Amersham Biosciences UK Limited, Buckinghamshire, UK). Nonspecific binding was determined by adding an excess of unlabeled phentolamine (10 µM, Sigma-Aldrich). The cell pellet was suspended in 50 mM K^+ phosphate buffer and homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) for 3 times 10 s. The association of radioligand took place during a 30 min incubation at 25 °C, this being terminated by dilution with ice-cold buffer (TM buffer, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) and separation of bound radioactivity by filtration on Whatman glass fiber micro filters (Whatman International Ltd., Maidstone, UK) with a cell harvester (Brandel Cell Harvester M48R, Gaithersburg, MD, USA, at 6 °C), followed by two washes with ice-cold TM buffer. The radioactivity on the filters was determined by liquid scintillation counting (Wallac 1410 Liquid Scintillation Counter), in OptiPhase HiSafe3 (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). The protein concentration of the cell homogenate was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as the reference.

4.5 Immunofluorescent labeling

Transfected cells (Study II) were seeded on sterile poly-D-lysine-coated glass coverslips. At 48 hours post-transfection, the cells were rinsed three times with PBS supplemented with Ca^{2+} and Mg^{2+} (PBS-CM). The cells were fixed for 5 min with 4% paraformaldehyde (PFA) at room temperature (RT). Blocking agent (5% dry milk, 2% goat serum, 50 mM HEPES pH 7.4 in PBS-CM) was used to reduce nonspecific antibody binding. In the experiments on permeabilized cells, NP40 at a final concentration of 0.2% was added to the blocking solution. Antibody applications were performed in blocking solution for 1 h at RT. The following antibodies were used: mouse monoclonal anti-HA (16B12) (1:500; Covance Research Products, Cumberland, VA, USA) and rabbit polyclonal anti-Flag (1:500; Cell Signaling Technology, Beverly, MA, USA). Stained cells were washed 3 times with PBS-CM at 5 min intervals and blocking solution was re-applied to the cells for 20 min. Secondary antibodies; goat anti-mouse Alexa 594 or goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen), were applied for 1 h in the dark (RT). After secondary labeling, cells were rinsed 3 times with PBS-CM at five min intervals in the dark and mounted using Vectashield Hard Set mounting medium H-1500 with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) examined by confocal immunofluorescent microscopy.

In Study IV, SVSMC cells were plated on FBS-pretreated coverslips and left to attach for 2 h before addition of culture medium. In the cell differentiation studies, differentiating culture medium was added for another 6 days. The coverslips were then

washed with PBS and fixed with either ice-cold methanol for 15 min at RT, then left to air-dry, or with 4% PFA for 5 min at RT. The coverslips were then thoroughly washed with PBS-CM. Non-specific binding was blocked by incubating with blocking buffer (0.2% NP40, 2% goat serum, 1% BSA in PBS-CM) for 30–40 min. Incubation with primary antibody lasted for 1 h at RT with the antibodies employed being monoclonal human anti-CD31 (Pecam-1) clone WM-59 (1:20, Sigma Aldrich), anti-smooth muscle α-actin (1:50, Sigma Aldrich) and anti-MLC (Sigma Aldrich). After labeling, the cells were washed three times at 5 min intervals with PBS-CM, followed by 15 min incubation in blocking buffer. The secondary antibody, Alexa Fluor anti mouse 546 (1:750) or 488 (1:500, Invitrogen), was applied for 60 min. After rinsing 3 times at 5 min intervals with PBS-CM, the cells were mounted for fluorescence microscopy using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

4.6 Functional assays

4.6.1 Measurement of intracellular cAMP

Plated CHO cells were cultured overnight (20 h) and subsequently incubated for 1 h in serum-free medium. For experiments on PTx-pretreated cells, CHO cells were first cultured for 8 h in growth medium, and then treated with serum-free medium with or without PTx (500 ng/ml, Sigma-Aldrich) for 16 h. All cells were incubated with the phosphodiesterase inhibitor, IBMX (0.1 mM, Sigma-Aldrich), for 10 min, and AC activity (except controls for basal cAMP levels) was then stimulated with forskolin (5 μM, Sigma). Increasing doses of noradrenaline (NA, [−]-arterenol, 1 nM–100 μM, Sigma) were added to all cells (except to control cells and cells used for the determination of maximal forskolin-stimulated cAMP production). The plate was incubated for 1 h at 37 °C and 5% CO_2, and the cells were subsequently lysed with a preheated detergent solution (0.03% digitonin in 19% DMSO). The concentration of cAMP in each sample was determined with a DELFIA cAMP kit using an acetylation protocol (PerkinElmer). Fluorescence was measured with a VICTOR^2 1420 Multilabel Counter (Wallac Oy). Inhibition of forskolin-stimulated cAMP accumulation was normalized to the effect of forskolin (5 μM). The E_{max} value was calculated from the means ± S.E. of the maximal inhibition/stimulation in the separate experiments. All measurements were carried out in at least in 3 individual experiments performed in duplicate.

4.6.2 Measurement of myosin light chain phosphorylation

A7r5-α_{2B} cells were plated on 96-well plates and grown to confluence (elongated shape), then serum-starved overnight in 50 μl DMEM / 0.5% FBS. Alternatively, SVSMC cells were plated on 96-well plates and grown to confluence (“hill and valley” morphology), and kept in differentiation medium for 6 days (changed once). In the

studies with agonists, plates were taken out of the incubator and instantly treated by adding 50 µl of dexmedetomidine, brimonidine or oxymetazoline solution in DMEM (1 µM final concentration, DMEM alone for controls) carefully on top of the medium for 5–120 s with a 12-well multichannel pipette. Receptor antagonists (atipamezole and rauwolscine; 100 µM final concentration) were added simultaneously with dexmedetomidine. In the studies with inhibitors, cells were pre-treated with 50 µl of each tested inhibitor in DMEM (vehicle for controls) at 37 °C. The concentrations of the inhibitors employed were based on published reports and were as follows: 30 µM ML-7 (10 min), 200 ng/ml PTx (overnight), 100 µM gallein (20 min), 20 µM nifedipine (20 min), 1 µM U73122 (15 min), 10 µM GF 109203X (10–20 min), 10 µM Gö 6976 (20 min) and 10 µM H-89 (15 min). Plates were taken out of the incubator and instantly stimulated with dexmedetomidine as above. Cells were then immediately fixed by adding room-temperature 3X (3.7% final concentration v/v) formaldehyde (20 min at RT) on top of the samples using a digital multichannel pipette with a repeater function to ensure accuracy in treatment timing. After fixation, the cells were washed three times with PBS-H (PBS supplemented with 20 mM Hepes), permeabilized with 0.1% Triton X-100 + 0.1 M glycine / PBS-H for 20 min at RT, and then washed three times with PBS-H. Cells were blocked with 2% BSA / PBS-H for 30 min at RT under gentle agitation, and then labeled with mouse anti-pSer19-MLC antibody (1:2000, Cell Signaling Technology) in DELFIA assay buffer (supplemented with BSA to a final concentration of 1% (PerkinElmer) for 1 h at RT under gentle agitation. The cells were washed 5 times (total 20 min) with TBST (TBS with 0.1% Tween20). Subsequently, cells were incubated with secondary Eu-N1-anti-mouse antibody at a final concentration of 300 ng/ml (PerkinElmer), diluted in DELFIA assay buffer (supplemented with 1% BSA and 2 mM $CaCl_2$), for 45 min at RT under agitation. The cells were washed 8 times with TBST (total 25 min), DELFIA Enhancement solution (PerkinElmer) was added and the plates were shaken vigorously for 15 min with a DELFIA plate shaker (Wallac). Time-resolved fluorescence was read with a Victor V2 multi-label plate reader (615/8.5 nm) (Wallac).

4.7 Data analysis

The results of Study I were analysed with standard methods using GraphPad Prism programs (GraphPad Software, San Diego, CA, USA). Statistical analyses were carried out with Student's *t*-test. *P* values smaller than 0.05 were considered to be statistically significant.

In the myosin light chain phosphorylation assay (Study IV), each treatment had three replicates, and Cook's distance estimation (Cook, 1977) was used to identify outlying replicates. Cook's distance was calculated according to the formula $(\text{mean}-\text{mean\_i})^2/\text{standard error}(\text{mean})^2$, where the mean was the average of all replicates and mean_i was the average of all replicates except replicate i. Replicates with a Cook's distance greater than 0.8 were omitted from further analysis. The area under the MLC

phosphorylation response curve (AUC) was determined for the different compounds used in the experiments according to the trapezoidal rule:

$$AUC = \sum_{t=0\ s}^{t=45\ s} \left(½*(y_1+y_2)*(t_2-t_1) \right)$$

where y_1, y_2 = averaged europium counts from successive samples, t_1, t_2 = successive time points in an experiment. The AUC values of different treatments were compared to the AUC of 1 µM dexmedetomidine in the same experiment and MLC phosphorylation responses were expressed as per cent of 1 µM dexmedetomidine. Graphs were plotted using GraphPad Prism programs. Statistical analyses were carried out with one-sample *t*-tests. *P* values smaller than 0.05 were considered to be statistically significant.

5 RESULTS

5.1 A biochemical test model based on transformed cells (Study I)

5.1.1 Hypothesis

Previous studies had investigated the contribution of a conserved cysteine in the third transmembrane domain ($C_{3.36}$) of the human α_{2A}-AR to ligand binding specificity, and had shown that receptors with a mutation at this residue became resistant to the irreversible α_2-AR antagonist phenoxybenzamine (Frang et al., 2001), and exhibited loss of agonist affinity due to a change in conformational flexibility, as well as a loss of affinity for imidazol(in)e antagonists because of changes in intramolecular interactions (Frang H., et al, unpublished data). It was concluded that $C_{3.36}$ is exposed in the ligand binding cavity and plays an important role in agonist and antagonist binding.

In order to investigate the effects of man-made or naturally occurring mutations on receptor activation and coupling mechanisms and second messenger pathway activation, recombinant protein expression in transformed mammalian cell lines has been commonly employed (Sautel and Milligan, 2000). Cells can be transfected with receptor constructs in plasmid vectors either stably, semistably or transiently. Stable expression involves the integration of the transfected cDNA into the host cell's genome, and most often employs selection of single-cell clones for further propagation (monoclonal cell populations). This technique is commonly used in the pharmacological and functional characterization of receptor subtypes (Marjamäki et al., 1992). Alternatively, the vector can be maintained as an extrachromosomal (episomal) element (termed semistable). If cells are not expanded from a single cell clone, the population is heterogeneous, and the individual cells in the population may express varying amounts of the transfected proteins. Both stable and semistable transfections include culturing the cells under conditions of chronic antibiotic selection. Transient expression systems are useful when experimental results are to be achieved within a short time frame or perhaps for mass-production of receptor proteins for use in receptor binding assays or biophysical structure analysis. The expression of recombinant proteins usually peaks at 24–72 hours post-transfection and is followed by a rapid deterioration in the expression of the transgene because of cell death or loss of the expression plasmid (Cullen, 1987; Colosimo et al., 2000).

The original aim of the work leading to Study I was to compare previously generated mutated receptor constructs, such as the mutation of the cysteine in TM3 ($C_{3.36}$) to valine, for possible effects on second messenger signaling, or more specifically to examine the effect of the mutations on the agonist-dependent inhibition of AC activity, in a similar manner as the effect on effector coupling of another mutation in the α_{2A}-AR had been investigated (Chabre et al., 1994).

5.1.2 Different adenylyl cyclase inhibition in two systems as a consequence of the transfection technique

In previous studies (Frang et al., 2001), the mutated receptor constructs were generated in the pREP4-plasmid, which is episomally maintained (Yates et al., 1985), and CHO cells were transfected semistably. For comparison, a monoclonal cell line stably expressing the human α_{2A}-AR (Pohjanoksa et al., 1997) had been used.

Two cell populations, expressing wild-type human α_{2A}-ARs either stably (Pohjanoksa et al., 1997) or semistably (in pREP4), were compared by receptor density determinations, which established that these two cell populations had almost equal densities of receptors (B_{max} = 1100 and 1200 fmol/mg total cellular protein, respectively). However, when looking closely at the semistable cell population by isolating single cell clones from the population, it was found that receptor density was only an average value of the heterogeneous cell population, as only a small number of the produced monoclonal cell populations expressed receptors at high density (24%) whereas most of the clonal cell populations (76%) had undetectable amounts of α_{2A}-ARs, although they still exhibited antibiotic resistance. Both confocal fluorescence microscopy and flow cytometric analysis supported the view of heterogeneous expression with the pREP4-based constructs.

Forskolin-stimulated AC activity was concentration-dependently inhibited by noradrenaline (NA) in the cell population stably transfected with α_{2A}-ARs, as expected. The extent of AC inhibition was maximally 75% (E_{max} = –74.9 ± 2.4%, $logEC_{50}$ = –7.9 ± 0.1). In contrast, in the other cell population transfected to express α_{2A}-ARs with a non-integrating vector, NA unexpectedly evoked almost no inhibition of forskolin-stimulated AC activity (E_{max} = –15.4 ± 4.3%, $logEC_{50}$ = –8.5 ± 0.1, not significant, P = 0.062), in spite of the identical α_{2A}-AR density in both cell populations (see Fig. 9). However, in the monoclonal cell lines originating from the pREP4-transfected semistable cell population (with B_{max} ranging from 3000 to 6100 fmol/mg protein), dose-dependent inhibition (67–76%) of forskolin-stimulated AC activity was observed, similarly to the stable cell line expressing α_{2A}-ARs. For simplicity, only the population expressing receptors at B_{max} = 5400 fmol/mg protein is visualized in Fig. 9. The extent of this inhibition was E_{max} = –67 ± 2.6%, $logEC_{50}$ = –8.5 ± 0.1. Here is also the stimulation of AC activity notable, as has been documented previously for receptor expression at high receptor density and at high agonist concentrations (Eason et al., 1992).

Cells were then pre-treated with pertussis toxin (PTx) in order to inactivate inhibitory G_i proteins. This resulted in complete loss of AC inhibition by NA both in the stable cell population and in the monoclonal cell populations expressing receptors (isolated from the semistable cell population).

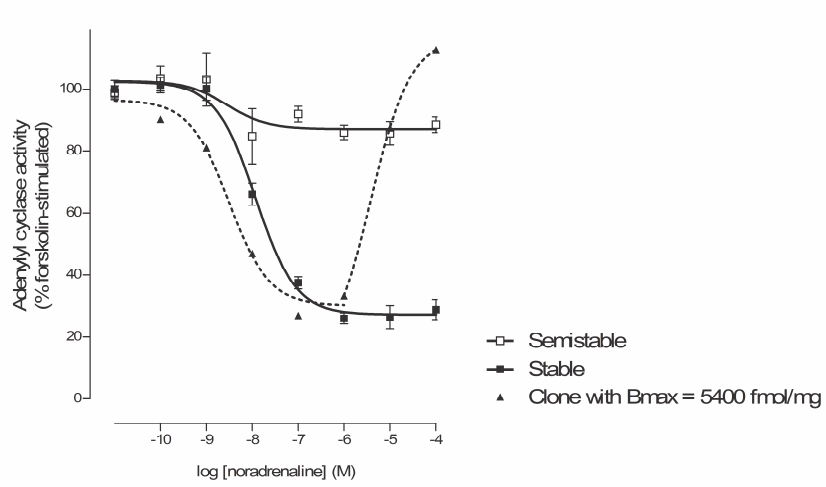

Figure 9. Comparison of the inhibition of forskolin-stimulated AC activity between stably and semi-stably transfected CHO cell populations, as well as in a monoclonal CHO cell population expressing receptors with a B_{max} of 5400 fmol/mg.

It was therefore concluded that in this particular functional assay, AC is stimulated by forskolin to produce cAMP in all cells, but as most cells in the semistable cell population lacked significant amount of receptors, the high receptor density in a few cells was not sufficient to evoke a significant inhibitory response and therefore a false negative pharmacological test result was obtained. Hence, attempts to screen semistably transfected mutant receptor constructs for the functional importance of the mutation in this kind of functional assay would result in incorrect interpretation of the results. Therefore, careful model validation is required, depending on the type of functional assay employed. In the assessment of the capacity of the investigated receptors to mediate inhibition of forskolin-stimulated AC activity, a clonal cell population expressing receptors in a homogeneous fashion is essential.

5.2 Neuronal-like cell models to study receptor trafficking (Studies II and III)

5.2.1 Hypothesis

One general concern in developing or using any functional cell-based screening assay, especially when using heterologous expression systems, is whether a GPCR is correctly targeted to the cell surface in a particular cellular setting, as the host cell might lack the appropriate chaperone and trafficking proteins particular for that GPCR (Allen and Roth, 2011). It has been shown that many neuronally relevant GPCRs, such as the olfactory and taste receptors, as well as α_{2C}-ARs are difficult to express in

heterologous cell systems (Daunt et al., 1997; Saito et al., 2004; Behrens et al., 2006). The α_{2C}-AR subtype is intracellularly retained in non-neuronal cell lines whereas it is targeted to the plasma membrane in neuronal-like cell types (Hurt et al., 2000), and in cultured sympathetic ganglion neurons it displays a distinct spatial and temporal expression pattern (Brum et al., 2006). Therefore, it was hypothesized that neuronal cells could express cell-specific accessory proteins necessary for membrane targeting of α_{2C}-ARs. As the receptor transporting proteins (RTP1–4) and receptor expression enhancing proteins (REEP1–6) were shown to rescue the plasma membrane expression of odorant receptors (Saito et al., 2004), it was speculated that co-expression of these proteins with the α_{2C}-ARs would target this receptor subtype to the cell surface in non-neuronal cell lines. As the nature of the cell plays a significant part in determining the receptor surface expression of α_{2A}- vs. α_{2C}-ARs, it was also hypothesized that this system would serve as a good model to investigate trafficking differences between these receptor subtypes as well as to further elucidate the differential properties of the α_{2A}- and α_{2C}-AR subtypes in neurons.

5.2.2 Differences in the capability to express and transport α_2-adrenoceptors in non-neuronal and neuronal cell models – roles of accessory proteins

RT-PCR was employed to identify differences in the cellular expression profiles of RTP1–4 and REEP1–6 in neuronal vs. non-neuronal cell lines. The results (see Table 5, unpublished data) revealed that REEP1, 2 and 6 mRNA were found in neuronal-like cell lines (PC12 and AtT20) but were absent in non-neuronal cell lines (NRK, Rat1). Consequently, REEP1, 2 and 6 were selected for further studies.

In order to delineate the specific roles that REEPs play in the trafficking of α_2-ARs, it is essential to determine their specific location of expression. Therefore co-localization was investigated with plasma membrane (avidin) or ER- (calreticulin) and Golgi-markers (giantin) and with both immunocytochemistry and live cell plasma membrane biotinylation. Avidin was co-localized with α_{2A}-ARs and to a minor extent with α_{2C}-ARs, but not at all with REEPs. Instead, REEPs showed extensive co-localization with ER- but not with the Golgi-markers, confirming that REEP1, 2 and 6 are ER-resident proteins. This was further corroborated with sucrose gradient experiments, in which none of the REEPs tested were found in the plasma membrane fraction (where the α_{2A}-ARs were localized), but instead in the ER-fraction. Therefore, this REEP subset resides in the ER and does not appear to traffic to or reside at the plasma membrane at detectable levels.

Table 5. mRNA expression profiles of rat or mouse RTP1−4 and REEP1−6 in several cell lines. Control data in white, neuronal cells in light grey and non-neuronal cells in dark grey. RB; rat brain cDNA, ME; mouse embryo cDNA, MB; mouse brain cDNA, PC12; pheochromocytoma cells from the rat adrenal medulla, AtT20; mouse pituitary gland tumor cell line, NRK; normal rat kidney epithelial cells, Rat1; rat fibroblast cell line. Symbols: +, present; −, absent (not detected); +/−, very weak expression.

	RB	ME/MB	PC12	AtT20	NRK	Rat1
RTP1	+	+	−	+	+	−
RTP2	+	+	−	+	−	−
RTP3	+	+	+/−	+	+	+
RTP4	+	+	−	+	+	+
REEP1	+	+	+	+	−	−
REEP2	+	+	+	+	−	−
REEP3	+	+	+	+	+	+
REEP4	+	+	+	+	+	+
REEP5	+	+	+	+	+	+
REEP6	+	+	+	+	−	−

To investigate the possible interaction of either α_2-AR subtype with REEPs in the ER, α_{2A}- or α_{2C}-ARs were co-expressed with either REEP1, 2 or 6 in HEK293A cells. It was demonstrated that of all REEPs examined, none showed any apparent co-localization with α_{2A}-ARs within the ER. However, α_{2C}-ARs and REEP1, 2 and 6 did show some small regions of co-localization and thus possible sites of interaction within the ER, which is the predominant site of α_{2C}-AR localization (Fig. 10).

To further quantitatively delineate the specific roles that REEPs play in α_2-AR processing, receptor binding studies and single cell fluorescence-activated cell sorting (FACS) analysis were performed. The results suggested that co-expression of α_2-ARs with REEP1, 2 or 6 enhanced the cargo capacity of ER (intracellular expression), but did not specifically elevate plasma membrane expression of either α_{2A}- or α_{2C}-ARs, but on the contrary REEP1, 2 and 6 decreased the total membrane expression of α_{2A}-ARs. Glycosylation analysis revealed that co-expression of REEPs with α_2-ARs could lead to specific alterations in cargo glycosylation, possibly due to changes in ER/Golgi processing or retention. Hence, this REEP subset appears to interact with and alters glycosidic processing of the α_{2C}-AR, but not the α_{2A}-AR, which demonstrates selective interactions of REEPs with their cargo proteins, in line with the results of Saito et al. (2004). Furthermore, co-immunoprecipitation studies revealed that REEPs selectively only interacted with α_{2C}-ARs, and more specifically, enhanced expression and their interaction with minimally/non-glycosylated forms of the α_{2C}-AR, and not the mature

glycosylated forms of α_{2A}- or α_{2C}-ARs. This indicates that REEP proteins are highly selective in their choice of which intracellular proteins they process.

The expression of REEPs at the protein level was investigated in cell lysates of some of the cell lines screened with RT-PCR. The analysis showed no expression of REEP1 in the tested cell lines (HEK293/A, Rat1, PC12, NRK). This was surprising, bearing in mind that REEP1 expression was detected in PC12 cells by RT-PCR. However, it is important to remember that RT-PCR is an extremely sensitive technique and can detect very low copy numbers of mRNA (Bartlett, 2002). REEP2 immunoblotting revealed expression in HEK293/A as well as PC12 cells but not in Rat1 or NRK cells, in line with RT-PCR data.

Detection of REEP expression in mouse tissues by immunoblotting demonstrated that REEP1 protein is not ubiquitous; its expression was relatively restricted to neuronal tissues whereas REEP2 protein expression was restricted to neuronal tissues (brain, spinal cord) and other cells that exhibit neuronal-like exocytosis mechanisms (pituitary, adrenal gland, testis). The time course of REEP1 expression in cultured SGNs was also determined and, interestingly, REEP1 expression was time-dependent and appeared at about the same time that α_{2C}-ARs became visible at the neuron plasma membrane (Brum et al., 2006), pointing to a possible role for REEPs in the neuron-specific processing of α_{2C}-ARs.

5.3 Vascular smooth muscle cell models (Study IV)

5.3.1 Hypothesis

Primary cells are regarded as fully differentiated cells with close approximation of native cell functions. It was decided to establish cultures of human vascular smooth muscle cells endogenously known to express α_2-ARs in order to examine the ability of α_2-AR agonists to evoke smooth muscle cell contraction, and to validate their use in a functional assay measuring the biochemical endpoint of contraction, e.g. phosphorylation of myosin light chains (MLC phosphorylation), as well as to compare these results to those acquired from a transformed smooth muscle cell line transfected to express the human α_{2B}-AR subtype (Huhtinen and Scheinin, 2008). The ultimate aim was to develop a functional assay to be used as a tool for the pharmacological characterization of the capacity of α_2-AR agonists and antagonists to regulate vascular smooth muscle cell contraction, since subtype-selective α_2-AR agonists and antagonists may have important applications in cardiovascular drug development.

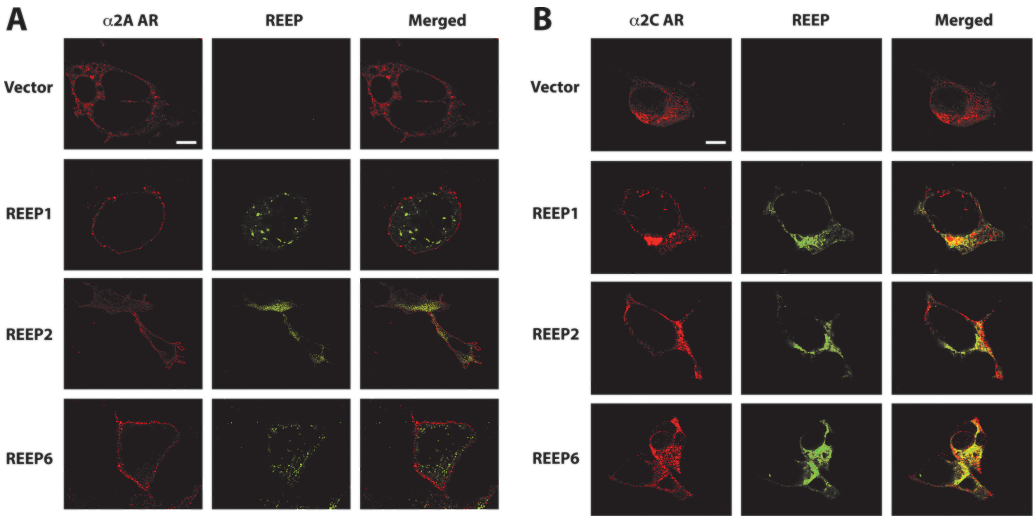

Figure 10. Co-localization of α_2-ARs and REEPs in permeabilized cells visualized with confocal fluorescence microscopy. HEK293A cells were co-transfected with HA-α_{2A}-AR (A) or α_{2C}-AR cDNAs (B) and either empty vector (pcDNA3.1), Flag-REEP1, -REEP2, or –REEP6 cDNAs. Cells were fixed, permeabilized, and examined by confocal microscopy 48 h hours post-transfection. Scale bars: 25 μm. Figure from Study II.

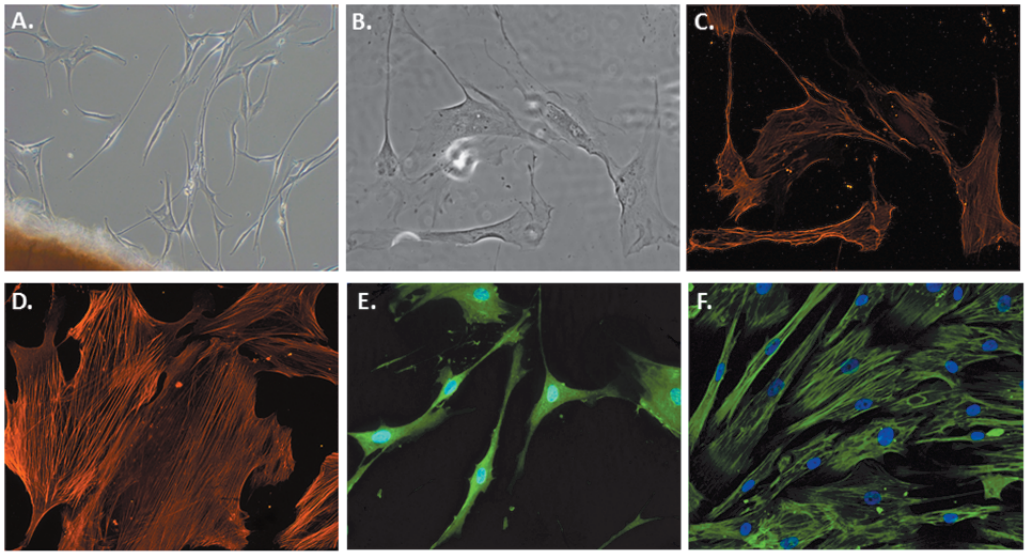

Figure 11. Immunofluorescent labeling of primary saphenous vein smooth muscle cells. (A) Phase contrast image of primary SMCs migrating out of saphenous vein media explants. (B) and (C) A population of SVSMC cells in visual light (B) and staining against smooth muscle α-actin (C). (D) Staining against α-actin in differentiated SVSMCs. (E) Proliferating and (F) differentiated SVSMCs stained against myosin light chains. Cell nuclei stained with DAPI shown in blue.

5.3.2 Establishment of primary cultures of human vascular smooth muscle cells and characterization of endogenous α_2-adrenoceptor expression

Prior to the establishment of routine primary culture of saphenous vein smooth muscle cells (SVSMC) several parameters had to be optimized. These included the validation of different culture vessel coating materials (plastic, fibronectin, collagen, Matrigel Matrix or poly-L-lysine) as well as different cell culture media formulations and supplements. As the cells are growing in culture, a number of criteria must be used to ascertain that the primary cultures indeed are composed of SMCs. Smooth muscle α-actin has been commonly used to identify SMCs (Skalli et al., 1986). However, both endothelial cells and fibroblasts have been reported to express small amounts of this protein and therefore additional markers are required. These include myosin heavy and light chains, calponin, SM22α, desmin, h-caldesmon, metavinculin and smoothelin (Shanahan et al., 1993; Owens, 1995; Proudfoot and Shanahan, 2012). Immunofluorescent labeling of SVSMCs revealed lack of expression of the endothelial marker CD31 (data not shown) which excluded the presence of endothelial cells in the culture. All cells were labeled with an antibody against smooth muscle α-actin, as visualized in panel C and D of Fig. 11. Fig. 11C is taken with the same acquisition window as the visual light image in panel B. The staining of cells in panel D extended over the entire length of the cell, crossing over the nuclear region and aligned in a non-parallel manner. Additionally, both proliferating and differentiated SVSMCs stained with an antibody against myosin light chains (MLC), as shown in panels E and F of Fig. 11. RT-PCR analysis confirmed the expression of smooth muscle α-actin and MLC, and also revealed the expression of calponin, further confirming the SMC specificity of the cell population. In accordance to the published literature (Chotani et al., 2004), RT-PCR analysis also disclosed that in cells isolated from human saphenous veins, the α_{2C}-AR subtype was the most prominent α_2-AR subtype expressed; this is also the subtype reported to mediate contractions of veins to NA stimulation *in vitro* (Docherty and Hyland, 1985; Gavin et al., 1997; Rizzo et al., 2001; Giessler et al., 2002). In addition, some α_{2A}-AR expression was also detected, but at lower levels compared to α_{2C}-AR expression (unpublished data), as also reported previously with RNase protection assay (Chotani et al., 2004)

5.3.3 α_2-Adrenoceptors evoke myosin light chain phosphorylation in vascular smooth muscle cell models

As phosphorylation of myosin light chains can be considered as a biochemical readout of smooth muscle cell contraction (Somlyo and Somlyo, 1968; Small and Sobieszek, 1977), we developed a cell-based quantitative assay to measure this end-point in order to be able to investigate α_2-AR-evoked contraction of both primary and transformed vascular smooth muscle cells.

Dexmedetomidine was chosen as the reference agonist, since it is the most selective α_2-AR agonist currently available (α_2/α_1-AR selectivity ratio = 1620) and a full agonist for the α_{2B}-AR (Peltonen et al., 1998). Therefore, as a control for cell responsiveness, a set of samples treated with dexmedetomidine was included in all experiments and all other compounds were related to the MLC phosphorylation response evoked by 1 μM dexmedetomidine. The dexmedetomidine-induced myosin light chain phosphorylation was assessed over a period of two minutes. In A7r5-α_{2B} cells, the dexmedetomidine response typically peaked at 20–45 s with an E_{max} value of approximately 60% over vehicle control, as shown in Fig. 12A. The treatment of wild-type (non-transfected) A7r5 cells with dexmedetomidine had no effect at all, and treatment with the α_2-AR antagonists atipamezole and rauwolscine blocked the dexmedetomidine-evoked MLC phosphorylation response, both strongly indicating that the MLC phosphorylation response seen in A7r5-α_{2B} cells was mediated by α_{2B}-ARs (Fig. 13). Finally, stimulation of endogenously expressed vasopressin V_1 receptors with 100 nM arginine vasopressin evoked similar MLC phosphorylation responses to dexmedetomidine in A7r5-α_{2B} cells (Fig. 12A). However, in differentiated human primary vascular smooth muscle cells from the saphenous vein, stimulation of endogenously expressed α_2-ARs with brimonidine (1 μM) or V_1 vasopressin receptors (Medina et al., 1998) with arginine vasopressin (100 nM) resulted in no more than 18 or 20% stimulation of the MLC phosphorylation response over vehicle control levels (Fig. 12B).

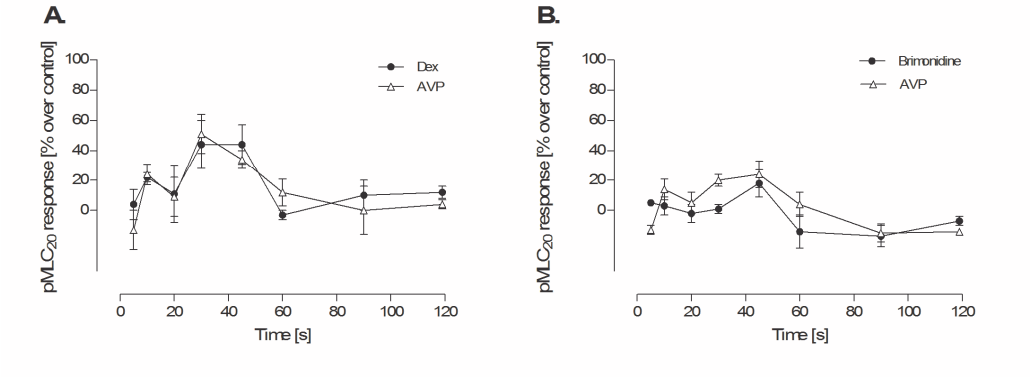

Figure 12. Agonist-induced myosin light chain phosphorylation in A7r5-α_{2B} cells (A) and in differentiated primary human vascular smooth muscle cells (B). Confluent and differentiated cells were treated with (A) dexmedetomidine (Dex, 1 μM) or arginine vasopressin (AVP, 100 nM), (B) with brimonidine (1 μM) or arginine vasopressin (100 nM). Normalized data are expressed as per cent over vehicle controls. The graphs are representative of individual experiments repeated at least three times in triplicate.

For the method validation, an inhibitor of myosin light chain kinase (ML-7) was used as a negative control, and the results showed that this completely abolished all dexmedetomidine-evoked MLC phosphorylation responses (Fig. 13). Immunoblotting experiments performed on cells treated in the same way as for the myosin light chain phosphorylation assay revealed that the antibody recognized proteins of the expected size, namely 18 kDa.

In order to explore the intracellular pathways involved in the α_{2B}-AR-mediated phosphorylation of myosin light chains, A7r5-α_{2B} cells were exposed to different inhibitor compounds. The results of all experiments are summarized in Fig. 13. Exposure to PTx eliminates the capacity of G_i type G proteins to be activated by their cognate receptors. Pre-treatment of the cells with PTx almost totally abolished the dexmedetomidine-evoked MLC phosphorylation response, suggesting that the response was G_i-mediated. An inhibitor of the Gβγ subunit (gallein) almost completely abolished (by 93 ± 6%) the dexmedetomidine-evoked MLC phosphorylation response, which points to an important role for Gβγ subunits in the MLC phosphorylation response. Not unexpectedly, also L-type Ca^{2+} channels seem to be crucial for the response, as treatment with nifedipine, an L-type Ca^{2+} channel blocker, attenuated the dexmedetomidine-evoked MLC phosphorylation response by 66 ± 12%. Inhibition of phospholipase C with U73122 completely abolished the dexmedetomidine-evoked MLC phosphorylation response, indicating that also phospholipase C is involved in the intracellular pathways leading to the phosphorylation of myosin light chains. Unexpectedly, the protein kinase C inhibitors GF109203X and Gö6976 very markedly potentiated the dexmedetomidine-induced MLC phosphorylation response (to 323 ± 74% and 300 ± 51% of the response to dexmedetomidine alone), while nifedipine blocked the combined effects of dexmedetomidine and protein kinase C inhibition, possibly indicating that increased influx of calcium ions via L-type Ca^{2+} channels is necessary for the potentiation of the dexmedetomidine-induced MLC phosphorylation response following pre-treatment with the protein kinase C inhibitors. Finally, blockade of protein kinase A with H-89 augmented the MLC phosphorylation response to 211 ± 22%. Control experiments were performed with the different inhibitors alone and all combination treatment results were normalized to their own controls (inhibitor alone).

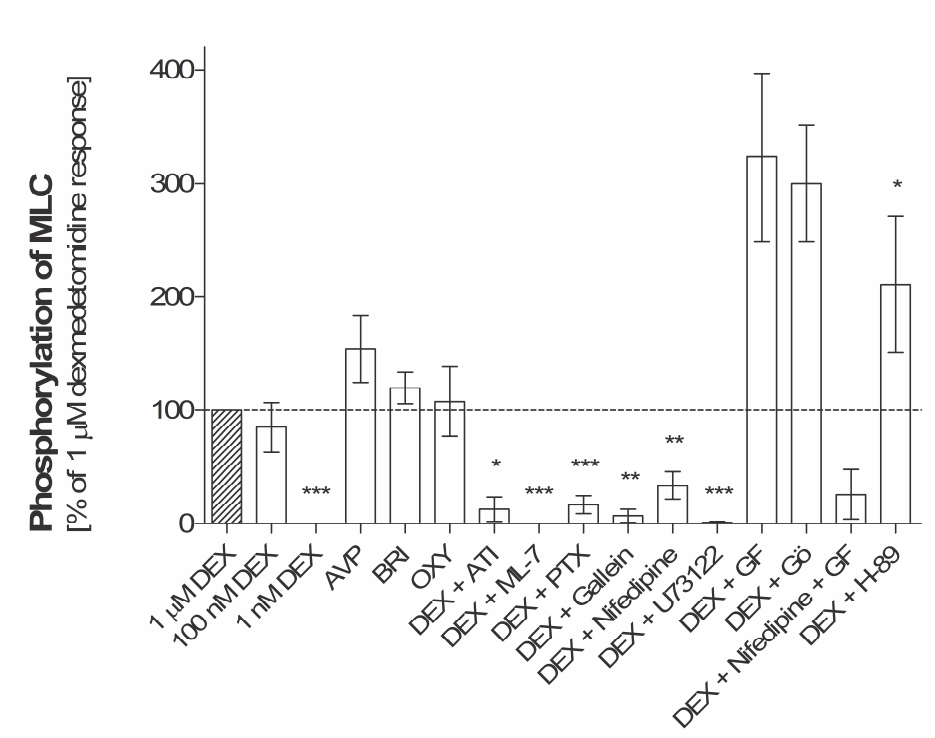

Figure 13. Effects of agonists, antagonists and inhibitors on the phosphorylation of myosin light chains in A7r5-α_{2B} cells, in relation to the MLC phosphorylation response (AUC) evoked by the α_2-AR agonist 1 μM dexmedetomidine (100%). Agonists included arginine vasopressin (AVP, 100 nM), brimonidine (BRI, 1 μM), oxymetazoline (OXY, 1 μM) and dexmedetomidine (DEX, 1 nM – 1 μM). The α_2-AR antagonist atipamezole (ATI, 100 μM) was added simultaneously with dexmedetomidine. Pretreatment times with the inhibitors were 10–20 min (overnight for PTx) and the employed inhibitors were: ML-7 (myosin light chain kinase inhibitor, 30 μM), pertussis toxin (PTx; G_i protein inhibitor, 200 ng/ml), gallein (Gβγ subunit inhibitor, 100 μM), nifedipine (L-type calcium channel blocker, 20 μM), U73122 (phospholipase C inhibitor, 1 μM), GF109203X (GF) and Gö6976 (Gö) (protein kinase C inhibitors, 10 μM) and H-89 (protein kinase A inhibitor, 10 μM). Results are means ± S.E.M. of at least three individual experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to 1 μM dexmedetomidine. Combined treatments with dexmedetomidine and GF and Gö failed to reach statistical significance ($p = 0.05 – 0.10$) because of the small number of observations. Figure from study IV.

6 DISCUSSION

6.1 Challenges and future prospects in drug discovery of novel subtype-selective compounds

GPCRs regulate a wide array of physiological processes and represent the largest group of targets for drug discovery for a broad spectrum of diseases (Kobilka, 2013). In the recent years the wealth of new structural information on GPCRs has broadened the view of receptor function, especially with regard to how receptors are activated by ligand binding and stabilized in different conformations that allow their messages to be conveyed further in the cells (Audet and Bouvier, 2012). Ten years ago, all model-based three-dimensional *in silico* predictions of drug-receptor interactions had been outlined from speculative homology models based on the structure of rhodopsin (Rosenbaum et al., 2009; Granier and Kobilka, 2012). Now that the crystal structure of the β_2-AR in complex with its ligand and associated G_s protein has been resolved, computational biologists have a whole new starting point, namely a high-resolution view of transmembrane signaling in a GPCR, allowing for much more detailed experimental design when investigating complex formation, ligand binding, G protein binding and complex dissociation (Granier and Kobilka, 2012). Structurally different ligands can stabilize functionally distinct receptor conformations, which in turn has consequences for intracellular signaling and the regulatory proteins which will be activated. Protein dynamics also are affected by the lipid environment and associated proteins (including other GPCRs) (Granier and Kobilka, 2012; Nygaard et al., 2013), and a better understanding of GPCR dynamics is therefore expected to contribute to the development of more selective and efficacious drugs. In contrast to conventional drug design where the ligand binding pocket is targeted, the G protein binding pocket can now additionally be targeted by designing diffusible ligands that are able to penetrate within the cells to modulate GPCR function (Granier and Kobilka, 2012), e.g. one could envisage drugs acting at the β and γ subunits of G proteins (Bonacci et al., 2006; Smrcka et al., 2008). Still, many questions remain unanswered, including the reasons why a GPCR preferentially couples to a certain G protein isoform and the specific intracellular pathways that are activated as a consequence. To gain such new knowledge, we need structures of GPCRs in complex with G_i, G_q and other G proteins. One can speculate that future structural work will be directed towards determining how GPCRs form complexes with other signaling proteins at the molecular level (Granier and Kobilka, 2012; Kobilka, 2013).

The different members of a GPCR subfamily may have unique but overlapping and sometimes even opposing functions, and the pharmaceutical industry therefore is attempting to design drugs that target a specific receptor subtype, in order to avoid adverse effects that often are a consequence of non-specific binding to some other receptor subtype. As the high homology between different members of the GPCRs was discovered, the challenges of developing subtype-selective drugs were revealed (Granier and Kobilka, 2012), e.g. the conserved transmembrane regions between

members of GPCR subfamilies, such as for the α_2-ARs, clearly represent significant restraints on drug structures (Bylund et al., 1992). Consequently, even today, the number of subtype-selective compounds for α_2-ARs is very limited and even though Orion Corporation has presented promising Phase II data on an α_{2C}-AR selective compound (ORM-12741) intended for treating Alzheimer's disease (poster presented at the American Academy of Neurology Annual Meeting, 2013), few subtype-selective drugs have entered clinical trials.

Cell-based models are employed as an initial screening tool in the development of these kinds of subtype-selective compounds into drugs,. The current drug discovery process has discovered numerous drugs using the target-focused approach, where a large chemical library is screened with biochemical and cell-based assays to identify lead compounds that affect this target. However, R&D efficiency, measured simply in terms of new drugs entering the market by the global biotechnology and pharmaceutical industries per billion US dollars spent on R&D, has declined (Scannell et al., 2012), and it has been claimed that the target-centric approach has contributed to the current high attrition rates and low productivity in pharmaceutical research and development (Swinney and Anthony, 2011). Many diseases, such as coronary heart disease, asthma and diabetes, are complex entities and therefore are unlikely to be cured by a single drug, a "magic bullet". If one wishes to improve drug development for complex diseases, more broadly based screens must be employed, making use of network medicine (Babcock and Li, 2013; Silverman and Loscalzo, 2013). This takes into account the off-target effects associated with the drug, which are often neglected in simple target-based screens, but subsequently prove to be the cause of an adverse effect triggered by the drug. Therefore, cell-based systems engineered to incorporate disease-relevant complexity may cast a wider net than the target-based approach. Here, the focus will be on the drug candidate itself and leads will be identified and optimized based on biological responses that they elicit in a complex cell system that models diverse active cells, pathways and networks in the disease state(s) of interest (Butcher, 2005). The leads are screened against multiple targets using various -omics readouts in cell-based assays, animal models and human trials. Clinical trials will be further adapted to incorporate newly discovered information on dynamic combinations of drug treatments, which is essential for the implementation of network medicine. Finally, these network and systems-based therapeutics coupled with well-defined disease phenotyping will optimize the successful achievement of personalized drug therapy (Silverman and Loscalzo, 2013). In this context, interest has emerged on developing inhibitor compounds for RGS proteins (Sjogren et al., 2010) and for α and $\beta\gamma$ subunits of G proteins, and thus targeting key steps in specific disease-related signaling pathways activated by multiple receptor subtypes (Smrcka, 2013).

For a drug screen to be relevant and predictive of the *in vivo* drug response, this *in vitro* response must be replicated in humans. In target-based drug discovery, lead compounds are not tested until later on in the pipeline for their efficiency in actual disease-relevant phenotypes, and even later against human disease, at the earliest in clinical Phase II trials. Apart from adapting the principles of network medicine,

induced pluripotent stem cell (iPSC) technology provides a possible solution to this issue as aspects of disease can be introduced already in the initial cell-based screens. Screening with "disease-in-dish" iPSC assays against focused libraries of molecules represents a powerful chemical and functional genomics approach to identify new drug targets and novel lead compounds, with the end-point monitoring some cellular alteration e.g. the morphology, behaviour or physiology of the cells in culture rather than a specific biochemical activity of a selected target protein. Systems biology tools are subsequently employed to design an interaction network, in which the candidate target together with its interacting genes/proteins are mapped. After validation and refinement, this network is then analyzed for druggable targets, using conventional target-based screening approaches (Mercola et al., 2013). This ability to provide systems that more closely approximate human cell biology and disease states mean that these "in-dish" iPSC disease models might allow earlier entry of patients into clinical trials (Bellin et al., 2012).

6.2 Aspects influencing the use of transformed cell line models

Immortalized, transformed cell lines have been employed as a reliable source of easily grown, infinitely-dividing cells that can be transfected to express the target receptor of interest which can clarify the ligand binding characteristics of a specific GPCR subtype as well as its coupling to different signaling components in a controlled well-defined cellular environment (Horrocks et al., 2003; Siehler, 2008; Kenakin, 2009). For these reasons, these cells are employed in the early screening processes of the drug development pipeline. However, many limitations are associated with the use of transformed cells, and careful model validation as well as data interpretation is essential, as demonstrated in Studies I–III of this thesis.

Twenty years ago, targeted mutagenesis was extensively employed to investigate receptor structure and function (Bikker et al., 1998; Sautel and Milligan, 2000). Molecular structures of GPCRs can be modeled with *in silico* methods and, together with data from sequence alignments, it can be used to elucidate important amino acid residues involved in either ligand docking or G protein activation. Mutant receptor constructs can then be designed to target these residues, and data from these experiments may provide valuable insights into molecular mechanisms of ligand binding, receptor folding, receptor activation, G protein coupling and regulation of GPCRs (Kristiansen, 2004; Hulme, 2013). The mutated receptor constructs are expressed in heterologous cell models and the effects of the mutation can be evaluated with receptor binding experiments and functional assays.

The starting point of Study I was to evaluate how a series of mutations in the third transmembrane region of the human α_{2A}-AR could interfere with second messenger generation, in a functional assay measuring coupling to adenylyl cyclase (AC) activity, an assay previously validated and optimized here and by others. However, it was noted that two cell populations containing similar numbers of recombinant human wild-type α_{2A}-ARs, but transfected with different techniques, yielded dramatically dissimilar

results. One of the cell populations, stably transfected with an integrating vector, exhibited the expected dose-dependent inhibition of forskolin-stimulated AC activity, whereas the other cell population, transfected with a non-integrating episomal vector (semistable) displayed no detectable AC inhibition. It was revealed that most cells in the latter cell population had undetectable amounts of α_{2A}-ARs, probably due to variable numbers of gene copies in the individual cells. When AC activity was stimulated with forskolin in all cells, a false negative pharmacological test result was achieved, due to the domination of cells expressing too few α_{2A}-AR gene copies. If one had utilized this kind of cell population with uneven receptor expression, it would consequently have led to a false negative pharmacological test result and incorrect interpretation of the impact of the induced mutation in the receptor construct. This highlights the importance of careful validation and optimization of the cell model, as well as emphasizes the importance of using control cell lines that have been treated as far as possible in a similar manner as the ones to be screened for the effect of the mutation.

When employing assays based on heterologous cell expression, it is essential to ensure that the GPCRs are correctly targeted to the cell surface in a particular cellular setting, and if not, to investigate whether this is due to a methodological concern such as the host cell lacking appropriate chaperone and trafficking proteins particular for that GPCR, or whether there is an underlying physiological reason. The expression of a GPCR is a complex process that includes protein folding, post-translational modifications, and transport through cellular compartments including the endoplasmic reticulum (ER) and Golgi apparatus to the final destination of the GPCR (most often the plasma membrane). The transport and delivery of GPCRs to their functional destination is governed by the cells' own exquisitely precise control system transporting and delivering cellular cargo, the importance of which was acknowledged recently; the discovery of the basic mechanisms behind this machinery was the focus for the award of the 2013 Nobel Prize in Physiology or Medicine. Disturbances in this transport system are involved in many diseases e.g. contributing to diabetes, immunological disorders and neurological ailments, such as hereditary spastic paraplegia (HSP).

With regard to the cellular localization of α_2-ARs, it has been shown that non-neuronal heterologous cell systems (e.g. NRK, HEK293 cells) are unable to traffic α_{2C}-ARs to the cell surface, whereas neuronal-like cells (such as PC12) as well as cultured mouse sympathetic ganglion neurons are capable of trafficking α_{2C}-ARs to the plasma membrane. In contrast, α_{2A}-ARs are efficiently targeted to the plasma membrane in both non-neuronal and neuronal cells (Hurt et al., 2000; Brum et al., 2006). Therefore, it seems evident that neuronal cells possess the appropriate factors/proteins that allow efficient delivery and targeting of the receptors to the plasma membrane, especially in view of the fact that the physiological expression of α_{2C}-ARs is primarily a neuronal phenomenon. Physiological differences in the functions of these receptor subtypes have also been documented, revealing that α_{2A}-ARs inhibit neurotransmitter release from sympathetic nerve endings at high stimulation frequencies, whereas α_{2C}-ARs

modulate neurotransmission at lower levels of nerve activity (Hein et al., 1999). This was utilized as a model to investigate trafficking differences between these receptor subtypes and to investigate the potential roles of accessory proteins in the transport of these receptor subtypes, attempting to clairfy the different properties of α_{2A}- and α_{2C}-ARs in neurons. This was done by comparing mRNA expression profiles of four receptor transporting protein (RTP) and six REEP family members (previously shown to play important roles in cellular transport), between several non-neuronal and neuronal cell lines. Apart from REEPs, several proteins have been identified as chaperone proteins possessing cell surface regulatory functions, e.g. receptor activity modifying proteins (RAMPs) have been reported to assist in the transport of the calcitonin-receptor-like receptor to the cell surface (McLatchie et al., 1998). However, these proteins did not appear to have any role in the transport of the α_{2C}-AR to the cell surface (Hurt et al., unpublished data). The present data revealed that REEP1, 2 and 6 were expressed in neuronal cells, but not in non-neuronal cells and the effect of these proteins on the intracellular regulation of $\alpha_{2A/C}$-ARs was therefore investigated. The results demonstrate that REEPs did not specifically enhance surface expression of either α_2-AR subtype, but influenced ER cargo capacity and thus the total expression of α_{2C}-ARs elevated. Immunofluorescence microscopy and biochemical assays revealed a consistent theme of ER localization of REEP1, 2, and 6, which was also shown to be the site for α_{2C}-AR interactions. REEP co-expression with α_2-ARs revealed that distinct REEP members were able to interact with and alter glycosidic processing of only α_{2C}- but not α_{2A}-ARs, demonstrating selective interactions with cargo proteins.

What determines the selectivity of REEPs for certain GPCRs? When these proteins were originally identified, it was noted that REEP1 preferentially enhanced plasma membrane expression of odorant receptors but not expression of the β_2-AR (Saito et al., 2004), and similarly, REEP members selectively interacted with one subtype over the other in the comparison α_{2A}- vs. α_{2C}-ARs. It is possible that the transit time through the ER may explain the relative responsiveness of different GPCRs to REEP modulation or interactions. Both α_{2A}- and β_2-ARs might be quickly processed in the ER and further transported to the plasma membrane, and therefore increasing the ER cargo capacity by REEP co-expression would not affect their plasma membrane delivery, whereas heterologously expressed α_{2C}-ARs are retained in the ER and thus have more time to interact with REEPs.

Previously performed RT-PCR analysis have revealed human REEP1 mRNA expression in many different tissues, e.g. brain, muscle, testis, liver, kidney, lung, and several endocrine organs (Zuchner et al., 2006). However, our results demonstrated that REEP1 and REEP2 protein expression was restricted to neuronal cells and other cells that exhibit neuronal-like exocytosis, which is in line with the involvement of these REEPs in neurodegenerative diseases, such as HSP (Park and Blackstone, 2010). Possible explanations for the differences in the results include methodological issues. Zuchner et al., (2006) utilized RT-PCR analysis of tissue samples, which might make possible the detection of low copy numbers of mRNAs, while a translated protein

encoded by the mRNA might not necessarily be detected. Furthermore, the results presented here, regarding the neuronal specificity of REEP expression and the REEPs' roles as ER adapter proteins, were corroborated in a concurrently published study, in which REEP1 knockout in mice revealed that loss of this protein evoked serious defects in ER organization. The authors of that report further showed that at the molecular level, REEP1 is a neuron-specific, membrane-binding, and membrane curvature-inducing protein that resides in the ER (Beetz et al., 2013).

It was concluded from the present studies, that REEPs are ER-resident proteins that have additional intracellular functions in addition to altering ER structure; these include enhancement of ER cargo capacity and the ability to selectively modulate membrane expression of some GPCRs, the regulation of ER-Golgi processing, as well as interactions with specific cargo proteins. Therefore, some REEPs can be further described as ER membrane shaping adapter proteins. Furthermore, expression of the REEP1/REEP2 subfamily appears to be restricted to neuronal and neuronal-like exocytotic tissues, consistent with the neuronally restricted symptoms of REEP1 genetic disorders (Park et al., 2010). Moreover, there is a parallel between the time course of appearance of REEP1 expression and α_{2C}-ARs localizing to the neuronal plasma membrane in cultured SGNs. It might be interesting to undertake a detailed biochemical analysis of possible interactions between REEP1 and α_{2C}-ARs in cultured SGN, at present this is not possible to perform since the amount of protein available from culture SGN is low. It therefore seems that REEPs significantly influence the regulation of α_{2C}-AR processing in neuronal cells, but the spectrum of other events required in the process still remains to be characterized. The present results suggest that REEPs may serve important functions in neuronal vesicle trafficking, especially in the ER-Golgi vesicular transport regulation, as has been observed with other Yip family members. Future aspects of investigating REEP functions could include the determination of the specific domains involved in the interaction of REEPs with its cargo, or to elucidate possible interactions of REEPs with transport/vesicle proteins and to what extent mutations associated with REEP1 can be connected to these processes.

6.3 Considerations and prospective applications of vascular smooth muscle cell models

Screening for drug efficacy or second messenger activation is a difficult task, and the more one can characterize different cell model systems with their potential benefits and drawbacks, the more likely the search for new and better drug compounds will be successfull. Primary cells more genuinely represent the *in vivo* physiological state of differentiated cell types, e.g. vascular smooth muscle cells (VSMC) are therefore considered important tools in research investigating how VSMCs function and contribute to blood vessel wall contraction under both normal and pathological conditions (Proudfoot and Shanahan, 2012). With that thought in mind, primary cultures of human VSMCs, endogenously expressing α_2-ARs, were established so that

they could be used as models for investigating α_2-AR expression, function and signaling in this important cardiovascular target tissue (unpublished observations by the author).

There are several different techniques of acquiring cultures of SMCs (Campbell and Campbell, 1993). The explant method was used here, since enzyme dispersion would not have been possible with the saphenous vein grafts due to their small size (~2 cm). The enzyme dispersion technique is more commonly used for example with the rat aorta. As the SMCs are placed in culture they undergo a spontaneous phenotypic change over the first five days and proliferate until they form a confluent layer (Chamley-Campbell et al., 1979; Proudfoot and Shanahan, 2012). Irrespective of whether one adopts the enzymatic or the explant outgrowth technique, the cells have been reported to de-differentiate to a synthetic proliferative phenotype (Proudfoot and Shanahan, 2012). If this phase is not extended over more than five cumulative population doublings, the cells tend to revert back to a contractile state within 1–2 days of confluency being reached. However, if they have been seeded too sparsely and need more than five doublings to reach confluency, they will never revert to the contractile phenotype (Chamley-Campbell et al., 1979; Chamley-Campbell and Campbell, 1981; Campbell and Campbell, 1993); the same phenomenon occurs with passaging the cells several times. Alterations in the expression of cytoskeletal proteins accompany the process of de-differentiation. Several studies have documented changes in the expression of contractile proteins, e.g. decreased expression of e.g. α-smooth muscle actin, smooth muscle myosin heavy chain, calponin and desmin (Chamley-Campbell et al., 1979; Shanahan et al., 1993; Owens, 1995; Worth et al., 2001). Furthermore, the change in phenotype is associated with reorganization of the proteins, with contractile cells displaying a distinct sorting of structural proteins within the cytoplasm that is consistent with the compartmentalization of these proteins into functional domains, whereas transition to the synthetic phenotype involves even spreading of cytoskeletal proteins that is accompanied by the loss of the cell's contractile properties (Worth et al., 2001). In addition, loss of expression of certain GPCRs may accompany the switch to the synthetic phenotype (Jarrousse et al., 2004). The change in phenotype is not only restricted to cultured cells, but there are previous observations that cells *in vivo* may also undergo this switch in the presence of different physiological disease conditions such as atherosclerosis (Ross and Glomset, 1973). However, evidence from studies on human vascular diseases *in vivo* has demonstrated that the definition of VSMCs as either "synthetic" or "contractile" is over-simplistic. VSMC heterogeneity has been observed in human blood vessels *in vivo* in different vascular beds in the sub-intimal layer, in different cell layers within the media as well as in atherosclerotic plaques. In cell culture, they can also exhibit heterogeneity in terms of size, protein expression and proliferation rates (Proudfoot and Shanahan, 2012). A challenge has been raised against the hypothesis that differentiated SMCs *in vivo* de-differentiate during vascular injury, with evidence that this role instead is due to the presence of multipotent vascular stem cells (Campbell et al., 2001; Tang et al., 2012) and that the heterogeneity of SMCs in culture and *in vivo* is attributable to multipotent vascular stem cells at different stages of differentiation (Tang et al., 2012).

The purpose of the present investigation was not to demonstrate differences between synthetic and contractile cells, as this has been done previously, but the change in phenotype is still fundamental to the use of this cell model. It became evident the that explant culture selects for subpopulations of SMC that migrate early and proliferate rapidly, leaving the more differentiated cells non-migratory and non-proliferating within the explant, surrounded by the matrix. Freshly dispersed (enzymatic harvesting) SMCs have a reduced lifetime of only about 4 h. However, if plated at a high density e.g. on laminin and maintained in differentiating medium (absence of serum and growth factors) without subculturing, they may retain their differentiated phenotype for a few days (Jarrousse et al., 2004). However, for a functional assay such as the present MLC phosphorylation assay, serial subculturing had to be done with these cells in order to obtain sufficient cells to cover a 96-well plate. As this yielded cells that did not revert to a contractile phenotype, the conclusion drawn was that primary cultures of SMCs from human saphenous vein grafts were not compatible with use in a 96-well functional assay due to loss of their contractile abilities. In support of this conclusion, there do not appear to be any previous reports with functional assays measuring contractility on primary cultures of VSMCs, only on single cells freshly isolated from blood vessels (Mironneau and Macrez-Lepretre, 1995; Hughes et al., 1996; Chotani et al., 2004). Instead, most VSMC contraction studies have involved whole or cultured blood vessel ring segments (Parkinson and Hughes, 1995; Roberts, 2001; Roberts, 2003).

The extent to which the SVSMCs were re-differentiated in the present experiments is hard to define and would require a more extensive study. For example RT-PCR analysis of differentiated cells showed expression of α-smooth muscle actin, myosin light chains and calponin, whereas no expression of smooth muscle myosin heavy chains (MHC) was detected. MHC is an important determinant of differentiated SMCs, but so is calponin, and the results were therefore not easily interpreted. Previous studies have tried to map the expression of α_2-ARs in SMCs in order to use these as primary cell models, but the findings have been contradictory. All three subtypes were reported to be expressed in primary cultures of rat aortic SMCs (Richman and Regan, 1998). However, these results were in contrast with the results of Ping and Faber, who used RT-PCR and identified only α_{2A}-AR mRNA in both vascular tissue and cultured vascular SMCs from rat aorta (Ping and Faber, 1993). The difference was speculated to arise from differences in cell isolation methods, which highlights the importance of using an appropriate isolation procedure, as the expression of receptors often is dramatically down-regulated after cell isolation (Faber et al., 2001). Vascular SMCs cultured from human arterioles and saphenous veins were previously shown to express high levels of α_{2C}-AR and low levels of α_{2A}-AR mRNA, but did not express any detectable levels of α_{2B}-AR mRNA (Chotani et al., 2004). In line with this, primary cell results in the investigated samples of proliferative and differentiated SVSMCs detected mRNA for the α_{2C}-AR subtype, which is also in agreement with reports claiming that subtype as the main mediator of contractions to noradrenaline in tissue experiments (Docherty and Hyland, 1985; Gavin et al., 1997; Rizzo et al., 2001; Giessler et al., 2002). The expression of this subtype was most often up-regulated in

differentiated cells (not consistently), perhaps indicating that the cells responded to the heparin differentiation treatment by increasing the expression of α_{2C}-ARs, a phenomenon also reported for the AT_1 angiotensin receptor in VSMCs (Hashimoto et al., 2005), but still not enough to regain contractile protein expression. In addition, some α_{2A}-AR expression was detected, but in lower amounts as compared to α_{2C}-AR expression, in line with other reports (Chotani et al., 2004) and not in a manner regulated by the differentiation treatment. Immunofluorescent labeling of differentiated SMCs against α-smooth muscle actin revealed staining that extended over the entire length of the cells, reaching to the nuclear region and being aligned in a non-parallel manner, a pattern associated with contractile cells (Worth et al., 2001). However, labeling of cells with an antibody against myosin light chains showed labeling of filaments in both proliferative and differentiated cells, with no increase in intensity in the differentiated cells that would be indicative of a contractile function (unpublished observations). The present myosin light chain phosphorylation assay only indicated 18–20% increases of MLC phosphorylation levels over control conditions, strongly suggesting that the primary cells did not represent a sufficiently sensitive model for comparing MLC phosphorylation responses to α_2-AR agonists and antagonists, nor to explore the effects of different inhibitor compounds in these cells. In spite of being kept in differentiating medium (with serum starvation) for a period of six days, the cells did not seem to re-differentiate back to a contractile phenotype. Personal communication with a SMC expert, Professor Julie Campbell (The University of Queensland, Australia) corroborated the hypothesis that the failure to induce the cells to a contractile phenotype could be traced to the large amount of cells needed for the assay, which required multiple passaging. The results of these experiments remain unpublished.

A functional assay measuring the biochemical end-point of contractility in primary cells with endogenous receptor expression is still appealing, but would require another source of cells. Contractile SMCs might possibly be derived from differentiated human bone marrow-derived mesenchymal stem cells (hMSC) (Galmiche et al., 1993; Pittenger et al., 1999). These cells are relatively easy to obtain, and have the potential to differentiate into diverse cell types. The differentiation of hMSCs into a SMC phenotype has been triggered by several manipulations, including addition of transforming growth factor β, mechanical stress, direct contact with vascular endothelial cells and interaction with an endothelial cell matrix (Kashiwakura et al., 2003; Gong et al., 2009; Lozito et al., 2009; Gong and Niklason, 2011). However, the proliferation and differentiation capacity of hMSCs decrease significantly with donor age and the cells suffer from culture senescence limiting their culture time to about 8–10 passages (Bajpai et al., 2012). This is probably still sufficient to acquire a sufficient number of cells for a 96-well plate and functional assays such as the one developed in the present studies, and hMSCs could therefore provide an attractive substitute for the non-contractile cultured primary SMCs. In an attempt to overcome limitations of old donor hMSC, experiments have been performed where human embryonic stem cells were differentiated into contractile SMCs (Ferreira et al., 2007; Vazao et al., 2011). Both endothelial and SMCs can in fact be derived from embryonic progenitor cells

(Hill et al., 2010) but there are, however, many practical limitations concerning the use of such cells. Several groups have successfully differentiated induced pluripotent stem cells (iPSC) into vascular SMCs (Lee et al., 2010; Cheung et al., 2012), and a recent paper described the differentiation of iPCS-derived mesenchymal stem cells into a homogeneous population of highly contractile SMCs. These cells may be useful for the development of cellular models of vascular diseases and for regenerative medicine (Bajpai et al., 2012). Interesting data has indicated that vascular cell types derived from the same human iPSC line may even be used to generate functional blood vessels *in vivo* in mice (Samuel et al., 2013). Perhaps stem cells induced into contractile SMC could be applied in label-free dynamic mass redistribution assays to monitor changes in cell shape as a measure of agonist-induced SMC contractility?

As noted above, the potential value of iPSCs in the drug discovery process is far-reaching, although not yet fully established. These cells have advantages over immortalized cell lines and primary cells as they provide a reproducible, inexhaustible, quantifiable and genetically relevant source of cells for drug-based screening platforms and functional assays. A major academic-industrial partnership project, StemBANCC, has emerged with the goal to provide 1500 high-quality, well characterized patient-derived iPSC lines from 500 patients and associated biomaterials being stored within a biobank (www.stembancc.org). The aim is to use these cell lines for testing of drug efficacy and safety and for improving drug development in certain diseases such as peripheral and central nervous system disorders and diabetes. The project is also investigating the use of human iPSCs for toxicity testing by generating liver, heart, nerve and kidney cells from iPSCs. Projects such as this are likely to make future drug discovery and toxicity estimation more effective, resulting in a reduced need for experimental animals and animal models as well as ensuring that a larger proportion of poor drug candidates are eliminated in the primary and secondary evaluation processes, which in turn will hopefully improve the quality of the candidates selected to be tested in clinical trials (Rubin and Haston, 2011; Mercola et al., 2013).

In their natural environment, vascular SMCs are surrounded by endothelial cells and adventitial fibroblasts in connective tissue. Endothelial cells (EC) and SMCs interact together to maintain the normal function of the blood vessel wall. The ECs create a barrier against the circulating blood and regulate the access of vasodilator and vasoconstrictor substances into the surrounding tissue, whereas the SMCs perform the actual contraction and relaxation of the blood vessel by regulating its diameter. It is conceivable that a system in which these cells are co-cultured would represent a more *in vivo*-like arrangement, even more so if they were cultured on a matrix consisting of the same connective tissue proteins that surround SMCs *in vivo*, such as collagen and elastin (Truskey, 2010). The first co-culture system of bovine ECs and rat SMCs, in which ECs were cultured directly on top of a SMC layer was already desribed more than 30 years ago (Jones, 1979). In this approach, still widely used to develop engineered blood vessels, the SMCs are seeded in a three-dimensional collagen or polymer gel scaffold that mimics the *in vivo* geometry (Niklason et al., 1999). Other methods of bilayer culture include culturing of ECs and SMCs on opposite sides of a

thin membrane (Fillinger et al., 1997), culture of ECs on top of a gel (made of collagen, fibrin or other polymer) in which SMCs are embedded (van Buul-Wortelboer et al., 1986), or culture of both cell types mixed together in equal amounts (Hirschi et al., 1998). Three-dimensional cell cultures in scaffolds more closely resemble the *in vivo* state, but this technique is more often employed for *in vivo* vessel generation intended for tissue grafts (Niklason et al., 1999; Isenberg et al., 2006), and it has not been commonly used in drug discovery and development. The improved feasibility of EC-SMC co-culture systems (e.g. by seeding ECs and SMCs on a 96-well plate coated with collagen matrix) makes them promising candidates for HTS/HCS assays focused on cardiovascular drug discovery, and would provide an attractive next step in the development of the functional assay measuring contractility by MLC phosphorylation responses reported here. Currently, there are no published reports describing such systems. Such a system would probably be of more physiological relevance than the currently developed method, especially for investigating intracellular signaling pathways.

6.4 Signaling routes involved in α_2-adrenoceptor-evoked myosin light chain phosphorylation

A vascular smooth muscle cell line previously transfected to express the human α_{2B}-AR subtype (Huhtinen and Scheinin, 2008) was employed to optimize and validate the MLC phosphorylation assay. These cells, A7r5, are derived from rat aorta and are commonly employed in cardiovascular research (Kimes and Brandt, 1976; Huhtinen and Scheinin, 2008). The present assay was set up based on the established linear correlation between the extent of phosphorylation of myosin light chains (MLC phosphorylation) and muscle contractility (Takashima, 2009), in order to biochemically quantify smooth muscle contraction, using phosphorylation of myosin light chains at Ser19 as the readout. Assay validation was performed with single-cell analysis of intracellular calcium responses, with Western blotting and with immunostaining. The MLC phosphorylation response was typically fully developed 20–45 seconds after agonist addition, and MLC phosphorylation levels returned to baseline levels by two minutes. The maximal response (E_{max}) to the α_2-AR agonist dexmedetomidine was approximately 60% over the control value seen in vehicle-treated control cells, and the endogenous vasoconstrictor, arginine vasopressin, evoked similar responses. The magnitude of the response, and hence the assay signal window, was not very large, but this can be explained by several factors. First, A7r5-α_{2B} cells exhibited relatively high basal levels of MLC phosphorylation, and different external stimuli (pipetting, shaking the plate, several liquid additions within a short time frame) may have further increased the levels of MLC phosphorylation, thus masking a substantial portion of the final outcome, since the results always were normalized to vehicle-treated controls. Therefore, only simultaneously treated samples are comparable, and it is important to design the layout of an assay plate in such a way that each condition contains all of the necessary controls in the same row. Pre-treatment of cells with inhibitors dissolved in DMSO also seemed to attenuate the maximal peak

response induced by dexmedetomidine. The use of inhibitors is well established when trying to deduce the involvement of specific proteins in the intracellular signaling cascades of receptors. However, these inhibitors may give very inconsistent results because of the complexity of the involved pathways, and also because of their potential toxicity, limited solubility and lack of specificity (Davies et al., 2000), and all results need therefore to be interpreted with care. We noted that the inhibitors gallein and U73122 needed lengthy procedures before full dissolution, and in the initial experiments, when they were not properly dissolved, these inhibitors erroneously appeared to produce no effects. In addition, fresh inhibitor solutions needed to be used in all experiments, and the cells should be inspected to confirm their viability after inhibitor treatments. The effects of the inhibitors alone on the extent of basal MLC phosphorylation (without agonist) were evaluated with *t-tests* for the AUC over 45 s after drug addition. The change in AUC was statistically non-significant for most of the tested inhibitors, but small reductions in MLC phosphorylation were observed after GF109203X (PKC inhibitor), H-89 (PKA inhibitor) and gallein (Gβγ inhibitor) (−17 %, −33 % and −16 %; $p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively). The use of inhibitors could partly be bypassed by using RNA silencing technology, but on the other hand, the use of this technology is hampered by the fact that A7r5 cells are rather resistant to the transfection procedure.

In addition to setting up an assay to assess the capacity of α_2-AR agonists and antagonists to evoke or inhibit myosin light chain phosphorylation leading to vascular SMC contraction, it was intended to elucidate the intracellular events being triggered by vascular α_{2B}-AR activation and which eventually lead to myosin light chain phosphorylation. These events have remained largely unresolved, in contrast to the intracellular events mapped for classical vasoconstrictors, e.g. angiotensin II and endothelin-1 (Wynne et al., 2009). Previous studies on vascular SMCs have shown that α_2-AR activation increases intracellular calcium levels (Chotani et al., 2004), which in turn is essential for MLC phosphorylation (Aburto et al., 1993). The mechanism behind this reaction has been suggested to involve pertussis toxin-sensitive, G_i-mediated activation of L-type calcium channels (Lepretre and Mironneau, 1994; Mironneau and Macrez-Lepretre, 1995; Hughes et al., 1996), a pathway that has been confirmed in studies using isometric tension measurements on blood vessels (Parkinson and Hughes, 1995; Roberts, 2001). These results are in agreement with these proposed mechanisms, as blocking the activation of G_i proteins with pertussis toxin resulted in almost complete abolishment of the MLC phosphorylation response evoked by dexmedetomidine, and addition of the L-type calcium channel blocker nifedipine resulted in significant decreases of MLC phosphorylation. Commonly, α_2-AR activation is coupled to inhibition of AC activity, but stimulation of phospholipase C (PLC) activity by pertussis toxin-sensitive G proteins in fibroblasts has also been reported, as described above. This activation of PLC has been proposed to be mediated by Gβγ subunits (Dorn et al., 1997), and it was therefore decided to evaluate the involvement of this signaling pathway by employing the βγ inhibitor gallein. This almost completely abolished of the dexmedetomidine-evoked MLC phosphorylation response, which indicates involvement of Gβγ signaling. Furthermore, it is believed

that Gβγ-mediated activation of phospholipase C is involved, since its inhibition with U73122 also resulted in complete abolishment of the MLC phosphorylation response. Thus, both intracellular and extracellular sources of calcium ions may be essential for this process.

Activation of protein kinase C (PKC) plays a central role in many cellular responses. PKC is activated subsequent to the activation of PLC, a process that results in the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, the latter activating PKC (Berridge and Irvine, 1984). Previous studies on α_2-AR activation in SMCs have demonstrated that blockade of PKC resulted in decreased calcium entry through calcium channels (Lepretre and Mironneau, 1994; Mironneau and Macrez-Lepretre, 1995). PKC possibly also plays a role in determining an individual subject's sensitivity to dexmedetomidine-induced blood vessel contraction, since marked differences in the potency of dexmedetomidine were associated with a polymorphism in the gene encoding PKCβ (Posti et al., 2013). In the present experiments, surprisingly, blockade of PKC activity markedly potentiated the dexmedetomidine-induced MLC phosphorylation response. There are several possible explanations for this phenomenon. First, PKC has been shown to directly phosphorylate IP_3 receptors (Ferris and Snyder, 1992), and therefore, blockade of PKC could result in increased IP_3 receptor activity, with more calcium being released from internal stores. Other studies have shown that blockade of PKC has resulted in increased responsiveness of the vasopressin- or angiotensin II-stimulated inositol-lipid signaling pathways, suggesting that PKC can exert a negative feedback control of phosphoinositide turnover to decrease calcium release and calcium entry, by terminating IP_3 formation and calcium mobilization in vascular SMCs (Pfeilschifter et al., 1989). Furthermore, PKC has been shown to directly inhibit PLC activity in myometrial SMCs (Zhong et al., 2008).

It is not possible to conclude that all the pathways involved in α_{2B}-AR mediated activation of MLC phosphorylation have now been elucidated, but instead that it is apparent that the network of involved pathways is complex and seems to involve many mediators, e.g. G_i proteins, Gβγ subunits, L-type calcium channels, PLC and PKC, as outlined in Fig. 14. Finally, the pathways activated as well as the magnitude and time-lines of the dexmedetomidine-induced MLC phosphorylation response in this present study appear to be strictly related to events taking place in an isolated SMC culture. However, the understanding of the role of α_2-ARs in blood vessels has been hampered by the fact that these receptors can mediate both contraction and relaxation. Regulation of the vascular responses elicited by dexmedetomidine *in vivo*, for instance, appears to be very complex; in endothelium-denuded arteries the drug has induced only contraction, whereas in intact vessels, the relaxing and contracting effects of dexmedetomidine appear to oppose each other (Snapir et al., 2009). The final outcome has been dependent on the dose, i.e. dexmedetomidine evoked endothelium-dependent relaxation at low concentrations in contracted small arteries and contraction at higher concentrations (Kim et al., 2009; Wong et al., 2010). In the physical environment of the blood vessel wall, there is an intense communication between ECs and SMCs. It is therefore conceivable that a more relevant way to study α_{2B}-AR-mediated contraction

with an assay as developed here would be in a co-culture system as suggested above; this would be truly innovative as no such studies have been conducted previously in this manner.

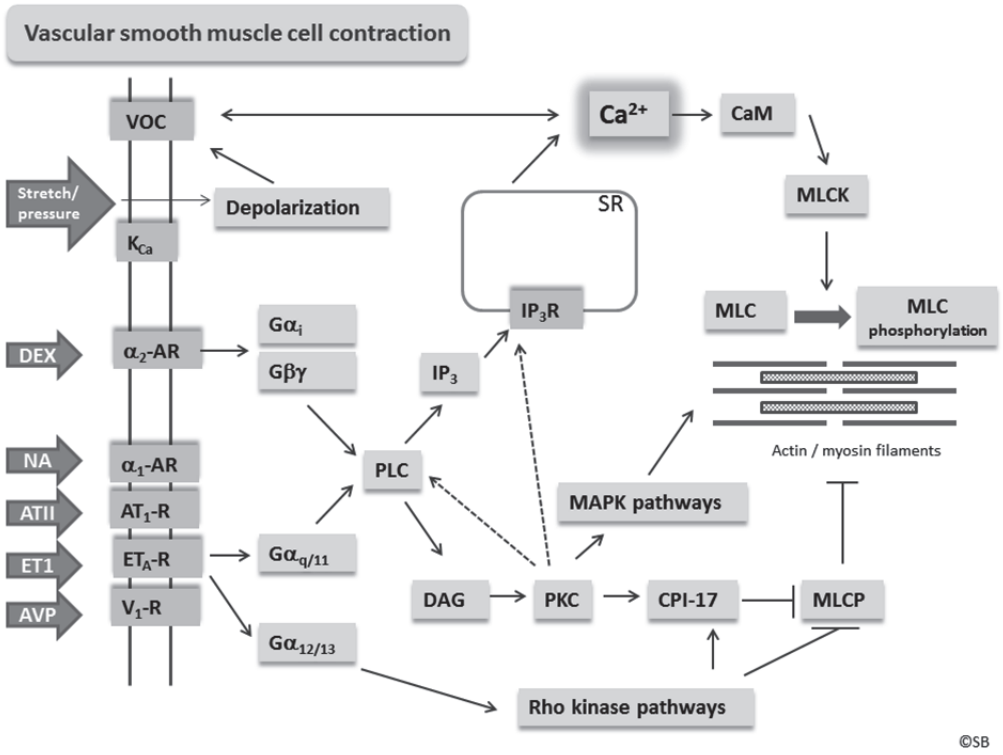

Figure 14. Overview of vascular smooth muscle cell contraction, modified to include the results of Study IV. Arrows indicate stimulation and T-shaped lines indicate inhibition, dashed arrows indicate negative feedback. Abbreviations: VOC; voltage-operated Ca^{2+} channel (L-type), K_{Ca}; K^+ channel regulated by Ca^{2+}, α_2-AR; α_2-adrenoceptor, α_1-AR; α_1-adrenoceptor, AT_1-R; angiotensin AT_1-receptor, ET_A-R; endothelin ET_A receptor, V_1-R; arginine vasopressin V_1 receptor, DEX; dexmedetomidine, NA; noradrenaline, ATII; angiotensin II, ET1; endothelin 1, AVP; arginine vasopressin, PLC; phospholipase C, IP_3; inositol trisphosphate, DAG; diacylglycerol; PKC; protein kinase C, CPI-17; C-kinase-activated protein phosphatase-1 inhibitor, MLCP; myosin light chain phosphatase, MAPK; mitogen-activated protein kinase, CaM; calmodulin, MLCK; myosin light chain kinase, MLC; myosin light chain. (Created by author, modified from KEGG pathways).

7 CONCLUSIONS

Cell models are employed in the early phases of drug discovery and development to screen potential drug candidates and to assess their properties in a biological context. However, cell models require careful validation, depending on the type of receptor subtype and effector system investigated as well as on the desired information output. This thesis investigated some aspects of biochemical, neuronal and cardiovascular cell models that are important to consider when investigating the properties of ligands interacting with α_2-ARs. The main results and conclusions were:

1. A functional assay monitoring receptor-mediated inhibition of AC activity is not compatible with a semistably transfected cell population. In the developed assay, AC was externally stimulated to produce cAMP (by forskolin) in all cells, but the majority of cells in the semistable cell population failed to display a significant amount of receptors and the high receptor density present in a few cells was not sufficient to evoke a significant inhibitory net response, and therefore, a false negative pharmacological test result was obtained. Hence, attempts to screen semistably transfected mutant receptor constructs for the functional importance of the mutation would have resulted in incorrect interpretation of the results. For the assessment of the capacity of the investigated receptors to mediate inhibition of forskolin-stimulated AC activity, it is recommended to use a cell population expressing receptors in a homogeneous fashion.

2. To investigate whether cell-surface delivery of recombinant α_{2C}-ARs (intracellularly retained in non-neuronal cells) could be rescued by accessory proteins, co-expression of REEPs with α_{2A}- and α_{2C}-ARs was performed. REEP co-expression did not specifically enhance cell surface expression of either α_2-AR subtype, but affected ER cargo capacity, and thus the total expression of α_{2C}-ARs was increased in non-neuronal cells. Immunofluorescent microscopy and biochemical analysis revealed a consistent theme of ER localization of REEP1, 2 and 6, which was also shown to be the site for interactions with α_{2C}-AR. In addition to enhancing ER cargo capacity, distinct REEP members may selectively modulate membrane expression of specific GPCRs; for the α_{2C}-AR, co-expression of REEPs only enhanced the expression of a non-glycosylated form. REEPs therefore regulate intracellular trafficking by affecting ER membrane structure, cargo capacity, and by acting as adapter proteins. REEPs are expressed in cultured neuronal cells in a time-dependent manner, pointing to a role in the regulation of α_{2C}-AR trafficking, but the complete network of proteins involved in the cell-surface delivery of α_{2C}-ARs in neuronal cells remains unresolved.

3. Validation and optimization of primary cultures of human vascular SMCs revealed that these cells are not compatible with a functional assay measuring contractility by myosin light chain phosphorylation in a 96-well format, due to the loss of contractile properties of the cells. A transformed cell line of rat aortic SMCs which had been transfected to express α_{2B}-ARs, however, showed myosin light chain phosphorylation in response to the α_2-AR agonist dexmedetomidine. The developed assay can be used in the assessment of the capacity of ligands to evoke or inhibit vascular SMC contraction.

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