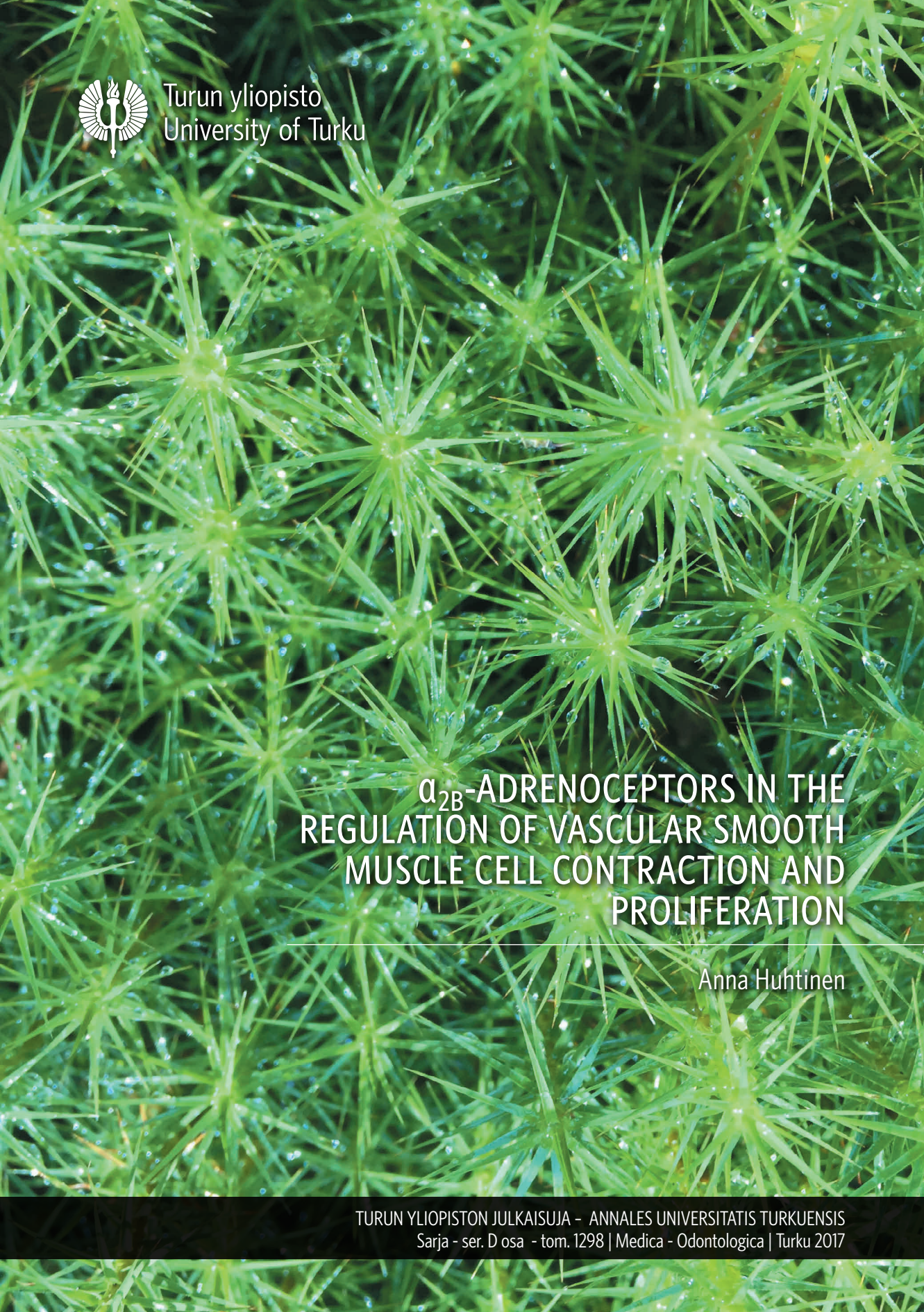

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To the wonderful people who have supported me during this journey.

“Oves luki vedä, mä työnsin sen auki.”

ABSTRACT

Anna Huhtinen

α_{2B}-Adrenoceptors in the regulation of vascular smooth muscle cell contraction and proliferation

University of Turku, Faculty of Medicine, Institute of Biomedicine, Department of Pharmacology, Drug Development and Therapeutics, Drug Research Doctoral Program and Unit of Clinical Pharmacology, Turku University Hospital, Turku, Finland.

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α_2-Adrenoceptors (α_2-ARs) belong to the large superfamily of G protein-coupled receptors (GPCRs). They mediate important actions of the endogenous catecholamines adrenaline and noradrenaline. All three α_2-AR subtypes are involved in the regulation of blood pressure and vascular tone. α_2-ARs can regulate both vascular smooth muscle contraction and remodeling of the blood vessel wall, but the intracellular signaling mechanisms involved in these functions have remained largely unknown. The aim of this thesis was to investigate the involvement of the α_{2B}-AR subtype in the regulation of the contraction and proliferation of vascular smooth muscle cells (VSMC), and to clarify the related cellular signaling mechanisms.

In order to characterize the effects of α_{2B}-AR activation on VSMC contraction and proliferation and to investigate whether these functions could be altered by drug treatment, a VSMC line stably expressing the human α_{2B}-AR was generated by transfection of rat A7r5 cells. Characterization of the novel A7r5-α_{2B} cell line indicated that the localization and ligand binding properties of the expressed α_{2B}-ARs were in line with earlier studies of α_{2B}-ARs in different host cell environments, and that the receptors had the expected pharmacological characteristics. Therefore, the generated A7r5-α_{2B} cell line was regarded as a useful tool for investigating the functions and regulation of α_{2B}-ARs in VSMCs. α_{2B}-ARs were demonstrated to be capable of mediating VSMC contraction by using a functional assay measuring myosin light chain phosphorylation, which is a biochemical readout of VSMC contraction. The network of signaling pathways involved in α_{2B}-AR-mediated contraction of A7r5 VSMCs appeared to be complex and seemed to involve many mediators, such as G_i proteins, Gβγ subunits, phospholipase C (PLC), protein kinase C (PKC) and L-type Ca^{2+} channels. Different screening assays, namely DNA microarray, small inhibitor compound library screening and kinase activity profiling, were used to investigate the genetic regulation and intracellular signaling mechanisms involved in α_{2B}AR-evoked proliferation of A7r5 VSMCs. The cellular mechanisms and signal transduction pathways participating in this response appeared to be complex and included redundancy. The employed screening assays and their respective data analysis approaches were found to be useful as tools to map the activation of cellular signaling networks in a situation where the exact mechanisms still remain unknown. These screening tools were considered suitable for hypothesis generation, but additional approaches will be required for further hypothesis testing.

Keywords: α_2-adrenoceptor, vascular smooth muscle, contraction, proliferation, intracellular signaling

TIIVISTELMÄ

Anna Huhtinen

α_{2B}-Adrenergiset reseptorit verisuonten sileälihassolujen supistuksen ja proliferaation säätelyssä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Farmakologia, lääkekehitys ja lääkehoito, Lääketutkimuksen tohtoriohjelma, ja Kliinisen farmakologian yksikkö, Tyks.

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α_2-Adrenergiset reseptorit (α_2-AR) ovat G-proteiinikytkentäisiä reseptoreita (GPCR). Ne aktivoituvat adrenaliinin ja noradrenaliinin vaikutuksesta ja välittävät monia tärkeitä elimistön säätelytehtäviä. Kaikki kolme α_2-AR-alatyyppiä osallistuvat verenpaineen säätelyyn. Ne voivat säädellä sekä verisuonen sileän lihaksen (VSL) supistumista että verisuonen seinämän rakenteessa tapahtuvia muutoksia. Näihin toimintoihin liittyvät solusisäiset viestintämekanismit ovat vielä suurelta osin tuntemattomia. Tämän väitöstutkimuksen tavoitteena oli selvittää α_{2B}-reseptorialatyypin merkitystä VSL-solujen supistumisen ja proliferaation säätelyssä ja näitä vaikutuksia välittäviä solutason viestintämekanismeja.

Rotan A7r5-solulinjan VSL-soluihin siirrettiin ihmisen α_{2B}-reseptoria koodittava geeni. Tämä tuotti VSL-solumallin, jonka avulla voitiin tutkia α_{2B}-reseptorien vaikutuksia VSL-solujen supistumiseen ja proliferaatioon. Kehitetyn A7r5-α_{2B} -solulinjan reseptorien sijainti ja farmakologiset ominaisuudet olivat odotuksia vastaavat. Tämän validoinnin perusteella A7r5-α_{2B}-solulinjan todettiin soveltuvan α_{2B}-reseptorien toiminnan tutkimiseen VSL-soluissa. Väitöstutkimuksessa osoitettiin, että α_{2B}-reseptorit välittävät VSL-solujen supistusvasteita. Tätä tutkittiin mittaamalla myosiinin kevytketjujen fosforylaatiota, sillä myosiinin kevytketjujen fosforylaatio on VSL-solujen supistuksen kannalta keskeinen biokemiallinen tapahtuma. A7r5-soluissa α_{2B}-välitteiseen supistusvasteeseen liittyvät solunsisäiset viestintämekanismit osoittautuivat monimutkaisiksi ja niihin osallistui monia viestinvälittäjiä, kuten G_i-proteiinit, Gβγ-alayksiköt, fosfolipaasi C, proteiinikinaasi C ja L-tyypin kalsiumkanavat. DNA-mikrosirumäärityksillä, kinaasi/fosfataasi-inhibiittorikirjaston seulonnalla ja kinaasiaktivaatiota mittaavilla mikrosirumäärityksillä selvitettiin α_{2B}-välitteiseen VSL-solujen proliferaatiovasteeseen liittyvää geenien ilmentymisen säätelyä ja solunsisäisiä viestintämekanismeja. VSL-solujen proliferaatiovasteen syntyyn liittyvät mekanismit ja viestintäreitit osoittautuivat monimutkaisiksi ja osittain päällekkäisiksi. Käytetyt seulontamenetelmät ja niihin sovellettavat analyysit todettiin käytännölliseksi lähestymistavaksi tilanteessa, jossa halutaan kartoittaa ennalta tuntemattomia solunsisäisiä viestintämekanismeja. Seulontamenetelmät todettiin hyödyllisiksi uusien havaintojen tuottamisessa, mutta havaintojen tuottamien hypoteesien testaamiseen tarvitaan muita, kohdennettuja menetelmiä.

Avainsanat: α_2-adrenerginen reseptori, verisuonen sileä lihas, supistus, proliferaatio, solunsisäinen viestintä

TABLE OF CONTENTS

ABBREVIATIONS

AC	adenylyl cyclase
AR	adrenoceptor
BrdU	5-bromo-2'-deoxyuridine
C-terminus	carboxyl terminus
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP response element-binding protein
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
EC	endothelial cell
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK	extracellular stimulus-regulated kinase
FBS	fetal bovine serum
FC	fold change
FGF	fibroblast growth factor
G protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
GO	Gene Ontology
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	hemagglutinin
HGF	hepatocyte growth factor
HCS	high-content screening
HTS	high-throughput screening
IP_3	inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
NO	nitric oxide
MAPK	mitogen-activated protein kinase
MEK	MAP/ERK kinase
MLC	myosin light chain

MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMP	matrix metalloproteinase
MVSC	multipotent vascular stem cell
NFAT	nuclear factor of activated T lymphocytes
N-terminus	amino-terminus
p38	a class of MAPKs
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI	phosphoinositide
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
PTX	pertussis toxin
ROC	receptor-operated Ca^{2+} channel
Ser/Thr	serine/threonine
α-SMA	smooth muscle α-actin
SMC	smooth muscle cell
SM-MHC	smooth muscle myosin heavy chain
SOC	store-operated Ca^{2+} channel
SR	sarcoplasmic reticulum
SRF	serum response factor
STK	serine/threonine kinase
TGF-β	transforming growth factor β
TM	transmembrane
VEGF	vascular endothelial growth factor
VOC	voltage-operated Ca^{2+} channel
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cell
wt	wild-type

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-III:

I **Huhtinen, A** and Scheinin, M (2008). Expression and characterization of the human α_{2B}-adrenoceptor in a vascular smooth muscle cell line. *European Journal of Pharmacology* 587: 48-56.

II Björk, S*, **Huhtinen, A***, Vuorenpää, A and Scheinin, M (2014). Quantitative determination of α_{2B}-adrenoceptor-evoked myosin light chain phosphorylation in vascular smooth muscle cells. *Journal of Pharmacological and Toxicological Methods* 70: 152-162.

III **Huhtinen, A**, Hongisto, V, Laiho, A, Löyttyniemi, E, Pijnenburg, D and Scheinin, M (2017). Gene expression profiles and signaling mechanisms in α_{2B}-adrenoceptor-evoked proliferation of vascular smooth muscle cells. *BMC Systems Biology* 11: 65.

*authors contributed equally

Additionally, this thesis also presents some unpublished data and the supplemental information published on the website of the publisher of Paper III.

Available from: https://bmcsystbiol.biomedcentral.com/articles/10.1186/s12918-017-0439-8

1 INTRODUCTION

Hypertension is one of the most prevalent chronic illnesses in Finland; it has been estimated that approximately half a million Finns are taking medication to treat high blood pressure (Mustajoki, 2017). The increased resistance of flow, which is typical in hypertension, may be caused by dynamic constriction of blood vessels or by static remodeling of the blood vessel wall, and α_2-adrenoceptors (α_2-ARs) may be involved in the regulation of both of these phenomena. α_2-ARs belong to the large family of G protein-coupled receptors (GPCRs). α_2-ARs are potential targets for cardiovascular drug development because they mediate important actions of the endogenous catecholamines, noradrenaline and adrenaline, in the regulation of vascular tone and blood pressure. The cardiovascular effects of α_2-AR activation include centrally-mediated inhibition of the sympathetic nervous system, inhibition of catecholamine release from the adrenal medulla and contraction and relaxation of vascular smooth muscle; this latter effect mediated by postsynaptic receptors located on vascular smooth muscle cells (VSMCs) and endothelial cells (Gilsbach and Hein, 2012).

Three mammalian α_2-AR subtypes have been identified by molecular cloning, and the roles of the individual receptor subtypes in short-term cardiovascular regulation are relatively well known. Intravenous administration of an α_2-AR agonist is associated with a biphasic blood pressure response, i.e. initial vasoconstriction and hypertension mediated by peripheral α_{2B}-ARs evoking vascular smooth muscle contraction (Link *et al.*, 1996; Paris *et al.*, 2003; Talke *et al.*, 2003), followed by sympathetic nervous system inhibition, bradycardia and prolonged hypotension mediated by central α_{2A}-ARs (MacMillan *et al.*, 1996; Ebert *et al.*, 2000). Indeed, since the 1960's, α_2-ARs have been employed as targets of clonidine-like antihypertensive drugs (Aantaa and Jalonen, 2006), first without any understanding of the mechanisms involved and only recently with an awareness of the role of central α_{2A}-ARs. In contrast, so far, the vascular α_{2B}-ARs have not been exploited as drug targets. Thus, subtype-selective α_2-AR agonists or antagonists might have therapeutic potential in cardiovascular diseases. The current, clinically available, α_2-AR agonists activate all α_2-AR subtypes and consequently, the antihypertensive therapy mediated by central α_{2A}-ARs is partially counteracted by vasoconstriction mediated by vascular α_{2B}-ARs. This emphasizes the need to discover more subtype-selective drugs; subtype-selective agonists could improve antihypertensive therapy, and on the other hand, antagonists of the α_{2B}-AR subtype could be used to treat vasoconstrictive disorders. Therefore, a more detailed understanding of the physiological roles and pharmacological properties of the α_2-AR subtypes is still needed.

Although vasoconstriction is a well known consequence of α_2-AR activation, much less is known about how these receptors are involved in the regulation of cell proliferation. Both stimulatory (Seuwen *et al.*, 1990; Cussac, *et al.*, 2002a; Vázquez *et al.*, 2006; Xia *et al.*, 2016a) and inhibitory effects (Kanno *et al.*, 2002; Karkoulias *et al.*, 2006a) on the proliferation of different cell types have been reported; however, little is known about the effects of α_2-AR activation on the proliferation of VSMCs. VSMC proliferation and differentiation are crucial physiological processes in vascular development and plasticity. In healthy vessels,

the main function of VSMCs is contraction and regulation of blood vessel diameter, and thus the cells exhibit very low rates of proliferation. However, VSMCs are not terminally differentiated, and in response to vascular injury, such as occurs in atherosclerosis, VSMCs may de-differentiate and start to migrate and proliferate. Increased VSMC proliferation is known to contribute to restenosis after coronary bypass surgery or angioplasty, limiting the long-term success of these clinical interventions designed to treat the complications of atherosclerosis. It is therefore essential to clarify the mechanisms regulating VSMC proliferation, if we are to develop new therapies (Campbell and Campbell, 1985; Shi and Chen, 2014). α_{2B}-AR-regulated VSMC proliferation may be important not only for the development and plasticity of blood vessels but also in the pathology and therapeutics of various cardiovascular disorders.

Although α_2-ARs in VSMCs may contribute to vascular diseases and to the undesired effects of such current drugs as clonidine and the sedative-hypnotic agent dexmedetomidine, almost all of the earlier *in vitro* studies on α_2-AR function and regulation have been carried out using fibroblast cell lines. The present series of studies was designed to further characterize the effects of α_{2B}-AR activation on VSMC contraction and proliferation, and to investigate whether these functions could be regulated with drugs. For this purpose, a recombinant VSMC line expressing human α_{2B}-ARs was developed in order to investigate α_{2B}-AR function and regulation in a cellular environment relevant for cardiovascular diseases. Generating new information about receptor functions in standardized conditions at the cellular level is essential in order to be able to predict receptor functions in the whole organism. A more detailed understanding of α_2-AR subtype functions at the cellular level could be beneficial for the discovery and development of future subtype-selective α_2-adrenergic drugs.

2 REVIEW OF THE LITERATURE

2.1 α_2-Adrenoceptors as members of the G protein-coupled receptor family

2.1.1 Historical background

Adrenergic receptors, or adrenoceptors (ARs), are cell membrane receptors that are present in almost every tissue of the body in various combinations. They mediate the physiological actions of the endogenous catecholamines noradrenaline and adrenaline. Adrenoceptors were first divided into two categories, α- and β-ARs, but are now appreciated to belong to three major subfamilies: the α_1-, α_2- and β-ARs. Adrenoceptors have been one of the most extensively investigated receptor families because of their major physiological importance and also because of their prominent position in the history of pharmacology.

The first steps leading to the discovery of adrenoceptors were made already in the beginning of the 20th century. In 1905, Langley presented the term "receptive substance" for the first time and he proposed that compounds such as nicotine, atropine and many other alkaloids produce their effects by combining with "receptive substances" on the cell surface (Langley, 1905). In 1906, Dale reported the first evidence of multiple receptor types in his studies on the effects of adrenaline on various physiologic responses in different mammals (Dale, 1906) (Figure 1). Since then, adrenoceptors have been a major focus of interest in biochemistry, physiology and pharmacology. However, the first attempts to classify adrenoceptors into different groups were not made until 42 years after the discoveries of Langley and Dale, when Ahlquist proposed a division of adrenoceptors into two types, termed α and β, based on their pharmacological properties (Ahlquist, 1948). He used a series of synthetic phenylethylamines with a close structural resemblance to adrenaline and evaluated their potency in different organs. In these studies, Ahlquist was the first to establish a pharmacological classification for adrenoceptors. The receptor he termed α mostly mediated excitatory functions including vasoconstriction, uterine muscle contraction, urethral contraction and pupil dilation and one important inhibitory function, intestinal relaxation. The receptor called β was mainly inhibitory, except in the heart. This concept of the presence of two distinct types of adrenoceptors, and adrenaline being the only endogenous mediator in the adrenergic system, was in direct opposition to the earlier hypothesis of Cannon and Rosenblueth (1937) who proposed the existence of two mediators, one being responsible for the excitatory functions and the other for the inhibitory functions of sympatho-adrenal activation (Cannon and Rosenblueth, 1937).

In the 1960's, Lands and his coworkers proposed a division of β-ARs into two subtypes, termed β_1 and β_2. The β_1-receptor mediated processes such as lipolysis and cardiac stimulation, and the β_2-receptor was involved in bronchial smooth muscle relaxation and stimulation of glycogenolysis in skeletal muscle (Lands *et al.*, 1966; Lands *et al.*, 1967a; Lands *et al.*, 1967b). This classification was widely approved by the research community. About ten years later, the α-ARs were also divided into subtypes. In the mid 1970's, two series of experiments revealed the existence of two types of α-ARs. Dubocovich and Langer (1974) concluded that pre- and postsynaptic α-ARs were not identical based on

the significant difference in the potency of phenoxybenzamine for inhibiting vascular effects and neurotransmitter release (Dubocovich and Langer, 1974). Based on this *in vitro* evidence, Langer suggested that postsynaptic α-ARs mediating responses in effector organs should be called α_1, while presynaptic α-ARs mediating inhibition of neurotransmitter release during nerve stimulation should be called α_2 (Langer, 1974). A few years later it became evident that α-ARs pharmacologically very similar to the presynaptic α_2-ARs are also found in postsynaptic locations. Therefore, Berthelsen and Pettinger refined Langer's purely anatomical classification into a pharmacological subclassification, independent of receptor location (Berthelsen and Pettinger, 1977). The α-ARs responsible for inhibitory responses were now termed α_2-ARs and the term α_1-AR was used for receptors responsible for excitatory responses, independent of their pre- or postsynaptic location. However, also this classification proved to be inadequate when Drew and Whiting established that while noradrenaline-induced vasoconstriction was inhibited by α_1-AR antagonists such as prazosin, also selective α_2-AR antagonists such as yohimbine were able to inhibit this response (Drew and Whiting, 1979). These results demonstrated that excitatory responses could also be mediated by α_2-ARs and, therefore, it was concluded that neither anatomical nor functional differentiation could adequately classify α-ARs.

In the 1980's, the development of new pharmacological methodologies for the study of receptors made it possible to broaden our understanding of α-ARs, and it became evident that there were more subtypes of adrenoceptors than previously appreciated. The first of these new techniques was the radioligand binding assay, which took advantage of newly introduced ^{3}H-labelled α-AR-selective ligands, such as clonidine, yohimbine, WB-4101 and prazosin, and enabled the direct measurement of the binding of these ligands to their specific receptors. By means of radioligand binding assays researchers were able to demonstrate that subtypes of both α_1-ARs (Morrow and Creese, 1986; Han *et al.*, 1987) and α_2-ARs (Bylund, 1985; Bylund, 1988; Murphy and Bylund, 1988; Simonneaux *et al.*, 1991) existed. However, it was not always easy to determine the relationship between ligand binding sites and functional receptors (Docherty, 1989).

In the late 1980's, the development of more selective drugs and the emergence of molecular cloning techniques revolutionized the study of α-AR structure and function. It became evident that neither α_1- nor α_2-ARs constitute homogenous groups, but instead, each category should be further subdivided into different receptor subtypes. The different α-AR subtypes were first defined pharmacologically by radioligand binding studies and by functional experiments (Bylund *et al.*, 1988; Murphy and Bylund, 1988; Bylund and Ray-Prenger, 1989; Lorenz *et al.*, 1990; Simonneaux *et al.*, 1991), and these conclusions were then confirmed by molecular cloning methods (Lorenz *et al.*, 1990; Alexander *et al.*, 1999). In the late 1980's and early 1990's, altogether six different mammalian genes for α-ARs − three subtypes each for both α_1- and α_2-ARs (α_{1A}, α_{1B}, α_{1D}; and α_{2A}, α_{2B}, α_{2C}) − were identified and sequenced. The current subclassification of α-ARs was established on the basis of these genetic receptor subtypes. Resolving and understanding the structure and function of adrenoceptors has been a long journey and can only be very briefly summarized here. Several comprehensive reviews can provide a more detailed picture of the whole endeavour (Harrison *et al.*, 1991; Bylund, 1992; Ruffolo and Hieble, 1994; Piascik *et al.*, 1996; Docherty, 1998; Civantos Calzada and Aleixandre de Artiñano, 2001; Guimarães and Moura, 2001).

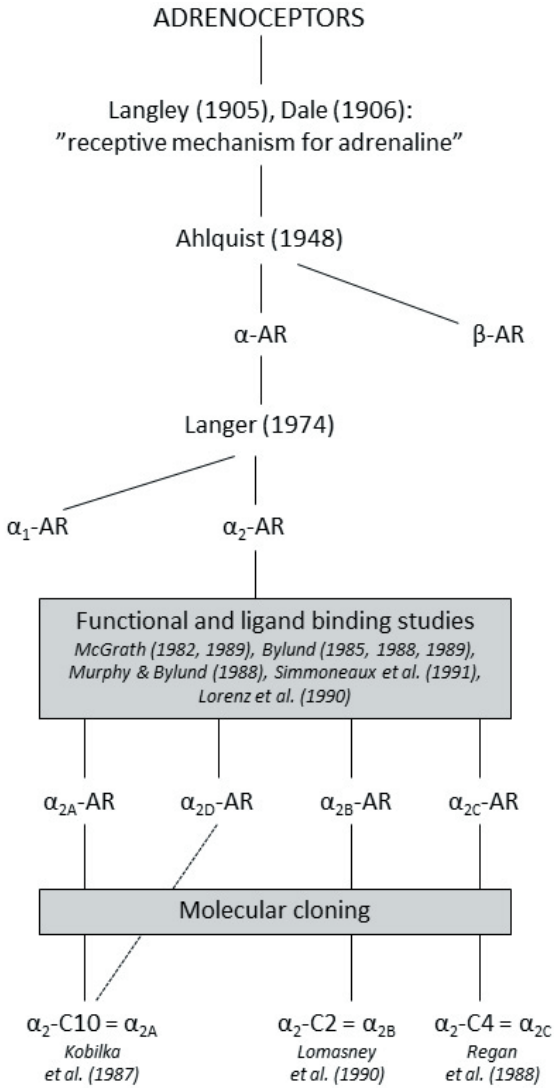

Figure 1. Important milestones in the discovery of α_2-AR subtypes [modified from (Civantos Calzada and Aleixandre de Artiñano, 2001)].

2.1.2 α_2-adrenoceptor subtypes, genes and structure

It is now established that α_2-ARs are members of the large superfamily of GPCRs. Mammalian GPCRs are encoded by approximately 800 genes and make up the largest and most diverse family of membrane proteins in mammalian genomes. GPCRs mediate many cellular responses to hormones, neurotransmitters and environmental stimulants and are even responsible for vision, olfaction and part of the sense of taste (Fredriksson *et al.*, 2003; Venkatakrishnan *et al.*, 2013). All GPCRs share a common structural template with seven α-helical transmembrane (TM) domains (Luttrell, 2006; Lefkowitz, 2007; Rosenbaum *et al.*, 2009). GPCRs are commonly divided into five distinct families based on their amino acid sequence and structural similarity: the rhodopsin-like (family A), secretin (family B), glutamate (family C), adhesion and frizzled/taste receptors. The rhodopsin

family is by far the largest and most diverse of these families; 90 % of all GPCRs, including the adrenoceptors, belong to the rhodopsin-like family (Fredriksson *et al.*, 2003; Latek *et al.*, 2012; Wolf and Grünewald, 2015).

2.1.2.1 α_2-adrenoceptor subtypes and genes

Endogenous catecholamines, adrenaline and noradrenaline, bind to and activate all members of the adrenoceptor family. Already in 1948, Ahlquist had suggested that "adrenotropic" receptors should be divided into two different types which he defined as α- and β-receptors (Ahlquist, 1948). As the amino acid sequences and biological and pharmacological properties of these receptors were untangled in the following decades, the receptors could be classified more precisely. Today, adrenoceptors are divided into three classes, namely α_1-, α_2- and β-ARs, and each adrenoceptor class is further divided into three subtypes: α_{1A}, α_{1B}, α_{1D}; α_{2A}, α_{2B}, α_{2C}; and β_1, β_2 and β_3, respectively (Bylund, 1992; Bylund *et al.*, 1994; Hieble *et al.*, 1995a; Ahles and Engelhardt, 2014). These different adrenoceptor subtypes are encoded by nine distinct genes in the genomes of humans and other mammalians (Fredriksson *et al.*, 2003; Ahles and Engelhardt, 2014).

It is now clear that mammals have three subtypes of α_2-ARs: α_{2A}, α_{2B} and α_{2C}. This classification of α_2-ARs was first established based on radioligand binding data, but was supported by preceding functional studies and finally confirmed by molecular cloning (Civantos Calzada and Aleixandre de Artiñano, 2001; Guimarães and Moura, 2001). A functional approach widely used to characterize the α_2-AR subtypes in different tissues has been to compare the functional potencies of sets of antagonists with their affinities to all receptor subtypes by means of radioligand binding in native tissues or transfected cells expressing only one subtype. The adrenoceptor subtype found in human platelets was identified as α_{2A}, while the α_{2B}-subtype was identified in neonatal rat lung, rat cerebral cortex and kidney (Bylund *et al.*, 1988). The α_{2C}-AR was identified by ligand binding studies in opossum kidney and in cell lines derived from this organ (Murphy and Bylund, 1988). Initially, also a fourth α_2-AR subtype, termed α_{2D}, was porposed to exist in rat submaxillary glands (Michel *et al.*, 1989) and also in the bovine pineal gland (Simonneaux *et al.*, 1991), but this was later recognized to represent species variants (orthologs) of the human α_{2A}-subtype, displaying some differences in its pharmacological properties (Bylund *et al.*, 1994; Docherty, 1998). Finally, with the introduction of molecular cloning techniques, which enabled the isolation and identification of the genes encoding different receptor subtypes, a consistent pharmacological classification of α_2-ARs could be confirmed.

The three human α_2-AR genes were cloned in the late 1980's and early 1990's, and the genetic 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. This molecular classification corresponded well with the earlier pharmacological classification of α_2-ARs into subtypes α_{2A}, α_{2B} and α_{2C} (Bylund *et al.*, 1995). By means of molecular cloning techniques, a true fourth α_2-AR subtype was identified in the zebrafish genome, where altogether five distinct α_2-AR genes were identified. Three of these genes code for orthologues of the mammalian α_2-ARs, while two genes encode a duplicated, fourth α_2-AR subtype (Ruuskanen *et al.*, 2004; Ruuskanen *et al.*, 2005). With respect to the α_{2B}- and α_{2C}-subtypes, the pharmacological characteristics are very similar across all investigated

mammalian species. However, the amino acid sequences of the α_{2A}-ARs cloned from certain species e.g. human or pig are slightly different compared with the homologous receptors cloned from rodents (rat, mouse, guinea pig); there is a serine residue in the rodent α_{2A}-ARs instead of a cysteine at the position corresponding to Cys^{201} of the human receptor. The pharmacological properties of the Ser^{201} receptor differ from the Cys^{201} receptor, and the two receptors were first distinguished as α_{2A}-AR (e.g. humans) and α_{2D}-AR (e.g. rodents) (Bylund *et al.*, 1992; Funk *et al.*, 1995; Limberger *et al.*, 1995a; Limberger *et al.*, 1995b; Trendelenburg *et al.*, 1995; Trendelenburg *et al.*, 1996; Paiva *et al.*, 1997; Guimarães *et al.*, 1998). At the molecular and cellular level, many significant differences exist between the α_2-AR subtypes, for example in their ligand binding properties and regulation, and they also have different tissue distributions (Eason and Liggett, 1993a; Eason *et al.*, 1994a; MacDonald and Scheinin, 1995; MacDonald *et al.*, 1997; Richman and Regan, 1998). These topics will be discussed in more detail in the upcoming chapters.

2.1.2.2 Structure of α_2-adrenoceptors

The current structural models of GPCRs were originally based on direct measurements made with the light-sensitive photoreceptor rhodopsin, and have quite recently been refined with information derived from X-ray crystallography of several crystallized GPCR proteins. Rhodopsin has been the most extensively investigated GPCR, which is easily explained by some of its unique properties: it is one of the most stable and detergent-tolerant GPCRs and, in contrast to most other GPCRs, it can be relatively easily isolated from a readily available tissue source (bovine retina) in large quantities, making it a convenient choice for structural and functional investigations (Khorana, 1992; Schertler, 1998). A low resolution model of bacteriorhodopsin provided the first sketch of a GPCR structure (Unwin and Henderson, 1975), but the initial insights into the true structure of GPCRs came from two-dimensional X-ray crystallography of bovine rhodopsin that revealed the general architecture of the seven TM helices (Schertler *et al.*, 1993; Krebs *et al.*, 1998). Major advances in the structural biology of GPCRs were made in the first decade of the 21st century. The first crystal-based structure of a GPCR, the crystal structure of bovine rhodopsin in complex with 11-cis-retinal, was resolved by Palczewski and colleagues in 2000 (Palczewski *et al.*, 2000). The resolution of the structure of other GPCRs was somewhat hindered by their low abundance, structural flexibility and instability in detergent solutions (Rosenbaum *et al.*, 2009; Zhang *et al.*, 2015). It took years of extensive research and technology development by many laboratories around the world before scientists succeeded in describing the first crystal structures of ligand-activated GPCRs: the human β_2-AR (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007; Rasmussen *et al.*, 2011; Rosenbaum *et al.*, 2011), the turkey β_1-AR (Warne *et al.*, 2008; Warne *et al.*, 2011; Warne *et al.*, 2012) and the human A_{2A} adenosine receptor (Jaakola *et al.*, 2008), as well as the first active-state GPCR structure of ligand-free rhodopsin (opsin) (Scheerer *et al.*, 2008). These crystal structures provided the first opportunity to understand how the structure of a receptor protein determines its unique functional properties and why these are such multifaceted signaling molecules. In the following years, technological advances in crystallography, such as the preparation of stable receptor – G protein complexes in detergent solution (Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b), and improved methods for membrane protein solubilization, stabilization and

crystallization (Chae *et al.*, 2010; Caffrey, 2011), led to an exponential increase in the number of GPCR structures resolved. At present, more than 100 structures of GPCRs, mainly from the rhodopsin-like subfamily, have been determined in complex with ligands of varied pharmacologies. These structures have provided many new insights into the structural similarities and the diversity of GPCRs as well as revealing details of important phenomena such as receptor conformation, ligand binding, activation, allosteric modulation and dimerization (Tebben and Schnur, 2011; Katritch *et al.*, 2012; Zhang *et al.*, 2015; Isberg *et al.*, 2016; Munk *et al.*, 2016).

In spite of the great advances made in receptor crystallization over the past decade, the crystal structures of α_2-ARs have not yet been resolved. Therefore, much of what is known about the structures and structure-function relationships of the α_2-ARs has been extrapolated from the related and well-studied structures of rhodopsin and, especially, the β-ARs. α_2-ARs, like other GPCRs, consist of seven TM-spanning α-helices (TM1−TM7) connected by three extracellular loops and three intracellular loops. The N-terminus of a GPCR is on the extracellular side of the cell membrane with the C-terminus located intracellularly (Figure 2). In general, the extracellular parts of the receptors recognize the huge spectrum of their cognate ligands and modulate ligand access into the binding site, the TM regions form the structural core, bind ligands and transduce extracellular signals to the intracellular regions through conformational changes, and the intracellular parts interact with cytosolic signaling proteins (Harrison *et al.*, 1991; Lefkowitz, 2007; Latek *et al.*, 2012; Venkatakrishnan *et al.*, 2013; Zhang *et al.*, 2015). The receptor core, formed by TM1−TM7, is well conserved among different rhodopsin-like GPCR family members, whereas the terminal regions and loop regions are much more divergent, even between closely related receptor subtypes.

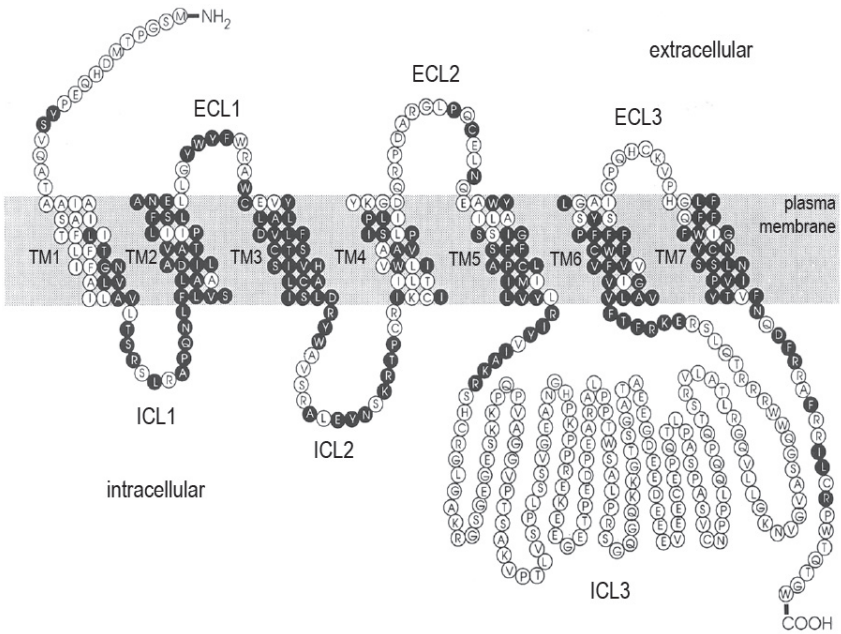

Figure 2. Schematic amino acid sequence representation of a rat α_{2B}-AR. The shaded residues are identical in all three α_2-AR subtypes. ECL = extracellular loop, ICL = intracellular loop, TM = transmembrane region [modified from (Harrison *et al*., 1991)].

As shown in Figure 2, the three α_2-AR subtypes share approximately 75 % amino acid identity in their TM regions, while the intra- and extracellular loop regions are more divergent (Harrison *et al.*, 1991; Bylund *et al.*, 1992; Saunders and Limbird, 1999; Venkatakrishnan *et al.*, 2013). The ligand binding pocket is located within the cell membrane and is formed by TM2−TM7. A high degree of structural homogeneity has been observed in the ligand binding pockets of related GPCR subtypes, for example the ligand binding pockets of β_1- and β_2-ARs are almost identical. This high conservation in receptor structure probably explains much of the difficulties in obtaining potent subtype-selective compounds for different classes of GPCRs (Rosenbaum *et al.*, 2009; Venkatakrishnan *et al.*, 2013). Agonist and antagonist selectivity between different receptor subtypes is due to structural differences within these TM domains. In the α_2-ARs, as well as in other adrenoceptors, a conserved aspartic residue (Asp^{113}) in TM3 is crucial for receptor function by providing an anchoring point for the charged amino group of agonist ligands (Ruffolo and Hieble, 1994; Venkatakrishnan *et al.*, 2013). The main binding site differences among the α_2-AR subtypes are located in TM5 and in the second extracellular loop (Laurila *et al.*, 2007; Ostopovici-Halip *et al.*, 2011), whereas TM1 is involved in determining the subtype-specific binding of some antagonists (Laurila *et al.*, 2011). Moreover, TM7 may be important in determining differences in antagonist binding specificity between α_2- and β_2-ARs (Kobilka *et al.*, 1988). The greatest structural differences between α_2-ARs and other members of the adrenoceptor family are found in the third intracellular loop and the C-terminal region. The third intracellular loop, which has been identified as an important site of receptor-G protein interaction in all adrenoceptors, is much longer in α_2-ARs as compared with α_1- and β-ARs, whereas the C-terminus of α_2-ARs is shorter than those of α_1- and β-ARs (Strosberg, 1993; Ruffolo and Hieble, 1994; Bylund *et al.*, 1995). This is in line with the observation that receptors involved in the regulation of adenylyl cyclase (AC) activity, like α_2-ARs, have shorter C-terminal sequences, whereas receptors coupling to other effector systems like phospholipase C (PLC) (e.g. α_1-ARs) have longer sequences in this region (Strosberg, 1993; Ruffolo and Hieble, 1994).

There are some post-translational protein modifications which differ slightly among the three α_2-AR subtypes: α_{2A} is glycosylated in its extracellular N-terminus and palmitoylated in its intracellular C-terminus, whereas the α_{2B}-subtype is palmitoylated in its C-terminus but is not glycosylated, and the α_{2C}-subtype is glycosylated in its N-terminus but is not palmitoylated (Keefer *et al.*, 1994). Mutations that eliminate these post-translational modifications do not perturb receptor-G protein coupling or receptor trafficking, and thus the functional significance of these differences in post-translational modifications among the different subtypes has remained uncertain (Kennedy and Limbird, 1993; Eason *et al.*, 1994b; Keefer *et al.*, 1994; Angelotti *et al.*, 2010). However, mutations eliminating palmitoylation of the α_{2A}-adrenoceptor completely abolished the downregulation of receptor number after prolonged agonist exposure (Eason *et al.*, 1994b).

Within an animal species, slight variations in the amino acid sequence of a receptor are quite common and may result in polymorphic receptors with altered structure or function. Indeed, all nine human adrenoceptor subtype genes display substantial variation, both in their coding sequences and in the adjacent regulatory regions. In

other words, receptor polymorphism may lead to altered affinity and potency of ligands, as a result of altered accessibility of the ligand binding pocket of the receptors due to variations in the N-terminus, the extracellular loops or the ligand binding pocket itself. Variations in the intracellular parts of the proteins may be expected to preferentially alter the interaction with G proteins or other interacting proteins. Approximately 4000 different variants of the genes encoding human adrenoceptors had been reported by 2014, including single nucleotide exchanges as well as insertion/deletion variants (Ahles and Engelhardt, 2014). Obviously, polymorphic variation exists for all three α_2-AR subtypes. The known variants of the α_{2A}-subtype include a single nucleotide polymorphism (asparagine to lysine substitution) in the third intracellular loop of the human α_{2A}-AR, leading to enhanced agonist-promoted G_i-coupling (Small *et al.*, 2000a), and a variant in the noncoding region of the α_{2A}-AR gene located 1252 nucleotides upstream of the start codon (−1291C/G polymorphism) suggested to be associated with enhanced receptor expression (Lario *et al.*, 1997; Sickert *et al.*, 2009). Three deletion variants of the human α_{2B}-AR gene have been identified. All of them are fairly common, occur in-frame and map to a limited region within the third intracellular loop. One of the common polymorphic variants consists of a deletion of three glutamic acids (residues 301-303) in the third intracellular loop (Heinonen *et al.*, 1999). This polymorphism shortens the stretch of acidic residues that is thought to establish the environment for agonist-promoted phosphorylation and desensitization of the receptor by G protein-coupled receptor kinases (GRKs). This deletion variant has shown significantly impaired agonist-promoted receptor desensitization in cultured transfected cells (Small *et al.*, 2001). In humans, this deletion variant has been associated with reduced basal metabolic rate (Heinonen *et al.*, 1999), and an increased risk of acute myocardial infarction (Snapir *et al.*, 2001; Snapir *et al.*, 2003a) and sudden cardiac death (Snapir *et al.*, 2003a). In addition, the human α_{2C}-AR has a common deletion variant in its third intracellular loop. Because of the deletion of four amino acids, this variant lacks an acidic motif in a region that is expected to be essential for G protein coupling. However, studies in mammalian cell lines have reported inconsistent findings: agonist-stimulated inhibition of AC was not affected in human embryonic kidney 293 (HEK-293) cells (Montgomery and Bylund, 2010), whereas in Chinese hamster ovary (CHO) cells, agonist-stimulated AC inhibition, extracellular stimulus-regulated kinase (ERK) 1/2 signaling and inositol phosphate production were significantly decreased (Small *et al.*, 2000b). Although some of these polymorphic receptor variants are rather common, the impact of genetic variation with regard to the efficacy of therapeutic drugs has not been systematically investigated (Ahles and Engelhardt, 2014).

2.1.3 Signaling of α_2-adrenoceptors

2.1.3.1 Coupling to G proteins

A major function of all cell membrane receptors is to recognize and bind the ligand molecules present in the extracellular environment and to mediate their signals into the cell. The interaction of external messengers with receptors at the cell surface represents the first step in a cascade of molecular events that underlies transmembrane (TM) signaling. Receptor activation typically results in subsequent activation of various effector proteins, such as enzymes and ion channels, which then mobilize chemical second

messengers that regulate various cellular functions. Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are key players in TM signaling. Research in the field of G proteins dates back more than 40 years and since then, it has become evident that G protein-mediated signal transduction is the most widely used TM signaling system, linking hundreds of different receptors to dozens of G proteins and effectors in the plasma membrane (Hepler and Gilman, 1992; Wettschureck and Offermanns, 2005; Luttrell, 2006; Syrovatkina *et al.*, 2016). Moreover, the actions of G proteins are not restricted to TM signaling at the cell surface; G proteins also appear to regulate pathways within cells, such as in Golgi membranes (Stow *et al.*, 1991; Ktistakis *et al.*, 1992).

GPCRs comprise a large and diverse superfamily - members of the family have been identified in organisms evolutionarily as distant from humans as yeast, and remarkably, G protein-mediated signaling is a general signal transduction mechanism shared by all GPCRs. G protein-mediated signaling is employed by virtually all cells in mammalian organisms and is crucially involved in such diverse physiological functions as perception of sensory information, modulation of synaptic transmission, hormone release and actions, regulation of cell contraction and migration, or cell growth and differentiation (Wettschureck and Offermanns, 2005). This huge diversity of functions is possible because of the substantial variation of GPCR family members in their ligand recognition properties and coupling to diverse signaling pathways. The repertoire of GPCR ligands is extensive and structurally diverse, ranging from light-activated retinal and odorants to many hormones and neurotransmitters. The combination of multiple isoforms of G proteins together with the large variety of receptors, effectors and regulatory proteins creates a highly versatile signal transduction system (Wettschureck and Offermanns, 2005; Lefkowitz, 2007; Rosenbaum *et al.*, 2009). About 800 GPCR genes have been identified in mammalian genomes (Fredriksson *et al.*, 2003). While about half of these GPCRs are sensory receptors like olfactory or taste receptors, the other half recognize non-sensory stimuli such as neurotransmitters, hormones or paracrine factors. For most GPCRs the physiological ligands are known, but there are about 100 so-called orphan GPCRs for which no endogenous ligand has been identified [http://gpcrdb.org/, 22.5.2017; (Isberg *et al.*, 2016; Munk *et al.*, 2016)] .

G proteins are composed three subunits, namely α, β and γ. The Gα subunit binds and hydrolyzes GTP. The β and γ subunits are tightly associated as a Gβγ dimer and can be regarded as one functional subunit. Heterotrimeric G proteins are part of a larger GTPase superfamily that includes the small GTP-binding proteins such as Ras and Rho (Gilman, 1987; Hepler and Gilman, 1992; Hamm and Gilchrist, 1996; Syrovatkina *et al.*, 2016). G proteins are characterized by their Gα subunits. More than 20 different Gα subunits have been identified in mammals, and based on their amino acid sequences (56 to 95 % identity) and functional similarities, these subunits have been grouped into four families: $G\alpha_s$ (s indicating stimulation of AC), $G\alpha_i$ (i indicating inhibition of AC), $G\alpha_q$ and $G\alpha_{12}$. Most Gα subunits are widely expressed and individual cells usually contain at least four or five types of Gα subunits (Neer, 1994; Neer, 1995). The molecular weight of α subunits ranges from 39 to 52 kDa (Hepler and Gilman, 1992; Neer, 1994). The $G\alpha_s$ family has two members: $G\alpha_s$, which is expressed in most types of cells and $G\alpha_{olf}$, which is specifically expressed in olfactory sensory neurons. The $G\alpha_i$ family is the largest and most varied family with seven members; it contains $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_t$, $G\alpha_g$ and $G\alpha_z$. $G\alpha_i$ proteins

have been detected in most types of cells. However, some of the subunits have a more restricted pattern of distribution: $G\alpha_o$ is highly expressed in neurons, the expression of $G\alpha_t$ is located in the eye, $G\alpha_g$ is found in taste receptor cells and $G\alpha_z$ is expressed in neuronal tissues and blood platelets. In humans, the $G\alpha_q$ family comprises four members: $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$ (the mouse equivalent is $G\alpha_{15}$). $G\alpha_q$ and $G\alpha_{11}$ are ubiquitously expressed, whereas the expression of the other two family members is more restricted. $G\alpha_{14}$ is found mainly in the kidney, lung and liver, and $G\alpha_{16}$ is specifically expressed in hematopoietic cells. The $G\alpha_{12}$ family is composed of two members, $G\alpha_{12}$ and $G\alpha_{13}$, which are expressed in most types of cells (Hepler and Gilman, 1992; Wettschureck and Offermanns, 2005; Syrovatkina *et al.*, 2016). There are five Gβ and twelve Gγ genes in the human and mouse genomes. Subunits $G\beta_1$–$G\beta_4$ share high amino acid sequence similarities (80-90 %) and are widely distributed throughout the body, while $G\beta_5$ is only approximately 50 % similar to the other Gβ subunits and is mainly found in the brain. Compared with the Gβ subunits, Gγ subunits are small (6-9 kDa), more diverse and much more different from each other with sequence similarities ranging from 26 % to 76 % (Hepler and Gilman, 1992; Neer, 1994; Neer, 1995; Khan *et al.*, 2013; Syrovatkina *et al.*, 2016). The Gβ and Gγ subunits do not act as monomers, but function as a tightly complexed Gβγ dimer which dissociates only under denaturing conditions. Gβγ dimers serve to increase the affinity of Gα for its receptor and to regulate a variety of effectors, either directly or in conjunction with Gα, and have also been implicated in the recruitment of GPCR kinases to the cell membrane (Hepler and Gilman, 1992; Hamm and Gilchrist, 1996; Vögler *et al.*, 2008; Khan *et al.*, 2013). The identified Gβ and Gγ isoforms could in theory form 60 different combinations, but not all of the possible pairs have been actually detected; e.g. $G\beta_1$ is able to interact with $G\gamma_1$ and $G\gamma_2$, but the very similar $G\beta_2$ molecule is able to form dimers only with $G\gamma_2$ (Neer, 1995). The different Gβγ pairs differ in their ability to bind Gα subunits and to activate effectors (Neer, 1994; Khan *et al.*, 2013); for example, $G\beta_1\gamma_2$ and $G\beta_1\gamma_3$ dimers were found to be 10–20 times more potent in stimulating AC type II than $G\beta1\gamma_1$ dimers (Iñiguez-Lluhi *et al.*, 1992). Moreover, the random association of the different Gα, Gβ and Gγ subunits would generate hundreds of different heterotrimeric G proteins, but it appears that there are preferred combinations of isoforms that interact to form a more limited number of distinct heterotrimeric complexes (Hamm and Gilchrist, 1996; Denis *et al.*, 2012).

The biological activity of G proteins is determined by the bound guanine nucleotide; i.e. GDP or GTP in the inactive or active state, respectively. Hence, G proteins act as molecular switches that are turned on and off via the GTPase cycle (Figure 3). In the inactive form, the GDP-bound Gα subunit is tightly associated with the Gβγ heterodimer. Downstream signaling is initiated after the agonist binds to a GPCR. Agonist binding activates the receptor, which in turn promotes the exchange of bound GDP on Gα with GTP. This leads to the dissociation of the Gα subunit from the Gβγ dimer, resulting in two functional subunits. Both Gα and free Gβγ are capable of signaling to various cellular pathways by interacting with downstream effector proteins (Gilman, 1987; Hepler and Gilman, 1992; Neer, 1994; Neer, 1995; Syrovatkina *et al.*, 2016).

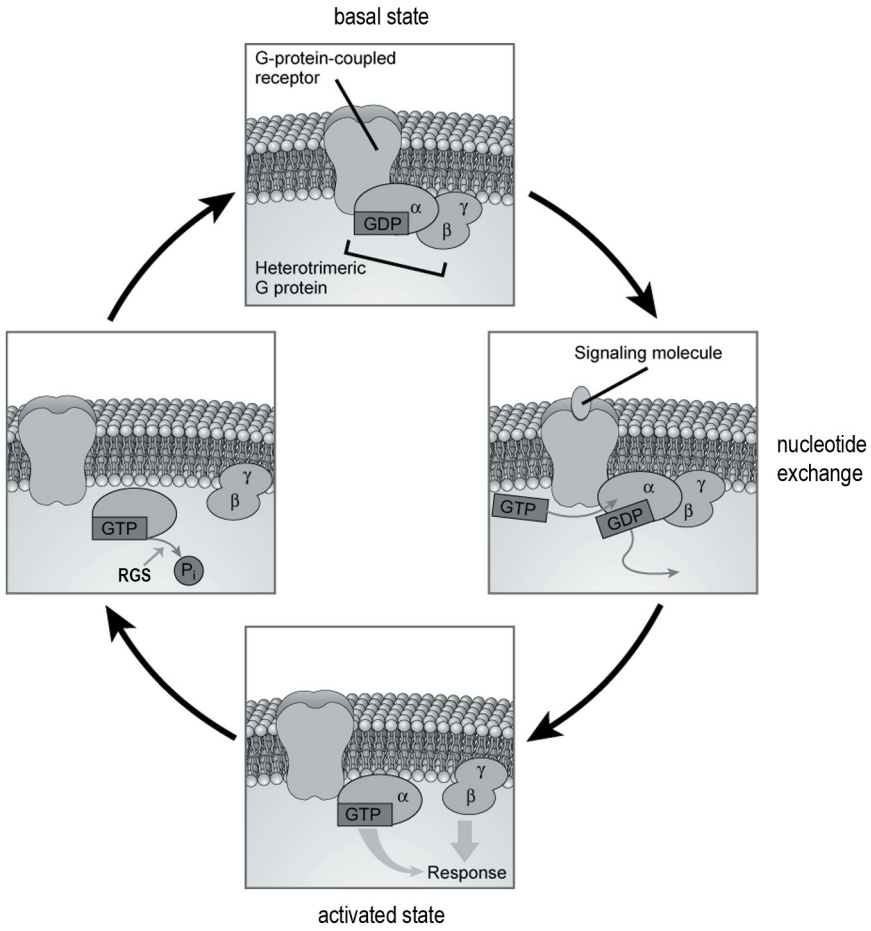

Figure 3. The G protein cycle. The binding of a signaling molecule to the GPCR leads to GDP-GTP exchange on the Gα subunit and dissociation of the GTP-bound Gα subunit from the Gβγ dimer. Both Gα and Gβγ may regulate downstream effectors to trigger cellular responses. GTP hydrolysis by the Gα subunit results in re-association of GDP-bound Gα with the Gβγ dimer, thus completing the G protein cycle. RGS (regulator of G protein signaling) proteins, as well as many effectors, can accelerate the rate of hydrolysis of GTP to GDP [modified from (OpenStax College, 2013)].

The Gβγ subunit may also serve another type of function: to protect the cell from overstimulation by promoting feedback inactivation of the receptor. Receptors are regulated by phosphorylation at serine and threonine residues on their cytoplasmic C-terminal tails by receptor-specific kinases and the phosphorylated receptors are less susceptible to subsequent activation. Thus, a feedback loop is created that controls the duration of receptor activation. This function has been most extensively studied for the β-ARs and muscarinic receptors; the Gβγ subunit has been shown to stimulate phosphorylation of these receptors by their respective kinases (Haga and Haga, 1992; Pitcher *et al.*, 1992; Neer, 1994; Wu *et al.*, 2000; Galés *et al.*, 2005). Gα signaling is terminated by the intrinsic GTPase activity of the subunit, which hydrolyzes bound GTP to GDP. Once GTP is cleaved to GDP, the Gα and Gβγ subunits reassociate, which in turn terminates Gβγ signaling. The reassociated, inactive

G protein returns to the receptor and primes the system to respond again. The rate of GTP hydrolysis is a timing mechanism that controls the duration of both Gα and Gβγ subunit activation. Different Gα subunits have different intrinsic rates of GTP hydrolysis. If a receptor activates two Gα subunits that have very different activated half-lives, one signal may be quickly extinguished while the other could be sustained and predominant (Neer, 1995). G proteins have a high intrinsic affinity for GDP (pM in some cases) that causes the GDP dissociation process to be slow. Guanine nucleotide exchange factors (GEFs) act to weaken the affinity of the Gα subunit for GDP; in this regard, activated GPCRs function as GEFs by promoting the exchange of GDP to GTP. In addition to GPCRs, G proteins can be regulated by non-receptor proteins such as Ric-8, G protein regulator (GPR) domain-containing proteins, Gα binding and activating (GBA) domain-containing proteins and regulator of G protein signaling (RGS) domain-containing proteins. These non-receptor proteins may facilitate or inhibit the exchange of GDP with GTP, regulate the membrane translocation of Gα subunits, compete with Gβγ for Gα binding, fine-tune the duration and extent of signal transduction and modulate the G protein deactivation cycle to achieve the desired cellular signal output (Syrovatkina *et al.*, 2016).

G proteins relay signals from GPCRs to a wide range of downstream effectors, including ACs, isoforms of phospholipases A, C and D, ion channels, protein tyrosine kinases and MAP kinases, among others. Several well-defined effector systems have been identified for G proteins (Figure 4). Both $G\alpha_s$ and $G\alpha_i$ regulate ACs; $G\alpha_s$ stimulates AC activity, resulting in increased formation of cyclic AMP (cAMP). Elevated cAMP, in turn, results in the activation of cAMP-regulated proteins such as protein kinase A (PKA) and cyclic nucleotide-gated ion channels. $G\alpha_i$, on the contrary, inhibits AC activity, leading to reduced intracellular cAMP levels. $G\alpha_q$ proteins activate β-isoforms of PLC (PLCβ) which further convert phosphatidylinositol-4,5-bisphosphate (PIP_2) into two second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). $G\alpha_{12/13}$ proteins can control a variety of signaling pathways leading to the activation of various downstream effectors including phospholipase A_2 (PLA_2), Na^+/H^+ exchanger and c-Jun N-terminal kinase (JNK). Moreover, $G\alpha_{12/13}$ family proteins are able to directly increase the activity of RhoGEF proteins and to interact with other effectors, such as tyrosine kinases and cadherins (Taylor *et al.*, 1991; Hepler and Gilman, 1992; Wettschureck and Offermanns, 2005; Syrovatkina *et al.*, 2016).

The Gα subunits of G_i and G_s proteins possess specific residues that can be covalently modified by certain bacterial toxins. Pertussis toxin (PTX) is capable of selectively modifying the function of G_i-proteins by ADP-ribosylating a cysteine residue on the α-subunit. This leads to the uncoupling of the G_i protein from its receptor and blocking of receptor-mediated responses (West *et al.*, 1985; Winslow *et al.*, 1987). Another bacterial product, cholera toxin, modifies the function of G_s proteins through an ADP-ribosylation reaction that acts to lock the G_s protein in its active, GTP-bound form, thereby continually stimulating ACs to produce cAMP (Bharati and Ganguly, 2011). Thus, PTX and cholera toxin may be used to study the roles of G_i and G_s proteins in receptor signaling. The fact that PTX blocks many cellular responses emphasizes the involvement of G_i proteins in a variety of signaling pathways and, indeed, the cellular concentrations of G_i proteins are much higher than those of G_s or G_q proteins. The activation of G_i proteins is, therefore, also believed to be the major coupling mechanism that results in the activation of Gβγ-mediated signaling processes (Clapham and Neer, 1997; Khan *et al.*, 2013).

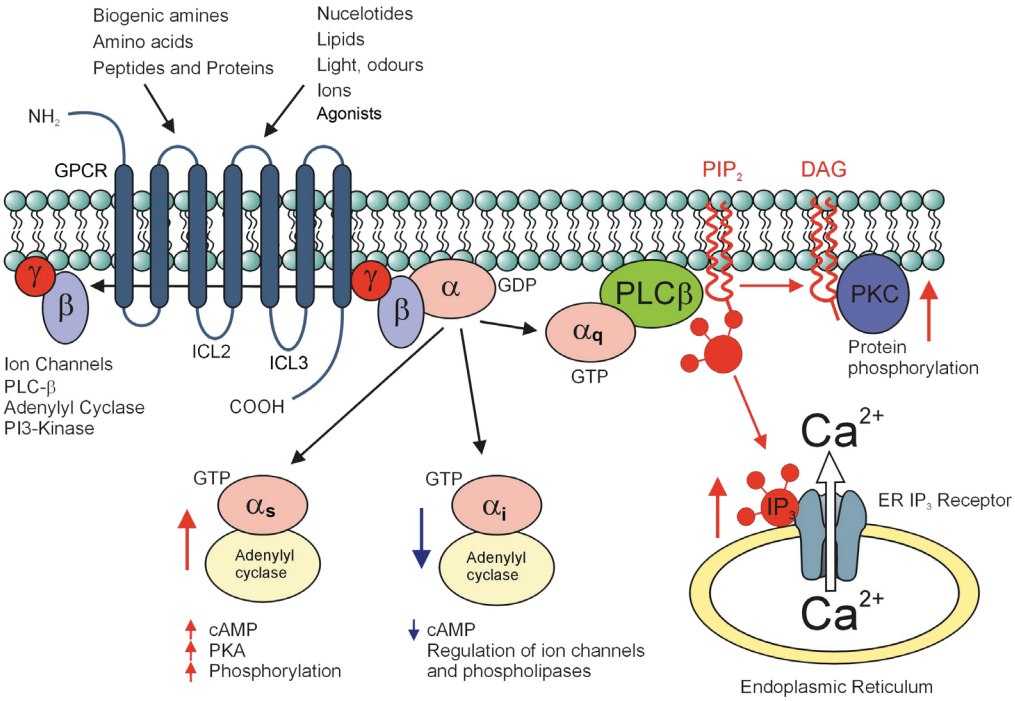

Figure 4. G proteins relay signals from activated GPCRs to regulate a variety of cellular responses. Different Gα subunits and Gβγ subunits are capable of regulating a wide range of downstream effectors, including adenylyl cyclases, cAMP, phospholipase C (PLC), protein kinases (PKC, PKA), phosphoinositide 3 (PI3)-kinases and ion channels [modified from (Summers, 2014)].

The pattern of G proteins activated by a given receptor determines the cellular and biological responses, and activated receptors that induce functionally similar or identical responses typically activate the same G protein subtypes (Wettschureck and Offermanns, 2005). Each subfamily of adrenoceptors preferentially couples to a certain type of G protein; α_1-ARs couple to G_q proteins, β-ARs primarily couple to stimulatory G_s proteins and α_2-ARs couple to inhibitory G_i proteins. The specificity with which G proteins and their subunits interact with receptors and effectors defines the range of responses a cell is able to elicit to a given external signal. G proteins act as a crucial control point determining whether a signal is focused to a single pathway or spreads through several pathways. If a receptor is capable of coupling to only one subtype of G proteins which, in turn, can activate only one effector, the cellular response may be quite focused. In contrast, if a receptor can interact with several G proteins, each of which can interact with more than one effector, the response would be expected to be spread broadly through several pathways (Neer, 1995; Neer, 1994). Coupling to alternative G proteins has been reported for several adrenoceptor subtypes, including β-ARs and α_2-ARs (Eason *et al.*, 1992; Pohjanoksa *et al.*, 1997; Soeder *et al.*, 1999; Xiao, 2001).

The dissociation of G protein subunits can generate parallel and/or interactive signals via both Gα and Gβγ subunits. However, the Gβγ subunit was originally thought to be necessary mainly for the inactivation of Gα subunits, allowing them to reassociate with the receptor for subsequent rounds of signaling. In this regard, Gβγ was considered to

be a negative regulator of Gα signaling [reviewed by (Neer, 1995; Khan *et al.*, 2013)]. In addition, Gβγ has been shown to play a role in agonist-induced receptor phosphorylation and desensitization (Haga and Haga, 1992). In the last 30 years, it has become clear that Gβγ subunits perform a vast array of functions in cells with respect to signaling (Figure 4), often independently but also in concert with Gα subunits. Gβγ subunits interact with receptors, Gα subunits, effectors and regulatory enzymes such as GRKs, highlighting the central role they play in all aspects of GPCR signaling (Smrcka, 2008; Khan *et al.*, 2013). Gβγ subunits have been shown to regulate a large number of downstream effectors including ACs, PLCβ, PLA_2, PI3-kinases, β-adrenergic receptor kinases, inwardly rectifying K^+ channels and voltage-gated Ca^{2+} channels (Clapham and Neer, 1993; Neer, 1995; Clapham and Neer, 1997; Khan *et al.*, 2013). Many of these effectors are regulated by both Gα and Gβγ subunits, although the pattern of regulation is specific to each effector protein. For example, one isoform of AC is activated by Gα and unaffected by Gβγ, whereas another isoform is activated by Gα and synergistically further activated by Gβγ, and a third isoform is activated by Gα but inhibited by Gβγ subunits (Tang and Gilman, 1991). In addition to the canonical effector pathways that have been established for Gβγ, Gβγ subunits may also directly interact with the cytoskeleton, thus influencing many cellular functions e.g. cell migration, intracellular trafficking, cell survival and proliferation (Roychowdhury and Rasenick, 2008; Dave *et al.*, 2009). Gβγ subunits are able to mediate calcium release from the endoplasmic reticulum (ER) independently of PLCβ activation by binding directly to the IP_3 receptor (Zeng *et al.*, 2003). Moreover, Gβγ has been functionally associated with the Golgi apparatus where it may regulate protein trafficking (Khan *et al.*, 2013).

In addition to the well-established downstream effectors of Gα and Gβγ subunits, there are other, non-canonical proteins reported to interact with G proteins, including tyrosine kinases, tubulin, actin, cadherins, protein kinase D and vinculin, among others. Although the physiological significance of these interactions is at present uncertain, these interactions might underlie cell-type-specific G protein functions and should be further explored. There are also many reports of physiological and pathological functions of G proteins that cannot be easily explained by the currently well-defined downstream effectors, for example the role of G proteins in cell division and receptor tyrosine kinase signaling. It is not clear whether GPCRs participate in these pathways; however, in some cases, transactivation of GPCRs by RTKs has been proposed [reviewed by (Syrovatkina *et al.*, 2016)].

2.1.3.2 Activation and pharmacology

Binding of endogenous catecholamines or exogenous agonists stabilizes adrenoceptors in an active conformation, coupling to and activating G proteins, and thereby initiating various intracellular signaling cascades. Most receptor theories trying to explain signaling events that occur by the interaction of a ligand with its specific receptor have their origin in the theories of Clark (1937), who recognized that the ability of a drug to induce an intracellular signal depended on the drug forming a reversible complex with the receptor (Clark, 1937). The widely accepted model to describe agonist activation of GPCRs is the so-called ternary complex model presented by De Lean and Lefkowitz in 1980, postulating that receptor activation requires agonist-promoted formation of an active, “ternary” complex of agonist, receptor and G protein (De Lean *et al.*, 1980). This model was then revised and

extended when it became evident that many receptors can activate G proteins also in the absence of agonists and that different classes of drugs (full agonists, partial agonists, antagonists and inverse agonists) have differential effects on receptor signaling (Samama *et al.*, 1993). The extended ternary complex model proposed that receptors exist in an equilibrium of two functionally different states, the inactive and the active state, and that ligands are able to shift this equilibrium based on their efficacies (Kenakin 1995; Gether & Kobilka 1998; Perez & Karnik 2005). As more evidence emerged, the picture of how drugs bind and activate receptors has become much more complex than was originally visualized. Receptors can adapt many different conformations, stabilized differentially by the binding of different ligands, activate more than one G protein isoform and also signal through G protein-independent pathways (Perez and Karnik, 2005; Kobilka and Deupi, 2007). Nowadays, GPCRs are considered to be structurally dynamic entities that exist in a range of conformations. GPCRs do not act as simple on-off switches, but rather like fluctuating integrators of information. Agonist binding and receptor activation are likely to occur through a series of conformational intermediates and structurally distinct ligands can stabilize different receptor conformations leading to differential effects on downstream signaling proteins and activation of specific signaling pathways. Moreover, ligands can differentially activate signaling pathways mediated through a single GPCR by stabilizing distinct active conformations of the receptor, a phenomenon termed biased agonism or functional selectivity. In the extreme, biased ligands may be both agonists and antagonists for different functions mediated by the same receptor (Urban *et al.*, 2007; Kenakin, 2011; Luttrell, 2014). The dynamic nature of GPCRs is likely to be essential to account for the wide variety of physiological functions mediated by these receptors (Kobilka and Deupi, 2007; Park *et al.*, 2008; Bockenhauer *et al.*, 2011).

The two catecholamines, adrenaline and noradrenaline, are full agonists of all three α_2-AR subtypes and their binding affinities are also quite similar for all subtypes. In contrast many synthetic α_2-AR agonists and antagonists with differing pharmacological properties have been discovered or developed. α_2-AR agonists can be ranked according to their selectivity for α_2-ARs over α_1-ARs. Clonidine was the first selective α_2-adrenergic drug approved for use in humans and other agonists can be compared to clonidine in terms of $\alpha_2 : \alpha_1$ selectivity. The $\alpha_2 : \alpha_1$ –ratio of clonidine is 200 : 1. Dexmedetomidine is the most selective clinically available α_2-agonist with an $\alpha_2 : \alpha_1$ –ratio of 1600 : 1, more than seven times greater than that of clonidine. Based on $\alpha_2 : \alpha_1$ selectivity, the rank order of α_2-AR agonists is dexmedetomidine >> brimonidine (UK 14.304), rilmenidine, BHT 920 > clonidine = ST-91, guanabenz, guanfacine, tizanidine > oxymetazoline (Khan *et al.*, 1999; Angelini *et al.*, 2000; Kamibayashi and Maze, 2000).

Many of currently employed α_2-AR agonists can be considered as subtype-preferring, but none of them is truly α_2-AR subtype-selective. Moreover, many of them also bind to other adrenergic and non-adrenergic receptors, in addition to α_2-ARs (Schoeffter and Hoyer, 1991; Gyires *et al.*, 2009). All clinically employed α_2-agonists share a five member heterocyclic ring in their structure, but vary in their relative efficacy, receptor specificity, duration of the effect and other pharmacological properties. Such agonists include clonidine, dexmedetomidine, guanfacine, oxymetazoline, moxonidine, rilmenidine and brimonidine. Oxymetazoline and guanfacine are rather selective for the α_{2A}-subtype, but no selective agonists exist for α_{2B}- or α_{2C}-ARs (Scheinin and Pihlavisto, 2000; Civantos

Calzada and Aleixandre de Artiñano, 2001; Gyires *et al.*, 2009). Dexmedetomidine and brimonidine demonstrate subtype-selectivity in terms of efficacy. Dexmedetomidine is a full agonist only at the α_{2B}-AR, and brimonidine is a full agonist at the α_{2A}-AR and only a partial agonist at the other two subtypes (Peltonen *et al.*, 1998).

Antagonist ligand development has been more fruitful in terms of subtype-selectivity, compared to agonists; antagonists showing preference for a certain subtype exist for all three α_2-ARs. BRL 44408 and BRL 48962 are selective for the α_{2A}-AR (Beeley *et al.*, 1995; Wikberg-Matsson *et al.*, 1995). Prazosin, ARC-239, imiloxan and chlorpromazine have relatively high affinity for the α_{2B}-AR (Bylund *et al.*, 1988; Michel *et al.*, 1990; Marjamäki *et al.*, 1993; Bylund *et al.*, 1994). MK-912 and rauwolscine show higher affinity for α_{2C}-ARs than for the other two subtypes (Hieble *et al.*, 1995b; Uhlén *et al.*, 1997). However, the subtype selectivity of these ligands is far from complete, e.g. rauwolscine is commonly used as a nonselective α_2-AR antagonist. Furthermore, atipamezole, RX821002, RS-79948, yohimbine and idaxozan show rather small differences in their binding affinity to the different α_2-AR subtypes (Marjamäki *et al.*, 1993; Bylund *et al.*, 1994; Halme *et al.*, 1995; Laurila *et al.*, 2011). Because of incomplete subtype selectivity, *in vivo* functional studies have not made it possible to establish a clear pharmacological differentiation of the functions mediated by each α_2-AR subtype.

2.1.3.3 Signal transduction mechanisms

The signal transduction mechanisms mediated by α_2-ARs have been extensively investigated both in recombinant cell expression systems and in native tissues. The properties of α_2-ARs appear to be similar among different animal species; the pharmacology of the cloned guinea pig α_2-AR subtypes was shown to be in agreement with data obtained for the native guinea pig receptors and was functionally similar to that of the cloned human α_2-AR subtypes (Svensson *et al.*, 1996). It is evident that α_2-ARs are involved in many important physiological functions throughout the body, reflecting their capability for multifaceted cellular signaling. α_2-ARs are capable of coupling to different types of G proteins and effectors depending e.g. on the receptor subtype, receptor density or cell/tissue type. Furthermore, α_2-ARs have also been demonstrated to signal *via* G protein-independent pathways via their interaction with several regulatory proteins, such as G protein-coupled receptor kinases (GRKs) and β-arrestins (Lefkowitz, 2007; Rosenbaum *et al.*, 2009).

Primarily, all three α_2-AR subtypes couple to PTX-sensitive, inhibitory G_i proteins and their main cellular responses are inhibition of AC activity, resulting in decreased cytosolic cAMP levels, inhibition of voltage-gated Ca^{2+} channels and activation of receptor-operated K^+ channels (Limbird, 1988; Cotecchia *et al.*, 1990; Surprenant *et al.*, 1992; Docherty, 1998; Saunders and Limbird, 1999). Inhibition of AC activity through α_2-AR activation was first reported in lysates of human blood platelets (Jakobs *et al.*, 1976) and has since been reported in many cells and tissues, including transfected cell lines, such as CHO, PC12, COS-7 and Sf9 (Fraser *et al.*, 1989; Cotecchia *et al.*, 1990; Jones *et al.*, 1991; Eason *et al.*, 1992; Eason *et al.*, 1994a; Eason and Liggett, 1995; Näsman *et al.*, 1997; Pohjanoksa *et al.*, 1997; Karkoulias *et al.*, 2007), NG108 neuroblastoma × glioma cells (Sabol and Nirenberg, 1979), HT29 human colon carcinoma cells (Turner *et al.*, 1985), OK opossum kidney cells (Murphy and Bylund, 1988; Kribben *et al.*, 1997), human and

hamster adipocytes (Burns *et al.*, 1981; Saulnier-Blache *et al.*, 1992), rat hepatocytes (Ogihara, 1995; Ogihara, 1996), rat and mouse striatum (Lu and Ordway, 1997; Zhang *et al.*, 1999), human erythroleukemia HEL 92.1.7 cells (Jansson *et al.*, 1998) and human neuroblastoma SH-SY5Y cells (Parsley *et al.*, 1999). For all α_2-AR subtypes, coupling has been demonstrated to G protein family members other than the preferential G_i proteins. Studies on recombinant α_2-ARs expressed in different cell lines have confirmed that the receptors have the potential to couple physically and functionally not only to G_i but also to G_s proteins. Coupling to stimulatory G_s proteins is affected by many factors, including receptor density (Fraser *et al.*, 1989), receptor subtype (Eason *et al.*, 1992; Pepperl and Regan, 1993; Jansson *et al.*, 1994; Jansson *et al.*, 1995; Näsman *et al.*, 1997; Pohjanoksa *et al.*, 1997), agonist concentration (Fraser *et al.*, 1989; Jones *et al.*, 1991; Eason *et al.*, 1992; Näsman *et al.*, 1997), ligand structure (Eason *et al.*, 1994a; Näsman *et al.*, 2001), G protein availability (Näsman *et al.*, 2001) and host cell type (Duzic and Lanier, 1992). This has also been demonstrated for endogenously expressed α_2-ARs coupling to G_s proteins. In late-pregnant rat uterus, cervical and myometrial α_{2B}-ARs couple, at least to some extent, to G_s proteins (Gál *et al.*, 2009; Hajagos-Tóth *et al.*, 2016a), and in rats, spinal α_2-AR-mediated analgesia in neuropathic pain involves an interaction with G_s proteins (Hayashida and Eisenach, 2010).

α_2-AR can also couple to other intracellular signaling pathways and even simultaneously activate different signal transduction systems (Limbird, 1988; Cotecchia *et al.*, 1990; Jones *et al.*, 1991; Saunders and Limbird, 1999), including regulation of Na^+/H^+ exchange (Nord *et al.*, 1987; Pihlavisto and Scheinin, 1999), increased PLC activation and phosphoinositide hydrolysis (Cotecchia *et al.*, 1990; Seuwen *et al.*, 1990; Koch *et al.*, 1994; Enkvist *et al.*, 1996; Dorn *et al.*, 1997; Holmberg *et al.*, 1998; Liang *et al.*, 2001; Karkoulias *et al.*, 2007), activation of PLA_2 and arachidonic acid mobilization (Jones *et al.*, 1991; Audubert *et al.*, 1999), and phospholipase D (PLD) activation (MacNulty *et al.*, 1992). Furthermore, at higher adrenaline concentrations, potentiation of cAMP production through a PTX-insensitive mechanism was observed (Jones *et al.*, 1991). The efficiency by which signaling pathways are activated may vary depending on the receptor subtype involved, as shown by Karkoulias and colleagues (2007) who demonstrated in stably transfected rat pheochromocytoma (PC12) cells that α_2-AR-evoked PLC activation and arachidonic acid release were involved in activation of the transcription factor cAMP-response element-binding protein (CREB) in a subtype-dependent manner, with the α_{2A}- and α_{2C}-subtypes exhibiting higher efficiencies compared with the α_{2B}-subtype (Karkoulias *et al.*, 2007). The responses obtained from recombinant cell systems may vary from one study to another because the host cell type affects the coupling of α_2-AR subtypes, due in part to differences in G protein availability (Näsman *et al.*, 2001). The receptors ultimately couple to a variety of effectors that can exist as multiple isoforms and may be independently regulated. The activation of one signaling system may also alter the response obtained from another as a result of cross-talk between two or more mechanisms (Scheinin and Pihlavisto, 2000).

Modulation of ion channel activity is an important function of α_2-ARs. In many types of cells, particularly in electrically excitable cells, α_2-ARs have been shown to couple to activation of receptor-operated K^+ channels, as well as to inhibition of voltage-gated Ca^{2+}channels, leading to attenuation of secretion from neuroendocrine and neuronal cells (Piascik *et al.*, 1996). α_2-ARs have been reported to inhibit different types of voltage-gated

Ca^{2+} channels in neurons (Waterman, 1997; Delmas *et al.*, 1999; Jeong and Ikeda, 2000) and in recombinant neuronal cell lines (Surprenant *et al.*, 1992; Soini *et al.*, 1998). The ability of α_2-ARs to modulate Ca^{2+} channel activity has also been investigated in other cell models, such as transfected insect cells, mammalian cells endogenously expressing α_2-ARs and cultured neurons. However, from the point of view of this study, the ability of α_2-ARs to activate Ca^{2+} channels in VSMCs is of specific interest. In vascular smooth muscle, where postsynaptic α_2-ARs mediate contraction, α_2-ARs have been shown to activate Ca^{2+} channels to allow the influx of extracellular calcium into the cell. The subsequent increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) then initiate a signaling cascade involving Ca^{2+}/calmodulin-mediated myosin light chain kinase (MLCK) activation, phosphorylation of the regulatory myosin light chain (MLC_{20}) and, eventually, smooth muscle cell (SMC) contraction (Somlyo and Somlyo, 1968; Small and Sobieszek, 1977).

This mechanism is supported by studies that have demonstrated, in *in vitro* preparations of rat tail artery and canine saphenous vein, that α_2-AR-mediated contraction is reduced by L-type Ca^{2+} channel antagonists and is nearly abolished in Ca^{2+}-free medium (Matthews *et al.*, 1984; Medgett and Rajanayagam, 1984). Furthermoe, in resistance vessels of pithed rats and cats, as well as in ganglion-blocked rabbits, Ca^{2+} channel antagonists, such as verapamil and nifedipine, have significantly reduced α_2-AR-evoked vasoconstriction, supporting the concept that the influx of extracellular Ca^{2+} is needed for α_2-AR-mediated vasoconstriction (van Zwieten *et al.*, 1982). In rabbit saphenous vein, a tissue with a relatively high density of postsynaptic α_2-ARs, activation of α_2-ARs causes a prominent increase in $[Ca^{2+}]_i$, and in Ca^{2+}-free medium in the presence of an α_2-AR agonist, $[Ca^{2+}]_i$ fell to resting levels (Aburto *et al.*, 1993). In rat portal vein SMCs, α_2-ARs have been shown to promote a sustained Ca^{2+} influx through voltage-gated Ca^{2+} channels by a mechanism involving G_i proteins and activation of protein kinase C (PKC) by DAG (Leprêtre *et al.*, 1994; Mironneau and Macrez-Leprêtre, 1995). In isolated human omental resistance artery SMCs and in human subcutaneous arteries, α_2-AR activation also induces an influx of Ca^{2+}, probably through voltage-gated Ca^{2+} channels, involving PTX-sensitive G_i proteins (Parkinson and Hughes, 1995; Hughes *et al.*, 1996). Furthermore, Chotani and colleagues (2004) demonstrated that α_2-AR activation caused differential responses in blood vessels of different origin; Ca^{2+} mobilization was detected in dermal arterioles and saphenous veins, but not in aorta. This appeared to be caused by differences in α_2-AR expression between VSMCs of these blood vessels, indicating the absence of functional α_2-ARs in the aortic blood vessel wall (Chotani *et al.*, 2004).

Another signal transduction mechanism of specific interest with regard to this study is the ability of α_2-ARs to activate mitogen-activated protein kinase (MAPK) pathways, which are essential for the regulation of cell growth and proliferation. Alblas and colleagues (1993) were the first to demonstrate that α_2-ARs could couple to the $p21^{ras}$-ERK2 pathway via G_i proteins, offering at least a partial explanation for the mitogenic potential of α_2-agonists in certain cell types. This G_i-mediated pathway was PTX-sensitive, but appeared to be independent of AC inhibition or PLD activation, suggesting that the Gβγ subunit could be the actual trigger of this pathway (Alblas *et al.*, 1993). This was confirmed one year later, when Koch and colleagues (1994) identified the Gβγ subunit as the primary mediator of Ras activation and the subsequent signaling via MAPK in response to activation of G_i-coupled receptors, including α_2-ARs (Koch *et al.*, 1994). α_2-ARs are capable of activating ERK1/2

pathways in many transfected cell lines, such as CHO, COS-7, HEK-293 and PC12 (Koch *et al.*, 1994; Flordellis *et al.*, 1995; Paris *et al.*, 2003; Karkoulias *et al.*, 2006b), and in different cell types endogenously expressing α_2-ARs, including rat preadipocytes, opossum kidney cells, rat aortic SMCs, astrocytes, renal tubular cells, intestinal epithelial cells, Müller cells and some breast cancer cell lines (Bouloumié *et al.*, 1994; Kribben *et al.*, 1997; Richman and Regan, 1998; Cussac *et al.*, 2002a; Buffin-Meyer *et al.*, 2007; Li *et al.*, 2008; Pérez Piñero *et al.*, 2012; Harun-Or-Rashid *et al.*, 2015; Xia *et al.*, 2016a). Some subtype-specific differences have been reported in α_2-AR-activated ERK1/2 signaling. The α_2-AR subtypes have been demonstrated to couple to ERK1/2 with different efficacies ($\alpha_{2B} \approx \alpha_{2A} >> \alpha_{2C}$), and the participation of other kinases/proteins/factors in the signal transduction may be subtype-dependent; PKC was shown to be involved in ERK1/2 signaling only with the α_{2A}- and α_{2B}-subtypes (Flordellis *et al.*, 1995), and the activation of ERK1/2 pathways by the α_{2B}-subtype was shown to be completely dependent on epidermal growth factor receptor (EGFR) transactivation, whereas the α_{2A}- and α_{2C}-AR subtypes activated ERK1/2 *via* at least two pathways in PC12 cells, with EGFR transactivation being the main one (Karkoulias *et al.*, 2006b).

Enhanced cell proliferation in response to α_2-AR -mediated activation of ERK1/2 pathways has been reported in different types of cells, e.g. opossum kidney cells, rat preadipocytes, human intestinal epithelial cells, and some breast cancer cell lines (Bouloumié *et al.*, 1994; Kribben *et al.*, 1997; Schaak *et al.*, 2000; Pérez Piñero *et al.*, 2012; Xia *et al.*, 2016a). However, also opposite effects have been reported; in rat aortic SMCs, ERK1/2 kinase activity was markedly stimulated by the α_2-AR agonist dexmedetomidine, but cell proliferation was not promoted (Richman and Regan, 1998), and in human cholangiocarcinoma cells, α_2-AR activation resulted in decreased ERK1/2 activity and inhibition of cell proliferation (Kanno *et al.*, 2002).

2.1.4 Regulation of α_2-adrenoceptors

2.1.4.1 Cellular localization and trafficking

GPCRs are generally considered as cell surface signaling proteins sensing extracellular signals and, indeed, most GPCRs are preferentially targeted to the cell membrane. Subtypes of α_2-ARs are differentially localized within the cell; α_{2A}- and α_{2B}-ARs localize primarily on the cell membrane, whereas α_{2C}-ARs have been found to be predominantly retained in intracellular organelles. Most studies on the localization of α_{2A}- and α_{2B}-ARs have been carried out with recombinant cell systems. In certain transfected cell lines, including COS-1, CHO, HEK-293, Madin-Darby canine kidney II (MCDK II) and PC12 cells, α_{2A}- and α_{2B}-ARs have been localized mainly to the cell membrane (Wozniak and Limbird, 1996; Daunt *et al.*, 1997; DeGraff *et al.*, 1999; Olli-Lähdesmäki *et al.*, 1999; Hurt *et al.*, 2000; Wilson and Limbird, 2000; Olli-Lähdesmäki *et al.*, 2003; Olli-Lähdesmäki *et al.*, 2004). There are only a few studies reporting on the subcellular localization of endogenously expressed α_{2A}-ARs. In neurons of rat and monkey brains, α_{2A}-AR labeling was found in the cytosol and in association with the ER and Golgi (sites of receptor synthesis and trafficking), as well as in the plasma membrane (Aoki *et al.*, 1994; Lee *et al.*, 1998a; Glass *et al.*, 2001). In cultured sympathetic neurons, endogenously expressed α_{2A}-ARs have been shown to localize to the cell body and to axonal and dendritic sites, and upon maturation, these receptors exhibited localization in the plasma membrane (Brum *et al.*, 2006). Moreover, rat aortic

SMCs have been demonstrated to endogenously express all three α_2-AR subtypes and all receptor subtypes were localized in the plasma membrane (Richman and Regan, 1998).

In contrast to α_{2A}- and α_{2B}-ARs, α_{2C}-ARs have a prominent intracellular localization when expressed endogenously or in recombinant cell systems. In regions of the rat brain, immunohistochemical staining revealed a perinuclear localization of the α_{2C}-AR (Rosin *et al.*, 1996; Holmberg *et al.*, 1999). In addition, in cultured sympathetic neurons and in rat aortic SMCs, endogenously expressed α_{2C}-ARs exhibited a prominent perinuclear staining pattern (Richman and Regan, 1998; Brum *et al.*, 2006). In transfected cell lines, extensive intracellular localization, e.g. in the ER and Golgi, of the α_{2C}-AR has been demonstrated in HEK-293 cells, rat-1 fibroblasts, MDCK II cells, COS-1 and COS-7 cells (Wozniak and Limbird, 1996; Daunt *et al.*, 1997; DeGraff *et al.*, 1999; Hurt *et al.*, 2000; Prinster *et al.*, 2006; Angelotti *et al.*, 2010; Filipeanu *et al.*, 2011). In contrast, two neuroendocrine cell lines, namely PC12 and AtT20, efficiently targeted α_{2C}-ARs to the plasma membrane (Hurt *et al.*, 2000), possibly reflecting the predominantly neuronal expression of this receptor subtype in humans and other mammals. The magnitude of the intracellular pool of α_{2C}-ARs and the ability of these receptors to access the plasma membrane are affected by different factors, such as the cell type, with the largest proportion of plasma membrane receptors occurring in neuroendocrine cell lines rather than fibroblast or epithelial cell lines (Hurt *et al.*, 2000). However, in another study on recombinant α_{2C}-ARs, when expressed in PC12 cells, the receptors were mostly located in an intracellular perinuclear compartment (Olli-Lähdesmäki *et al.*, 1999). In addition, external factors may affect α_{2C}-AR trafficking: exposure to lower temperatures has been shown to enhance α_{2C}-AR transport to the cell membrane (Filipeanu *et al.*, 2011; Filipeanu, 2015), co-expression with β_2-ARs has been shown to increase the surface localization of α_{2C}-ARs (Prinster *et al.*, 2006) and maturation has been demonstrated to alter the localization of α_{2C}-ARs from intracellular vesicles (day 8) to sites of synaptic contact (day 16) in cultured sympathetic neurons (Brum *et al.*, 2006).

Although the different α_2-AR subtypes share a high degree of amino acid identity and functional similarity with respect to ligand binding properties and G protein coupling, the intracellular trafficking of these subtypes does reveal significant differences. This may reflect the need for precise targeting of receptor subtypes for many physiological processes. In addition to the targeting of different α_2-AR subtypes to specific cellular organelles, the receptor distributions may be restricted to subdomains within a membrane compartment. It has been proposed that receptor subtypes may be targeted to particular compartments of the cell membrane (e.g. apical vs. basolateral) where receptors are found in close proximity with specific G proteins and effector molecules, contributing to a higher degree of specificity in receptor-mediated signal transduction events (Hein and Kobilka, 1995; Saunders and Limbird, 1999). The three α_2-AR subtypes differ from each other in their steady-state localizations; in MDCK II kidney cells, the α_{2A}-AR subtype is almost exclusively localized on the basolateral cell surface (exposed to the bloodstream) and the localization of the α_{2B}-AR subtype is also almost completely basolateral, but under certain conditions a small endocytosed compartment may be present. At a given moment, about half of the α_{2C}-ARs are found on the basolateral cell surface and the other half are in intracellular compartments. There are differences in the trafficking of newly synthesized α_2-AR subtypes; the α_{2A}-subtype is directly delivered to the basolateral membrane (Keefer and Limbird, 1993), whereas the α_{2B}-subtype is randomly transported to both the apical

and basolateral surfaces, but is selectively retained on the basolateral surface (Wozniak and Limbird, 1996). The α_{2C}-subtype is also directly delivered to the basolateral surface, but a substantial amount of the receptors become retained inside the cell (von Zastrow *et al.*, 1993; Wozniak and Limbird, 1996).

Over the past decades, our understanding of receptor trafficking has greatly advanced, but most studies have focused on the events involved in the internalization, recycling and degradation of receptors. In contrast, the molecular mechanisms underlying the exit of newly synthesized receptors from the ER and its subsequent transport to the cell surface have remained largely unknown. In recent years, researchers have succeeded in shedding light on this matter by recognizing specific sequences in the loop/terminal regions of α_2-ARs that affect the trafficking properties of these receptors. Dong and colleagues (2012) identified a triple arginine motif in the third intracellular loop of the α_{2B}-AR which modulated ER exit, cell surface transport and function of the receptor. A mutation of this motif attenuated α_{2B}-AR exit from the ER, transport to the cell surface and activation of downstream signaling molecules (Dong *et al.*, 2012). In addition, a single leusine residue in the first intracellular loop and a hydrophobic motif (F(x)6IL), as well as a positively charged motif (R(x)3R(x)4R) in the C-terminus have all been identified to be crucial for α_{2B}-AR export from the ER (Wu *et al.*, 2015a). Angelotti and colleagues (2010) identified an evolutionarily conserved hydrophobic sequence in the extracellular N-terminal region of the α_{2C}-AR that was responsible for the subtype-specific trafficking of this receptor. Removal or disruption of this sequence dramatically increased plasma membrane expression and decreased ER retention of the α_{2C}-AR (Angelotti *et al.*, 2010).

2.1.4.2 Desensitization, internalization and downregulation

To enable precise and dynamic homeostatic regulation of cellular functions, signaling pathways that are switched on also need to be switched off, and to accomplish this, agonist-induced activation of receptors can initiate a series of processes controlling the temporal and spatial receptor input. There are three such regulatory processes i.e. desensitization, internalization and downregulation (Figure 5). Desensitization is a phenomenon described as a diminishment of receptor responses upon continuous agonist exposure. It is characterized by receptor−G protein uncoupling, and often occurs rapidly within seconds or minutes. Desensitization may involve multiple mechanisms including receptor phosphorylation events and interactions with other proteins, especially arrestins, which by binding to the G protein recognition site, block the receptor−G protein interaction. Internalization, receptor recycling and lysosomal degradation may also be involved in the desensitization process, and these activities are further complicated by factors such as GPCR oligomerization and localization to specific membrane compartments (Finch *et al.*, 2006; Luttrell, 2006; Rosenbaum *et al.*, 2009). Desensitization can be homologous or heterologous. Homologous desensitization is considered to be strictly agonist-dependent, and therefore only activated receptors can be desensitized. In contrast, in heterologous desensitization, the activation of one receptor can alter the responses of other unrelated receptors, particularly if the affected receptors activate common signaling pathways or effectors (Bünemann *et al.*, 1999). Phosphorylation of receptors by specific G protein-coupled receptor kinases (GRKs)

plays a crucial role in eliciting rapid, short-term desensitization. Phosphorylation of agonist-occupied receptors by GRKs is followed by binding of proteins called arrestins, which not only uncouple receptors from G proteins but also target many receptors for internalization (Goodman *et al.*, 1996; Heck and Bylund, 1997; Bünemann *et al.*, 1999; Luttrell *et al.*, 1999; Saunders and Limbird, 1999; Ferguson, 2001). Phosphorylation of GPCRs can also be mediated by second messenger-dependent kinases, such as PKA and PKC, which not only phosphorylate agonist-activated receptors, but may also indiscriminately phosphorylate receptor proteins that have not been exposed to an agonist. This kind of agonist-independent phosphorylation of receptors is a feature that has typically been ascribed only to second messenger-dependent protein kinases and not to GRKs (Ferguson, 2001; Finch *et al.*, 2006).

Downregulation, in turn, is caused by a decrease in receptor density as a consequence to long-term exposure to an agonist. Compared with desensitization, it displays a much longer time course (hours) and is thought to result from an actual loss of receptor proteins. The decrease in receptor density may result from either a decrease in receptor synthesis or an increase in receptor degradation. Termination of agonist exposure allows the receptor density to recover, but this may require a long time because, in most cases, synthesis of new receptors will be needed (Hein and Kobilka, 1995; Heck and Bylund, 1997; Pitcher *et al.*, 1998; Saunders and Limbird, 1999; Finch *et al.*, 2006).

Internalization or sequestration of receptors away from the cell membrane is also a much slower process than functional desensitization, and takes place in minutes to hours. Receptor phosphorylation may facilitate internalization, but internalization can also occur without phosphorylation. Typically, internalization depends on an interaction of receptors with arrestins, which then target phosphorylated receptors for endocytosis from the cell membrane through clathrin-coated pits. Internalized receptors may be either dephosphorylated and recycled back to the cell surface (resensitization) or targeted for degradation in lysosomes (Bünemann *et al.*, 1999; Finch *et al.*, 2006). Arrestins do not only play a role in the desensitization and internalization of receptors, but are also essential for activating some signaling pathways by interacting with other regulatory proteins and kinases, such as Src kinases, JNKs and p38 MAPKs (Minneman, 2006). Negative regulation of cell signaling through receptor desensitization, downregulation and internalization is encountered in a wide variety of receptors, and these mechanisms have traditionally been considered to exist in order to enable the signaling systems to adapt to protracted agonist exposure. Using mathematical modeling, Shankaran and colleagues (2007) demonstrated that downregulation and desensitization confer GPCR systems with an increased ability to process spatio-temporal variations in ligand concentration. Therefore, in addition to serving as mechanisms for adaptation, these regulatory mechanisms may also play important modulatory roles in the context of information processing (Shankaran *et al.*, 2007).

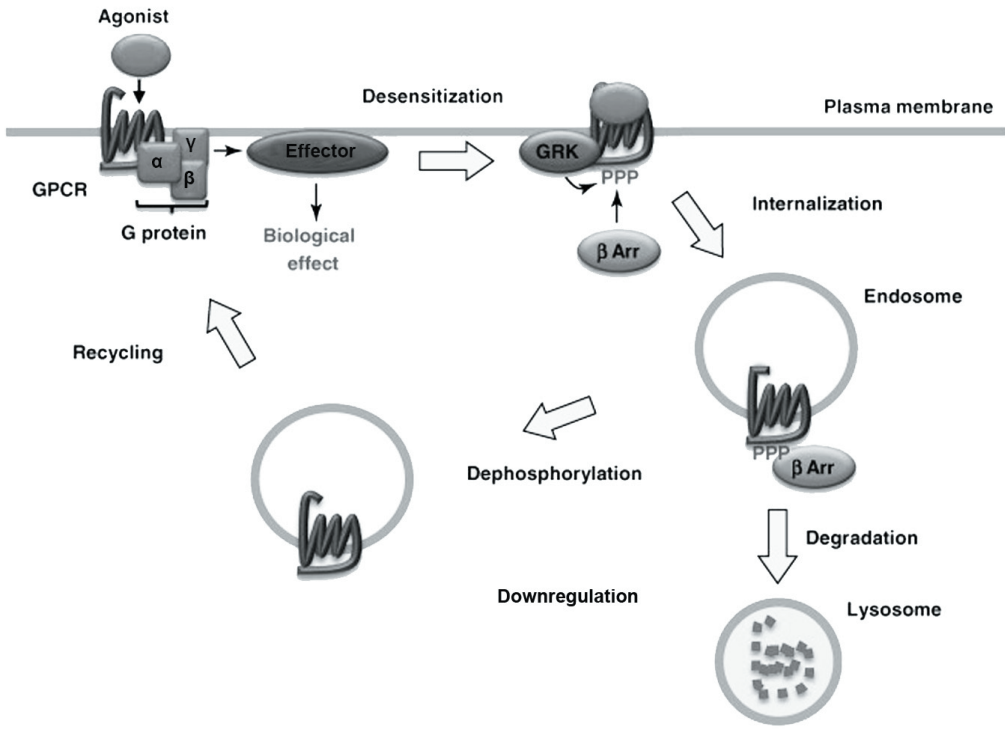

Figure 5. Conventional model of GPCR desensitization, internalization and downregulation. Binding of an agonist to a GPCR leads to the activation of G proteins, which in turn regulate effector proteins. The activation of downstream signaling cascades ultimately produces cellular responses. Upon prolonged agonist exposure, GPCRs are phosphorylated by GRKs and recruit β-arrestins (βArr); these are mechanisms responsible for fast desensitization. Subsequently, GPCRs may be internalized into endosomes. Internalized GPCRs are either targeted to lysosomes for degradation or dephosphorylated and recycled back to the cell surface to undergo a new cycle of activation [modified from (Calebiro *et al*., 2010)].

The propensity for desensitization varies substantially between the α_2-AR subtypes. Tissue distributions and cellular backgrounds may lead to differences in the susceptibility of receptors to be desensitized (Saunders and Limbird, 1999). In CHO and COS cells, α_{2A}- and α_{2B}-ARs have been shown to be effectively desensitized by phosphorylation upon short-term agonist exposure, whereas α_{2C}-ARs have remained unaffected (Eason and Liggett, 1992; Kurose and Lefkowitz, 1994). α_2-ARs also undergo agonist-mediated desensitization after long-term agonist exposure; after 24 h of adrenaline exposure, the α_{2A}- and α_{2B}-ARs, and to a lesser extent the α_{2C}-AR, were desensitized with respect to receptor-evoked inhibition of AC (Eason and Liggett, 1992). Attenuation of α_{2A}- and α_{2B}-AR-elicited function was accompanied by a decrease in cellular G_i content, and a 30–40 % decrease in functional α_{2A}-AR ligand binding capacity in CHO cells. Later, these findings were corroborated by studies in rat adipocytes, which demonstrated that prolonged exposure to the α_2-AR agonist brimonidine (4 days in culture) led to desensitization of lipolysis that paralleled a 15–30 % decrease in the $G_{i\alpha}$ content of the cells (Gasic and Green, 1995). Although α_{2C}-ARs are largely found in intracellular compartments, when present in the plasma membrane, these receptors are resistant to phosphorylation by

GRKs resulting in decreased desensitization and internalization when compared to the other two α_2-AR subtypes (Chotani and Flavahan, 2011). When it became evident that α_2-ARs are capable of undergoing subtype-selective desensitization upon agonist exposure, it was suggested that the different α_2-AR subtypes have possibly evolved to meet differing needs for adaptive regulation (Eason and Liggett, 1992).

Agonist-mediated desensitization of α_2-ARs appears to involve GRK-dependent phosphorylation events. Phosphorylation sites for GRKs have been identified in the third intracellular loop of both α_{2A}-AR (Liggett, 1998) and α_{2B}-AR (Kurose and Lefkowitz, 1994; Jewell-Motz and Liggett, 1995); these phosphorylation sites are important for short-term desensitization of these receptor subtypes. In contrast, studies with the human α_{2C}-AR indicate that this receptor subtype lacks GRK substrates in its intracellular domains and is neither phosphorylated nor desensitized upon agonist activation (Eason and Liggett, 1992; Eason and Liggett, 1993b; Jewell-Motz and Liggett, 1996). Interestingly, the opossum α_{2C}-AR has been shown to be phosphorylated by GRKs and to undergo agonist-induced desensitization (Jones *et al.*, 1990; Deupree *et al.*, 2002). Jewell-Motz and Liggett (1996) investigated the specificity of GRKs for phosphorylation and desensitization of α_2-AR subtypes and found that β-AR kinases 1 and 2 (BARK1 and BARK2, also known as GRK2 and GRK3) were capable of phosphorylating the α_{2A}-AR, whereas GRK5 and GRK6 did not phosphorylate this receptor subtype. In contrast to the findings with the α_{2A}-subtype, the α_{2C}-AR was not phosphorylated by any of these kinases, indicating that desensitization of α_2-ARs depends not only on the expressed GRK isoform, but also on the receptor subtype (Jewell-Motz and Liggett, 1996).

Heterologous desensitization has not been studied in depth for α_2-ARs, but it has been speculated to include negative feedback regulation by signaling pathways activated through G_i proteins (Saunders and Limbird, 1999). However, it has been demonstrated that α_{2A}-ARs can be desensitized by PKC-mediated phosphorylation of the third intracellular loop. PKC activation either by short-term exposure to phorbol 12-myristate-13-acetate (PMA) or through activation of α_{1B}-ARs co-expressed with α_{2A}-ARs resulted in marked desensitization of α_{2A}-AR function (Liang *et al.*, 1998; Liang *et al.*, 2002). These results indicated that the cellular events triggering PKC activation can lead to phosphorylation and desensitization of receptor function. Such heterologous regulation also represents a possible mechanism by which rapid crosstalk between the α_{2A}-AR and other receptors can occur. In fact, in a human neuroblastoma cell line (BE(2)-C) that endogenously expresses α_{2A}- and β_2-ARs, these receptors have been demonstrated to interact with each other. Long-term exposure to adrenaline induced α_{2A}-AR desensitization by mechanisms that involved β_2-AR-dependent upregulation of GRK3 and downregulation of α_{2A}-AR levels (Bawa *et al.*, 2003).

Downregulation as a result of long-term agonist exposure has been commonly described for many GPCRs, including α_2-ARs, and it is a well-known side effect of long-term treatment with agonist drugs. The molecular events involved in downregulation are not fully understood, but accelerated removal of receptors from the cell surface or decreased rates of receptor synthesis or both have been suggested to be possible mechanisms for agonist-induced downregulation. For some receptors, agonist stimulation leads to receptor internalization by means of endocytotic vesicles, after which these receptors

can either be recycled back to the plasma membrane or be targeted to lysosomes for degradation. The steady-state levels of receptors at the cell surface represent the dynamic consequence of *de novo* receptor synthesis/maturation and regulated receptor removal from the cell surface (Heck and Bylund, 1997; Bünemann *et al.*, 1999; Saunders and Limbird, 1999). Agonist-evoked downregulation of α_2-ARs has been shown to occur both *in vivo* for endogenously expressed receptors and *in vitro* for both endogenous and transfected receptors. *In vivo* studies conducted in rats have demonstrated that after prolonged noradrenaline exposure, α_2-ARs are downregulated due to increased receptor degradation. Although these studies were successful in exploring the mechanism of α_2-AR downregulation, they did not make any distinction between the receptor subtypes (Barturen and Garcia-Sevilla, 1992; Ribas *et al.*, 1993). To investigate the behavior of individual receptor subtypes, *in vitro* studies have proved necessary. It has become evident that also in terms of downregulation, the α_2-AR subtypes are regulated differently. The α_{2B}- and α_{2C}-subtypes appear to be more sensitive to agonist-induced downregulation compared with the α_{2A}-subtype, depending on the cell type and animal species of origin. Rat α_{2B}-AR endogenously expressed in NG-108 cells or transfected into CHO cells has been shown to be downregulated by about 50 % following agonist treatment (Thomas and Hoffman, 1986). Likewise, the α_{2C}-AR endogenously expressed in opossum kidney cells has been shown to downregulate to a similar extent when exposed to 0.3 µM noradrenaline (Pleus *et al.*, 1993). The human α_{2A}-AR endogenously expressed in NG-108 cells or transfected into CHO cells has also been demonstrated to be capable of being downregulated by about 50 %, but downregulation of this receptor subtype required approximately 100-fold greater concentrations of noradrenaline than those needed for the downregulation of the other two subtypes (Pleus *et al.*, 1993). These *in vitro* findings were replicated by Heck and Bylund (1997), who showed that human α_{2A}-ARs endogenously expressed in HT29 human adenocarcinoma cells or transfected into CHO cells and rat α_{2B}-ARs transfected into CHO cells were all downregulated by about 50 % upon long-term exposure to noradrenaline. Here, a concentration of 30 µM was required for downregulation of the α_{2A}-subtype, whereas a concentration of only 0.3 µM was sufficient for the α_{2B}-subtype (Heck and Bylund, 1997). However, contradictory results on the extent of downregulation of α_2-AR subtypes have also been presented. In the study of Eason and Liggett (1992), human α_{2A}- and α_{2B}-AR subtypes transfected into CHO cells exhibited only 20 - 25 % downregulation even though the receptors were exposed to 100 µM adrenaline (Eason and Liggett, 1992). As for the α_{2C}-subtype, although it has been demonstrated to be capable of downregulation, there are some studies reporting that this subtype should not be downregulated in response to agonist exposure (Eason and Liggett, 1992; Kurose and Lefkowitz, 1994). Differential regulation of α_2-AR subtypes is evident when considering the concentration of noradrenaline required to induce half-maximal downregulation (EC_{50}). For the α_{2A}- subtype, an EC_{50} of 2 µM was reported, whereas the EC_{50} was only about 100 nM for the other two subtypes. This 20-fold difference in the potency of noradrenaline to produce downregulation may represent a fundamental difference in the way in which the α_2-AR subtypes are regulated (Cerutis *et al.*, 1996; Heck and Bylund, 1998). Interestingly, simultaneous activation of α_{2B}- and β_2-ARs has been demonstrated to result in a 67-fold increase in the potency of adrenaline to evoke α_{2B}-AR downregulation, and α_{2B}- and β_2-AR-dependent upregulation of GRK3 expression was shown to be associated with this potentiation (Desai *et al.*, 2004).

The mechanism of agonist-induced downregulation of α_2-ARs appears to involve an increased rate of receptor degradation, with no change in the rate of appearance of new receptors (Cerutis *et al.*, 1996; Heck and Bylund, 1998). Less is known about the molecular determinants of α_2-AR downregulation, but certain structural differences between the subtypes may be important. Mutagenesis experiments have revealed regions of the α_{2A}-AR important for downregulation. The human α_{2A}-AR has a cysteine residue in its cytoplasmic tail that serves as a palmitoylation site. Substitution of this residue, which also prevents the site from being palmitoylated, has been shown to result in attenuation of downregulation of the receptor (Eason *et al.*, 1994b). However, based on other evidence, this cysteine residue would not be required for downregulation, since the α_{2C}-AR endogenously expressed in opossum kidney cells does not contain such a cysteine residue, but nevertheless is desensitized and downregulated upon agonist exposure (Jones *et al.*, 1990). Another important motif appears to exist in the amino terminal region of the third intracellular loop of the α_{2A}-AR, necessary for agonist-promoted degradation of the receptor protein following a 24-h exposure to adrenaline (Heck and Bylund, 1997). Functional coupling of α_2-AR to G proteins is not necessary for downregulation to occur, as has been demonstrated with rat NG-108 cells endogenously expressing α_{2B}-ARs; PTX treatment did not affect the extent of receptor downregulation (Thomas and Hoffman, 1986). Interestingly, PTX has been shown to change the rate constants for receptor appearance and disappearance for endogenously expressed α_{2A}-ARs, but not for transfected receptors (Heck and Bylund, 1997).

One important aspect of receptor activity and regulation is the internalization of agonist-activated receptors into intracellular compartments. Traditionally, internalized receptors have been considered to be incapable of signaling because they are relocated away from their signaling partners, G proteins and effectors, and are commonly inaccessible to their ligands. After accumulation of new evidence, some additional roles have been postulated for receptor internalization; it might play a role in delivering receptors to intracellular compartments where they might participate in alternative signaling pathways, such as MAPK-mediated events (Daaka *et al.*, 1998), or it may allow for dephosphorylation and consequent resensitization of the receptors, as demonstrated with α_{2C}-ARs that were dephosphorylated by a protein phosphatase, type 2A GPCR phosphatase, in purified Sf9 cell membranes (Pitcher *et al.*, 1995). Agonist-elicited redistribution of α_2-ARs has been studied in different cell models for both endogenous and transfected receptors. In human erythroleukemia cells, a suspension-grown cell line related to human blood platelets, 75 % of the α_{2A}-ARs on the cell surface were internalized after 1 h exposure to 100 µM adrenaline (McKernan *et al.*, 1987). In HT29 cells as well as in opossum kidney cells expressing endogenous α_{2A}- and α_{2C}-ARs long-term exposure to an α_2-agonist lead to internalization of ca. 40 % of the total receptor population (Jones *et al.*, 1990). Interestingly, the α_{2C}-subtype, when endogenously expressed in opossum kidney cells, was downregulated after long-term exposure to agonist, but did not appear to downregulate in transfected heterologous CHO cells (Eason *et al.*, 1994b).

Heterologous systems have demonstrated cell type-dependent profiles for agonist-elicited redistribution of the α_2-AR subtypes. In CHO cells, the α_{2A}- and α_{2B}-AR subtypes have been demonstrated to undergo 26 % and 35 % internalization, respectively, in response to 30 min exposure to adrenaline, whereas the α_{2C}-AR subtype did not appreciably redistribute (12 %)

under the same conditions (Eason and Liggett, 1992). In transfected PC12 cells, marked agonist-induced internalization of human α_2-AR subtypes has been observed. Taraviras and colleagues (2002) reported internalization of all three subtypes (Taraviras *et al.*, 2002), whereas, Olli-Lähdesmäki and colleagues (1999) reported marked internalization of the α_{2A}- and α_{2B}-AR subtypes into partly overlapping populations of intracellular vesicles, although the α_{2C}-AR was mostly located in an intracellular perinuclear compartment even without agonist exposure (Olli-Lähdesmäki *et al.*, 1999). A few years later, Olli-Lähdesmäki and colleagues (2003) demonstrated that trafficking of human α_2-ARs was subtype-specific, but independent of the cell type where they were expressed (HEK-293 vs. PC12); agonist-induced internalization of the α_{2B}-AR subtype depended on the formation of clathrin-coated vesicles, whereas internalization of the α_{2A}-AR subtype appeared to involve both clathrin-coated and caveolin vesicles (Olli-Lähdesmäki *et al.*, 2003). For transfected HEK-293 cells, conflicting reports exist on agonist-induced trafficking of the α_2-AR subtypes. In some studies, the α_{2A}-AR did not appear to undergo agonist-elicited endocytosis upon stimulation, whereas the α_{2B}-AR subtype was rapidly and extensively internalized. A majority of the α_{2C}-AR resided in the ER and Golgi compartments with only relatively little cell surface localization. Nevertheless, some internalization of the α_{2C}-AR could be detected after prolonged agonist exposure (Daunt *et al.*, 1997; Schramm and Limbird, 1999). In another study, both α_{2A}-ARs and α_{2B}-ARs were shown to exhibit rapid dose-dependent internalization in HEK cells. The extent of internalization was directly proportional to agonist efficacy; brimonidine exhibited subtype-specific high efficacy at α_{2A}-ARs and dexmedetomidine at α_{2B}-ARs. Although agonist-induced G_i protein activation was totally blocked by pretreatment with PTX for both receptor subtypes, only about 50 % of the internalization was blocked by PTX, indicating that agonist-induced receptor internalization was only partly dependent on G_i protein coupling (Olli-Lähdesmäki *et al.*, 2004). Taken together, for the α_{2B}-AR, there is consistent evidence indicating that this receptor subtype is rapidly internalized in response to agonist exposure, the α_{2C}-AR subtype only rarely undergoes internalization, and internalization of the α_{2A}-AR subtype varies depending on the host cell type. Interestingly, when expressed in COS-1 cells, all α_2-AR subtypes demonstrated modest levels of agonist-mediated internalization and the α_{2C}-AR subtype was sequestered more than the other two receptor subtypes ($\alpha_{2C} > \alpha_{2B} > \alpha_{2A}$). Co-expression of β-arrestin-1 or β-arrestin-2 dramatically enhanced internalization of the α_{2B}-AR into clathrin-coated vesicles. In contrast, internalization of the α_{2C}-AR subtype was selectively promoted by co-expression of β-arrestin-2, while α_{2A}-AR internalization was only slightly stimulated by co-expression of either arrestin (DeGraff *et al.*, 1999).

In recent years, more light has been shed on the details of the regulatory mechanisms of receptor desensitization and trafficking. Although desensitization and internalization are two closely linked events, both requiring participation of GRKs and arrestins, the causative connection between these two events has been controversial. Lu and colleagues (2009) demonstrated that different agonists differentially regulated the trafficking and signaling duration of α_{2A}-ARs in primary neurons. Clonidine induced a more pronounced receptor desensitization than guanfacine, and this desensitization was largely attributable to α_{2A}-AR internalization. These observations might provide a potential cellular mechanism explaining the longer duration of clinical efficacy of guanfacine as compared with clonidine, namely clonidine-evoked acceleration of internalization and desensitization of the α_{2A}-

AR, which is temporally and quantitatively different from that achieved by guanfacine (Lu *et al.*, 2009). Functionally related receptors have been shown to be simultaneously desensitized and internalized upon activation of only one of the receptors. Tan and colleagues (2009) demonstrated that long-term treatment with either an opioid receptor agonist (DAMGO) or an α_2-AR agonist (clonidine) induced mutual cross-desensitization between μ-opioid receptors and α_{2A}-ARs, and this cross-desensitization was closely associated with simultaneous internalization of both receptors. By promoting receptor cross-desensitization and co-internalization, such functional interactions may serve as negative feedback triggered by prolonged agonist exposure to modulate the signaling of functionally related GPCRs (Tan *et al.*, 2009). Although trafficking of α_2-ARs has been investigated by many research groups, most studies have focused on the events involved in the internalization, recycling and degradation of the receptors. The mechanisms involved in cell surface transport of newly synthesized receptors have remained a less extensively investigated topic. Only very recently, Zhang and colleagues (2016) examined the regulation of cell surface transport of α_2-AR subtypes by an adaptor protein (GGA3 = Golgi-localized, γ-adaptin ear domain homology, ADP ribosylation factor-binding protein 3) that sorts cargo proteins at the trans-Golgi network to the endosome/lysosome pathway. GGA3 knockdown significantly inhibited the cell surface expression of newly synthesized α_{2B}-ARs by arresting the receptors in the trans-Golgi network without altering overall receptor synthesis and internalization. In contrast, α_{2A}-ARs did not interact with GGA3 and their cell surface export and signaling were not affected by GGA3 knockdown (Zhang *et al.*, 2016a).

2.1.4.3 Regulation of α_2-adrenoceptor gene expression

Mechanisms involved in the regulation of α_2-AR gene expression are complex. Receptor expression is likely to be regulated in a cell-specific manner and may be influenced by various factors. Important changes in α_2-AR expression may occur for many reasons e.g. ontogeny and aging, with nutritional changes, obesity, and stress, and with physiologic or pharmacologic changes in sex steroids (Insel and Motulsky, 1988).

The density and distribution of α_2-ARs may change significantly during ontogeny. During fetal development, α_2-ARs are present in some tissues that fail to express receptors in adult animals, whereas other tissues express more receptors as the animal matures. The lung is a well-known tissue where α_2-ARs are expressed most prominently during the early stages of life; in the neonatal rat lung, a high density of α_{2B}-ARs can be found, but adult rat lungs do not express these receptors at detectable levels (Latifpour and Bylund, 1983). In addition, in rat iris arterioles, α_2-ARs are expressed only during early development as has been demonstrated by Sandow and Hill (1999); sympathetic nerve-mediated vasoconstriction of iris arterioles of mature rats was shown to occur through the activation of α_{1B}-ARs alone, while in immature rat iris arterioles, vasoconstriction occurred through activation of both α_1- and α_2-ARs (Sandow and Hill, 1999). The expression of α_2-AR subtypes in the developing and adult rat brain has been demonstrated to exhibit temporal and spatial expression patterns. The level of α_{2A}-AR mRNA was markedly increased by embryonic day 19 in sites corresponding to layers of migrating and differentiating neurons. During the first postnatal week, the α_{2A}-AR mRNA expression changed to a more mature anatomical distribution (Winzer-Serhan and Leslie, 1999). Intense, transient α_{2B}-AR mRNA expression

has been detected in the developing vascular plexus and vasculature, but it disappeared by birth. In most brain areas which exhibit mRNA expression in the adult, expression starts during late embryonic development. During late embryonic and early postnatal development, α_{2B}-AR mRNA expression was transiently upregulated in the caudate putamen and cerebellar Purkinje cells, respectively. This transient expression correlated with the time of neuronal migration and differentiation in these structures, and mirrored the developmental expression of α_{2A}- and α_{2C}-ARs. Based on these results, α_{2B}-ARs may play a role in angiogenesis and in mediating neurotrophic functions of noradrenaline in some brain areas (Winzer-Serhan and Leslie, 1997). The normal aging process appears to be associated with changes in the expression and regulation of adrenergic receptors. In humans, blood platelets obtained from newborns expressed about 50 % less α_2-ARs than platelets obtained from their mothers, and in brains from aged rats, decreased α_2-AR levels were detected, suggesting that with aging, these receptors may be synthesized more slowly in the rat brain (Insel and Motulsky, 1988).

The effects of nutrition and obesity on the expression of α_2-ARs have been examined in different settings. In adipocytes from several species, obesity has been associated with increased numbers of α_2-ARs and decreased lipolytic responses to catecholamines. A low calorie diet (500 kcal/d less than usual diet) has been demonstrated to reduce α_2-AR mRNA levels in adipose tissue of obese women. Furthermore, the α_2-AR-mediated antilipolytic effect was reduced by calorie restriction; downregulation of α_2-AR mRNA levels was suggested to mediate this effect (Stich *et al.*, 2002). Mild prenatal protein malnutrition (from 25 % to 8 %) has been shown to cause a threefold increase in α_{2C}-AR density in the cerebral cortex of 8-day old rat pups. High expression of α_{2C}-ARs during postnatal life was speculated to play a role in the function of synaptic networks and visuo-spatial memory formation (Soto-Moyano *et al.*, 2005; Sierralta *et al.*, 2006). In addition, the effect of high fat diet (cafeteria diet) and the resulting hypertension on hypothalamic tyrosine hydroxylase and α_2-AR gene expression in male, female and neonatally testosterone-imprinted female rats has been investigated (Plut *et al.*, 2002). The cafeteria diet (15 % protein, 49 % fat, 36 % carbohydrate vs. standard chow with 25 % protein, 6 % fat, 69 % carbohydrate) differentially affected hypothalamic gene expression of the α_2-AR subtypes: α_{2A}-AR expression was increased in intact normotensive female rats, whereas expression of α_{2B}-AR was upregulated only in male and testosterone-treated female rats. Moreover, α_{2C}-AR overexpression was also induced, but only in male rats on cafeteria diet. If the upregulation of tyrosine hydroxylase and α_{2B}-AR gene expression were indications of increased sympathetic activity, then this kind of altered gene expression could be responsible for the maintenance of high blood pressure in male and testosterone-treated female rats on cafeteria diet. On the other hand, in intact female rats, the absence of these increases and the upregulation of α_{2A}-AR gene expression could reflect an adaptive response to the diet and, thus, could be protective against diet-induced hypertension.

Estrogen and progesterone regulate many aspects of the adrenergic system and evoke prominent changes in the expression of α_2-ARs in the myometrium of several species, including humans. In late pregnancy in rats, estrogen has been shown to decrease the myometrial expression of all α_2-AR subtypes (significantly decreased mRNA levels) and to decrease myometrial contractile responses to noradrenaline by reducing the coupling of α_{2B}-ARs to G_i proteins. Thus, estrogen dysregulation may be involved in some cases of

preterm labor or uterine inertia with this effect mediated via the α_2-ARs (Hajagos-Tóth *et al.*, 2016b). Progesterone has been demonstrated to increase the mRNA and protein expression of all α_2-AR subtypes in the late-pregnant rat uterus. It decreased contractile responses to noradrenaline through α_{2B}-ARs, blocked the function of the α_{2A}-AR subtype and altered G protein coupling of these receptors by promoting a G_s-dependent pathway (Hajagos-Tóth *et al.*, 2016a). In humans, prominent changes in myometrial α_2-AR density have been detected during the menstrual cycle. The increases in receptor density paralleled similar increases in the plasma estrogen (17-β-estradiol) concentration. Progesterone, a hormone whose levels are markedly increased during pregnancy, may counteract the ability of estrogens to increase α_2-AR expression in the myometrium, since during pregnancy, α_2-AR density is not elevated even though there are high estrogen levels (Insel and Motulsky, 1988). Interestingly, estrogen has also been shown to increase the expression of α_{2C}-ARs in human cultured dermal VSMCs. α_{2C}-ARs located on VSMCs of cutaneous arteries are key mediators of cold-induced vasoconstriction e.g. in Raynaud's disease. An estrogen-dependent increase in the expression of α_{2C}-ARs may contribute to the increased occurrence of cold-induced vasoconstriction under estrogen-replete conditions. This may also explain why Raynaud's disease is more common in women than in men (Eid *et al.*, 2007). Testosterone has also been demonstrated to influence the expression of α_2-ARs. In male hamsters, adaptation to a short daily photoperiod reduced α_2-AR expression in adipocytes, which was related to sexual involution and could be reversed by testosterone administration (Saulnier-Blache *et al.*, 1992).

In addition to ontogenic development, aging, obesity, nutritional influences and sex steroids, also other factors affecting the expression of α_2-ARs have been identified. α_2-ARs are important regulatory elements in the responses mounted to combat stress. In male tree shrews (*Tupaia belangeri*), chronic stress has been shown to decrease the expression of presynaptic α_{2A}-ARs (autoreceptors) in the locus coeruleus and the expression of α_{2A}-heteroreceptors in glutamatergic neurons (Meyer *et al.*, 2000). Denervation or treatments that deplete tissue catecholamines can also mediate upregulation of α_2-ARs, especially in the brain. Depletion of brain monoamines (noradrenaline, serotonin, dopamine) with reserpine has been demonstrated to markedly increase the expression of α_{2A}-AR mRNA in rat brain (by 125 %). This transcriptional activation of receptor gene expression was identified as the cellular mechanism by which reserpine induces upregulation in the density of brain α_2-ARs (Ribas *et al.*, 2001). Likewise, diseases or disturbed physiological conditions may alter the expression of α_2-ARs. Based on studies conducted with a rat neuropathic pain model, the expression of α_{2A}- and α_{2C}-ARs (mRNA) in the spinal cord is decreased with neuropathic pain. This decrease was possibly due to tonic descending noradrenergic input or loss of inhibitory interneurons and it was speculated to be more significant for the maintenance of the neuropathic pain state than its alleviation (Leiphart *et al.*, 2003). Moreover, in rats with genetic forms of hypertension, substantial increases in the density of α_2-ARs in the kidney have been detected. Increased receptor numbers preceded the increase in blood pressure, suggesting that increased receptor density may be of pathogenic significance rather than a consequence of the disease (Insel and Motulsky, 1988). Activation of α_2-ARs in pancreatic β-cells inhibits insulin secretion in response to various stimuli, and acute or long-term regulation of α_2-AR-mediated effects may influence the responses to disturbances of glucose control. Investigation of the effects

of various hormonal and metabolic manipulations on α_{2A}-AR expression in two different pancreatic β-cell lines revealed that glucocorticoids (dexamethasone, hydrocortisone, prednisolone) upregulated α_{2A}-AR expression, augmenting agonist-induced regulation of effector molecules. In contrast, different growth conditions (altered serum or glucose concentration), insulin, testosterone or progesterone did not alter receptor density. This level of regulation may operate in a cell type-specific manner, allowing maximized signaling specificity and efficiency and discrete modulation of tissue responses to glucose disturbances (Hamamdzic *et al.*, 1995).

Growth factors and cAMP may also modulate α_2-AR expression. In VSMCs isolated and cultured from human cutaneous arterioles, serum stimulation increased the expression of α_{2C}-ARs, which was paralleled by an increased intracellular concentration of cAMP. Elevated levels of intracellular cAMP increased the expression of α_{2C}-ARs, whereas abolishing the increase of cAMP also markedly reduced α_{2C}-AR induction in response to serum stimulation. cAMP signaling was shown to have dual effects in cutaneous VSMCs: activation of α_{2C}-AR transcription mediated by the GTP-binding protein Rap1 and suppression mediated by PKA. The former effect predominated in serum-stimulated VSMCs leading to increased α_{2C}-AR expression, thus possibly contributing to enhanced cold-induced vasoconstriction (Chotani *et al.*, 2005). In contrast, in human colonic adenocarcinoma HT29 cells, serum deprivation resulted in an increase in the density of α_{2A}-ARs, whereas re-exposing the cells to serum led again to a decrease in receptor density (in a concentration-dependent manner). Insulin and other growth factors were identified as the components of serum that were responsible for this phenomenon. The decrease in the α_2-AR number was mediated through stimulation of insulin receptors or corresponding tyrosine kinase receptors of other growth factors (Devedjian *et al.*, 1991). Furthermore, cAMP has been shown to modulate the expression of α_{2A}-ARs in human corpus cavernosum smooth muscle, where the mRNA expression of α_{2A}-ARs was decreased in response to increased intracellular cAMP (Traish *et al.*, 2000).

2.1.5 Distributions and physiological functions of α_2-adrenoceptors

α_2-ARs are located both pre- and postsynaptically and generally mediate inhibitory actions in the central and peripheral nervous systems. All adrenoceptors, including α_2-ARs, are primary targets of the endogenous catecholamines adrenaline and noradrenaline. They are essential mediators in the sympathetic nervous system, helping to maintain homeostasis under resting conditions and mediating signaling to activate the body's fight-or-flight responses, i.e. the ability to react to acute stress. In stress conditions, adrenaline is released from the adrenal medulla into the bloodstream and noradrenaline is released from sympathetic nerve endings (Westfall and Westfall, 2011). α_2-ARs located in the presynaptic membrane of adrenergic/noradrenergic neurons are called autoreceptors and α_2-ARs located in the presynaptic membrane of non-adrenergic neurons are called heteroreceptors. The main function of α_2-autoreceptors is to inhibit the release of noradrenaline or adrenaline (from sympathetic nerves and adrenal chromaffin cells, respectively), as part of a negative feedback loop. These receptors are desensitized upon prolonged activation, such as in chronic heart failure, where sympathetic activity is enhanced. Still, many of the pharmacological effects of α_2-AR agonists such as analgesia,

sedation, hypothermia, bradycardia and hypotension require the activation of α_2-heteroreceptors or postsynaptic α_2-ARs (Starke, 2001; Gilsbach and Hein, 2012).

In rodents and in humans, α_2-AR expression is widely distributed throughout the central nervous system (CNS) and peripheral tissues (Table 1). Most cells of the human body express at least one of the nine AR subtypes. In many cases, one receptor subtype predominates in a certain type of cells in effector organs of the sympathetic nervous system or in the CNS. Moreover, different cell types express different compositions of G proteins and downstream signaling molecules allowing for diverse responses of tissues and organs to the adrenaline or noradrenaline released from the sympathetic nervous system or within the CNS (Westfall and Westfall, 2011). For example, sympathetic activation (adrenaline and noradrenaline) may cause both contraction and relaxation of vascular smooth muscle depending on the AR subtype that is expressed in the cells of the investigated vascular bed. In dermal blood vessels, VSMCs contract in response to adrenaline, and this is mainly mediated by α_1-ARs, but also α_{2C}-ARs contribute to vasoconstriction (especially cold-induced vasoconstriction). In skeletal muscle arteries, β_2-ARs are dominant and mediate relaxation upon exposure to adrenaline (Chotani *et al.*, 2000; Jeyaraj *et al.*, 2001; Westfall and Westfall, 2011).

The lack of sufficiently selective ligands has hampered the resolution of the functions specific to each individual α_2-AR subtype by pharmacological methods. It has been established that all three α_2-AR subtypes, whether pre- or postsynaptic, have very similar pharmacological properties in all different animal species studied, and also in humans (Svensson *et al.*, 1996). Subtype-specific knockout mouse lines have provided an alternative approach for the evaluation of the specific physiological roles of the different α_2-AR subtypes. However, it should be kept in mind that compensatory mechanisms, such as up- or downregulation of other components of the involved signaling pathways may modify the effects seen in such genetically engineered mice (Kable *et al.*, 2000; Gyires *et al.*, 2009).

Table 1. Tissue distribution of α_2-adrenoceptor subtypes in different animal species, as evaluated with various methods. The term α_{2D} refers to the rodent equivalent of the human α_{2A}-AR subtype.

Subtype	Tissue	Species	Reference
$\alpha_{2A/D}$			
	heart*	rat	Smith *et al.*, 1995; Trendelenburg *et al.*, 1997; Ho *et al.*, 1998
		mouse	Wahl *et al.*, 1996; Hein *et al.*, 1999; Trendelenburg *et al.*, 2001
		human	Eason and Liggett, 1993a
		zebrafish	Ruuskanen *et al.*, 2005
	aorta	human	Eason and Liggett, 1993a
		rat	Faber *et al.*, 2001
		pig	Wright *et al.*, 1995a
	pulmonary artery*	rabbit	Molderings and Göthert, 1995
	mesenteric artery*	dog	Daniel *et al.*, 1995
	tail artery**	mouse	Chotani *et al.*, 2000
	gastric arteries*	human	Guimarães *et al.*, 1998
	ileocolic arteries*	human	Guimarães *et al.*, 1998
		dog	Paiva *et al.*, 1997
	palmar common digital artery**	pig	Blaylock and Wilson, 1995; Wright *et al.*, 1995a
	ear artery	pig	Wright *et al.*, 1995a
	splenic artery	pig	Wright *et al.*, 1995a
	cutaneous arterioles	human	Chotani *et al.*, 2004
	cremaster muscle arterioles and venules**	rat, rabbit	Leech and Faber, 1996
	saphenous vein**	dog	Hicks *et al.*, 1991; MacLennan *et al.*, 1997
	saphenous vein*	human	Molderings and Göthert, 1995; Chotani *et al.*, 2004
	mesenteric vein**	dog	Paiva *et al.*, 1999
	pulmonary vein	pig	Görnemann *et al.*, 2007
	vena cava*	rat	Trendelenburg *et al.*, 1997
	vena cava	human	Eason and Liggett, 1993a
	femoral vein**	rat	Paiva *et al.*, 1999
	palmar lateral vein**	pig	Blaylock and Wilson, 1995; Wright *et al.*, 1995a
	marginal ear vein	pig	Wright *et al.*, 1995a
	blood platelets**	human	Bylund, 1985; Bylund *et al.*, 1988
	brain*	rat	Bylund, 1985; Handy *et al.*, 1993; Blaxall *et al.*, 1994
	brain	zebrafish	Ruuskanen *et al.*, 2005
	locus coeruleus	rat	Nicholas *et al.*, 1993; MacDonald and Scheinin, 1995
		mouse	Wang *et al.*, 1996
	brain stem	rat	Scheinin *et al.*, 1994
	cerebral cortex*	rat	Brown *et al.*, 1990; Uhlén *et al.*, 1992; Nicholas *et al.*, 1993; Trendelenburg *et al.*, 1993; Renouard *et al.*, 1994; Scheinin *et al.*, 1994; Ho *et al.*, 1998
		rabbit	Trendelenburg *et al.*, 1993
		human	Petrash and Bylund, 1986; Raiteri *et al.*, 1992
		mouse	Wang *et al.*, 1996; Hein *et al.*, 1999; Bücheler *et al.*, 2002
	cerebellum	human	Petrash and Bylund, 1986
	hippocampus*	mouse	Trendelenburg *et al.*, 2001
		rat	Brown *et al.*, 1990; Scheinin *et al.*, 1994
	septum	rat	Scheinin *et al.*, 1994
	hypothalamus	rat	Nicholas *et al.*, 1993; Scheinin *et al.*, 1994
	amygdala	rat	Scheinin *et al.*, 1994
	occipito-parietal cortex*	mouse	Trendelenburg *et al.*, 2001
	caudate nucleus*	human	Petrash and Bylund, 1986; Bylund, 1988
	reticular thalamic nucleus	rat	Nicholas *et al.*, 1993
	ventrolateral medullary reticular formation	rat	Nicholas *et al.*, 1993
	nucleus tractus solitarii	rat	Nicholas *et al.*, 1993
	deep cerebellar nuclei	rat	Nicholas *et al.*, 1993
	trapezoid nuclei	rat	Nicholas *et al.*, 1993
	vestibular nuclei	rat	Nicholas *et al.*, 1993
	pontine nuclei	rat	Nicholas *et al.*, 1993
	spinal cord*	rat	Uhlén and Wikberg, 1991; Uhlén *et al.*, 1992; Nicholas *et al.*, 1993; Blaxall *et al.*, 1994; Shi *et al.*, 1999
	skeletal muscle	human	Eason and Liggett, 1993a
	muscle	zebrafish	Ruuskanen *et al.*, 2005
	kidney*	rat	Bohmann *et al.*, 1993; Handy *et al.*, 1993; Blaxall *et al.*, 1994
	kidney (cortex + medulla)	human	Eason and Liggett, 1993a; Trendelenburg *et al.*, 1997
	kidney (MDCK cells)	dog	Okusa *et al.*, 1994
	liver	human	Eason and Liggett, 1993a
	adult lung	rat	Handy *et al.*, 1993

Sub-type	Tissue	Species	Reference
	lung	human	Eason and Liggett, 1993a
	pineal gland*	bovine	Simonneaux *et al.*, 1991
	urethra*	guinea pig	Trendelenburg *et al.*, 1997
	non-pregnant myometrium	rat	Gáspár *et al.*, 2007
	pregnant myometrium**	rat	Bouet-Alard *et al.*, 1997; Gáspár *et al.*, 2007
		human	Bouet-Alard *et al.*, 1997
	ovary	zebrafish	Ruuskanen *et al.*, 2005
	pancreatic islets**	rat	Niddam *et al.*, 1990
	pancreas	human	Eason and Liggett, 1993a; Lacey *et al.*, 1996
	stomach	human	Eason and Liggett, 1993a
	gut / intestine wall	zebrafish	Ruuskanen *et al.*, 2005
	intestine	human	Eason and Liggett, 1993a
	spleen**	rabbit	Michel *et al.*, 1990
	spleen	rat	Handy *et al.*, 1993
	thymus	rat	Handy *et al.*, 1993
	eye**	human	Bylund and Chacko, 1999
		bovine	Berlie *et al.*, 1995; Bylund *et al.*, 1997
	eye	zebrafish	Ruuskanen *et al.*, 2005
	prostate	human	Eason and Liggett, 1993a
	vas deferens*	rat	Smith *et al.*, 1992; Smith and Docherty, 1992
		mouse	Trendelenburg *et al.*, 2001
	adrenal gland	human	Eason and Liggett, 1993a
	submandibular gland*	rat	Bylund, 1985; Michel *et al.*, 1989; Limberger *et al.*, 1992; Smith *et al.*, 1992; Smith and Docherty, 1992; Handy *et al.*, 1993; Renouard *et al.*, 1994
	fat, perinephric	human	Eason and Liggett, 1993a
	skin	zebrafish	Ruuskanen *et al.*, 2005
	colonic adenocarcinoma cell line HT29**	human	Bylund, 1988; Bylund and Ray-Prenger, 1989
	hypothalamic cell line GT1	mouse	Lee *et al.*, 1995
α_{2B}			
	heart	rat	Handy *et al.*, 1993
		zebrafish	Ruuskanen *et al.*, 2005
	heart (endocardium)	human	Eason and Liggett, 1993a
	aorta	human	Eason and Liggett, 1993a
	vena cava	human	Eason and Liggett, 1993a
	vasculature	zebrafish	Ruuskanen *et al.*, 2005
	brain	rat	Handy *et al.*, 1993; Blaxall *et al.*, 1994
		zebrafish	Ruuskanen *et al.*, 2005
	cerebral cortex (neonatal) **	rat	Bylund *et al.*, 1988
	cerebellum	mouse	Wang *et al.*, 1996
	caudate nucleus	human	Petrash and Bylund, 1986
	thalamus	rat	Nicholas *et al.*, 1993; MacDonald and Scheinin, 1995
		mouse	Wang *et al.*, 1996
	spinal cord	rat	Blaxall *et al.*, 1994
	skeletal muscle	human	Eason and Liggett, 1993a
	muscle	zebrafish	Ruuskanen *et al.*, 2005
	kidney **	rat	Bylund *et al.*, 1988; Michel *et al.*, 1990; Handy *et al.*, 1993; Blaxall *et al.*, 1994
	kidney (cortex + medulla)	human	Eason and Liggett, 1993a
	neonatal lung**	rat	Bylund, 1985; Bylund *et al.*, 1988; Handy *et al.*, 1993
	adult lung	rat	Handy *et al.*, 1993
	lung	human	Eason and Liggett, 1993a
	liver	rat	Handy *et al.*, 1993
		human	Eason and Liggett, 1993a
		zebrafish	Ruuskanen *et al.*, 2005
	spleen	human	Eason and Liggett, 1993a
	stomach	human	Eason and Liggett, 1993a
	gut / intestine wall	zebrafish	Ruuskanen *et al.*, 2005
	intestine	human	Eason and Liggett, 1993a
	pancreas	human	Eason and Liggett, 1993a; Lacey *et al.*, 1996
	non-pregnant myometrium	rat	Gáspár *et al.*, 2007
	pregnant myometrium**	rat	Bouet-Alard *et al.*, 1997; Gáspár *et al.*, 2007
		human	Bouet-Alard *et al.*, 1997
	ovary	zebrafish	Ruuskanen *et al.*, 2005
	prostate	human	Eason and Liggett, 1993a
	adrenal gland	human	Eason and Liggett, 1993a
	fat, subcutaneous	human	Eason and Liggett, 1993a
	fat, perinephric	human	Eason and Liggett, 1993a

Sub-type	Tissue	Species	Reference
	skin	zebrafish	Ruuskanen *et al.*, 2005
	eye	zebrafish	Ruuskanen *et al.*, 2005
	neuroblastoma×glioma hybrid cell line NG108-15**	mouse/rat	Bylund, 1988; Bylund and Ray-Prenger, 1989
α_{2C}			
	heart atrium*	mouse	Hein *et al.*, 1999; Trendelenburg *et al.*, 2001
		rat	Ho *et al.*, 1998
	heart (right) atrium*	human	Rump *et al.*, 1995
	heart (epicardium + endocardium)	human	Eason and Liggett, 1993a
	aorta	human	Eason and Liggett, 1993a
		rat	Richman and Regan, 1998
	adventitial arterioles of the aorta	human	Chotani *et al.*, 2004
	tail artery**	mouse	Chotani *et al.*, 2000
		rat	Jantschak *et al.*, 2010
	cutaneous arteries	mouse	Chotani *et al.*, 2000; Bailey *et al.*, 2004
	cutaneous arterioles	human	Chotani *et al.*, 2004
	cutaneous veins	horse	Zerpa *et al.*, 2010
		dog	Flavahan *et al.*, 1985
	vena cava	human	Eason and Liggett, 1993a
	vas deferens*	mouse	Trendelenburg *et al.*, 2001
	saphenous vein**	human	Gavin *et al.*, 1997; Chotani *et al.*, 2004
	pulmonary vein**	pig	Görnemann *et al.*, 2007; Jantschak and Pertz, 2012
	brain	rat	Handy *et al.*, 1993; Blaxall *et al.*, 1994; Rosin *et al.*, 1996; Lee *et al.*, 1998b
		zebrafish	Ruuskanen *et al.*, 2005
	basal ganglia	rat	MacDonald and Scheinin, 1995
	olfactory tubercle	rat	MacDonald and Scheinin, 1995
		mouse	Dossin *et al.*, 2000
	olfactory bulb	rat	Nicholas *et al.*, 1993
	anterior olfactory + septal areas	rat	Rosin *et al.*, 1996
	hippocampus	rat	MacDonald and Scheinin, 1995; Rosin *et al.*, 1996
		mouse	Dossin *et al.*, 2000
	hippocampal formation	rat	Nicholas *et al.*, 1993
	cerebral cortex	rat	MacDonald and Scheinin, 1995
	cerebellar cortex	rat	Nicholas *et al.*, 1993
	cerebral cortex*	rat	Uhlén *et al.*, 1992; Nicholas *et al.*, 1993
		mouse	Hein *et al.*, 1999; Bücheler *et al.*, 2002
	piriform cortex	rat	Rosin *et al.*, 1996
	substantia nigra	rat	Rosin *et al.*, 1996
	amygdala	rat	Rosin *et al.*, 1996
		mouse	Dossin *et al.*, 2000
	thalamic nuclei	rat	Rosin *et al.*, 1996
	anterodorsal nucleus of the thalamus	mouse	Dossin *et al.*, 2000
	dorsal root ganglia	rat	Nicholas *et al.*, 1993
	islands of Calleja	rat	Nicholas *et al.*, 1993
		mouse	Dossin *et al.*, 2000
	nucleus accumbens	mouse	Dossin *et al.*, 2000
	striatum	rat	Nicholas *et al.*, 1993
	caudate–putamen	mouse	Wang *et al.*, 1996; Dossin *et al.*, 2000
	spinal cord*	rat	Uhlén *et al.*, 1992; Blaxall *et al.*, 1994; Shi *et al.*, 1999
	skeletal muscle	human	Eason and Liggett, 1993a
	kidney	rat	Blaxall *et al.*, 1994
	kidney**	opossum	Murphy and Bylund, 1988
	kidney (cortex + medulla)	human	Eason and Liggett, 1993a
	spleen	human	Eason and Liggett, 1993a
	pancreas	human	Eason and Liggett, 1993a; Lacey *et al.*, 1996
	intestine	human	Eason and Liggett, 1993a
	non-pregnant myometrium	rat	Gáspár *et al.*, 2007
	pregnant myometrium**	human	Bouet-Alard *et al.*, 1997
		rat	Gáspár *et al.*, 2007
	ovary	zebrafish	Ruuskanen *et al.*, 2005
	prostate	human	Eason and Liggett, 1993a
	adrenal gland	human	Eason and Liggett, 1993a
	lung	human	Eason and Liggett, 1993a
	eye	zebrafish	Ruuskanen *et al.*, 2005
	retinoblastoma cell line Y79**	human	Gleason and Hieble, 1992

* *claimed to be presynaptically located, according to various criteria*
** *claimed to be postsynaptically located, according to various criteria*

2.1.5.1 α_2-Adrenoceptors in the central nervous system

In the CNS, all three α_2-AR subtypes have been identified and their discrete localizations in the CNS suggests that the different subtypes mediate different processes. The most abundant subtype is the α_{2A}-AR [90 % in the mouse brain; see (Bücheler *et al.*, 2002)] and it has a predominant role in mediating many of the pharmacological and therapeutic effects of α_2-AR agonists. Activation of α_{2B}- and/or α_{2C}-ARs may contribute to the α_{2A}-AR-mediated classic and established therapeutic effects of α_2-AR agonists (e.g. analgesia, sedation, hypotension, inhibition of neurotransmitter release), but in some cases they may counteract their therapeutic actions (e.g. α_{2B}-AR-evoked vasoconstriction counteracts the α_{2A}-AR-mediated hypotensive action) or attenuate the side effects (activation of α_{2C}-ARs can inhibit the α_{2A}-AR-mediated sedative action) (Gyires *et al.*, 2009). α_{2A}-ARs are localized both pre- and postsynaptically, and α_{2A}-ARs are extensively distributed both in the locus coeruleus and other noradrenergic brain nuclei and in noradrenergic projection areas, such as the cerebral cortex and hippocampus (Brown *et al.*, 1990; Uhlén *et al.*, 1992; Nicholas *et al.*, 1993; Trendelenburg *et al.*, 1993; Renouard *et al.*, 1994; Scheinin *et al.*, 1994; MacDonald and Scheinin, 1995; Wang *et al.*, 1996; Ho *et al.*, 1998; Hein *et al.*, 1999; Bücheler *et al.*, 2002). α_{2A}-ARs are also expressed in the brain stem, medulla and spinal cord (Uhlén and Wikberg, 1991; Uhlén *et al.*, 1992; Nicholas *et al.*, 1993; Blaxall *et al.*, 1994; Scheinin *et al.*, 1994; Nicholas *et al.*, 1996; Rosin *et al.*, 1996; Shi *et al.*, 1999). Presynaptic α_{2A}-ARs play an important inhibitory role in the regulation of noradrenaline release from central noradrenergic neurons and sympathetic neurons, and they may also mediate presynaptic inhibition of the release of other neurotransmitters in the central and peripheral nervous systems (Aantaa *et al.*, 1995; Hein *et al.*, 1999; Westfall and Westfall, 2011). Initially, the α_{2A}-AR was presumed to be the major inhibitory autoreceptor (Funk *et al.*, 1995; MacDonald *et al.*, 1997). However, it was soon discovered that also the α_{2C}-AR could act as an autoreceptor in certain tissues, and that it was required for the normal presynaptic regulation of noradrenaline release in the CNS (Philipp *et al.*, 2002a). Based on studies with knockout mice lacking α_{2A}- or α_{2C}-ARs, distinct neuronal localizations and functional roles for these two types of presynaptic autoreceptors have been identified. In contrast to the α_{2A}-AR, the α_{2C}-AR localizes more in sympathetic nerve endings than in central noradrenergic neurons, and each receptor subtype appears to control neurotransmitter release in a different way: the α_{2A}-AR inhibits noradrenaline release significantly faster and at higher stimulation frequencies, whereas α_{2C}-ARs modulate neurotransmission at lower stimulation frequencies (Altman *et al.*, 1999; Hein *et al.*, 1999; Bücheler *et al.*, 2002).

Based on studies with knockout mice, the α_{2A}-AR subtype has been identified as the predominant subtype mediating the central hypotensive, analgesic/antinociceptive, sedative, hypothermic and behavioral actions of α_2-AR agonists (MacMillan *et al.*, 1996; Hunter *et al.*, 1997; Lakhlani *et al.*, 1997; Sallinen *et al.*, 1997; Altman *et al.*, 1999; Guo *et al.*, 1999; Lähdesmäki *et al.*, 2002; Lähdesmäki *et al.*, 2003; Pertovaara, 2006; Nazarian *et al.*, 2008). α_{2A}-ARs may also enhance the cognitive functions mediated by the prefrontal cortex (Franowicz *et al.*, 2002). Depending on their localization, pre- or postsynaptic, α_{2A}-ARs may cause opposing effects in the brain. Szot and colleagues (2004) investigated the inconsistent effects of α_2-AR agonists on seizure activity. Their findings led them to hypothesize that presynaptic α_{2A}-ARs would be responsible for proconvulsive effects of

α_2-AR agonists, while postsynaptic α_{2A}-ARs would account for the anticonvulsant effects of α_2-AR agonists (Szot *et al.*, 2004).

α_2-AR-evoked analgesia in the spinal cord is largely mediated by the α_{2A}-subtype (Nazarian *et al.*, 2008), but there is also evidence that α_{2C}-ARs can contribute to this analgesic effect and that their role in the modulation of pain is rather complex (Fairbanks *et al.*, 2002; Chen *et al.*, 2008). Chen and colleagues (2008) were the first to demonstrate the existence of adrenergic inhibition of opioid peptide release in the spinal dorsal horn, mediated by α_{2C}-ARs. Opioid peptides released in the dorsal horn are known to evoke analgesia (Budai and Fields, 1998), and therefore, inhibition of their release by α_{2C}-ARs would rather be expected to increase pain sensations. The α_{2C}-AR-mediated inhibition of opioid peptide release may serve to shut down the opioid system when the adrenergic system is active. The highest densities of α_{2C}-ARs in the CNS are found in the basal ganglia, olfactory tubercle and bulb, hippocampus, cerebral cortex, amygdala and spinal cord (Uhlén *et al.*, 1992; Nicholas *et al.*, 1993; Blaxall *et al.*, 1994; MacDonald and Scheinin, 1995; Rosin *et al.*, 1996; Wang *et al.*, 1996; Shi *et al.*, 1999; Dossin *et al.*, 2000; Bücheler *et al.*, 2002; Holmberg *et al.*, 2003). Many of these brain regions are involved in the processing of sensory information and in the control of motor- and emotion-related activities.

α_{2C}-ARs also appear to play a role in the regulation of cortical arousal (increased wakefulness, vigilance, muscle tone, heart rate, and minute ventilation) and α_{2C}-ARs may antagonize the sedative effect of α_2-AR agonists mediated mainly by activation of α_{2A}-ARs (Puoliväli *et al.*, 2002). Based on behavioral studies with transgenic mouse models, the α_{2C}-subtype also appears to be implicated in the modulation of motor behavior and memory processes (Björklund *et al.*, 1999; Tanila *et al.*, 1999), startle reflex, stress responses and stress-dependent depression (Sallinen *et al.*, 1998a; Sallinen *et al.*, 1998b; Sallinen *et al.*, 1999). Moreover, an association between a polymorphism of the human α_{2C}-AR gene [ADRA2C (GT)n repeat polymorphism] and attention-deficit/hyperactivity disorder (ADHD) has been suggested (Cho *et al.*, 2008).

The α_{2B}-AR subtype is mainly expressed outside the CNS, in many peripheral tissues (Docherty, 1998; Philipp *et al.*, 2002a). In the CNS, α_{2B}-AR expression has only been identified in the thalamus, (neonatal rat) cerebral cortex, caudate nucleus and spinal cord (Petrash and Bylund, 1986; Bylund *et al.*, 1988; Nicholas *et al.*, 1993; Blaxall *et al.*, 1994; Scheinin *et al.*, 1994; MacDonald and Scheinin, 1995; Wang *et al.*, 1996), and the physiological significance of this subtype in the regulation of CNS functions appears to be minor. It has been suggested that central α_{2B}-ARs could play a role in salt-induced hypertension (Makaritsis *et al.*, 1999). In studies on genetically engineered mice, it has been demonstrated that the antinociceptive response to an exogenous α_2-AR agonist is mediated by α_{2A}-ARs, but there seems to be a role for α_{2B}- or α_{2C}-ARs, or both, in the analgesic response to nitrous oxide (N_2O) (Guo *et al.*, 1999). Furthermore, in a double knockout mouse model lacking α_{2A}- and α_{2C}-ARs, the α_{2B}-AR may contribute to presynaptic feedback regulation (Philipp and Hein, 2004).

2.1.5.2 α_2-Adrenoceptors in peripheral tissues

α_2-ARs have a widespread distribution in peripheral tissues (for details, see Table 1); they are found in the heart, in vascular and other smooth muscle cells, adipocytes and

different types of secretory epithelial cells, including intestinal, renal and endocrine cells. Peripheral tissue distributions have been examined using both radioligand binding assays, mRNA expression analysis and pharmacological studies of receptor-mediated responses. However, it should be borne in mind that radioligand binding assays may not detect receptors expressed at low density or in a small subset of cells relative to the mass of the tissue. In addition, the employed pharmacological agents have limited subtype selective and may not allow absolute identification of individual α_2-AR subtypes (Nicholas *et al.*, 1996; MacDonald *et al.*, 1997). In contrast to α_1-ARs that seem to be located close to sympathetic adrenergic nerve terminals in peripheral target organs, α_2-ARs may be located relatively far away from noradrenaline-releasing nerve terminals. Such extrasynaptical receptors exist for example in VSMCs and blood platelets, and they may be preferentially activated by circulating catecholamines, particularly adrenaline, rather than locally released noradrenaline (Westfall and Westfall, 2011).

Most smooth muscles appear to contain several subtypes of adrenoceptors (Bülbring and Tomita, 1987; Martinotti, 1991). α_2-ARs are expressed at least in vascular, intestinal and myometrial smooth muscle cells (Table 1), where they mediate contraction and relaxation in response to catecholamine stimulation. α_2-ARs have physiological roles in the regulation of gastrointestinal functions. Activation of α_2-ARs has been reported to inhibit gastric acid secretion and gastric motility (Kunchandy *et al.*, 1985; Asai *et al.*, 1997). These functions appear to be mediated by different α_2-AR subtypes: α_{2B}-ARs mediate a gastroprotective effect, whereas α_{2A}-ARs appear to inhibit gastric emptying and motility. However, the sites of action (central, peripheral or both) remain to be established (Fülöp *et al.*, 2005). Myometrial α_2-ARs are involved in the regulation of motor activity of the uterus (Bouet-Alard *et al.*, 1997; Gáspár *et al.*, 2007; Hajagos-Tóth *et al.*, 2016a). In the pregnant rat uterus, the α_{2B}-AR is the predominant subtype mediating contraction, whereas the α_{2A}- and α_{2C}-subtypes have been reported to inhibit contractions (Gáspár *et al.*, 2007). Vascular α_2-ARs are involved in the regulation of contraction of VSMCs (Bylund *et al.*, 1995). The α_{2A}- and α_{2B}-subtypes are involved in the regulation of vascular functions, whereas α_{2C}-ARs seem to play only a minor role in cardiovascular regulation. The functions of vascular α_2-ARs will be discussed in more detail in the next paragraph.

In addition to smooth muscle, α_2-ARs are expressed abundantly in many peripheral organs, such as heart, lung, liver, kidney, pancreas, adipose tissue, platelets and eye. In the liver, α_2-ARs have been shown to participate in the regulation of carbohydrate metabolism (Kulcsár-Gergely and Kulcsár, 1989). Based on recent studies, α_2-ARs appear to be involved in both the promotion and the prevention of liver damage. In mice, restraint stress has been demonstrated to increase the number of apoptotic hepatocytes, and treatment with an α_2-AR antagonist has been reported to reduce stress-induced hepatocyte apoptosis. Based on these findings, it can be hypothesized that during restraint stress, catecholamines released into the circulation reach the liver where they trigger an unfavorable stress response, and that α_2-ARs are involved in the promotion of this stress response by mediating hepatocyte apoptosis (Zhu *et al.*, 2014). In contrast, α_2-ARs appear to play a protective role in sepsis [by lipopolysaccharide (LPS) treatment]-induced liver damage, since activation of α_2-ARs by dexmedetomidine attenuated LPS-induced deleterious effects on the liver, and blockade of α_2-ARs with the antagonist yohimbine provoked LPS-induced liver damage (Chen *et al.*, 2015). In the kidney, α_2-ARs are involved

in modulation of salt and water balance (water clearance and natriuresis), and α_2-ARs also mediate tonic renal vasoconstriction and inhibition of renin release (Blandford and Smyth, 1988; de Leeuw and Birkenhäger, 1988; Ruffolo *et al.*, 1993; Michel and Rump, 1996; Miller *et al.*, 2001). In adipose tissue, α_2-ARs inhibit lipolysis, and also α_2-AR-mediated stimulation of white preadipocyte proliferation has been reported (Lafontan and Berlan, 1995; Stich *et al.*, 2003). In pancreatic β-cells, α_2-ARs inhibit insulin secretion (Angel *et al.*, 1990; Niddam *et al.*, 1990; Ruffolo *et al.*, 1991; Fagerholm *et al.*, 2004; Fagerholm *et al.*, 2008; Fagerholm *et al.*, 2011). In postganglionic sympathetic neurons of peripheral tissues, all three α_2-AR subtypes have been shown to play a role in the feedback regulation of transmitter release (Trendelenburg *et al.*, 2003). Moreover, the α_{2B}-AR has been proven to be important in developmental processes. Because all α_2-AR knockout mice survive and are viable, no single receptor subtype is absolutely necessary for development. However, homozygous α_{2B}-knockout mice do not breed well, indicating that this receptor subtype plays some developmental or reproductive role (Link *et al.*, 1996). This finding is corroborated by the reported inability to produce α_{2AB}- or α_{2BC}-double knockout mice, whereas α_{2AC}-double knockout mice are viable (Hein *et al.*, 1999). Philipp and colleagues (2002) demonstrated that knockout mice deficient of all three α_2-AR subtypes die during embryonic development due to a defect in the establishment of the placental circulatory system. The α_{2B}-AR was defined as important for normal vascular development of the placenta and yolk sac, establishing placental circulation and maintenance of pregnancy (Philipp *et al.*, 2002b; Muthig *et al.*, 2007).

Another consequence of α_2-AR activation that is of specific interest for the present study is the regulation of proliferation of different types of cells. α_2-AR activation has been reported to increase the proliferation of primary rat proximal tubule cells (Cussac *et al.*, 2002a), opossum kidney cells (Kribben *et al.*, 1997), Chinese hamster lung fibroblasts (CCL39 cells) (Seuwen *et al.*, 1990) and several breast cancer cell lines (Vázquez *et al.*, 2006; Bruzzone *et al.*, 2008; Pérez Piñero *et al.*, 2012; Xia *et al.*, 2016b). In contrast to the above situation, in cholangiocarcinoma cells, PC12 cells, and in adult hippocampal progenitor cells (*in vivo* and *in vitro*) α_2-AR activation led to inhibition of proliferation (Kanno *et al.*, 2002; Karkoulias *et al.*, 2006a; Yanpallewar *et al.*, 2010). In many cases, the investigated cells expressed all three α_2-AR subtypes, making it difficult to specify the subtype(s) involved in the regulation of cell proliferation. Some of the studies indicated that the enhanced proliferation was caused by activation of α_{2A}-ARs (Seuwen *et al.*, 1990) or α_{2B}-ARs (Cussac *et al.*, 2002a; Vázquez *et al.*, 2006), whereas inhibition of proliferation was attributed to α_{2C}-ARs (Karkoulias *et al.*, 2006a; Karkoulias *et al.*, 2006b). In CCL39 cells transfected to express α_{2A}-ARs, the α_2-AR agonists adrenaline and clonidine stimulated cell proliferation (measured as increased [^{3}H]-thymidine incorporation), and this response was efficiently inhibited both by the α_2-AR antagonist yohimbine and by PTX (Seuwen *et al.*, 1990). In primary rat proximal tubule cells endogenously expressing α_{2B}-ARs and in a transfected porcine renal tubular cell line (LLC-PK$_1$), activation of α_{2B}-ARs stimulated cell proliferation most likely through activation of the ERK pathway (Cussac *et al.*, 2002a). Furthermore, in human breast cancer cell lines, the α_{2B}-AR was identified as the probable subtype stimulating cell proliferation upon receptor activation (Vázquez *et al.*, 2006). Interestingly, in the mouse placenta, the α_{2B}-AR appears to be differentially involved in the proliferation of different cell types. Lack of α_{2B}-ARs led to increased proliferation

[measured as bromodeoxyuridine (BrdU) incorporation] of labyrinth trophoblast cells and significantly decreased proliferation of endothelial cells in Adra2b$^{-/-}$ embryos (Muthig *et al.*, 2007). In PC12 cells stably transfected with the human α_{2C}-AR gene: α_{2C}-AR activation promoted differentiation (neurite outgrowth) and decreased cell proliferation (Karkoulias *et al.*, 2006a; Karkoulias *et al.*, 2006b).

Adrenaline and noradrenaline stimulated VSMC proliferation in culture, as has been demonstrated for rat thoracic aorta SMCs and bovine aortic SMCs in secondary culture (Blaes and Boissel, 1983; Bauch *et al.*, 1987; Bell and Madri, 1989). Proliferation of the A7r5 VSMC line was also stimulated by noradrenaline (Kuriyama *et al.*, 1988). Later, it was demonstrated that both α_1- and β-ARs inhibited the proliferation of A7r5 cells (Wang *et al.*, 2004). However, little is known about the effects of α_2-AR activation on the proliferation of VSMCs, as can be perceived from the lack of publications on this topic.

2.1.5.3 Vascular functions of α_2-adrenoceptors

VSMCs express several AR subtypes that are involved in various aspects of blood vessel function, including contraction, cellular growth and proliferation. Many physiological factors can selectively influence responses to a particular receptor subtype, and the relative roles of each subtype may vary between different vascular beds and even along an individual blood vessel as its diameter changes (Bylund *et al.*, 1995). Therefore, the net response to agonists, such as adrenaline and noradrenaline that activate all AR subtypes, depends on the relative expression of each receptor population. In most vascular tissues, α_1- and α_2-AR-mediated effects predominate. The concentration threshold for α_1- and α_2-AR-mediated effects in large arteries and veins is approximately 1–10 nM for noradrenaline. The levels of noradrenaline and adrenaline in human plasma at rest have been reported to be approximately 1−2 and 0.1−0.5 nM, respectively. During exercise, these concentrations may be elevated by as much as 30-fold compared to those at rest, and these levels may exert significant effects on blood vessels (Dodt *et al.*, 1997; Guimarães and Moura, 2001; Ambade *et al.*, 2009). Typically, α- and β-ARs have different locations in blood vessels. Therefore, when the concentrations of circulating catecholamines or that of noradrenaline released from sympathetic nerves change, α- and β-ARs may be affected differentially. Both α_2-ARs and α_1-ARs mediate vasoconstrictor responses, but postsynaptic α_1- and α_2-ARs are located differentially in relation to sympathetic nerve endings. In arteries, α_1-ARs are found in the vicinity of sympathetic nerve endings, where they can be activated by noradrenaline released from the nerves, whereas α_2-ARs are located extrasynaptically and may be activated preferentially by circulating catecholamines, especially adrenaline (Ruffolo, 1985; Jie *et al.*, 1987; Guimarães and Moura, 2001). In contrast, in veins, α_2-ARs may be situated closer to the sympathetic nerve endings than α_1-ARs (Ruffolo, 1985; Flavahan *et al.*, 1987a).

Role of individual receptor subtypes in α_2-adrenoceptor-mediated cardiovascular responses. α_2-ARs mediate important actions of noradrenaline and adrenaline in the regulation of vascular tone and blood pressure, including centrally mediated bradycardia and hypotension, regulation of myocardial contractility and vasoconstriction of small arteries (Talke *et al.*, 2001; Gilsbach and Hein, 2012). In mice, activation of α_2-ARs by intravenous or intra-arterial administration of clonidine-like α_2-agonists is typically

associated with a biphasic blood pressure response: after a transient increase in blood pressure, there is a subsequent decrease in blood pressure, the arterial pressure falls below the baseline at the same time as the animal experiences severe bradycardia. This is a typical cardiovascular response to intravenous administration of α_2-AR agonists also in humans and other species, e.g. rats and dogs (Savola, 1989; Bloor *et al.*, 1992; Ruffolo *et al.*, 1993; Urban *et al.*, 1995; Ebert *et al.*, 2000; Guimarães and Moura, 2001; Talke *et al.*, 2001). For long, the lack of subtype-selective ligands and the possible contribution of imidazoline receptors hampered the elucidation of the roles of individual α_2-AR subtypes (Esbenshade and Minneman, 1994). The development of different knockout mouse models has enabled the study of the functions of the different α_2-AR subtypes. Five different mouse strains with genetic alterations of α_2-AR expression have been generated and exploited in the investigation of α_2-AR-elicited cardiovascular responses (Guimarães and Moura, 2001; Philipp and Hein, 2004; Knaus *et al.*, 2007). These consist of knockout mouse strains lacking either α_{2A}- (Altman *et al.*, 1999), α_{2B}- (Link *et al.*, 1996) or α_{2C}-ARs (Link *et al.*, 1995), an α_{2AC}-double knockout strain (Hein *et al.*, 1999) and a mouse model with a point mutation of the α_{2A}-AR gene (α_{2A}-D79N), which is considered to be a functional knockout due to altered receptor expression and agonist activation properties (Surprenant *et al.*, 1992; MacMillan *et al.*, 1996).

Based on pharmacological analysis and studies with α_{2A}-knockout (α_{2A}-KO) and α_{2A}-D79N functional knockout mice, it has been established that most of the pharmacological functions (analgesic, sedative, hypotensive, hypothermic and behavioral actions) of α_2-AR agonists are mediated by α_{2A}-ARs (Gyires *et al.*, 2009). Both of these mouse models, α_{2A}-KO and α_{2A}-D79N, failed to become hypotensive in response to intra-arterially administered α_2-AR agonists, while the initial hypertensive vasoconstrictor response was unchanged. These results suggested that central α_{2A}-ARs were responsible for mediating the central hypotensive response not only to endogenous catecholamines but also to imidazoline-based α_2-AR agonists (MacMillan *et al.*, 1996; Altman *et al.*, 1999; Hein *et al.*, 1999). In addition, the α_{2A}-AR appears to be, at least in certain vascular compartments, also involved in the vasoconstrictor effect of α_2-AR agonists, as was demonstrated by the finding that the hypertensive response in α_{2A}-D79N mice was lacking after femoral administration of brimonidine, an α_2-AR agonist (MacMillan *et al.*, 1996). In α_{2B}-KO mice, the initial hypertensive response to α_2-AR agonists was abolished, and the hypotensive response was instantaneous and more pronounced than in control mice. The bradycardia evoked by α_2-AR agonists was not changed in α_{2B}-KO mice. It was concluded that the initial hypertensive phase was due to vasoconstriction elicited by activation of α_{2B}-ARs on vascular smooth muscle (Link *et al.*, 1996). Other studies with knockout mice indicated that this subtype also plays a significant role in the onset of salt-induced hypertension (Link *et al.*, 1996; Makaritsis *et al.*, 1999). Studies with α_{2C}-KO mice have demonstrated that the α_{2C}-AR subtype is necessary for normal control of adrenaline release from adrenal chromaffin cells, since mice lacking this receptor subtype develop more pronounced cardiac hypertrophy and heart failure after cardiac pressure overload than control mice (Philipp and Hein, 2004). As for cardiovascular regulation, the α_{2C}-AR appears to play only a minor role, since α_{2C}-KO mice showed no differences in their hypertensive, hypotensive and bradycardic responses to α_2-AR agonists when compared with wild-type mice (Link *et al.*, 1996).

Based on studies with different knockout mouse models, different roles have been suggested for the individual α_2-AR subtypes in the cardiovascular responses to α_2-AR activation by agonists (Figure 6). Three main elements have been identified in the acute biphasic blood pressure responses *in vivo*: activation of peripheral α_{2B}-ARs in vascular smooth muscle that is responsible for the initial vasoconstriction and transient hypertension; activation of central α_{2A}-ARs leading to a reduction in sympathetic tone resulting in bradycardia and prolonged hypotension; and augmentation of the hypotensive effect by activation of presynaptic α_2-ARs located on sympathetic nerve endings innervating the VSMCs (Ruffolo *et al.*, 1993; Urban *et al.*, 1995; Piascik *et al.*, 1996; Guimarães and Moura, 2001). Therefore, central α_{2A}-ARs are employed as targets of antihypertensive therapy (Aantaa and Jalonen, 2006). As the currently used clonidine-like antihypertensive drugs are not subtype-selective, the same drugs also activate α_{2B}-ARs in the blood vessel wall causing vasoconstriction, and thus partially counteract the therapeutic central hypotensive action of systemically administered α_2-AR agonists.

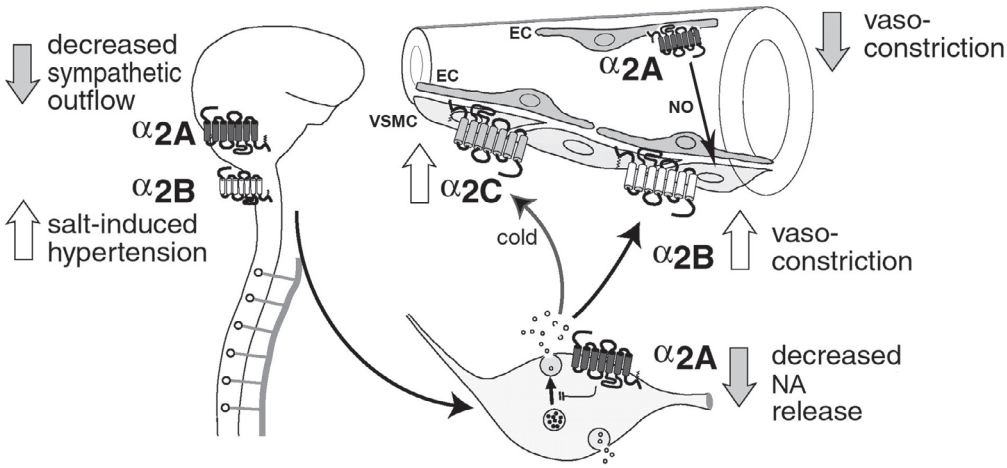

Figure 6. Regulation of blood pressure by different α_2-adrenoceptor subtypes. Activation of α_{2A}-ARs in the brain inhibits central sympathetic outflow as well as noradrenaline (NA) release from sympathetic nerves, leading to a decrease in blood pressure. Peripheral α_{2A}-ARs on vascular endothelial cells (EC) futher increase the hypotensive effect by regulating nitric oxide (NO) release, which in turn exerts a relaxing effect on closely located vascular smooth muscle cells (VSMCs). This effect is counteracted by vasoconstriction evoked by peripheral α_{2B}-ARs on VSMCs in the blood vessel wall, and perhaps also regulated by central α_{2B}-ARs that may mediate salt-induced hypertension. Silent α_{2C}-ARs onVSMCs may participate in vasoconstriction upon exposure to cold temperatures [modified from (Philipp *et al*., 2002a)].

α_2-adrenoceptor-mediated responses in different vascular tissues. Already more than 30 years ago, postsynaptic α_2-ARs were demonstrated to exist in vascular smooth muscle and to mediate vasoconstrictor responses (Docherty *et al.*, 1979; Drew and Whiting, 1979; Docherty and McGrath, 1980). In the pithed rat model, a preparation that excludes central mechanisms, α_2-ARs on vascular smooth muscle were demonstrated to mediate vasoconstriction, indicating that the vasoconstrictor response occurred independently of the peripheral sympathetic nervous system (Docherty *et al.*, 1979; Drew and Whiting, 1979; Docherty and McGrath, 1980). Initially, it was proposed that vascular α_2-ARs would

be primarily extrasynaptic and mediate responses to circulating catecholamines (Langer *et al.*, 1980; Langer *et al.*, 1981), but it soon became evident that in certain vascular tissues, such as the human saphenous vein, also contractions to nerve stimulation could be mediated by α_2-ARs. As vasoconstriction ensued from activation of α_2-ARs by noradrenaline released from sympathetic neurons, the receptors involved had to be located in the vicinity of the synaptic cleft (Docherty and Hyland, 1985; Ruffolo, 1985; Flavahan *et al.*, 1987a).

A vast number of *in vivo* and *in vitro* studies have been performed to investigate the contractile responses mediated by α_2-ARs, revealing the large variation in the contractile responses to postsynaptic α_2-AR activation, depending on the animal species, the diameter of the vessel and its anatomical origin. Typically, α_2-ARs are not present in large arteries such as the aorta, and constrictor activity of α_2-AR agonists is generally restricted to small arteries and veins (Hughes *et al.*, 1988; Nielsen *et al.*, 1989; Parkinson and Hughes, 1995; Leech and Faber, 1996; Chotani, 2004; Flavahan, 2005). The postsynaptic response to noradrenaline is influenced by the vessel diameter, with usually a slow contraction of large blood vessels and a rapid and intense contraction of small vessels. Other factors also contribute to the force and duration of vasoconstriction, including the proportions of smooth muscle and collagen in the vessel wall, the density of its innervation, being richer in small arterioles, and the distance between nerve terminals and VSMCs, which is greater in larger vessels (Bülbring and Tomita, 1987; Martinotti, 1991). The contribution of α_2-ARs to noradrenaline-induced vasoconstriction (in addition to α_1-AR-mediated vasoconstriction) may change along the length of a single vessel as the vessel diameter decreases. It has been demonstrated in human limb arteries that the participation of α_2-ARs in noradrenaline-induced vasoconstriction increases from the proximal to the distal parts of these vessels (Thom *et al.*, 1985; Flavahan *et al.*, 1987b). Similarly, in the mouse tail artery, α_2-AR-evoked vasoconstriction is greater in the distal parts compared with the proximal parts, whereas α_1-AR effects exhibit an opposite pattern (Chotani *et al.*, 2000). In contrast, in cerebral arteries, α_2-AR-elicited responses have been reported to weaken from proximal to distal arteries (Bevan *et al.*, 1987). Furthermore, in veins, such as the dog saphenous and cephalic veins, the magnitude of α_2-AR-evoked vasoconstriction was markedly smaller in the distal parts compared with the proximal parts. This was apparently due to higher α_2-AR densities in the proximal parts, since agonist potencies did not change along the length of the vessels (Guimaraes and Nunes, 1990). In general, for contraction of veins evoked by noradrenaline, the predominant receptor is of the α_2-AR class, unlike the arteries, where α_1-AR-mediated vasoconstriction predominates in larger vessels and α_2-AR-mediated vasoconstriction is restricted to smaller arteries and arterioles. Therefore, vein preparations in particular have been useful in determining the roles of the different α_2-AR subtypes in vasoconstriction (Civantos Calzada and Aleixandre de Artiñano, 2001).

Differences exist between animal species in the distribution of α_2-ARs, and therefore, results obtained in one species cannot be directly extrapolated to another (Badino *et al.*, 2005). In arteries, α_2-AR-mediated vasoconstriction has been reported in human resistance arteries (Hughes *et al.*, 1988), rat tail artery (Hicks *et al.*, 1984; Medgett *et al.*, 1984; Xiao and Rand, 1989), immature rat iris arterioles (Sandow and Hill, 1999), dog external carotid circulation (Willems *et al.*, 2001), pig palmar common digital artery (Blaylock and Wilson,

1995; Wright *et al.*, 1995a), pig thoracic aorta and splenic artery (Wright *et al.*, 1995a), pig ear artery (Bhattacharya and Roberts, 2003; Bhattacharya *et al.*, 2005), rabbit abdominal aorta (Gnus *et al.*, 2013), rabbit distal saphenous artery (Dunn *et al.*, 1991) and rabbit femoral artery (García-Villalón *et al.*, 1992). In veins, α_2-AR-mediated vasoconstriction has been reported in e.g. dog saphenous vein (Shoji *et al.*, 1983; MacLennan *et al.*, 1997), pig palmar lateral vein (Blaylock and Wilson, 1995; Wright *et al.*, 1995a), pig marginal ear vein (Wright *et al.*, 1995a), rabbit ear vein, saphenous vein and plantaris vein of the foot (Daly *et al.*, 1988) and in the vasculature (veins and sinusoids) of the nasal mucosa of several species including dog, pig and monkey (Berridge and Roach, 1986; Lacroix and Lundberg, 1989; Corboz *et al.*, 2007).

Often, vasoconstriction of these vessels is mediated by both α_1- and α_2-ARs (Shoji *et al.*, 1983; Daly *et al.*, 1988; Xiao and Rand, 1989; Dunn *et al.*, 1991; García-Villalón *et al.*, 1992; Blaylock and Wilson, 1995; Elliott, 1997; MacLennan *et al.*, 1997; Willems *et al.*, 2001; Gnus *et al.*, 2013). In the pig palmar lateral vein and palmar common digital artery both α_1- and α_2-ARs participated in the vasoconstriction, but the contribution of α_2-ARs was larger in the palmar lateral vein (Blaylock and Wilson, 1995). In addition, in the carotid arteriovenous anastomoses in anaesthetized pigs, both receptor types were found to mediate vasoconstriction (Willems *et al.*, 1999). In the rabbit abdominal aorta, α_1-ARs were identified as the predominant receptor type controlling contractility, but α_2-ARs were also involved in maintaining muscle tone (Gnus *et al.*, 2013). In the rat tail artery, vasoconstrictor responses to the relatively selective α_1-AR agonist phenylephrine were enhanced by α_2-AR agonists (Xiao and Rand, 1989). In the rabbit distal saphenous artery, inducing a small degree of vascular tone with a low concentration of phenylephrine, significantly increased the threshold sensitivity of the selective α_2-AR agonist, brimonidine (Dunn *et al.*, 1991). Both the saphenous vein and the plantaris vein of the rabbit foot expressed a mixture of α_1- and α_2-ARs, but the contribution of α_1-ARs appeared to be more prominent in the larger saphenous vein, while α_2-ARs appeared to predominate in the smaller plantaris vein (Daly *et al.*, 1988).

In vivo studies have enabled the assessment of central and peripheral factors in α_2-AR-elicited vasoconstriction. α_2-ARs mediate both sympatholytic and vasoconstrictive hemodynamic effects through the activation of central α_2-ARs in the brain or peripheral α_2-ARs in the blood vessel wall, respectively. In a series of studies on human subjects, Talke and colleagues (2003, 2005) have profiled the peripheral vasoconstrictive effects of the selective α_2-AR agonist dexmedetomidine in isolation from its sympatholytic effects. When the central sympatholytic effects of dexmedetomidine were attenuated by general anesthesia, dexmedetomidine evoked vasoconstriction and elevated systolic blood pressure already at low plasma concentrations. In awake subjects, dexmedetomidine decreased systolic blood pressure. In the intact hand, low plasma concentrations of dexmedetomidine (≤ 0.6 ng/ml) caused vasodilatation, whereas in the opposite hand of the same volunteer subjects that had been temporarily denervated by axillary block, the same concentrations of dexmedetomidine caused vasoconstriction. Thus, by denervation of the vascular bed of interest (hand) or by decreasing sympathetic nervous system activity, it was possible to detect vasoconstriction induced by small concentrations of systemically administered dexmedetomidine (Talke *et al.*, 2003). In another study, Talke and colleagues (2005) investigated whether α_2-AR activation induced enhanced vasoconstriction in

carriers of the α_{2B}-AR deletion-deletion (D/D) genotype compared with carriers of the insertion-insertion (I/I) or insertion-deletion (I/D) genotypes. The D/D deletion variant has been associated with a loss of short-term agonist-promoted receptor desensitization, which might lead to increased vasoconstriction upon α_2-AR activation. The results of this study confirmed that dexmedetomidine induced marked peripheral vasoconstriction. In this experiment with a rather short duration (15 min) of agonist exposure, differences in vasoconstriction responses could not be identified between carriers of the α_{2B}-AR DD genotype and the other subjects (Talke *et al.*, 2005a).

α_2-adrenoceptor subtypes involved in vasoconstriction. α_2-ARs play an important physiological role in modulating blood vessel diameter and flow and peripheral resistance. Understanding the roles of the different α_2-AR subtypes in the regulation of vasoconstrictor responses has been sought by many research groups. The α_{2A}-AR has been the predominant subtype in almost all veins studied, including the dog saphenous vein (Hicks *et al.*, 1991; MacLennan *et al.*, 1997), rabbit skeletal muscle venules (Leech and Faber, 1996), rat cremaster venules (Leech and Faber, 1996), pig marginal ear vein (Wright *et al.*, 1995a) and pig palmar lateral vein (Blaylock and Wilson, 1995; Wright *et al.*, 1995a). However, α_{2A}-AR-mediated contraction of arteries has also been demonstrated in the pig common digital artery (Blaylock and Wilson, 1995; Wright *et al.*, 1995a), pig thoracic aorta, ear artery and splenic artery (Wright *et al.*, 1995a), as well as in rat cremaster arterioles (Leech and Faber, 1996). Based on studies with pithed rat and mouse models, the major α_2-AR subtype implicated in vasoconstrictor responses is the α_{2A}-AR. This subtype is also the predominant mediator of the hypotensive actions of α_2-AR agonists, as shown in knockout mice lacking the α_{2A}-AR subtype (MacMillan *et al.*, 1996). Hence, the α_{2A}-AR subtype may mediate both central hypotensive responses and peripheral vasoconstrictor responses to α_2-AR agonists, but in conscious animals, the central effects predominate. Initially, in the pithed rat model, the α_{2A}-AR appeared to be the subtype mediating vasoconstriction, although the involvement of the α_{2B}-AR subtype was also considered possible [reviewed by (Docherty, 1998; Civantos Calzada and Aleixandre de Artiñano, 2001)]. In the pithed mouse model, the α_{2B}-AR subtype was suggested to take part in the vasoconstrictor response upon α_2-AR agonist stimulation (McCafferty *et al.*, 1999). The important role of the α_{2B}-AR subtype in eliciting vasoconstrictor responses to systemically administered α_2-AR agonists was first demonstrated in α_{2A}-AR and α_{2B}-AR knockout mice (Link *et al.*, 1996) and later confirmed also in humans and other species (Snapir *et al.*, 2001; Snapir *et al.*, 2003b; Talke *et al.*, 2005b; Wong *et al.*, 2010; Ok *et al.*, 2011).

Initially, the α_{2C}-AR was not considered to be involved in vascular regulation; it was rather thought to represent a vestigial or silent receptor subtype (Chotani and Flavahan, 2011). However, Gavin and colleagues (1997) first suggested, based on comparisons of antagonist potencies, that the postsynaptic α_2-ARs mediating contractions of the human saphenous vein appeared to be of the α_{2C}-AR subtype (Gavin *et al.*, 1997). Soon thereafter, Richman and Regan (1998) were able to demonstrate the expression of α_{2C}-ARs in primary rat aortic SMCs by means of RT-PCR, although the role of this subtype in the regulation of vascular functions remained unclear (Richman and Regan, 1998). Postsynaptic α_{2C}-ARs have been shown to mediate contraction of pig pulmonary veins (Görnemann *et al.*, 2007; Jantschak and Pertz, 2012), and it has been confirmed by RT-PCR analysis that these veins express

both α_{2A}- and α_{2C}-ARs, but not α_{2B}-ARs (Görnemann *et al.*, 2007). A similar composition of α_2-AR subtypes has been demonstrated in the mouse and rat tail arteries (McNeill *et al.*, 1999; Chotani *et al.*, 2000). The isolated rat tail artery represents an *in vitro* model of the cutaneous circulation, and in this model, α_{2C}-ARs were identified as the predominant receptor mediating vasoconstriction both at cooler (27 °C) and at warmer (32 °C and 37 °C) temperatures. Precontraction of the arteries enhanced the maximal contraction response to brimonidine stimulation which was further enhanced by rapid cooling (Jantschak *et al.*, 2010). Many other studies have also demonstrated the predominance of the α_{2C}-AR in cold-induced vasoconstriction of cutaneous arteries (Chotani *et al.*, 2000; Bailey *et al.*, 2004; Eid *et al.*, 2007), and also in cutaneous veins (Flavahan *et al.*, 1985; Zerpa *et al.*, 2010). The role of α_{2C}-ARs in the local cooling-induced reduction of skin blood flow also has been confirmed in the murine foot *in vivo* (Honda *et al.*, 2007). Constriction of cutaneous arteries is an essential part of thermoregulation. In cold temperatures, cutaneous vasoconstriction protects the body from heat loss, whereas vasodilatation occurs when the body experiences heat stress (Charkoudian, 2003). Thus, cutaneous α_{2C}-ARs have a unique physiological function and role in mediating vasoconstriction in response to cooling. Abnormal activity of α_{2C}-ARs has been proposed to contribute to cold-induced vasospasm as seen in Raynaud's disease (Chotani *et al.*, 2000; Chotani *et al.*, 2004; Chotani *et al.*, 2005).

Chotani and colleagues (2004) investigated the expression and regulation of α_2-AR subtypes in VSMCs derived from human saphenous veins and cutaneous arterioles, displaying α_2-AR-mediated contractile activity, and from human aorta that lacks α_2-AR responsiveness. They discovered high expression levels of functional α_2-ARs ($\alpha_{2C} > \alpha_{2A}$) in VSMCs derived from saphenous veins and cutaneous arterioles, whereas VSMCs cultured from aorta did not express detectable levels of α_2-ARs. However, functional α_{2C}-ARs were detected in VSMCs of adventitial arterioles of the aorta. They also found promoters of the α_{2A}- and α_{2C}-AR genes to exhibit higher activities in venous and arteriolar VSMCs compared with aortic VSMCs, resulting in site-specific expression of these α_2-AR subtypes in human VSMCs (Chotani *et al.*, 2004).

Endothelial α_2-adrenoceptors. The vascular endothelium is an important regulator of the cardiovascular system. The endothelial layer (intima) can regulate vascular tone and growth by releasing vasodilating factors (NO and prostacyclin), contraction-inducing factors [e.g. endothelin and angiotensin II (AT-II)] and growth inhibitors or promoters [e.g. heparin and platelet-derived growth factor (PDGF)] (Rajendran *et al.*, 2013). The endothelium-dependent response to catecholamines involves activation of α_2-ARs in endothelial cells. In many blood vessels, activation of endothelial α_2-ARs stimulates the release of NO, leading to relaxation of the vascular smooth muscle layer, an action that would tend to attenuate vasoconstriction produced by activation of postsynaptic vascular α_1- and α_2-ARs. Typically such responses have been detected in blood vessels that are chronically exposed to high partial pressure of oxygen, such as systemic arteries (coronary, femoral) and pulmonary veins (Vanhoutte and Miller, 1989; Nishina *et al.*, 1999), but α_2-AR-mediated release of NO has also been reported in the microcirculation, namely in coronary microvessels (Angus *et al.*, 1986). Moreover, in human pulmonary resistance arteries, activation of endothelial α_2-ARs and the subsequent release of NO inhibited the dexmedetomidine-induced vasoconstriction seen in endothelium-denuded pulmonary

arteries (Nong *et al.*, 2016). Characterization of the α_2-AR subtypes present on vascular endothelium has been carried out in different vascular tissues. In pig coronary arteries, endothelial cells (ECs) expressed both α_{2A}-ARs (23 %) and α_{2C}-ARs (77 %). However, despite the predominance of the α_{2C}-AR subtype, the α_{2A}-AR was the subtype mediating endothelium-dependent relaxation (Bockman *et al.*, 1993). In the rat mesenteric artery, the α_2-AR subtype mediating endothelium-dependent NO-mediated relaxation was identified as α_{2A} (Bockman *et al.*, 1996; Figueroa *et al.*, 2001; Wong *et al.*, 2010), although the involvement of α_{2B}-ARs in this response was also speculated (Wong *et al.*, 2010).

Factors affecting α_2-adrenoceptor-mediated vascular responses. Different factors such as temperature, age, gender, genes/genetic background and diseases may affect the cardiovascular responses evoked by α_2-AR activation. α_2-ARs are sensitive to alterations in temperature and their role in thermoregulation appears to be important (Guimarães and Moura, 2001). One well established response is the enhanced α_2-AR-evoked vasoconstriction of cutaneous arteries when they are exposed to colder temperatures (Chotani *et al.*, 2000; Bailey *et al.*, 2004; Eid *et al.*, 2007).

Aging is associated with many changes in the vascular system, and has been linked with an increase in the incidence of cardiovascular diseases, such as hypertension and coronary artery disease. Changes occur especially in the structure and function of arteries, e.g. endothelial function and smooth muscle cells of the vessels are affected (Marín and Rodríguez–Martínez, 1999). In human subcutaneous resistance arteries, the contractile response to BHT-933 (an α_2-AR agonist) has been reported to increase with age, whereas α_1-AR-mediated responses decrease (Nielsen *et al.*, 1992). However, vasoconstrictor responses mediated by α_1-ARs appear to be relatively well maintained with aging, whereas α_2-AR-mediated responses (both pre- and postsynaptic) have often been reported to be attenuated (Marín and Rodríguez–Martínez, 1999). In rats, aging has been associated with a decreased sensitivity of α_2-ARs to α_2-AR agonists in the vas deferens (Docherty and O'Malley, 1983), and also in humans, α_2-AR responsiveness is altered with age, as demonstrated by reduced postsynaptic α_2-AR responsiveness in the leg circulation upon endogenous noradrenaline release (Smith *et al.*, 2007). In rats, presynaptic α_2-AR-mediated inhibitory control of noradrenaline release has been shown to decrease with age (Daly *et al.*, 1989). The resulting increase in the release of noradrenaline might serve to maintain noradrenergic neurotransmission although postsynaptic responsiveness may deteriorate with age (Docherty, 2002).

Gender-related differences in vasoconstrictor responses upon postsynaptic α_2-AR activation were investigated in isolated tail arteries from male and female rats. The response to brimonidine was more pronounced in male arteries. Removal of sex hormones (by gonadectomy) did not alter the male-female differences, suggesting that sex differences in α_2-AR function are not directly caused by either male or female sex hormones but could be developmentally regulated (Chen *et al.*, 1999). However, Li and colleagues (1997) demonstrated the influence of sex hormones on vasoconstrictor responses in tail arteries isolated from intact and gonadectomized male and female rats. After ovariectomy, the contractile responses of female rat tail arteries to sympathetic nerve stimulation were increased to levels similar to those observed in male arteries. Orchiectomy had no effect on neurally evoked contraction. Low concentrations of noradrenaline produced greater

contractile responses in male compared with female arteries; however, this gender-related difference was eliminated by orchiectomy but not by ovariectomy. Taken together, the results suggested that circulating sex hormones contributed to the gender differences observed in the rat tail artery. Vasoconstrictor responses to exogenous noradrenaline appeared to be enhanced by testicular hormones. In contrast, the vasoconstriction induced by sympathetic nerve stimulation appeared to be influenced by chronic exposure to ovarian hormones, resulting in smaller vascular responses in female arteries (Li *et al.*, 1997).

Furthermore, also genetic differences in the components of the signal transduction pathways mediating the vascular responses evoked by α_2-AR activation might contribute to the observed differences in the responses. Posti and colleagues (2013) investigated the genetic factors contributing to the substantial inter-individual variability observed in α_2-AR-mediated vascular constriction induced by dexmedetomidine. They identified PKCβ as a possibly important player in the signaling pathways of venous constriction evoked by dexmedetomidine, as a variant form of the PKCβ gene (rs9922316) was associated with decreased vasoconstrictor responses to dexmedetomidine (Posti *et al.*, 2013). Interestingly, a variant form of the human α_{2B}-AR gene, the D/D genotype, has been associated with acute coronary events and cardiovascular morbidity and mortality (Snapir *et al.*, 2001; Snapir *et al.*, 2003a).

In addition, vascular α_2-ARs can be affected in certain diseases, either as a result of alterations suffered by the vessels or by contributing to the generation of diseases or by being their primary cause (Guimarães and Moura, 2001). α_2-ARs appear to have a role in the development of Raynaud's disease which is characterized by vasospastic episodes due to cold-induced vasoconstriction upon α_2-AR activation (Chotani *et al.*, 2000). In scleroderma, the constrictor responses to α_2-AR agonists in arterioles are also increased due to enhancement of the α_2-AR-mediated VSMC responses (Flavahan *et al.*, 2000). Salt loading is known to cause hypertension, and this has been proposed to involve α_{2B}-ARs as demonstrated with α_{2B}-AR knockout mice which are salt-resistant compared with control mice (Makaritsis *et al.*, 1999). Furthermore, the functional role of extrasynaptic α_2-ARs may be pronounced in disease states such as hypertension and congestive heart failure where circulating levels of catecholamines can be significantly elevated and, thus, contribute to the maintenance of elevated vascular resistance (Ruffolo, 1985).

2.1.6 Therapeutic applications of α_2-adrenoceptor agonists and antagonists

α_2-ARs are important drug targets mediating multiple effects at various anatomical sites. α_2-AR agonists have been used as sedative and analgesic agents in veterinary medicine since the 1970s, and for human use α_2-adrenergic agonists were introduced for the treatment of hypertension a few years later, in the late 1970s to early 1980s (Khan *et al.*, 1999; Angelini *et al.*, 2000). α_2-AR agonists are also useful as sedative, hypnotic and anesthetic-sparing drugs e.g. to lower sympathetic tone during cardiac surgery and to prevent postoperative cardiovascular complications. Furthermore, clonidine and some other α_2-AR agonists have been used for decades to treat alcohol and drug withdrawal symptoms, ADHD, pain, panic disorder and glaucoma, and some α_2-AR agonists are frequently used as over-the-counter nasal decongestants (Gilsbach and Hein, 2012; Giovannitti *et al.*, 2015). Hypotension and bradycardia have limited the use of α_2-agonists for sedation, although these side effects

are dose-dependent and occur mainly in hypovolemic patients (Khan *et al.*, 1999; Goyal, 2016).

2.1.6.1 α_2-Adrenoceptor agonists

Clinically employed α_2-AR agonists vary not only in their duration of action but also in their potency and affinity for the different α_2-AR subtypes. Agonists that clearly favour α_2-ARs over α_1-ARs include clonidine, dexmedetomidine, guanabenz, guanfacine, tizanidine and brimonidine (Angelini *et al.*, 2000; Giovannitti *et al.*, 2015). Clonidine, the prototype α_2-agonist, activates α_2-ARs with an approximately 200-fold selectivity over α_1-ARs. However, it is only a partial α_2-AR agonist (Angelini *et al.*, 2000; Giovannitti *et al.*, 2015). Dexmedetomidine is more potent and efficacious as an α_2-AR agonist than clonidine; also, it is the most selective α_2-agonist clinically available with a selectivity ratio of 1620:1 for α_2-ARs versus α_1-ARs (Virtanen *et al.*, 1988; Gertler *et al.*, 2001; Mantz *et al.*, 2011). It has a shorter elimination half-life than clonidine (2 h vs 9-12 h, respectively) (Angelini *et al.*, 2000; Mantz, 2000). Clonidine, dexmedetomidine and tizanidine have had the most wide-spread clinical use, however, none of these α_2-agonists in the clinic is subtype-selective (Angelini *et al.*, 2000; Giovannitti *et al.*, 2015). Most of the clinical effects of α_2-AR agonists are mediated by the α_{2A}-AR subtype, but also α_{2B}- and α_{2C}-ARs may contribute to some of the therapeutic actions of α_2-agonists. Therefore, it is doubtful whether a selective agonist of the α_{2A}-AR subtype would provide any clear advantage over the current non-selective α_2-AR agonists. A selective agonist of the α_{2B}- or α_{2C}-AR subtypes might be devoid of most of the typical side effects of α_2-AR agonists mediated by the α_{2A}-AR subtype (e.g. sedation, bradycardia), but there is still uncertainty of the precise roles of α_{2B}- and α_{2C}-ARs in the regulation of physiological and pharmacological processes and further studies are needed to address this issue (Gyires *et al.*, 2009). Many attempts have been made to improve α_2-AR agonist properties, but since both the antihypertensive action and the major side effects are mediated by the α_{2A}-AR subtype, these attempts have been unsuccessful (Crassous *et al.*, 2007).

Central α_{2A}-ARs have long been employed as targets of antihypertensive drugs (Aantaa and Jalonen, 2006), and the primary clinical use of α_2-AR agonists, especially clonidine, has been the treatment of hypertension. Despite its good efficacy in decreasing blood pressure, because of its prominent sedative and bradycardic side effects, clonidine has largely been replaced by other drugs, such as ACE inhibitors or AT_1-antagonists, in the treatment of hypertension (Crassous *et al.*, 2007; Giovannitti *et al.*, 2015). So far, vascular α_2-ARs have not been exploited as cardiovascular drug targets. Recently, a peripherally acting subtype-nonselective α_2-AR antagonist, MK-467, was demonstrated to effectively counteract dexmedetomidine-evoked vasoconstriction (Honkavaara *et al.*, 2012). However, prolonged subtype-nonselective α_2-AR blockade can be expected to lead to adverse cardiovascular effects as a consequence of loss of presynaptic α_{2A}-AR autoreceptor function. This situation underlines the need to discover and evaluate new α_{2B}-AR subtype-selective antagonists.

Based on their capacity to enhance the effects of traditional anesthetics, the α_2-AR agonists dexmedetomidine and clonidine have acquired widespread use as adjuncts for intra- and perioperative sedation and anxiolysis. Dexmedetomidine and clonidine exhibit rather similar,

broad patterns of action, but dexmedetomidine is more potent and more selective for α_2-ARs (Mantz *et al.*, 2011). Dexmedetomidine is the pharmacologically active enantiomer of racemic medetomidine, a veterinary anesthetic drug, and, in 1999, dexmedetomidine was approved by the US Food and Drug Administration (FDA) for use in the intensive care unit (ICU) in critically ill patients. In 2008, the approval was extended for procedures outside the operating room in non-intubated patients (Angelini *et al.*, 2000; Goyal, 2016). Dexmedetomidine possesses many of the properties of an ideal sedative: it is analgesic and anxiolytic, has a rapid onset of action, is titratable, largely lacks respiratory depressant effects and produces sedation with hemodynamic stability. In addition, even if clinically this property has so far been applied only in veterinary medicine, α_2-AR antagonists make a quick reversal of sedation an option (Mantz, 2000; Giovannitti *et al.*, 2015). α_2-AR agonists, including dexmedetomidine, mimic some aspects of natural sleep by causing sedation through a mechanism involving inhibition of the locus coeruleus, and because of this, patients sedated with dexmedetomidine can be aroused by touching and speaking to them (Crassous *et al.*, 2007). Dexmedetomidine is also widely used as an adjunct for sedation in combination with agents such as opioids, benzodiazepines and propofol, and to reduce anesthetic requirements. In addition to permitting significant reductions of the doses of anesthetic agents, dexmedetomidine enhances sedation and promotes and maintains hemodynamic stability (Crassous *et al.*, 2007; Giovannitti *et al.*, 2015; Goyal, 2016). In some animal studies, dexmedetomidine has been found to be neuroprotective and, therefore, can be probably used safely also in children (Sanders *et al.*, 2010; Goyal, 2016). Clonidine is also used for ICU sedation in some European countries; however, it is not easily titratable to the target level of sedation, and it has been claimed to evoke rebound hypertension, making gradual discontinuation of the drug infusion necessary (Khan *et al.*, 1999; Angelini *et al.*, 2000; Mantz, 2000; Giovannitti *et al.*, 2015).

Particularly clonidine and guanfacine have been administered to treat ADHD in children and adolescents, where reduced release of noradrenaline in the prefrontal cortex has been suggested to improve the impulsive and hyperactive behavior typical for ADHD (Sallee, 2008; Sallee, 2010; Bidwell *et al.*, 2010; Giovannitti *et al.*, 2015). α_2-AR agonists also appear to be useful in the treatment of post-traumatic stress disorder (PTSD). PTSD is associated with increased noradrenergic tone; thus, agents dampening the sympathetic nervous system provide a potential treatment for certain aspects of this condition. Clonidine seems to be preferred in clinical practice, possibly because of its sedative side effects, which can actually be considered as desirable, as they may induce sleep and promote relaxation in agitated PTSD patients (Belkin and Schwartz, 2015). Brimonidine in particular has proven to be effective for long-term treatment of glaucoma (Burke and Schwartz, 1996; Crassous *et al.*, 2007), and tizanidine is being used in the treatment of myofascial pain disorders of the head and neck. Tizanidine can reduce spasticity by increasing the presynaptic inhibition of motor neurons in the brain and spinal cord, and by reducing painful muscle spasms. Patients with cerebral palsy or other spastic disorders may also benefit from tizanidine (Malanga *et al.*, 2002; Crassous *et al.*, 2007; Giovannitti *et al.*, 2015).

2.1.6.2 α_2-Adrenoceptor antagonists

Atipamezole, idazoxan and yohimbine are selective α_2-AR antagonists; atipamezole is used as a reversal agent in veterinary practice (Khan *et al.*, 1999). α_2-AR antagonists have

been suggested to be potentially useful for the treatment of several diseases, including Raynaud's disease, type 2 diabetes and depression. However, apart from being effective antidotes of α_2-AR agonists, the clinical use of α_2-AR antagonists has been very limited. Yohimbine has had some use in the treatment of sexual dysfunction in men, despite the fact that its efficacy has not been clearly demonstrated, and it may have a beneficial effect in orthostatic hypotension triggered by other drugs or pathological conditions (Crassous *et al.*, 2007). Preclinical and clinical evidence suggests that noradrenaline plays an important role in the etiology of depressive disorders; hence, α_2-AR antagonists may be useful in the treatment of such conditions. Mianserin and mirtazapine, two clinically available antidepressant drugs, have been demonstrated to act also as antagonists at the α_2-ARs. These drugs inhibit presynaptic α_2-AR auto- and heteroreceptors, but have no effects on noradrenaline re-uptake, thus increasing noradrenergic neurotransmission. However, selective α_2-AR antagonists such as idazoxan have not exhibited convincing properties in the treatment of depression (Davis and Wilde, 1996; de Boer, 1996; Crassous *et al.*, 2007).

Cold-induced vasoconstriction is a typical symptom of Raynaud's disease and it has been suggested to be mediated by α_{2C}-ARs. Therefore, selective inhibition of this receptor subtype has been speculated to be useful in the treatment of the vasospastic attacks. Clinical trials of this concept, however, have yielded unconvincing results. A trial with the α_{2C}-AR antagonist OPC-28326 indicated that selective inhibition of this subtype improved recovery from cold-induced vasoconstriction (Wise *et al.*, 2004), whereas another trial with another α_{2C}-AR antagonist, ORM-12741, was negative and did not provide any proof of this concept (Herrick *et al.*, 2014). Selective inhibition of the α_{2C}-AR may also have therapeutic potential in the treatment of CNS disorders, such as schizophrenia or Alzheimer's disease, and indeed, two selective α_{2C}-AR antagonists, JP-1302 and ORM-10921, have shown preclinical potential of α_{2C}-AR antagonism to treat such disorders (Sallinen *et al.*, 2007; Sallinen *et al.*, 2013). Since the year 2000, almost 100 patents have been placed regarding different strategies of α_{2C}-AR modulation for diagnosis, prognosis and treatment of disorders involving this receptor subtype. Nevertheless, despite the numerous patents, no highly selective α_{2C}-AR antagonists have emerged (Quaglia *et al.*, 2011). This describes well the enormous challenges in attempting to develop novel subtype-selective α_2-AR ligands; the drugs have tantalizing therapeutic prospects but, all-too-often, this vision has proved to be a mirage.

2.2 Vascular smooth muscle – phenotype, contraction and cell models

VSMCs are a major component of the blood vessel wall, where they exist in a differentiated contractile phenotype. The blood vessel wall consists of three distinct layers (Figure 7), with minor variation in the composition of these layers between individual arteries and veins. The innermost layer exposed to the circulating blood is the tunica intima composed of a single layer of endothelial cells lying on a basement membrane composed of collagen. In some arteries, such as the aorta and coronary arteries, also VSMCs may be present in the intima. The medial layer, tunica media, is primarily composed of spirally arranged VSMCs and some multipotent vascular stem cells (MVSCs), elastin fibers and extracellular matrix (ECM). The outermost layer is the tunica adventitia, which contains fibroblasts and some VSMC-related progenitor cells surrounded by collagen, proteoglycan matrix,

vasa vasorum and innervation (Chamley-Campbell *et al.*, 1979; Ross, 1986; Proudfoot and Shanahan, 2012; Kennedy *et al.*, 2014a). VSMCs in adult blood vessels are highly specialized cells and their main function is contraction and regulation of blood vessel diameter, blood pressure and blood flow distribution. They proliferate at a very low rate, exhibit low synthetic activity and express contractile proteins, such as smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain (SM-MHC) and calponin, ion channels, and signaling molecules required for the cell's contractile function. Unlike skeletal or cardiac muscle, VSMCs within adult animals are not terminally differentiated, and in response to vascular injury, such as atherosclerosis, VSMCs may de-differentiate and begin to migrate and proliferate. VSMCs are also capable of producing ECM; this plasticity is essential for vascular repair at sites of injury (Chamley-Campbell *et al.*, 1979; Ross, 1986; Owens, 1995; Owens *et al.*, 2004; Alexander and Owens, 2012; Proudfoot and Shanahan, 2012; Shi and Chen, 2014).

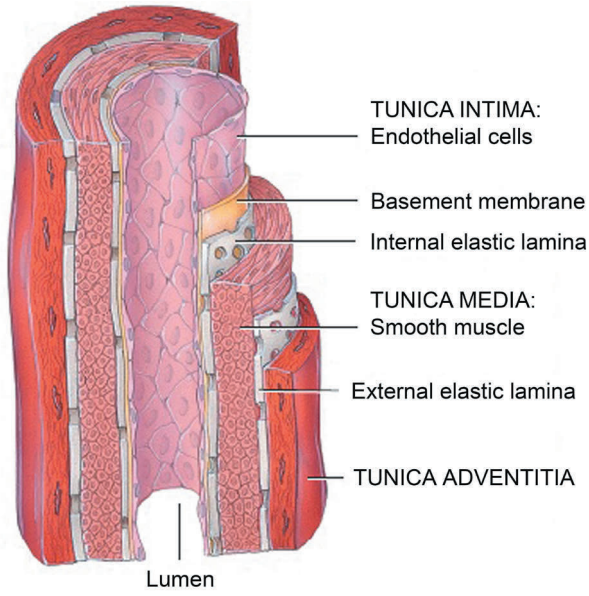

Figure 7. Structure of the arterial blood vessel wall. The blood vessel wall consists of three layers: the innermost layer tunica intima composed of endothelial cells, the middle layer tunica media mainly composed of vascular smooth muscle cells and the outermost layer tunica adventitia composed of fibroblasts, collagen and proteoglycan matrix among others [modified from (Tortora and Derrickson, 2012)].

2.2.1 Phenotypic modulation of VSMCs

2.2.1.1 VSMC phenotypes

VSMC proliferation and differentiation are important physiological processes during vascular development. The main function of fully differentiated VSMCs is the contraction of the blood vessel. The contractile phenotype is characterized by a distinct spindle shape form, highly differentiated contractile machinery and an extremely low proliferation rate. The majority of VSMCs within the vascular wall exhibit this phenotype under normal physiological conditions. However, even in adult animals, VSMCs retain remarkable plasticity. They are very sensitive to different environmental

stimuli, such as growth factors, inflammatory mediators and mechanical influences, and upon stimulation, they can undergo rapid changes in their functional and morphological properties. During such changes, VSMCs show markedly reduced expression of smooth muscle-specific differentiation marker genes, and they lose their ability to contract, start to proliferate, migrate and synthesize ECM proteins – a phenomenon called phenotypic modulation or switching (Campbell and Campbell, 1985; Alexander and Owens, 2012; Kudryavtseva *et al.*, 2013). In the literature, the terms "contractile" and "synthetic" have been widely used to describe the VSMC phenotypes. However, it has become apparent that a simple two-state model is insufficient to explain the diverse range of phenotypes that can be exhibited by SMCs under different physiological and pathological conditions. Indeed, these two phenotypes represent the extremes of a wide spectrum of possible VSMC phenotypes, and it may actually be rather artificial to assign the cells to distinct subcategories. For instance, during vascular development, when VSMCs are first acquiring their contractile properties, but also simultaneously participating in vessel growth and remodeling, it becomes difficult to distinguish between the contractile and synthetic phenotypes (Owens *et al.*, 2004). The contractile phenotype has well-defined characteristics, whereas the noncontractile phenotype resembles more a large cluster of different phenotypes in accordance with the specific functional characteristics of these cells. Different stimuli can give rise to distinct VSMC phenotypes, such as proliferative, migratory, synthetic and inflammatory phenotypes (Owens *et al.*, 2004; Alexander and Owens, 2012; Kudryavtseva *et al.*, 2013). A very recent study, employing prolonged time-lapse imaging, demonstrated the phenotypic modulation of contractile VSMCs to a migratory phenotype with phagocytic capabilities, suggesting that VSMCs, in addition to their other functional phenotypes, may act as macrophage-like cells in vascular remodeling (Sandison *et al.*, 2016). Phenotypic modulation can be adaptive, e.g. during angiogenesis, pregnancy and injury repair; however, these changes can also be underlying factors in the development of different diseases. Indeed, due to the wide spectrum of VSMC phenotypes with varying functions, phenotypic switching may play important roles in many major diseases in humans, including systemic hypertension, atherosclerosis, restenosis, asthma and cancer (Campbell and Campbell, 1985; Owens *et al.*, 2004; Rzucidlo *et al.*, 2007; Cecchettini *et al.*, 2011; Alexander and Owens, 2012; Shi and Chen, 2014).

2.2.1.2 VSMC-specific markers

VSMCs express several markers indicative of their relative state of differentiation, but no single marker is sufficient to exclusively identify VSMCs to the exclusion of all other cell types. A variety of SMC-selective or-specific proteins have been identified that can be used as markers of the relative state of differentiation of the VSMCs. VSMC contraction depends on the interaction between different contractile proteins, including α-SMA, SM-MHC, h1-calponin and smooth muscle α-tropomyosin. Therefore, these proteins are appropriate markers of the differentiated, contractile phenotype. In addition, some other proteins that are part of the cytoskeleton are believed to be involved in the regulation of contraction; these proteins include e.g. SM22α, h-caldesmon, metavinculin, telokin, smoothelin, and desmin (Rzucidlo *et al.*, 2007; Cecchettini *et al.*, 2011; Alexander and Owens, 2012). Typically, differentiated, contractile VSMCs express a set of these upregulated contractile and cytoskeleton proteins and, after the phenotypic switch,

the expression of these markers is remarkably downregulated in the dedifferentiated, proliferating VSMCs (Salmon *et al.*, 2012; Wang *et al.*, 2015). The most widely used VSMC marker is α-SMA, probably at least in part because of many commercially available high-affinity and highly selective α-SMA antibodies. There are many features that contribute to the usefulness of α-SMA as a differentiation marker: it is the first protein known to be expressed in VSMC differentiation during development, it is highly selective for VSMCs in adult animals under normal conditions, it is required for the high force development properties of fully differentiated VSMCs and it is the single most abundant protein in differentiated VSMCs, making up to 40 % of the total cellular protein (Fatigati and Murphy, 1984; Hungerford and Little, 1999; Owens *et al.*, 2004). However, α-SMA is known to be expressed in a wide variety of non-SMC cell types under certain conditions, and therefore, α-SMA expression alone is not sufficient evidence for the identification of isolated primary VSMCs. With cell lines this is not a problem, because the cell type is known and there are only cells of one kind in the culture. In established cell lines, α-SMA is used more as a differentiation marker, for which it is well suited (Owens *et al.*, 2004). The SM-MHC isoforms have been widely regarded as the most specific differentiation marker of VSMCs, since virtually all other markers have been shown to be expressed in other cell types either during development or in response to pathophysiological stimuli (Miano *et al.*, 1994; Owens *et al.*, 2004). However, more recently, it has been discovered that tropomyosin 1 plays an important role in the regulation VSMC contraction and actin reorganization (Gunning *et al.*, 2008; Marston and El-Mezgueldi, 2008; Baker, 2011), and it has been suggested to be a better phenotypic marker for quiescent VSMCs than the traditional markers α-SMA and SM-MHC (Vrhovski *et al.*, 2005; Wang *et al.*, 2011).

2.2.1.3 Regulation of VSMC phenotypic modulation

Although the role of phenotypic modulation in the development of many vascular diseases is recognized, less is known about the cellular and molecular mechanisms involved in phenotypic modulation. VSMC proliferation and differentiation have been generally considered as opposite processes. However, it has become evident that these processes are in fact distinct but concurrent, and that they may be regulated both independently and simultaneously with a high possibility of crosstalk between their regulatory mechanisms. Today, it is well-known that VSMCs can acquire a broad spectrum of different phenotypes in response to different physiological or pathological stimuli. It is reasonable when considering e.g. a situation where a blood vessel is injured; some VSMCs must be recruited to repair the injury, while at the same time VSMCs must also maintain the contractile function of the blood vessel. Depending on the signals present in their local environment, contractile VSMCs can rapidly change their functional and morphological properties and acquire distinct phenotypes, e.g. proliferative, migratory or secretory. Overall, the control of VSMC differentiation and phenotypic modulation is dependent on the complex interaction of a multitude of local environmental cues, and involve cooperative interaction of many factors and signaling pathways. Possible stimuli triggering phenotypic modulation include mechanical forces (stretch, shear stress, injury), oxidative stress (reactive oxygen species, hypoxia), growth factors, inflammatory mediators, contraction-evoking receptor activation and cell−cell and cell−matrix interactions (Figure 8) (Campbell and Campbell, 1985; Owens, 1995; Owens *et al.*, 2004; Cecchettini *et al.*, 2011; Alexander and Owens, 2012; Lacolley *et al.*, 2012; Shi and Chen, 2014; Zhang *et al.*, 2016b). Mitochondrial

metabolism is an example of a newly identified mechanism that has been demonstrated to be involved in the complex regulation of the VSMC phenotype, especially involving VSMC proliferation (Chiong *et al.*, 2014).

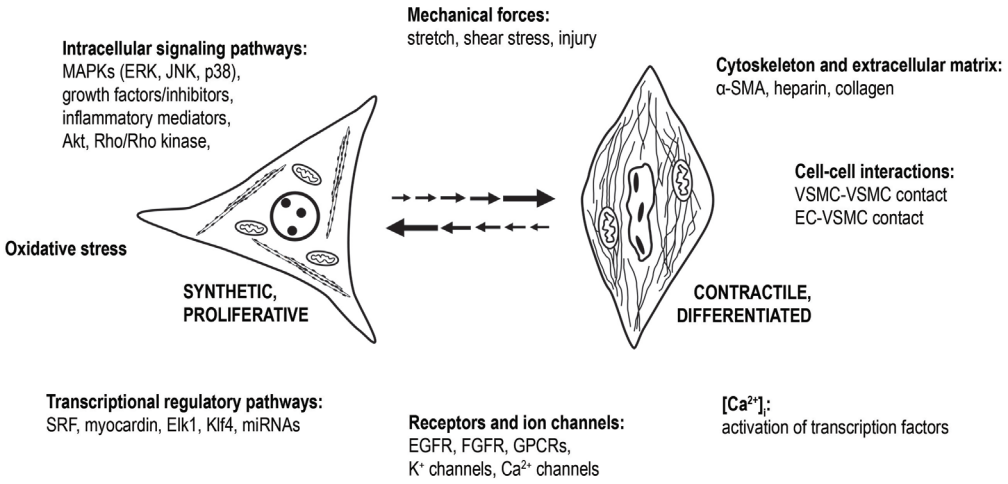

Figure 8. Regulation of vascular smooth muscle cell (VSMC) phenotypic modulation. VSMC proliferation and differentiation are separate but concurrent processes that may be regulated both independently and simultaneously with a high possibility of crosstalk between their regulatory mechanisms. Control of VSMC phenotypic modulation depends on the complex interaction of a multitude of local environmental cues and involves the interaction of many factors and signaling pathways, including mechanical forces, oxidative stress, cell-cell interactions, cell-matrix interactions, intracellular signaling pathways and transcriptional regulatory pathways. VSMCs can acquire a spectrum of phenotypic states ranging from the highly synthetic/proliferative VSMC depicted on the left to the highly contractile, fully differentiated VSMC depicted on the right, depending on the variable expression of SMC-specific differentiation markers. MAPK = mitogen-activated protein kinase; ERK = extracellular signal-regulated kinase; JNK = c-Jun N-terminal kinase; Akt = protein kinase B; SRF = serum response factor; Klf4 = Krüppel-like factor 4; miRNA = microRNA; EGFR = epidermal growth factor receptor; FGFR = fibroblast growth factor receptor, GPCR = G protein-coupled receptor; $[Ca^{2+}]_i$ = intracellular Ca^{2+} concentration; EC = endothelial cell; α-SMA = smooth muscle α-actin [created by author, modified from (Owens *et al*., 2004)].

VSMCs constantly integrate the signals present in their local environment and it is the combination of these signals that determines the patterns of gene expression and cell function. Therefore, regulation of VSMC-specific gene expression cannot depend on any single intracellular factor which would be completely specific for VSMCs but rather depends on combinatorial interactions of several factors that are either ubiquitously expressed (e.g. serum response factor) or selective for VSMCs (e.g. myocardin) (Alexander and Owens, 2012). Both transcriptional regulatory pathways and intracellular signaling pathways are involved in the regulation of phenotypic modulation. There is a growing body of evidence suggesting that a variety of molecules including transcription factors, microRNAs, cytokines and growth factors, membrane receptors and ion channels, components of the cytoskeleton and ECM play important roles in the regulation of VSMC phenotypic modulation. However, it should be noted that many of the studies investigating the mechanisms that regulate phenotypic modulation of VSMCs have been

performed using cultured VSMCs; therefore, their results should be evaluated with appropriate caution, since cultured VSMC systems may not adequately recapitulate all of the regulatory pathways that are critical *in vivo* (Owens *et al.*, 2004). One major limitation of cultured VSMCs is that the cells have already undergone profound phenotypic switching as a result of isolation and culture and the loss of many of the critical environmental cues that control differentiation and maturation of these cells *in vivo* (Alexander and Owens, 2012).

Transcriptional regulatory pathways. Phenotypic modulation is controlled by many transcriptional regulatory pathways, in particular serum response factor (SRF) and its main cofactor, myocardin. SRF binds to a sequence called the CArG box located within the promoters of the majority of VSMC contraction-associated genes and it represents a key transcription factor required for VSMC gene expression, differentiation, cell proliferation and motility (Miano *et al.*, 2007; Lacolley *et al.*, 2012; Zhang *et al.*, 2016b). Myocardin is a potent SRF transcription coactivator that is exclusively expressed in cardiac myocytes and differentiated SMCs. Myocardin potently and selectively induces expression of all CArG-dependent SMC marker genes tested to date, including α-SMA, SM-MHC, SM22α and calponin in cultured VSMCs (Wang *et al.*, 2001; Owens *et al.*, 2004; Alexander and Owens, 2012; Wang *et al.*, 2015). It has been demonstrated that downregulation of myocardin leads to a reduction of SMC marker gene expression by up to 80 % in cultured SMCs (Du *et al.*, 2003; Yoshida *et al.*, 2003; Alexander and Owens, 2012). SRF creates a system together with its coactivators and corepressors, where growth signals may increase or repress VSMC-specific gene expression by promoting or inhibiting, respectively, the binding of myocardin to SRF. Elk-1, a ternary complex factor, and Klf-4, a member of the Krüppel-like family of transcriptional repressors, are important regulators of the SRF-myocardin system, thus playing critical roles in the molecular program regulating the SMC phenotype. Elk-1 is capable of displacing myocardin from SRF, leading to repression of SMC contraction-associated genes, and Klf-4 has been shown attenuate SRF/myocardin binding to CArG boxes of VSMC contraction-associated genes (Alexander and Owens, 2012; Huang and Parmacek, 2012; Zhang *et al.*, 2016b). MicroRNAs (miRNAs) represent a class of endogenous, small (20–25 nucleotides), non-coding RNAs that act as regulators of many cellular events, ensuring that there is a balance between proliferation and differentiation. miRNAs modulate gene expression by inhibiting mRNA translation or by reducing mRNA stability (Bushati and Cohen, 2007; Wang *et al.*, 2015; Zhang *et al.*, 2016b). miRNAs are also necessary for the regulation of VSMC differentiation and phenotypic modulation; miRNAs miR-143/145, miR-21 and miR-1 promote the contractile phenotype, whereas, miR-221, miR-146a, miR-24 and miR-26a are involved in the switch to the synthetic phenotype and cell proliferation after vascular injury (Davis-Dusenbery *et al.*, 2011). Especially miR-143 and miR-145 have been investigated intensively; they have been shown to cooperatively target a network of transcription factors, including myocardin (↑), Klf4 (↓) and Elk-1 (↓), to promote differentiation and repress proliferation of smooth muscle cells (Cordes *et al.*, 2009; Alexander and Owens, 2012; Lacolley *et al.*, 2012; Zhang *et al.*, 2016b). In summary, phenotypic modulation of VSMCs requires a specific combination of transcriptional factors, such as SRF/myocardin, Klf4 and Elk-1, and miRNA expression, mechanisms employed by which are gradually beginning to emerge.

Intracellular signaling pathways. Different intracellular signaling pathways participate in the modulation of the VSMC phenotype. The activity of many transcription factors and cofactors controlling the differentiation/de-differentiation of VSMCs is regulated by a wide range of signaling pathways, including ERK, JNK, p38 mitogen activated protein kinases, Akt, Rho/Rho kinase (ROCK) and calcineurin and calmodulin kinases. The SRF/myocardin system has been shown to be activated by the Rho/ROCK pathway (Lacolley *et al.*, 2012). Two cytokines that have been identified as key mediators of VSMC phenotypic modulation are platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). PDGF is involved in the downregulation of multiple VSMC differentiation markers, such as α-SMA, SM-MHC and calponin, and in the upregulation of pro-migratory genes such as matrix metalloproteinases (MMPs). In contrast, TGF-β promotes VSMC differentiation and maintains the contractile phenotype by upregulating the expression of α-SMA, SM-MHC and calponin (Hao *et al.*, 2003; Owens *et al.*, 2004; Alexander and Owens, 2012; Lacolley *et al.*, 2012; Wang *et al.*, 2015; Zhang *et al.*, 2016b). Results from *in vitro* experiments have indicated that these pathways also interact with each other, and the net response of gene regulation is the result of a complicated interplay between several factors, e.g. upregulation of MPPs in response to PDGF can be suppressed by a threshold level of active TGF-β, thus promoting a contractile phenotype (Risinger *et al.*, 2010). Moreover, PDGF and other cytokines have been shown to affect a wide range of signaling pathways, including pathways involving ERK, p38 MAPK and Akt, which represent potential regulatory mechanisms that may contribute to VSMC phenotypic modulation (Alexander and Owens, 2012). Notch and Wnt signaling also play prominent roles in regulating VSMC differentiation and modulating the phenotypic response following different stimuli through the influence on positive and/or negative transcription factors and co-factors that determine gene expression patterns of VSMCs. For instance, Notch signaling may interact with PDGF and TGF-β signaling to regulate VSMC migration and differentiation, and Wnt inhibition has an inhibitory effect on PDGF-induced VSMC proliferation (Morrow *et al.*, 2008; Tsaousi *et al.*, 2011; Boucher *et al.*, 2012; Lacolley *et al.*, 2012; Wang *et al.*, 2015).

Receptors and ion channels. Different membrane receptors and ion channels are also involved in VSMC phenotypic modulation. For instance, transactivation of the fibroblast growth factor receptor (FGFR) and the epidermal growth factor receptor (EGFR) is required for the induction of ERK, which is a key pathway controlling VSMC cell cycle progression (Lacolley *et al.*, 2012; Zhang *et al.*, 2016b). In addition, decreased ERK1/2-mediated phosphorylation inhibits VSMC contraction during phenotypic modulation (Carrillo-Sepúlveda and Barreto-Chaves, 2010). PDGF has been shown to act synergistically with fibroblast growth factor-2 (FGF-2) in the phenotypic modulation via a multi-protein complex involving growth factor receptors, such as PDGF receptor-β and FGF receptor-1 to downregulate the expression of contractile proteins (α-SMA and SM22) in VSMCs (Zhang *et al.*, 2016b). Many mediators and drugs act through GPCRs to induce different downstream signaling pathways and thereby regulate VSMC differentiation and proliferation. Two major pathways involving the pro-contractile G proteins G_{q11} and $G_{12/13}$ antagonistically regulate VSMC differentiation. $G_{12/13}$ promote VSMC differentiation through a pathway involving RhoA and SRF, whereas G_q/G_{11} promote VSMC proliferation via ERK1/2 and Elk-1, suggesting that the balanced effects of both G protein-mediated signaling pathways govern VSMC regulation (Althoff *et al.*, 2012; Althoff and Offermanns,

2015). Furthermore, changes in the expression levels of different ion channels, such as voltage-dependent K^+ channels and voltage-dependent Ca^{2+} channels, have been shown to be associated with many occlusive vascular diseases involving VSMC dedifferentiation and proliferation (Zhang *et al.*, 2016b).

Intracellular Ca^{2+}. Intracellular Ca^{2+} has a dual role in VSMCs; it is necessary for contraction but it is also a prerequisite for cell cycle progression. Intracellular Ca^{2+} can activate several transcription factors, such as cAMP response element-binding protein (CREB), nuclear factor of activated T lymphocytes (NFAT) and SRF, that selectively regulate the genes that define the VSMC phenotype (Matchkov *et al.*, 2012; Kudryavtseva *et al.*, 2013; Zhang *et al.*, 2016b). CREB regulates gene expression by binding to cAMP response elements in the promoter regions of target genes in a variety of tissues including VSMCs. Genes regulated by CREB in VSMCs include those involved in the regulation of VSMC proliferation and phenotypic modulation, including early response genes (e.g. c-fos and Egr-1) and genes characteristic for both contractile and synthetic phenotypes. The net effect of CREB activation on VSMC phenotype is therefore controversial (Cartin *et al.*, 2000; Kudryavtseva *et al.*, 2013). NFAT activation is generally associated with VSMC phenotypic modulation, proliferation and migration, however, regulation of NFAT activity in VSMCs is very sensitive to dynamic changes in $[Ca^{2+}]_i$ and Ca^{2+} oscillations (Matchkov *et al.*, 2012; Kudryavtseva *et al.*, 2013). Unlike CREB and NFAT, SRF is not directly activated by Ca^{2+}-dependent kinase activity, but it is indirectly regulated by Ca^{2+} influx. Ca^{2+} entry through L-type Ca^{2+} channels, followed by activation of the RhoA/ROCK pathway promotes the binding of SRF to the CArG promoter regions of VSMC-specific contractile protein genes but not early response genes, such as c-fos. The SRF regulatory pathway can therefore lead to expression of genes that maintain the contractile phenotype or those that induce cell proliferation. The Ca^{2+}-dependent RhoA/ROCK pathway plays a crucial role in the differentiation between these two potential outcomes (Wamhoff *et al.*, 2004; Matchkov *et al.*, 2012; Kudryavtseva *et al.*, 2013). In general, Ca^{2+} signaling appears to be tightly associated with gene transcription in a stimulus-specific manner, so that different Ca^{2+} signaling patterns may activate different transcription factors and lead to specific functional outcomes. Activation of the same transcription factor by Ca^{2+} from different sources yields different outcomes; both voltage-dependent and -independent (i.e. store-operated) Ca^{2+} entry can activate CREB, but differentially regulate gene transcription patterns (Pulver-Kaste *et al.*, 2006). It has been suggested that multiple Ca^{2+}-dependent transcription factors can be activated by the same stimulus, whereas the overall $[Ca^{2+}]_i$ will determine the balance between different factors and the final transcriptional outcome. The interplay between different Ca^{2+} sources, signaling pathways, transcription factor recruitment and selective gene expression patterns helps to maintain tight VSMC transcription control under normal physiological conditions, and provides a basis for the adaptive and pathological transition of VSMCs from contractile to noncontractile phenotypes during vascular diseases (Kudryavtseva *et al.*, 2013).

Cytoskeleton and extracellular matrix. Alterations of the cytoskeleton and extracellular matrix (ECM) play important roles in the regulation of VSMC phenotypic modulation. Apart from its important mechanical functions, including contraction, movement, cell growth, maintenance of cell shape and resistance to extracellular forces, the cytoskeleton can function as a molecular framework providing support for the spatial organization of

signaling proteins and miRNAs, and for mediating information through specific receptors from the cell surface to the nucleus (Worth *et al.*, 2001). Consequently, rearrangements of the cytoskeletal network may affect molecular expression and signal transduction, and ultimately alter gene expression and VSMC phenotype. This has been demonstrated with differentiated VSMCs that contain an organized actin cytoskeleton, where α-SMA is significantly downregulated in proliferating VSMCs (Zhang *et al.*, 2016b). In the blood vessel wall, VSMCs are practically embedded in the ECM and are, indeed, highly sensitive to the composition and organization of the ECM. The ECM is consists mostly of collagen isoforms (mostly type I and III), elastin, fibronectin and proteoglycans. In particular, the proteoglycan heparin has proven to be important for the regulation of VSMC phenotype; it is known to promote the contractile phenotype e.g. by increasing α-SMA expression, and to slow down VSMC proliferation (Owens, 1995; Rensen *et al.*, 2007). Collagen provides a good example of the complex relationship between ECM composition and VSMC phenotype. Fibrillar collagen type I promotes the contractile phenotype, whereas monomeric collagen type I induces VSMC proliferation. Furthermore, VSMCs cultured on either monomeric or polymerised collagen exhibit very different gene expression profiles, with many of the differentially expressed genes coding for ECM or cytoskeletal proteins (Ichii *et al.*, 2001; Rensen *et al.*, 2007). While most ECM proteins appear to be required for induction or maintenance of the contractile phenotype, fibronectin has been demonstrated to promote the synthetic phenotype (Rensen *et al.*, 2007).

Physical factors. VSMCs continuously encounter mechanical stress that has a major impact on their phenotype, for example by altering the nature of cell-cell interactions. Flow causes shear stress and pressure causes stretch, both of which induce remodeling of the vessel wall by changing VSMC characteristics. Shear stress effects are mediated by the endothelial layer, which coordinates the response of VSMCs to this type of mechanical stress. This occurs in part through nitric oxide (NO) release, but also through direct cell–cell interactions. Cultured endothelial cells (ECs) have been shown to secrete a variety of growth factors, such as PDGF and FGF2, as well as growth inhibitors, such as heparin and TGF-β. EC-conditioned culture medium or co-culture of VSMCs with confluent ECs have been shown to inhibit VSMCs growth and to some extent, prevent the phenotypic changes that occur in VSMCs when placed in culture (Owens, 1995). On the other hand, it has also been shown that co-culture with ECs induces the modulation of VSMCs towards a synthetic phenotype, as demonstrated by a shift from a spindle-shaped to a rhomboid morphology and reduced α-SMA and SM-MHC expression (Hao *et al.*, 2002). This exemplifies how difficult it is to mimic the normal physical and biochemical interactions between ECs and VSMCs with cell cultures. In contrast to endothelium-modulated shear stress, pressure-induced stretch acts directly on VSMCs. Mechanical forces have been shown to increase the expression of both ECM and contractile proteins in VSMCs, and consequently, cause reorientation of cells, increased protein and DNA synthesis, production of ECM and remodeling of the vessel wall (Owens, 1995; Rensen *et al.*, 2007).

2.2.1.4 Origin of VSMCs

VSMCs have been extensively studied both *in vitro* and *in vivo* for more than 50 years. It is widely accepted that the dedifferentiation of VSMCs from the contractile to the

proliferative/synthetic phenotype plays an important role during vascular remodeling; upon vascular injury, VSMCs in the medial layer may de-differentiate, proliferate and migrate into the neointima (Chamley-Campbell *et al.*, 1979; Owens *et al.*, 2004; Alexander and Owens, 2012). However, in the past ten years, several studies have suggested that also vascular stem cells could participate in vascular remodeling and repair (Sainz *et al.*, 2006; Majesky *et al.*, 2011; Tang *et al.*, 2012; Tang *et al.*, 2013; Wang *et al.*, 2015). There has been a lively debate about whether vascular stem cell activation and differentiation, instead of VSMC dedifferentiation, results in the proliferative and synthetic VSMCs in the vascular wall (see below).

Ten to fifteen years ago, evidence started to accumulate that circulating stem cell populations and progenitor cells could give rise to smooth muscle-like cells in association with vascular injury (Owens *et al.*, 2004; Sinha *et al.*, 2004; Sainz *et al.*, 2006). More recently, multipotent vascular stem cells (MVSCs) have been proposed to contribute to VSMC accumulation and vascular disease progression. MVSCs are resident stem cells located in the medial and adventitial layers of the vascular wall that express stem cell and glia markers (Sox10, Sox17, S100β), can be cloned, have telomerase activity and are capable of differentiating into neural cells and mesenchymal stem cell-like cells that subsequently differentiate into VSMCs (Owens *et al.*, 2004; Gomez and Owens, 2012; Tang *et al.*, 2012; Tang *et al.*, 2013). Tang and colleagues (2012, 2013) performed genetic lineage tracing studies with SM-MHC as a marker, and found that MVSCs and proliferative/synthetic VSMCs did not arise from the dedifferentiation of mature VSMCs. They speculated that MVSCs, instead of dedifferentiated VSMCs, would repopulate the neointima upon vascular injury, become proliferative and differentiate into VSMCs. Moreover, they proposed that MVSCs at different stages of differentiation could also explain the heterogeneity of VSMC phenotypes in culture and *in vivo*. Shortly thereafter, a group of leaders in the field of VSMC biology wrote a commentary strongly criticizing the studies of Tang *et al.* (Nguyen *et al.*, 2013). According to Nguyen *et al.* (2013), prior studies in the field clearly refuted the major conclusions made by Tang *et al.* (2012), and established that mature VSMCs are not terminally differentiated and are capable of transitions in phenotype, including cell proliferation and loss of differentiation markers. Both sides agreed on the fact that the definitive characterization of the origin of VSMC cultures was highly important, and that there was a need for more rigorous and complete VSMC lineage tracing studies *in vivo*. Indeed, a common shortcoming of many previous studies has been that it was assumed that vascular cells in primary culture and in injured blood vessels are mostly derived from VSMCs without sufficient confirmation of the origin of the cells.

Today, this debate is still ongoing, and conflicting points of view exist about whether the VSMCs in the vessel wall are heterogeneous or derived from MVSCs, that have differentiated into specific subpopulations with different functions. There is compelling evidence that MVSCs can be activated upon vascular injury and that the rapid expansion and the aberrant differentiation of MVSCs contribute to neointima formation. However, the mechanisms for stem cell differentiation into VSMCs are not completely defined, but appear to be similar to the mechanisms regulating phenotypic modulation (Wang *et al.*, 2015). Recently, Kennedy *et al.* (2014) investigated VSMCs from three different species (rat, murine and bovine) to establish whether they exhibit neural stem cell characteristics

typical of MVSCs. All VSMCs examined expressed both VSMC differentiation markers (α-SMA, SM-MHC, and calponin) and stem cell markers (Sox10+, Sox17+), and serum deprivation considerably increased VSMC marker expression and decreased stem cell marker expression. Upon adipogenic or osteogenic induction stimulation, VSMCs did not differentiate into adipocytes or osteoblasts, respectively, indicating that VSMCs in culture did not retain the multipotency of MVSCs. However, the ultimate origin of these cells remained uncertain, as they could be reminiscent of VSMCs derived from differentiated VSMCs, MVSCs or both (Kennedy *et al.*, 2014b). In another recent study, the contribution of VSMCs and MVSCs to neointimal thickening upon vascular injury was investigated by means of genetic lineage tracing. This study showed that VSMCs and MVSCs were actually two distinct cell populations, and for the first time, defined two distinct types of neointima. Type-I neointimal cells expressed VSMC markers (α-SMA; SM-MHC, calponin) and may have been derived from medial VSMCs or from other, non-SMCs that differentiated into VSMCs upon injury. In contrast, type-II neointima consisted mostly of other cell types, most of which were SM-MHC-negative. Only 7 % of the cells were identified as MVSCs. Based on these results, it is evident that VSMCs are not the only major cell type in neointima formation, but that neointimal cells rather appear to be a heterogeneous population. Both VSMCs and MVSCs, and even endothelial cells, significantly contribute to the neointima formation (Yuan *et al.*, 2017).

2.2.2 Regulation of vascular smooth muscle contraction

2.2.2.1 Mechanism of VSMC contraction

VSM contraction plays an important role in the regulation of blood pressure and organ blood flow. Different stimuli, including drugs, circulating neurotransmitters, hormones, endothelium-derived factors and even shear stress, are involved in the modulation of vascular smooth muscle tone, and consequently blood vessel diameter. VSMCs contain thin and thick filaments of myosin and actin, forming organized structures that enable the maintenance of tonic contractions and reduction of lumen diameter. Regardless of the stimulus, VSMCs produce force or contraction through cross-bridge cycling between actin and myosin filaments. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activation are the main determinants of VSMC contraction. In VSMCs, an increase in $[Ca^{2+}]_i$ due to its release from intracellular stores and entry from the extracellular space through plasma membrane Ca^{2+} channels initiates the process leading to contraction. The free Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complexes activate MLCK which is capable of phosphorylating the 20 kDa regulatory subunits of myosin light chains (MLC_{20}). The phosphorylation of MLC_{20} together with ATP hydrolysis allows cross-bridge formation with actin filaments, and consequently smooth muscle contraction. VSMC relaxation occurs when there is reduced phosphorylation of MLC_{20}, which can result from decreased $[Ca^{2+}]_i$ due to Ca^{2+} uptake into the sarcoplasmic reticulum (SR) and Ca^{2+} extrusion from the cell, inhibition of MLCK e.g. by an increased intracellular concentration of cAMP and subsequent activation of PKA, or MLCP-mediated MLC_{20} dephosphorylation (Somlyo and Somlyo, 1968; Kamm and Stull, 1985; Wynne *et al.*, 2009; Khalil, 2010; Goulopoulou and Webb, 2014).

2.2.2.2 Signaling pathways of VSMC contraction

The intracellular signaling pathways involved in the regulation of VSMC contraction vary depending on the GPCR that is activated (Figure 9). In the vasculature, noradrenaline released from sympathetic nerve endings activates postjunctional α_1-ARs in large arteries and α_2-ARs in small arteries and arterioles, leading to vasoconstriction. Activation of G_q proteins, e.g. by α_1-AR stimulation, activates PLCβ, which stimulates the breakdown of the plasma membrane component PIP_2 into IP_3 and DAG. IP_3 is a water-soluble compound that diffuses in the cytosol and activates specific receptors on the SR, leading to Ca^{2+} release from intracellular stores and consequently, VSM contraction. DAG, on the other hand, is a lipophilic compound that remains in the plasma membrane where it binds to and activates PKC, which further promotes VSM contraction. Indeed, receptor-dependent activation of PLC is one of the major signaling routes leading to increased $[Ca^{2+}]_i$ and vasoconstriction. G_q protein activation also activates ROCK, which in turn inhibits MLCP, leading to enhanced VSMC contraction.

Activation of G_i proteins, e.g. by α_2-AR stimulation, results in inhibition of AC activity with a resultant reduction in intracellular cAMP levels and reduced activation of PKA, which subsequently increases the activity of MLCK (and reduces the activity of MLCP), and hence promotes VSMC contraction. Moreover, α_2-ARs also indirectly activate voltage-gated Ca^{2+} channels, promoting the influx of extracellular Ca^{2+} ions, and thereby increase $[Ca^{2+}]_i$ and enhance VSMC contraction. Therefore, the effects of activated α_1- and α_2-ARs in VSMCs are similar but are elicited via different signaling pathways.

In contrast, activation of G_s proteins, e.g. by β_2-AR stimulation, activates AC resulting in increased cAMP concentrations and a consequent increase in PKA activity. Both cAMP and PKA interfere with VSMC contraction through inhibition of MLCK. In addition, activated PKA can inhibit the influx of extracellular Ca^{2+} by activating K^+ channels (K_{ATP}), resulting in hyperpolarization. The net effect of G_s activation is VSMC relaxation (Ogut and Brozovich, 2003; Khalil, 2010; Goulopoulou and Webb, 2014; Klabunde, 2014; Brozovich *et al.*, 2016; King, 2017). However, it should be kept in mind that the mechanisms regulating VSMC contraction are very complex, and may be influenced by receptors and ion channels on both VSMCs and endothelial cells, mechanical sensors that respond to changes in blood flow or intravascular pressure, and by signaling molecules such as NO that enter VSMCs to directly activate second messenger systems that induce changes in vascular tone. The signaling routes presented in Figure 9 should therefore be considered as a simplified representation of the actual complex network regulating VSMC contraction. Although each of these receptor-mediated pathways leading to VSMC contraction are different, they converge at the point of increased $[Ca^{2+}]_i$.

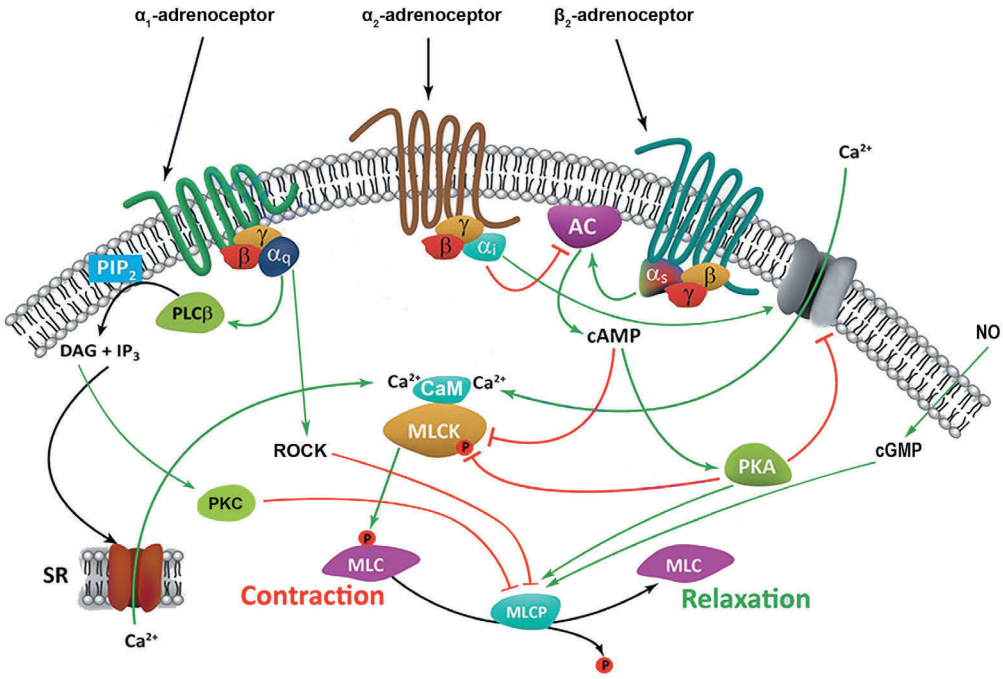

Figure 9. Adrenoceptor-mediated regulation of vascular smooth muscle contraction and relaxation. Activation of α_1-ARs leads to increased PLCβ activity resulting in release of Ca^{2+} stored in the sarcoplasmic reticulum (SR) and activation of PKC. α_2-AR activation results in inhibition of adenylyl cyclase (AC) activity with a resultant decrease in cAMP levels and reduced activation of PKA. Activation of β_2-ARs leads to increased AC activity, increased cAMP levels, and a resultant increase in the activity of PKA. α_1- and α_2-AR activation leads to VSMC contraction, whereas β_2-AR activation results in VSMC relaxation. PLCβ = phospholipase C, β-isoform; PKA = protein kinase A; PKC = protein kinase C; MLCK = myosin light chain kinase; CaM = calmodulin; MLCP = myosin light chain phosphatase; DAG = diacylglycerol; IP_3 = inositol-1,4,5-trisphosphate; PIP_2 = phosphatidylinositol-4,5-bisphosphate; ROCK = Rho kinase; NO = nitric oxide. Green arrows represent activation processes and red T-shaped lines represent inhibitory processes [modified from (King, 2017)].

2.2.2.3 Regulation of intracellular calcium levels

Ca^{2+} is an important 2nd messenger and a major determinant of VSMC functions. *In vivo*, the average intracellular Ca^{2+} concentration of VSMCs (0.1–1 µM) is several orders of magnitude lower than that in the extracellular space (≈1.6–2 mM) or within the SR (Goulopoulou and Webb, 2014; Klabunde, 2016). VSMCs exercise tight control of $[Ca^{2+}]_i$ by expressing a wide repertoire of Ca^{2+} channels and transporters; the presence of several pathways for Ca^{2+} influx and efflux provides many possibilities for controlling $[Ca^{2+}]_i$ in a temporal and spatial manner. Basically, $[Ca^{2+}]_i$ depends on Ca^{2+} that enters the cell, Ca^{2+} that is released from intracellular storage sites (e.g. SR) and removal of Ca^{2+} either back into storage sites or out of the cell. The balance between these mechanisms maintains resting $[Ca^{2+}]_i$ constant, and upon stimulation, this balance is perturbed leading to an increase in $[Ca^{2+}]_i$ and VSMC contraction (Wynne *et al.*, 2009; Khalil, 2010; Kudryavtseva *et al.*, 2013; Brozovich *et al.*, 2016). Increases in $[Ca^{2+}]_i$ result either from release of Ca^{2+} from intracellular stores or from increased Ca^{2+} influx into the VSMCs. With respect to the different Ca^{2+} entry channels, the voltage-operated Ca^{2+} (VOC), receptor-operated Ca^{2+}

(ROC) and store-operated Ca^{2+} (SOC) channels modulate most Ca^{2+} mobilization within the cell.

VOC channels represent a major route for Ca^{2+} entry in VSMCs. Membrane potential regulates VOC channel function by closing them upon hyperpolarization and opening them upon depolarization, with the latter leading to vasoconstriction. There are several types of VOC channels (L-, T-, P-, N- and R-types), but Ca^{2+} influx into VSMCs is mainly mediated by the opening of L-type VOC channels. L-type VOC channels are highly expressed in VSMCs and therefore they have the largest influence on the overall $[Ca^{2+}]_i$: their activity largely determines the contractile state of VSMCs. VOC channels can also depolarize in response to membrane stretch, indicating that these channels presumably are important for the myogenic response and maintenance of vascular tone (Jackson, 2000; Wynne *et al.*, 2009; Khalil, 2010; Goulopoulou and Webb, 2014; Brozovich *et al.*, 2016).

ROC channels are loosely defined as Ca^{2+} channels that are neither voltage-operated nor store-operated and that require binding of (physiological) agonists to their membrane receptors for activation (McFadzean and Gibson, 2002; Miwa *et al.*, 2005; Wynne *et al.*, 2009; Khalil, 2010; Fernandez *et al.*, 2012). ROC channels are activated via ligand-mediated activation of a range of GPCRs and receptor tyrosine kinases. Receptor-mediated activation of PLC leads to the generation of IP_3 and DAG; while IP_3 is capable of releasing Ca^{2+} from intracellular stores (ER or SR), DAG remains in the plasma membrane and then activates a select group of plasma membrane-localized Ca^{2+} channels resulting in Ca^{2+} influx into the cell (Fernandez *et al.*, 2012). Members of the transient receptor membrane potential (TRP) channel family, such as TRPC3, TRPC6 and TRPC7, have been shown to be components of ROC channels (Goulopoulou and Webb, 2014).

SOC channels represent yet another mechanism of excitation–contraction coupling in VSMCs. Release of Ca^{2+} from intracellular stores is typically followed by sustained Ca^{2+} influx from the extracellular space and, therefore, it has been suggested that depleted Ca^{2+} stores in the SR act as a capacitor for stimulated maintained Ca^{2+} influx from the extracellular space. This process, earlier defined as "capacitative Ca^{2+} entry", is now known as store-operated Ca^{2+} entry (SOCE) (Parekh and Putney, 2005; Wynne *et al.*, 2009; Goulopoulou and Webb, 2014). However, it should be noted that this does not refer to any single mechanism of Ca^{2+} entry, or to any particular Ca^{2+} channel. Cellular mechanisms by which SOC channels are activated have not been fully established, but a membrane-spanning protein called stromal-interacting molecule 1 (STIM1) appears to play a crucial role in the activation of SOC channels in VSMCs. Although STIM1 acts as the sensor of ER/SR Ca^{2+} levels, and STIM1 oligomerization is crucial for activation of SOCE, it is ultimately the interaction between STIM1 and plasma membrane proteins that is required for facilitating Ca^{2+} entry into the cell. Two types of such plasma membrane proteins have been described: the SOC pore-forming subunits Orai1, a Ca^{2+} release-activated Ca^{2+} modulator, and TRPCs (Jackson, 2000; McFadzean and Gibson, 2002; Parekh and Putney, 2005; Leung *et al.*, 2008; Wynne *et al.*, 2009; Khalil, 2010; Collins *et al.*, 2013; Goulopoulou and Webb, 2014).

The SMC plasma membrane and intracellular organelles also have roles in the maintenance of Ca^{2+} homeostasis. Plasma membrane Ca^{2+}-ATPase, an ATP-dependent Ca^{2+} extrusion pump, plays a major role in maintaining $[Ca^{2+}]_i$ at basal levels; inhibition of this pump leads to accumulation of Ca^{2+} inside the cell. The Na^+–Ca^{2+} exchanger provides

an alternative plasma membrane mechanism through which excess intracellular Ca^{2+} is extruded (Khalil, 2010). In addition to these plasma membrane mechanisms, the SR and mitochondria participate in the regulation of the cytosolic Ca^{2+} concentration. These intracellular organelles have a pump−leak system that involves active uptake of Ca^{2+} from the cytosol and passive leak of Ca^{2+} back into the cytosol. The SR can accumulate Ca^{2+} from solutions containing micromolar concentrations of Ca^{2+} (≈ 1 μM), whereas the threshold for mitochondrial Ca^{2+} uptake is 10−17 μM. Hence, the SR appears to be the predominant Ca^{2+} storage site under physiological conditions, and mitochondria take up Ca^{2+} only when the cytosolic [Ca^{2+}] is unusually high, exceeding 5 μM (Yamamoto and van Breemen, 1986; Khalil, 2010).

2.2.2.4 Contractile properties of VSMCs

The contractile properties of VSMCs are very different from cardiac and skeletal myocytes. VSMCs undergo both fast, phasic contractions and slow, sustained, tonic contractions, whereas cardiac and skeletal muscle contractions are rapid and of relatively short duration. In VSMCs, actin and myosin are not arranged into distinct bands as they are in cardiac myocytes, instead they are highly organized in a manner that is well-suited for their role in maintaining tonic contractions and regulating lumen diameter. Under physiological circumstances, agonist stimulation of VSMCs results in an initial phasic contraction followed by a tonic contraction. The initial agonist-induced contractile response is believed to be due to Ca^{2+} release from intracellular stores, mainly the SR. The ability of VSMCs to maintain a tonic contraction for extended periods of time with minimal energy expense is unique; however, the related regulatory mechanisms are not fully understood. Ca^{2+}-dependent MLC_{20} phosphorylation plays a major role in the sustained agonist-induced contraction of VSMCs, but it is clear that also other mechanisms must be involved (Ogut and Brozovich, 2003; Khalil, 2010). Studies with fluorescent Ca^{2+} indicators have demonstrated that the degree of MLC_{20} phosphorylation and force of contraction at a given $[Ca^{2+}]_i$ level can vary depending on the type of stimulus; agonist-induced force is higher than depolarization-induced force at similar, or even lower, $[Ca^{2+}]_i$ - a phenomenon called Ca^{2+} sensitization (Somlyo and Somlyo, 2003). Different signaling pathways contribute to Ca^{2+} sensitization in VSMCs. Activation of PKC may increase the myofilament force sensitivity to $[Ca^{2+}]_i$ and MLC_{20} phosphorylation, and thereby maintain VSMC contraction with smaller increases in $[Ca^{2+}]_i$. Furthermore, PKC translocation to the cell surface may initiate a cascade of protein kinases, such as MAPKs, that eventually interact with the contractile myofilaments and cause VSMC contraction (Khalil, 2010). CPI-17 (C-kinase potentiated protein phosphatase-1 inhibitor, 17 kDa) is another potential mediator of Ca^{2+} sensitization. Several kinases, including PKC, can phosphorylate CPI-17, which enhances its potency for inhibiting MLCP, and thereby promotes VSMC contraction (Somlyo and Somlyo, 2003). The Rho/ROCK pathway plays a crucial role in Ca^{2+} sensitization of VSMC contraction by modulating the level of MLC_{20} phosphorylation, mainly through inhibition of MLCP (Fukata *et al.*, 2001; Somlyo and Somlyo, 2003; Khalil, 2010). These Ca^{2+}-dependent and Ca^{2+}-sensitization pathways work synergistically during the development and maintenance of agonist-induced VSMC contraction.

Another specific feature of VSMC contraction is diphosphorylation of MLC_{20}. VSMC contraction is activated primarily by phosphorylation at Ser19 of the regulatory MLC_{20}

subunits ($pMLC_{20}$), but under certain conditions $pMLC_{20}$ can be further phosphorylated at Thr18 ($ppMLC_{20}$). MLC_{20} diphosphorylation has been detected in mammalian VSMCs in response to specific contractile stimuli and in pathological conditions associated with hypercontractility, such as vasospasms and hypertension (Shimokawa *et al.*, 1999; Walsh, 2011; Takeya *et al.*, 2014). MLC_{20} diphosphorylation does not increase the contractile force but it has been shown to decelerate the relaxation of arterial smooth muscle (Sutherland and Walsh, 2012). MLC_{20} diphosphorylation has also been observed frequently in cultured cells where it activates force generation (Walsh, 2011). The possibility of MLC_{20} diphosphorylation was originally demonstrated *in vitro* when it was shown that MLCK was capable of phosphorylating the neighboring Thr18 in addition to Ser19, but this occurred only at very high (unphysiological) concentrations of MLCK (Ikebe and Hartshorne, 1985; Ikebe *et al.*, 1986). Thus, it is obvious that MLCK does not phosphorylate Thr18 of MLC_{20} in intact VSMCs. Moreover, MLC_{20} diphosphorylation and contraction do not require Ca^{2+}, suggesting that other protein kinases must mediate these events, because MLCK activity is absolutely dependent on Ca^{2+} and calmodulin (Walsh, 2011; Takeya *et al.*, 2014). Indeed, several other protein kinases have been demonstrated to diphosphorylate MLC_{20} at Thr18 and Ser19 in a Ca^{2+}-independent manner. Evidence from *in vitro* studies points to integrin-linked kinase (ILK) and zipper-interacting protein kinase (ZIPK) as the primary candidates, but other kinases may also be involved (Deng *et al.*, 2001; Niiro and Ikebe, 2001; Wilson *et al.*, 2005; Moffat *et al.*, 2011; Walsh, 2011; Takeya *et al.*, 2014). ILK- and/or ZIPK-mediated MLC_{20} diphosphorylation has been shown to occur in concert with MLCP inhibition (Sutherland and Walsh, 2012). It remains to be elucidated if these kinases are responsible for MLC_{20} diphosphorylation also *in vivo*.

2.2.3 VSMC models

The history of SMC culture dates back to the beginning of the 20th century. The first reports of SMCs in culture were published already in 1913−1914, and the first papers describing SMC proliferation originate from the late 1960s−early 1970s and factors affecting SMC development and differentiation were reported in the 1970s (Chamley-Campbell *et al.*, 1979). VSMC models can be divided into immortalized cell lines and primary cell cultures, and the introduction of these cell models has remarkably expanded our understanding of the structural and functional biologic processes of vascular smooth muscle. In particular, VSMC differentiation has been a widely studied topic that has benefited greatly from these cell models. VSMCs exhibit plasticity in their phenotype by phenotypic switching or modulation, as described earlier. Phenotypic modulation is associated with many pathological conditions, such as atherosclerosis and systemic hypertension, and therefore, VSMCs have been of great interest from both basic and biomedical points of view (Owens, 1995). Rapid dedifferentiation of primary VSMCs in culture has proved to be a hindrance in many studies; therefore, these studies have often been carried out with primary cells or very early subcultures of VSMCs. Consequently, a number of VSMC lines have been developed in order to generate cell models with a long lifespan and a more predictable phenotype than primary VSMC cultures (Firulli *et al.*, 1998). However, it has to be kept in mind that no cell line can authentically reproduce the complex *in vivo* pattern of cellular regulation during development and differentiation and that cell lines may not be able to retain critical properties of the differentiated contractile VSMCs.

2.2.3.1 VSMC lines

The first reports of smooth muscle cell lines of infinite lifespans were published in the 1970s (Chamley-Campbell *et al.*, 1979). Kimes and Brandt (1976) described the generation of three clonal VSMC lines from the thoracic aorta of 14−17-day-old embryonic rats, namely A7r5, A10 and A9. They described all three cell lines as tetraploid (having four sets of chromosomes) and, while proliferating, oblong, flat or ribbon-like in appearance. Upon growth cessation, the cell lines assumed different morphologies, A7r5 cells becoming spindle-shaped and lined-up, A9 cells remaining mostly flat and oblong and A10 cells exhibiting both flat and narrow, spindle-shaped structures (Kimes and Brandt, 1976). However, it took 20 years until the cell-specific characteristics of different VSMC lines were first studied in depth. A10 cells were identified to express the smooth muscle-specific markers α-SMA, SM22, calponin and vimentin, characteristic of *in vivo* rat VSMCs, but they expressed non-muscle myosin rather than SM-MHC. Therefore, A10 cells appeared to be non-differentiated VSMCs differing from neonatal cells but bearing a significant resemblance to neointimal cells (Rao *et al.*, 1997). Firulli and colleagues (1998) compared the cell-specific marker profiles, transfectability, promoter activity and growth characteristics of four rat VSMC lines: A7r5, adult and pup aortic cells and PAC1 (Firulli *et al.*, 1998). They found that despite alterations in chromosomal number and structure, A7r5, adult aortic and PAC1 cells expressed all SMC markers studied, α-SMA, calponin, SM22, tropoelastin, and to a lesser extent, SM-MHC (expression of this protein is especially sensitive to *in vitro* culture conditions). These cell lines also showed expression of differentiation genes during active growth, indicating that growth and differentiation are not necessarily mutually exclusive events in cultured VSMCs. The A7r5 cell line was found to differ from the other cell lines in terms of growth rate and transfectability; it grew much more slowly but appeared to be most efficiently transfected compared with the other three cell lines. In 2004, a novel VSMC line, U8A4, derived from rabbit primary aortic smooth muscle cells was described by Pasquet and colleagues. The U8A4 cell line was reported to exhibit a conserved smooth muscle phenotype through multiple passages, and to be well-suited for smooth muscle differentiation and gene expression studies employing recombinant DNA methods. U8A4 cells expressed SMC-specific markers (SM-MHC, SM22, α-SMA, calponin and tropomyosin) to a level comparable with other widely used VSMC lines, like A7r5 and PAC1. Moreover, these cells were able to grow under serum-free conditions, which is in contrast to other known VSMC lines, thus providing interesting perspectives in terms of growth control analysis. The U8A4 cell line has been proven to be stable over time; even after growing them for several years, the cells did not show any noticeable differences in their properties upon differentiation (Pasquet *et al.*, 2004).

It has been recently observed that multipotent vascular stem cells (MVSCs) reside within the blood vessel wall, and it has been suggested that traditionally defined proliferative VSMCs, such as A7r5 and A10, could be derived from the differentiation of resident stem cells in culture rather than the dedifferentiation of mature VSMCs (Tang *et al.*, 2012; Tang *et al.*, 2013). Therefore, Kennedy and colleagues (2014) examined the identity and origin of A7r5 and A10 cell lines by determining their differentiation state, stem cell marker expression and multipotency potential *in vitro*. Both VSMC lines expressed the smooth muscle markers α-SMA, SM-MHC and calponin, whereas, MVSCs isolated from rat aortic

explants were negative for SM-MHC but positive for several neural stem cell markers such as Sox10+ and Sox17+. A similar MVSC marker profile was also detected in both A7r5 and A10 cells, and serum deprivation significantly increased the expression of both SMC and stem cell markers. Based on the expression of smooth muscle markers and neural stem cell markers in A7r5 and A10 cells, Kennedy and colleagues concluded that these cell lines could represent MVSC-derived VSMCs, dedifferentiated VSMCs or a combination of both (Kennedy *et al.*, 2014a).

2.2.3.2 Primary VSMCs

Primary VSMCs are considered to more authentically represent the physiological phenotype of differentiated cells, and are therefore valued as important research tools in the investigation of VSMC function and regulation in normal and pathological conditions (Proudfoot and Shanahan, 2012). Common sources of primary VSMCs include rat mesenteric arteries (Gunther *et al.*, 1982), rat and bovine thoracic aorta (Bellas *et al.*, 1995; Szöcs *et al.*, 2007), and human saphenous vein (Bond *et al.*, 2001), coronary artery (Kiyan *et al.*, 2007) and umbilical cord arteries and veins (Okker-Reitsma *et al.*, 1985; Abramowitz *et al.*, 2003). Two techniques are routinely used for VSMC isolation from the medial layer of the blood vessel wall i.e. the enzymatic dispersion method and the explant method [originally described by (Ross, 1971; Chamley *et al.*, 1977)]. The enzymatic dispersion method is relatively fast but it requires surgical dissection of the medial layer and large amounts of proteolytic enzymes, whereas in the explant method, VSMCs are allowed to migrate from the explanted pieces of tissue, making it a simple and cost-effective, but time-consuming, method. With either method, the cells undergo a phenotypic change, and de-differentiate and proliferate to become synthetic cells (Campbell and Campbell, 1993; Xu *et al.*, 2009; Proudfoot and Shanahan, 2012). Interestingly, it has been observed that depending on the isolation method used, the primary VSMCs in culture differentially express SMC-specific and stem cell-specific markers; enzymatically dispersed VSMCs expressed the SMC-specific marker SM-MHC and only very little stem cell markers, whereas explant-derived VSMCs were negative for SM-MHC and positive for several stem cell markers (Tang *et al.*, 2012; Tang *et al.*, 2013; Kennedy *et al.*, 2014b). Although primary VSMCs isolated from different species are widely used, human VSMCs should be preferred when studying human diseases, as differences in gene expression between human and rodent VSMCs have been demonstrated (Shanahan *et al.*, 1993; Newman *et al.*, 1995; Proudfoot and Shanahan, 2012). Human blood vessels for the isolation of VSMCs are obtained from organ donors and after surgical procedures, and the choice of blood vessel used typically depends on local availability. Therefore, a battery of smooth muscle markers must be used, together with knowledge of the origin of the isolated cells, in order to identify the cells as VSMCs. VSMCs themselves may express different phenotypes in culture and also other cell types, such as myofibroblasts and mesenchymal cells, may express different smooth muscle contractile proteins. Therefore, at least α-SMA and one other smooth muscle marker (SM-MHC, calponin, SM22, desmin, h-caldesmon, metavinculin, smoothelin) should be positive to confirm that the cells in culture actually are VSMCs (Proudfoot and Shanahan, 2012).

Freshly isolated VSMCs in primary culture represent the contractile, non-proliferative phenotype characterized by abundant actin and myosin filaments. After 5 to 8 days

in culture, VSMCs typically undergo spontaneous modulation of the phenotype into a synthetic, proliferative state, characterized by a cytoplasm filled with SR and Golgi complexes. However, these phenotypes represent only the extremes of a series of phenotypes (Chamley-Campbell *et al.*, 1981; Proudfoot and Shanahan, 2012). VSMCs have heterogeneous features in different situations *in vivo*, e.g. VSMCs from animals of different age or from normal vs injured blood vessels may differ in their growth properties and phenotype, and these features can be maintained, at least in part, in culture. VSMCs obtained from different blood vessels may also exhibit heterogeneous characteristics *in vitro*, such as different growth patterns and rates, cell shapes and sizes, and combinations of expressed proteins, possibly reflecting the different embryonic origins of the cells. Human VSMCs isolated from young arteries have been demonstrated to exhibit higher proliferative rates than VSMCs from adult arteries (Proudfoot and Shanahan, 2012). In addition, VSMC populations isolated from the aorta of newborn, young adult or old rats exhibited differing proliferation rates and cytoskeletal features (Bochaton-Piallat *et al.*, 1993). Primary VSMCs isolated from balloon-induced intimal thickenings exhibited differential contractilities at 15 days vs 60 days after the injury. After 15 days, the isolated VSMCs showed decreased contractility compared with control VSMCs, but after 60 days these cells had returned to a phenotype closely resembling the control VSMCs (Orlandi *et al.*, 1994). Primary VSMC morphology varies to some extent with the tissue of origin, age and species of donor animal and even the length of time that the cell has been in culture. Generally freshly isolated VSMCs in primary culture are ribbon- or spindle-shaped cells that form a classic hill-and-valley pattern at confluence. Other morphological types of primary VSMCs in culture include large, rounded senescent cells; small, fast-growing cells; and cells that are contact-inhibited with a cobblestone-like appearance at confluency (Chamley-Campbell *et al.*, 1979; Proudfoot and Shanahan, 2012). Different phenotypes of VSMCs may be present in the blood vessel wall, and some of these different morphological types can also coexist in cultures obtained from the same tissue explant. However, many of these morphologies are not stable over time in culture. For example, from the coronary artery of young pigs, two distinct VSMC populations have been isolated: spindle-shaped cells exhibit a classic hill-and-valley growth pattern and lower proliferation rates, whereas rhomboid cells grow as mono- or multi-layers and display increased proliferation rates. Interestingly, spindle-shaped VSMCs can be converted into rhomboid VSMCs by growth factor treatment. Fast-growing rhomboid VSMCs were identified as the major cell population isolated from stent-induced restenosis arterial tissue (Zhang *et al.*, 2016c).

Different factors, such as seeding density, number of population doublings, serum and insulin may affect the phenotype of primary VSMCs in culture. The phenotypic change of the VSMCs can be either reversible or irreversible, depending on the initial seeding density and the time required to achieve confluence. If VSMCs are seeded at a sufficiently high density (>10^6 cells/ml), they form a confluent monolayer during the first day in culture and remain in the contractile state. If the cells are seeded at a lower density, they spontaneously undergo a change in phenotype over the first five days; they lose their contractility and start to proliferate. If it does not take more than five population doublings to reach confluence, the cells revert back to the contractile phenotype within a few days. However, if the cells are seeded too sparsely and more than five population doublings are required to reach confluence, the cells will permanently remain in the synthetic state

(Campbell and Campbell, 1985; Campbell and Campbell, 1993). Growth media used for primary VSMC culture are typically supplemented with serum and insulin. Indeed, it has long been known that serum and insulin promote the proliferation of primary VSMCs. The proliferative responses of VSMCs to both serum and insulin are concentration-dependent; however, insulin acts only as a cofactor for growth since it alone cannot replace serum (Chamley-Campbell *et al.*, 1979).

3 AIMS OF THE STUDY

It is well known that all three α_2-AR subtypes participate in the normal regulation of vascular tone and blood pressure. The increased resistance of flow occurring in hypertension can be caused by vasoconstriction, or alternatively it may involve a structural remodelling of the blood vessels. Vascular α_2-ARs may regulate both VSMC contraction and remodelling of the vascular wall. Studies on knockout mice lacking α_{2A}, α_{2B}- or α_{2C}-ARs have suggested that α_{2B}-ARs on VSMCs represent the receptor subtype that is mainly responsible for the vasoconstrictor response, whereas central α_{2A}-ARs mediate the hypotensive effects of clonidine-like drugs. The α_2-AR subtypes involved in vascular remodelling have not been identified. Current clinically available α_2-AR agonists activate all α_2-AR subtypes and consequently, antihypertensive therapy mediated by central α_{2A}-ARs is partially counteracted by vasoconstriction mediated by vascular α_{2B}-ARs. Therefore, considerable research efforts have been extended to develop α_2-AR subtype-selective drugs, in order to avoid unwanted effects. However, the development of such drugs must be based on an in-depth understanding of the functional roles of the individual α_2-AR subtypes. At present, details of α_2-AR regulation and functions at the cellular level have mainly originated from studies performed with fibroblast cell lines. However, α_2-AR regulation and functions may significantly depend on the host cell type. To date, rather few studies on α_2-ARs have been performed with cultured VSMCs. The primary aim of this thesis was to investigate the functions of α_{2B}-ARs in a VSMC model, and more specifically, to gain a better understanding of the possible role of α_{2B}-ARs in the regulation of VSMC contraction and proliferation.

The specific aims of the studies presented in this thesis were:

1. To develop and characterize a VSMC line stably expressing human α_{2B}-ARs in order to investigate α_{2B}-AR functions in a cellular environment that would be relevant for cardiovascular diseases. The generated α_{2B}-AR-expressing VSMC line was used in Studies II and III to further characterize the effects of α_{2B}-AR activation on VSMC contraction and proliferation.

2. To devise a functional assay suitable for quantitative monitoring of VSMC contraction in order to investigate the role of α_{2B}-ARs in the regulation of VSMC contraction, and to identify possible signal transduction mechanisms related to the α_{2B}-AR-evoked contraction response in VSMCs.

3. To investigate the role of α_{2B}-ARs in the regulation of VSMC proliferation and to explore pathways of regulation of gene expression and intracellular signaling related to the α_{2B}-AR-evoked proliferation of VSMCs.

4 MATERIALS AND METHODS

4.1 Cell culture

4.1.1 A7r5 cell line

All studies (I, II and III) utilized the A7r5 rat aortic smooth muscle cell line obtained from the American Type Culture Collection (Manassas, VA, USA). A7r5 cells, referred to as wild-type (wt) cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Corporation, St. Louis, MO, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (PAA laboratories GmbH, Pasching, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin, referred to as growth medium. The transfected A7r5-α_{2B} cell line stably expressing the human α_{2B}-AR was cultured in growth medium supplemented with Geneticin® (400 µg/ml, Sigma-Aldrich). Both wt and transfected A7r5 cells were grown to approximately 90 % confluence in 75 cm^2 culture flasks. Cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. The medium was changed every 3 to 4 days, and the cells were passaged approximately once a week by dissociation in a phosphate-buffered saline (PBS) solution containing 0.025 % trypsin and 0.1 % EDTA.

4.1.2 Generation of an A7r5 cell line expressing the human α_{2B}-adrenoceptor subtype

In Study I, the cDNA encoding the human α_{2B}-AR with an N-terminal triple hemagglutinin (3·HA) tag was purchased from the UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO, USA). The cDNA was cloned into a pcDNA3.1+ expression vector (Invitrogen, Carlsbad, CA, USA). A7r5 cells were transfected using nucleofection, an electroporation-based technique, which delivers DNA, in addition to the cytoplasm, directly into the nucleus (Johnson *et al.*, 2005). Transfected cells were cultured and expanded in antibiotic selection for 8–12 weeks (passages 1 and 2: 800 µg/ml Geneticin, passage 3 and thereafter: 400 µg/ml Geneticin), and the resulting heterogeneous cell populations were screened for α_{2B}-AR expression with preliminary radioligand binding experiments (only 3 concentration points). One of the resulting cell lines, named A7r5-α_{2B}, expressing an α_{2B}-AR density of approximately 1.5 pmol/mg membrane protein, was chosen for further studies. Non-transfected (wt) A7r5 cells did not express endogenous α_2-ARs, as evidenced by non-detectable radioligand binding.

4.2 Receptor density determination

In Study I and occasionally also later, to confirm the stability of receptor expression levels, receptor densities were determined with saturation binding experiments as described previously (Halme *et al.*, 1995), using the α_2-AR antagonist radioligand [^{3}H]RS-79948-197 ([ethyl-^{3}H]RS-79948-197, 82 Ci/mmol; Amersham Biosciences UK, Buckinghamshire, UK). A7r5-α_{2B} cell homogenates or isolated cell membranes (5 µg protein/tube) were incubated in 50 mM K^+-phosphate buffer with 0.03–4.0 nM [^{3}H]RS-79948-197 for 30 min at 25 °C. Non-specific binding was determined by adding an excess of unlabeled phentolamine

(10 µM; Sigma-Aldrich). Reactions were terminated by dilution with ice-cold buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4) , and bound radioactivity was separated by filtration through Whatman glass fibre (GF/B) filters (Whatman International, Maidstone, UK) with a cell harvester (Brandel Cell Harvester M48R, Gaithersburg, MD, USA), followed by two washes with ice-cold buffer. Filters were transferred into scintillation tubes, OptiPhase 'HiSafe'3 liquid scintillation cocktail (PerkinElmer, Boston, MA, USA) was added and the incorporated radioactivity was measured using a Wallac 1410 liquid scintillation counter (PerkinElmer). Protein concentrations were determined according to the method of Bradford, using bovine serum albumin (BSA) as the reference (Bradford, 1976). Receptor densities (B_{max}) were calculated from saturation binding data using GraphPad Prism Software (San Diego, CA, USA).

4.3 Functional assays

4.3.1 [^{35}S]-GTPγS binding assay

Agonist-induced stimulation of [^{35}S]GTPγS binding (Study I) was measured as described previously (Olli-Lähdesmäki *et al.*, 2004). All [^{35}S]GTPγS binding experiments were performed on isolated A7r5-α_{2B} cell membranes in binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 10 µM GDP, 1 mM dithiothreitol, 30 µM ascorbic acid, pH 7.4). Incubations were performed on 96-well Millipore MultiScreen MAFB glass fiber filter plates (Millipore Corp., Bedford, MA, USA). Samples containing 5 µg of membrane protein were preincubated with α_2-AR agonists for 30 min at 37 °C prior to addition of 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol, PerkinElmer). Reactions were terminated after 60 min incubation at 37 °C by vacuum filtration using a Millipore MultiScreen Vacuum Manifold. The filter plate was washed three times with cold wash buffer (20 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4). Filters were dried and 50 µl of SuperMix scintillation cocktail (PerkinElmer) was added into each well. The incorporated radioactivity was measured using a MicroBeta scintillation counter (PerkinElmer).

4.3.2 Measurement of intracellular calcium levels

In Study II, A7r5-α_{2B} cells were cultured on poly-L-lysine-coated coverslips for 48 h before being used in the experiments. Cells were washed twice with imaging buffer (138 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 2 mM TES, 10 mM glucose, 1 mM $CaCl_2$, and 1 mM probenecid, pH 7.4). Cells were incubated with loading buffer (imaging buffer supplemented with 2 µM fura-2 acetoxymethyl ester and 0.01 % pluronic F-127) for 30 min at 37 °C and then washed with imaging buffer. The coverslips were placed in a perfusion chamber that was mounted on a fluorescence microscope (Zeiss Axiovert 35). The 340 and 380 nm excitation filters were used and emission was measured at 510 nm. The light source was an XBO 75W/2 xenon lamp. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA, USA), and images were collected with a SensiCam CCD camera (PCO/CD Imaging, Kelheim, Germany). When a stable baseline was obtained (after 1 min), the cells were stimulated with agonists at RT. Pre-treatment with nimodipine (1 µM) lasted for 5 min. The ΔF340/F380 ratio was used to evaluate the increases in intracellular Ca^{2+} levels.

4.3.3 Measurement of myosin light chain phosphorylation

In Study II, 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. For studies with agonists, plates were taken out of the incubator and instantly treated at RT by carefully adding 50 µl of dexmedetomidine, brimonidine or oxymetazoline solution (1 µM final, DMEM alone for controls) 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. For studies with inhibitors, cells were pre-treated with 50 µl of each tested inhibitor in DMEM (vehicle for controls) at 37 °C. The employed concentrations of the inhibitors 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 treated with dexmedetomidine as above. Cells were then immediately fixed by adding 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 and 0.1 M glycine in PBS-H for 20 min at RT, and then washed three times with PBS-H. Cells were blocked with 2 % BSA in PBS-H for 30 min at RT under gentle agitation and then labelled with mouse anti-pSer19-MLC antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA) in DELFIA® assay buffer (supplemented with BSA to a final concentration of 1 %; PerkinElmer) for 1 h at RT under gentle agitation. Cells were washed five times (total 20 min) with TBST (TBS with 0.1 % Tween20). Subsequently, cells were incubated with a secondary Eu-N1-anti-mouse antibody at a final concentration of 300 ng/ml, diluted in DELFIA® assay buffer (supplemented with 1 % BSA and 2 mM $CaCl_2$), for 45 min at RT under agitation. Cells were washed eight times with TBST (total 25 min), DELFIA® Enhancement solution (PerkinElmer) was added and plates were shaken vigorously for 15 min on a DELFIA® plate shaker (PerkinElmer). Time-resolved fluorescence was read with a Victor V2 multi-label plate reader (615/8.5 nm) (PerkinElmer).

4.3.4 Proliferation assays

4.3.4.1 [^{3}H]-thymidine incorporation assay

In Study I, A7r5 wt and A7r5-α_{2B} cells were cultured on 48-well plates for 24 h, then serum-starved for 24 h and incubated with different concentrations of agonists and other agents for 24 h in DMEM containing 0.5 % FBS. [^{3}H]Thymidine (2 Ci/mmol, Amersham Biosciences UK) was added during the last 4 h of the incubation period. The cells were then washed twice with ice-cold PBS and incubated with 10 % trichloroacetic acid at 4 °C for 30 min, and the acid-soluble material was removed. The wells were washed once with PBS, and the radioactivity incorporated into the acid-insoluble fraction was dissolved in 0.5 M NaOH / 0.5 % sodium dodecyl sulphate, transferred into scintillation tubes containing an excess of scintillation fluid and quantitated using a Wallac 1410 liquid scintillation counter (PerkinElmer).

4.3.4.2 BrdU incorporation assay

In Study III, the DELFIA® Cell Proliferation kit (PerkinElmer), which is based on the measurement of incorporation of the nucleoside analogue 5-bromo-2'-deoxyuridine

(BrdU) during DNA synthesis in proliferating cells, was used to determine the effects of different inhibitors on the dexmedetomidine-evoked proliferation response of A7r5-α_{2B} cells. Briefly, A7r5-α_{2B} cells were serum-deprived overnight in DMEM supplemented with 0.5 % FBS and seeded into 384-well plates on top of pre-plated inhibitors using a Multidrop™ Combi Reagent Dispenser (Thermo Fischer Scientific, Rockford, IL, USA). Cells were allowed to attach for 2 h at 37 °C before the addition of 100 nM (final concentration) dexmedetomidine or vehicle (DMEM supplemented with 0.5 % FBS), each treatment on individual plates. Plates were incubated for 24 h and BrdU (10 µM) was added during the last 4 h. The cells were then fixed and labelled with an anti-BrdU-Eu antibody (0.5 µg/ml) for 75 min at RT under gentle agitation. Cells were washed five times (total 25 min), DELFIA® Inducer solution was added and the plates were shaken vigorously for 30 min on a DELFIA® plate shaker. An EnSight Multimode plate reader (PerkinElmer) was used for signal quantification.

4.4 Immunofluorescent labeling

In Study I, A7r5-α_{2B} cells were plated on poly-L-lysine-coated coverslips and cultured for three days. Subsequently, differentiating culture medium (DMEM supplemented with 2 % FBS and 0.04 % heparin) was added for another 3 to 5 days. The coverslips were then washed with PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, fixed with 4 % paraformaldehyde for 5 min at RT and again thoroughly washed with PBS. Non-specific binding was blocked by incubating the cells with blocking buffer (Nonidet P40, 2 % goat serum and non-fat dry milk in PBS). 3·HA-tagged α_{2B}-ARs were labeled with 5−7 µg/ml anti-HA antibody (HA.11 monoclonal antibody (clone 16B12); Covance Research Products, Cumberland, VA, USA) in blocking buffer for 60 min. Smooth muscle α-actin (α-SMA) was labelled with a monoclonal antiαSMA antibody (1:50, Novocastra Laboratories, Newcastle, UK) in blocking buffer for 60 min. The coverslips were then washed thoroughly with PBS, followed by 15 min incubation in blocking buffer and 60 min incubation with the secondary antibody Alexa Fluor 546 (1:700, Fluor546-linked anti-mouse-IgG antibody (goat), Molecular Probes, Eugene, OR, USA) in blocking buffer. After six washes with PBS, the labeled cells were mounted for fluorescence microscopy using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA). As negative control, non-transfected A7r5 cells were treated according to the same protocol. Cells were observed with a laser-scanning confocal microscope (Zeiss LSM 510 meta, Plan-Apochromat 40×/1.40 or 63×/1.40 oil DIC objective) or with a fluorescence microscope (Leica DMR, Leica HCX PL Fluotar 40×/0.75 objective).

In Study II, immunofluorescent labeling of A7r5-α_{2B} cells was carried out (with minor modifications) as described in Study I. Briefly, A7r5-α_{2B} cells, cultured on poly-L-lysine-coated coverslips, were treated with 1 µM dexmedetomidine (final concentration) or DMEM (control) for 10−45 s and fixed with 3.7 % formaldehyde for 20 min. The cells were subsequently thoroughly washed with PBS (supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and immunolabeled either directly or stored at +4 °C. Non-specific binding was blocked by incubating the cells with blocking buffer (0.2 % Nonidet P40, 2 % goat serum, 1 % BSA in PBS). Phosphorylated myosin light chains ($pMLC_{20}$) were labeled with a monoclonal anti-pMLC antibody (monoclonal mouse anti-pSer19-MLC antibody,

1:50 dilution, Cell Signaling Technology) in blocking buffer for 1 h at RT. The secondary antibody, Alexa Fluor 546 (1:1000), was applied for 45 min at RT. Nuclei were stained with DAPI (included in the Vectashield® mounting medium, Vector Laboratories). Cells were observed with a fluorescence microscope (Zeiss AxioVert 200M, AxioVision 4.8.1 software, Plan-NEOFLUAR 40x/0.75 objective).

4.5 Western blotting

In Study II, A7r5-α_{2B} cells were plated in 60 mm dishes and grown to confluence, then serum-starved overnight in DMEM supplemented with 0.5 % FBS. Dishes were taken out of the incubator and the cells treated instantly at RT with 1 µM (final concentration) dexmedetomidine (or vehicle, or other drugs, or combinations of drugs) added on top of the medium for 10−45 s. The medium was then aspirated, the plates were placed on ice and the cells were lysed by scraping them into ice-cold RIPA buffer containing inhibitors of phosphatases and proteases. Samples were maintained at constant agitation for 20 min at 4 °C and then centrifuged (20 min, 16 000 x g, +4 °C). The supernatants were frozen at −20 °C and protein concentrations were determined with a protein assay kit (BioRad DC™ Protein Assay, BioRad, Hercules, California, USA). For SDS-PAGE, samples were boiled, centrifuged and loaded onto 15 or 18 % acrylamide SDS-PAGE gels with a 4 % stacking gel. SDS-PAGE was carried out in running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) using 60 V until the samples reached the separating gel and then at 150 V in a BioRad Mini Protean Electrophoresis Cell. The separated proteins in the gel were then transferred to a PVDF membrane (pre-soaked in MeOH for 2 min) in ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20 % MeOH, pH 8.3) at 15 V for 50 min using a BioRad Trans Blot® SD Semi-dry Transfer Cell. The membrane was washed for 5 min in PBS and then incubated in 0.5 % formaldehyde for 1 h to improve the retention of phosphoproteins in the membrane (Takeya *et al.*, 2008), and then washed twice in TBS for 5 min. Membranes were then blocked in Odyssey® blocking buffer (OBB, Li-Cor Biosciences, Lincoln, NE, USA) for 1 h at RT. Incubation with mouse anti-pSer19-MLC antibody (1:2000) and anti-α-SMA antibody (1:20000) in OBB was then performed overnight at +4 °C. Subsequently, the membranes were washed seven times (total, 25 min) in TBST (TBS with 0.02 % Tween-20) at RT on a bench-top shaker. Membranes were then blocked again with 5 % milk in TBST for 30 min at RT, and finally incubated with the HRP-conjugated anti-mouse secondary antibody (1:1000, Sigma Aldrich) in 5 % milk / TBST for 1 h at RT. Membranes were then again washed seven times with TBST (total, 40 min). Chemiluminescence was developed with the Amersham™ ECL Western blotting reagent.

In Study III, protein extraction and Western blot analysis of A7r5-α_{2B} cells was carried out (with minor modifications) as described in Study II. A7r5-α_{2B} cells were plated on 35 mm dishes and allowed to multiply until they reached a density of 80−90 %. The cells were serum-deprived overnight in DMEM supplemented with 0.5 % FBS and then stimulated with 1 µM dexmedetomidine for 5−120 min. The cell extracts were separated by SDS-PAGE and transferred to a nitrocellulose blotting membrane. Western blot analysis was done using specific antibodies against phospho-ERK1/2 (1:1000, mouse, New England Biolabs, Ipswich, MA, USA) and phospho-p38 (1:1000, rabbit, New England Biolabs).

Signals were detected with 1:200.000 dilutions of appropriate horseradish peroxidase-conjugated secondary antibodies, followed by visualization with SuperSignalTM West Femto ultrasensitive ECL Western blotting reagent (ThermoScientific, Rockford, IL, USA).

4.6 DNA microarray

A7r5-α_{2B} cells were treated with 100 nM dexmedetomidine or vehicle for 24 h and total RNA was isolated using the NucleoSpin® RNA II mini spin kit (Macherey-Nagel, Düren, Germany). RNA concentration and purity were determined using optical density (OD) measurements at 260 nm and 280 nm. Only RNA of high quality was used (OD260/OD280 ratio ≈ 2.0) for microarray analysis and quantitative RT-PCR. DNA microarray experiments were performed at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. 200 ng of total RNA from each sample was amplified with Ambion's Illumina™ RNA TotalPrep Amplification kit (Thermo Fisher Scientific). During the overnight *in vitro* transcription reaction, cRNA was labeled by biotinylation. Both before and after the amplifications, the RNA/cRNA concentrations were checked with Nanodrop ND-1000 (Thermo Fisher Scientific), and cRNA quality was controlled with the use of BioRad's (Hercules, CA, USA) Electrophoresis station. Labelled and amplified material (0.75 µg/array) was hybridized to Illumina's Sentrix® RatRef-12 BeadChips™ (Illumina Inc., San Diego, CA, USA) at 58 °C overnight according to Illumina® Whole Genome Gene Expression with IntelliHyb Seal protocol (Revision B). Hybridization was detected with 1 µg/ml cyanine3-streptavidine (GE Healthcare Biosciences, Buckinghamshire, UK). The chips were scanned with the Illumina BeadArray™ reader. Numerical results were extracted using Illumina's BeadStudio™ software without any normalization or background subtraction. The hybridization control report indicated that all hybridizations were successful.

To validate the DNA microarray results, 1 µg of the RNA was transcribed to cDNA using the DyNAmo™ cDNA synthesis kit (Thermo Fischer Scientific), including controls with no reverse transcriptase enzyme included. Quantitative RT-PCR was performed using the SYBR green kit (Kapa Biosystems, Wilmington, MA, USA). Primers specific for the selected genes (Table 2) were designed by using Universal Probe Library Assay Design Center (Roche, Basel, Switzerland). The final concentration of forward and reverse primers in the reaction was 0.2 µM. All measurements were done in triplicate.

Table 2. Oligonucleotide sequences for quantitative RT-PCR analysis.

Gene symbol	Left primer	Right primer
Glrx	GGC TCA GGA GTT TGT GAA CTG CAA G	ATC TGC TTC AGC CGG GCC GT
Cx3cl1	CCA CAA GAT GAC CTC GCC AAT C	TCC ACT GTG GCT GAC TCA GGC T
Cav1	AAC GAC GAC GTG GTC AAG A	CAC AGT GAA GGT GGT GAA GC
Prrx1	CTT CTC CGT CAG TCA CCT GC	CGT GCA AGA TCT TCC CGT AC
GAPDH	CAA CTC CCT CAA GAT TGT CAG CAA	GGC ATG GAC TGT GGT CAT GA

Glrx = glutaredoxin, Cx3cl1 = chemokine (C-X3-C motif) ligand / fractalkine, Cav1 = caveolin 1, Prrx1 = paired related homeobox 1, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

4.7 Small compound library screening

A compound library (former CAT# 2831A) from BioMol (Hamburg, Germany) with 84 known kinase and phosphatase inhibitors (annotations are provided in Supplemental file 1 of Paper III) was screened. The compounds [and dimethyl sulfoxide (DMSO) as control] were transferred to 384-well plates at four different concentrations using an automated liquid handling station (Hamilton, Bonaduz, Switzerland). The final concentration range of the compounds was 0.0143 µM, 0.143 µM, 1.43 µM and 14.3 µM. The library screen was performed four times as separate biological replicates. The DELFIA® Cell Proliferation kit was used to determine the effects of kinase and phosphatase inhibitors on the dexmedetomidine-evoked proliferation response of A7r5-α_{2B} cells as described in section 4.3.4.2. Treatments (dexmedetomidine or vehicle) were performed on separate sample plates, and proliferation responses were determined by comparing the inhibitor-treated samples to the DMSO-treated samples (baseline) on each sample plate separately. Total inhibitor effects were determined as an average of four inhibitor concentrations and statistical significance was determined based on these average values.

4.8 Kinase activity profiling

A7r5-α_{2B} cells were plated on 60 mm dishes and grown to approximately 90 % confluence followed by serum deprivation overnight in DMEM supplemented with 0.5 % FBS. Two series of dishes were treated in parallel with 100 nM dexmedetomidine (or vehicle) by replacing the entire medium for 5 min, 30 min, 2 h or 24 h. After exposure for the desired time, the dexmedetomidine (or vehicle) solution was aspirated from the first series of samples, then the dishes were placed on ice and the cells were washed twice with ice-cold PBS. Cells were lysed with ice-cold M-PER Mammalian Extraction Buffer (Thermo Fischer Scientific) containing Halt™ phosphatase (1/100) and protease inhibitors (1/100) (both from Thermo Fischer Scientific). Lysates were incubated for 15 min in a shaking ice bath. Cell lysis was confirmed visually and completed by scraping. The lysates from the first series were transferred to the replicate dishes so as to lyse the contents of both dishes in the same buffer. Cell lysates were centrifuged (15 min, 16 000 × g, +4 °C) and supernatants were collected, snap-frozen with liquid nitrogen and stored at −70 °C. Protein concentrations were determined with a protein assay kit (Pierce™ BCA protein assay kit, Thermo Fischer Scientific).

Kinase activity profiles were determined using the PamChip® 12 serine/threonine (STK) and protein tyrosine (PTK) peptide microarray system (PamGene International B.V., ‘s-Hertogenbosch, The Netherlands) (Hilhorst *et al.*, 2009; Sikkema *et al.*, 2009; Versele *et al.*, 2009; Hilhorst *et al.*, 2013). To prevent non-specific binding, the arrays on the PamChip® 12 STK chips were incubated with 2 % BSA in water for 30 cycles (15 min). Arrays were then washed three times with kinase assay (KA) buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM EGTA, 2 mM DTT, 0.01 % Brij35, a detergent). The reaction mixtures contained 0.01 % BSA in KA buffer supplemented with anti-phospho-Ser/Thr antibodies [PamGene International BV, (Hilhorst *et al.*, 2013)] and 0.5 µg of protein sample. The reaction was initiated by the addition of ATP (final concentration 400 µM) just prior to application onto the arrays. Samples were pumped up and down through the porous membrane of the arrays for 60 cycles (for a total of 60 min). Arrays were washed and then incubated with a secondary

antibody (polyclonal pig anti-rabbit Immunoglobulin/FITC) for 30 min. Images (10, 50 and 200 msec exposure time) were captured every 5 minutes with an integrated CCD-based optical system combined with Evolve software (version 1.5, PamGene International BV). The secondary antibody was removed and arrays were washed before post-wash images were taken at different exposure times (20, 50, 100 and 200 msec). The PTK assay mixture contained kinase assay buffer, 0.01 % BSA and 400 µM ATP, supplemented with 10 mM DTT, a proprietary protein kinase-additive and FITC-labeled anti-phospho-Tyr antibody (both from PamGene International BV). For each PTK assay, 5 µg of protein sample was used. Since a labeled antibody is present in the PTK assay mixture, peptide phosphorylation was monitored during incubation with the assay mixture by capturing images every 5 minutes (10, 20, 50 and 200 msec exposure time), allowing real time recording of the reaction kinetics (one-step reaction). Arrays were washed and fluorescence was detected at different exposure times (20, 50, 100 and 200 msec). Fluorescence signal intensities for all peptides were analyzed using BioNavigator 6.1 software (PamGene International BV, https://www.pamgene.com/en/bionavigator.htm), a statistical analysis and visualization software tool. Around each spot, a local background was calculated, and then this value was subtracted from the signal intensity, resulting in SigmBg (signal minus background). For signal quantification, the slope of the SigmBg versus exposure times was calculated to increase the dynamic range. Visual quality control was done to exclude defective arrays from the analysis. Peptides with CV < 30 % for the replicates were included in the analysis, resulting in 96 peptides for PTK and 98 peptides for STK analysis.

4.9 Data analysis

In Study I, the results were analyzed with standard methods using GraphPad Prism programs (GraphPad Software, San Diego, CA, USA). Statistical analyses were carried out with two-tailed, one-sample *t*-tests. *P* values smaller than 0.05 were considered to be statistically significant.

For Western blot densitometry (Studies II and III), bands were analyzed and fluorescence intensities were quantitated using the Image-J 1.45s software [National Institutes of Health, Bethesda, MD, USA; (Schneider *et al.*, 2012)]. The images for measurement of intracellular calcium (Study II) were processed using Axon Imaging Workbench 5.1 software (Axon Instruments, Foster City, CA, USA).

In the myosin light chain phosphorylation ($pMLC_{20}$) assay (Study II), 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 $(\text{mean} - \text{mean\_i})^2 / \text{standard error(mean)}^2$, 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 $pMLC_{20}$ response curve (AUC) was determined for the different compounds used in the experiments according to the trapezoidal rule:

$$\mathrm{AUC} = \sum_{t=0\,s}^{t=45\,s} \left(\tfrac{1}{2} * (y_1 + y_2) * (t_2 - t_1) \right)$$

where y_1, y_2 = averaged europium counts from successive samples, and 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 $pMLC_{20}$ responses were expressed as per cent of 1 μM dexmedetomidine. Graphs were plotted using GraphPad Prism programs. Statistical analyses were carried out with two-tailed, one-sample *t*-tests. *P* values smaller than 0.05 were considered to be statistically significant.

The DNA microarray data (Study III) were analyzed using R statistical analysis software (R Core Team, 2013) and the Limma package of the related Bioconductor module [http://www.bioconductor.org/, (Gentleman *et al.*, 2004; Ritchie *et al.*, 2015). The data were normalized using the quantile normalization method. After statistical testing with Limma, the differentially expressed genes were filtered requiring false discovery rates < 0.05 and absolute fold-changes > 1.3. The GeneFuncster tool was used to carry out enrichment analysis of all differentially expressed genes towards both Gene Ontology (GO) categories and KEGG pathways [http://bioinfo.utu.fi/GeneFuncster, (Laiho *et al.*, 2012)]. Functional associations of the differentially expressed genes were further analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity®Systems, http://www.ingenuity.com/). The RT-PCR data were analyzed according to the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference gene (relative expression to GAPDH) (Livak and Schmittgen, 2001).

In the inhibitor assay (Study III), for each inhibitor, two-way analysis of variance (ANOVA) was employed to evaluate how concentration and treatment were associated with the proliferation response. All statistical tests were performed as 2-sided, with a significance level set at 0.05. The analyses were performed using SAS System, version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA).

In the kinase activity profiling experiments (Study III), statistically significant effects ($P < 0.05$) were identified by employing a linear mixed model and then performing Dunnet's tests for multiple conditions against a single control. Peptides found to be significantly differently phosphorylated between dexmedetomidine-treated samples and their vehicle-treated controls (t = 30 min and t = 24 h) were used for pathway analysis using the canonical pathway analysis program MetaCore™ (Thomson Reuters, St. Joseph, MI, USA). The top 10 most significant process networks were identified and relevant signaling networks were assembled based on manually curated objects generated by log fold-change data. Pathways were ranked by −log (*P*); −log (*P*) > 4 were considered significant. BioNavigator software was used to perform upstream kinase analysis by comparing differentially phosphorylated peptides between dexmedetomidine-treated samples and their vehicle-treated controls (t = 30 min and t = 24 h) and linking them to the putative upstream kinases (derived from Kinexus phosphoNET database; http://www.phosphonet.ca/) responsible for their phosphorylation. The upstream kinase analysis tool generated hypotheses about kinases that were differentially active between dexmedetomidine-treated samples and their vehicle-treated controls.

5 RESULTS

5.1 A VSMC line stably expressing the human α_{2B}-adrenoceptor (Study I)

Different conventional transfection methods were tested for the transient transfection of A7r5 cells, including calcium phosphate precipitation, cationic polymer transfection reagents (jetPEI, ExGen500) and liposomal transfection reagents (Lipofectamine 2000, Transfectin, FuGene6) (Table 3). Transfection optimizations were carried out using LacZ as a reporter gene and transfection efficiencies were determined by β-galactosidase staining 48–72 h after transfection. Complex formation at different temperatures and for different periods of time was monitored in order to optimize the calcium phosphate precipitation method. Cationic polymer and liposomal transfection optimizations were carried out by varying the amount of plasmid DNA (1–3 µg), the volume of transfection reagent (3–6 µl), the DNA : reagent ratio (liposomal reagents) and the nitrogen to phosphate (N/P) ratio (cationic polymers). Despite comprehensive optimization efforts, all transfection reagents proved to be inefficient and/or toxic and the best transfection efficiencies remained below 10 %. To study α_{2B}-AR function and regulation in A7r5 cells, transient transfection would have been useful only if transfection efficiencies greater than 50 % could have been produced reliably and repeatedly. Therefore, stable transfection using the electroporation-based nucleofection technique combined with extended antibiotic selection was considered necessary.

Table 3. Tested transfection methods for the transient transfection of A7r5 cells, their optimization, achieved transfection efficiencies and observed toxicity.

Method	Optimization	Transfection efficiency	Toxicity
Liposomal reagents			
Lipofectamine 2000	plasmid DNA [µg] transfection reagent [µl] DNA:reagent ratio	poor	high
Transfectin		poor	high
FuGene6		< 10 %	non-toxic
Cationic polymers			
jetPEI	plasmid DNA [µg] transfection reagent [µl] N/P ratio	poor	medium
ExGen500		poor	high
Calcium phosphate precipitation			
	temperature time of complex formation	very poor	non-toxic

The procedure with nucleofection and antibiotic selection resulted in several heterogeneous cell populations with different expression levels of human α_{2B}-ARs (140 ± 20, 460 ± 30 and 860 ± 180 fmol/mg total cellular protein; means ± SD); the population expressing the highest average receptor density (called A7r5-α_{2B}) was selected for further studies. Cell membranes were isolated from A7r5-α_{2B} cells with and without PTX treatment and receptor densities were determined. Results indicated that cells with or without PTX treatment had comparable receptor densities (1900 and 1500 fmol/mg membrane protein, respectively). As expected, in A7r5 wt cells, no α_2-AR-specific radioligand binding was detected (limit of assay sensitivity: ≈ 30 fmol/mg protein). Receptor density determinations performed several passages later confirmed that the receptor density remained stable (Figure 10).

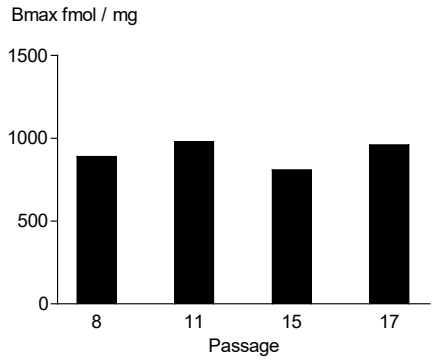

Figure 10. Receptor densities (B_{max}) in A7r5-α_{2B} cell homogenates with increasing passage number. B_{max} refers to receptor density/mg total cellular protein; *n* = 1.

The localization of α_{2B}-ARs expressed in A7r5 cells was determined by immunofluorescent labelling and confocal fluorescence microscopy. α_{2B}-ARs (red staining, anti-HA antibody) appeared to localize to the cell membrane which tightly envelopes the nucleus, as can be seen from the specific labelling on top of and below the nucleus (blue) (Figure 11D). Vesicular structures with specific labelling were also detected (Figure 11B). The A7r5-α_{2B} cell line is of heterogeneous origin, which was demonstrated by the varying fluorescence intensities in different cells, indicating that the cells expressed receptors at varying densities (Figure 11A). As expected, no specific staining could be detected in A7r5 wt cells (Figure 11C). However, the expression of α-SMA was similar in A7r5-α_{2B} cells and A7r5 wt cells.

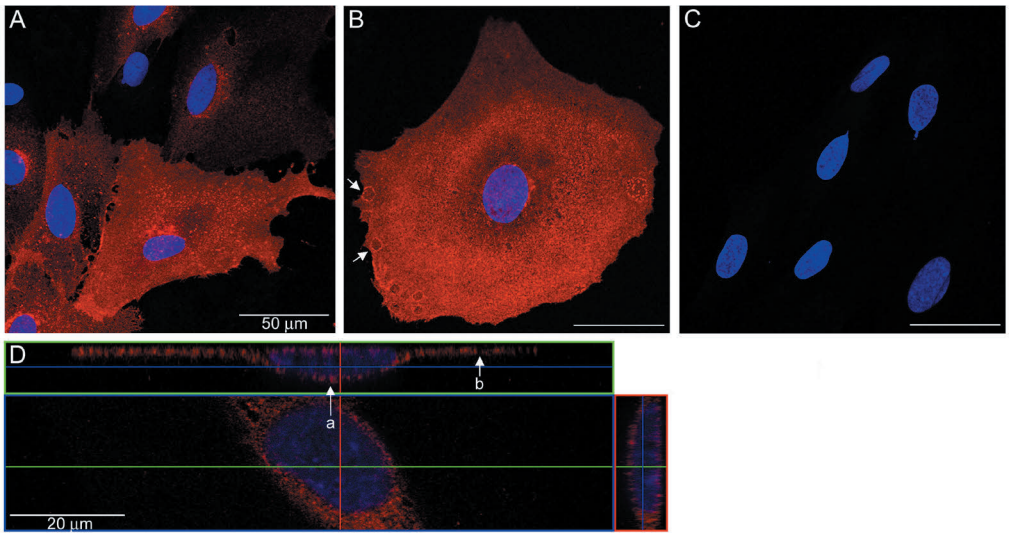

Figure 11. Confocal fluorescence microscopy of the A7r5-α_{2B} cells with heterogeneous cellular receptor expression. Fluorescence intensity is relative to the receptor expression level and is higher in some of the cells and lower in others (A). Arrows indicate vesicular structures detected in A7r5-α_{2B} cells (B). A7r5 wt cells as negative controls, only DAPI-stained nuclei are visible (C). A cross-section image of an A7r5-α_{2B} cell to visualize the cellular localization of the receptors (D). The thickness of the cell by the arrows is 6 µm (arrow a) 2 µm (arrow b). The figure is from Study I.

Receptor function was assessed by [^{35}S]GTPγS binding and [^{3}H]thymidine incorporation assays. [^{35}S]GTPγS binding assays were performed to determine the functionality and pharmacological properties of the expressed human α_{2B}-ARs in A7r5 cells. Agonist-induced [^{35}S]GTPγS binding provides a measure of agonist efficacy and potency at the first level of signal transduction, activation of G_i type G proteins. The rank order of agonist efficacy (intrinsic activity relative to adrenaline) for the investigated agonists was adrenaline = noradrenaline (100 %) > dexmedetomidine (74 %) >> oxymetazoline (49 %) > brimonidine (17 %) > clonidine (9 %). The rank order of potency (EC_{50} = the agonist concentration causing 50 % of the maximal effect) for the same agonists was dexmedetomidine (10 nM) > clonidine (0.2 μM) ≥ brimonidine (0.6 μM) ≥ oxymetazoline (0.9 μM) > noradrenaline (1.5 μM) = adrenaline (2 μM). PTX pre-treatment abolished the capacity of all studied agonists to stimulate [^{35}S]GTPγS binding, indicating that α_{2B}ARs expressed in A7r5 cells were coupled to PTX-sensitive G_i proteins.

The effect of α_{2B}-AR activation on cell proliferation was determined by measuring the capability of dexmedetomidine to stimulate the incorporation of [^{3}H]thymidine into newly synthesized DNA. Dexmedetomidine concentration-dependently stimulated [^{3}H] thymidine incorporation with an EC_{50} of 0.85 nM in A7r5-α_{2B} cells, but not in A7r5 wt cells (Figure 12). Cells were serum-deprived for 24 h before the experiments. As shown in the right-hand part of Figure 12, 10 % FBS stimulated [^{3}H]thymidine incorporation to a comparable extent in both A7r5-α_{2B} and A7r5 wt cells. Under normal cell culture conditions, A7r5-α_{2B} and A7r5 wt cells exhibited comparable growth rates, indicating that α_{2B}-AR expression as such exerted no effect on the growth characteristics of A7r5 cells.

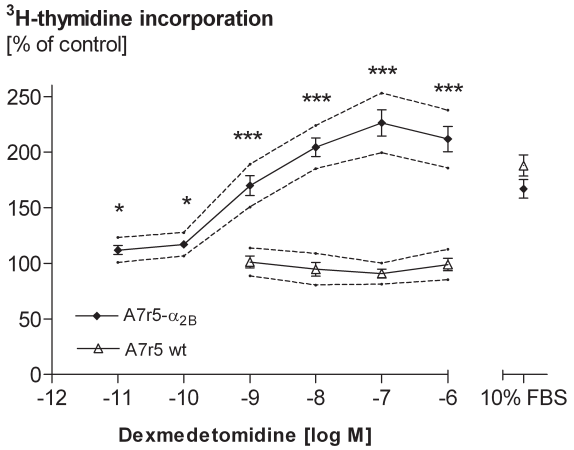

Figure 12. Agonist-induced [^{3}H]thymidine incorporation in A7r5-α_{2B} (◆) and A7r5 wt(Δ) cells. A7r5-α_{2B} and A7r5 wt cells were incubated in serum-free medium for 24 h with increasing concentrations of dexmedetomidine and with 10 % FBS. Dashed lines indicate a confidence interval of 95%. Results are means ± S.E.M. of three individual experiments performed in triplicate. The figure is from Study I.

It was concluded that the generated A7r5-α_{2B} cell line expressed functional human α_{2B}-ARs that exhibited a localization pattern and ligand binding properties similar to results obtained in earlier studies conducted with α_{2B}-ARs in different host cell types. Furthermore, functional characterization indicated that the receptors had the expected pharmacological characteristics in terms of efficacy and potency for a set of α_2AR agonists, and that the α_{2B}-ARs expressed in A7r5 cells were functional also in intact living cells, as evidenced by the marked increase in cell proliferation occurring after α_{2B}-AR activation. It was also concluded that due to the heterogeneous receptor expression of the generated A7r5-α_{2B} cell line, it was better suited for multi-cell than single-cell assays.

5.2 α_{2B}-adrenoceptor-evoked contraction of VSMCs (Study II)

5.2.1 Cell-based quantitative assay for measuring myosin light chain phosphorylation

Phosphorylation of regulatory myosin light chains can be considered to represent a biochemical readout of smooth muscle cell contraction (Somlyo and Somlyo, 1968; Small and Sobieszek, 1977). Therefore, a cell-based quantitative assay was developed to measure this readout of the contraction process, to be used as a tool for the investigation of α_2-AR-evoked VSMC contraction. Dexmedetomidine was chosen as a reference agonist, since it is the most selective (α_2/α_1-AR selectivity ratio 1620) α_2-AR agonist currently available (Virtanen *et al.*, 1988) and a potent, almost full agonist of the α_{2B}-AR as demonstrated in Study I. Dexmedetomidine-induced MLC_{20} phosphorylation ($pMLC_{20}$) in A7r5-α_{2B} cells was measured over a period of 5−120 s. Typically, the dexmedetomidine response peaked at 20−45 s with an $E_{max} \approx 60$ % over vehicle control (Figure 13).

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 $pMLC_{20}$ response evoked by 1 µM dexmedetomidine. The dexmedetomidine response was dose-dependent (Figure 14), and could be blocked by the α_2-AR antagonists atipamezole (Figure 14) and

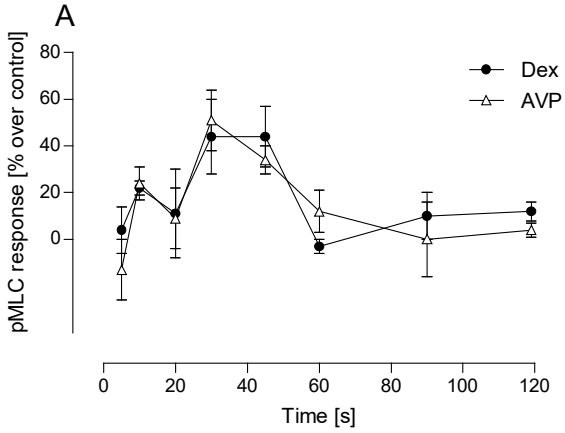

Figure 13. Agonist-induced myosin light chain phosphorylation in A7r5-α_{2B} cells. Confluent and serum-starved A7r5-α_{2B} cells were treated with dexmedetomidine (Dex, 1 µM) or arginine vasopressin (AVP, 100 nM). The figure is from Study II.

rauwolscine (both 100 µM), indicating that the $pMLC_{20}$ response was mediated by α_{2B}-ARs. This was further supported by the observation that treatment of A7r5 wt cells with dexmedetomidine had no effect on MLC_{20} phosphorylation. Two additional α_2-AR agonists, brimonidine and oxymetazoline, at 1 µM, evoked $pMLC_{20}$ responses that were comparable with dexmedetomidine-evoked responses in A7r5-α_{2B} cells (Figure 14). Furthermore, stimulation of endogenously expressed vasopressin V_1-receptors with 100 nM AVP evoked $pMLC_{20}$ responses similar to dexmedetomidine-evoked responses in A7r5-α_{2B} cells (Figure 13). ML-7, an inhibitor of MLCK, was used as a negative control to prove that the observed response actually was phosphorylation of myosin light chains, and as expected, ML-7 treatment completely abolished the dexmedetomidine-evoked $pMLC_{20}$ response (Figure 14).

For method validation, Western blotting and immunofluorescent labelling experiments were performed on cells treated in a similar way as in the MLC_{20} phosphorylation assay. The results indicated that the antibody against phosphorylated Ser19 of MLC_{20} detected proteins of the expected molecular mass (18 kDa) and that dexmedetomidine treatment clearly increased the $pMLC_{20}$ immunoreactivity compared with vehicle-treated controls.

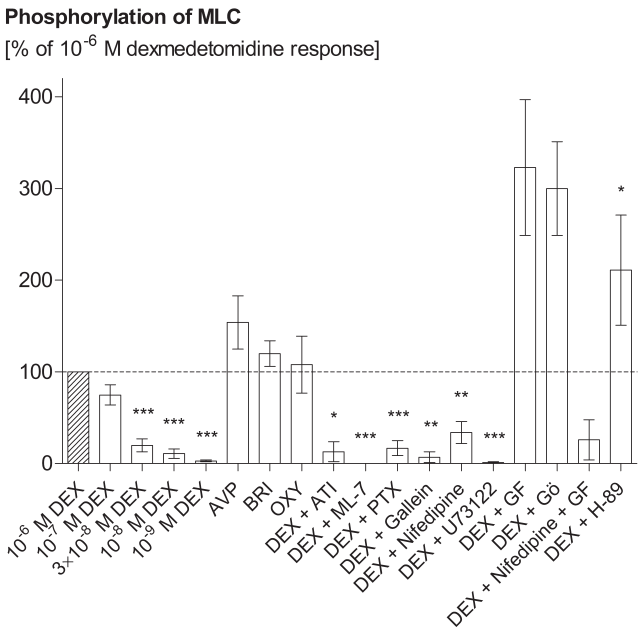

Figure 14. Effects of agonists, antagonists and inhibitors on the phosphorylation of myosin light chains in A7r5-α_{2B} cells, in relation to the $pMLC_{20}$ response (AUC) induced by the α_2-AR agonist 1 µM dexmedetomidine (100 %). Agonists included arginine vasopressin (AVP, 100 nM), brimonidine (BRI, 1 µM), oxymetazoline (OXY, 1 µM) and dexmedetomidine (DEX, 1 nM–1 µM). The antagonist atipamezole (ATI, 100 µM) was added simultaneously with dexmedetomidine. Pretreatment times for inhibitors were 10–20 min (overnight for PTX) and the inhibitors used were: ML-7 (MLCK 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 (PLC inhibitor, 1 µM), GF109203X (GF) and Gö6976 (Gö) (PKC inhibitors, 10 µM) and H-89 (PKA inhibitor, 10 µM). Results are AUC means ± S.E.M. (up to 45 s) 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 ($0.05 < p < - 0.10$), possibly because of the small number of observations. The figure is from Study II.

Measurements of intracellular Ca^{2+} levels in A7r5-α_{2B} cells were performed to validate the method with an alternative read-out than $pMLC_{20}$. Dexmedetomidine stimulation increased intracellular Ca^{2+} levels in a concentration-dependent manner in A7r5-α_{2B} cells, but not in A7r5 wt cells, indicating that the responses were mediated by α_{2B}-ARs.

5.2.2 Intracellular pathways involved in α_{2B}-adrenoceptor-evoked VSMC contraction

A7r5-α_{2B} cells were exposed to different inhibitor compounds in order to explore the intracellular pathways involved in the α_{2B}-AR mediated phosphorylation of myosin light chains. The results of all experiments are summarized in Figure 14. Pre-treatment with PTX almost completely abolished the dexmedetomidine-evoked $pMLC_{20}$ response (by 83 ± 8 %), indicating that the response was G_i protein-mediated. Pre-treatment with gallein, an inhibitor of Gβγ subunit-dependent signalling (Lehmann *et al.*, 2008), almost completely abolished the dexmedetomidine-evoked $pMLC_{20}$ response (by 93 ± 6 %), suggesting that Gβγ subunits played an important role in the $pMLC_{20}$ response. Pre-treatment with nifedipine, an L-type Ca^{2+} channel blocker, attenuated the dexmedetomidine-evoked $pMLC_{20}$ response by 66 ± 12 %, revealing that also L-type Ca^{2+} channels were important for the phosphorylation response. PLC appeared to be crucially involved in the intracellular pathways leading to the phosphorylation of MLC, since U73122 completely abolished the dexmedetomidine-evoked $pMLC_{20}$ response. Unexpectedly, the PKC inhibitors GF109203X and Gö6976 very markedly potentiated the dexmedetomidine-induced $pMLC_{20}$ response to 323 ± 74 % and 300 ± 51 % of the response to dexmedetomidine alone. Nifedipine antagonized also the combined effects of dexmedetomidine and PKC inhibition, suggesting that increased influx of Ca^{2+} ions via L-type Ca^{2+} channels is required for the potentiation of the dexmedetomidine-induced $pMLC_{20}$ response upon pre-treatment with the PKC inhibitors. Finally, inhibition of PKA with H-89 increased the $pMLC_{20}$ response up 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). The effects of the inhibitors alone on the extent of basal MLC phosphorylation (without agonist) were statistically non-significant for most of the tested inhibitors (ML-7, PTX, U73122, Gö6976 and nifedipine), but significant decreases in the basal MLC phosphorylation were observed after GF109203X, H-89 and gallein treatments.

It was concluded that the developed assay was suitable for the quantitation of MLC phosphorylation responses in VSMCs. Furthermore, this study demonstrated that activation of α_{2B}-ARs induced MLC phosphorylation, i.e. a surrogate for contraction, in A7r5-α_{2B} VSMCs. The signaling mechanisms related to α_{2B}-AR-evoked VSMC contraction appeared to involve at least G_i proteins, Gβγ subunits, PLC and L-type calcium channels, since their inhibition led to almost complete inhibition of the contraction response.

5.3 α_{2B}-adrenoceptor-evoked proliferation of VSMCs (Study III)

As shown in Study I, prolonged stimulation (24 h) of α_{2B}-ARs with the selective agonist dexmedetomidine had prominent stimulatory effects on the proliferation of cultured A7r5-α_{2B} VSMCs. However, based on published literature, the cellular mechanisms and signal transduction pathways underlying this phenomenon have remained largely unknown. Therefore, three different types of screening assays were employed as tools to

investigate changes in gene expression, signaling pathways and kinase activation profiles related to α_{2B}-AR-evoked proliferation in A7r5-α_{2B} VSMCs.

5.3.1 Differentially expressed genes related to the α_{2B}-adrenoceptor-evoked proliferation response

In order to identify the specific genes involved in α_{2B}-AR-evoked proliferation of A7r5-α_{2B} VSMCs, Illumina DNA microarray experiments were performed. To define the effects of activated α_{2B}-ARs on gene expression profiles, A7r5α_{2B} VSMCs were incubated with 100 nM dexmedetomidine for 24 h. Of the 22 000 genes analyzed, 55 genes were differentially expressed in dexmedetomidine-treated cells compared with vehicle-treated controls (FC > 1.3 and $P < 0.05$; P-values corrected for false positive discovery rates). Out of these genes, 29 were upregulated with fold change (FC) values ranging from 1.32 to 2.01, and 26 were downregulated with FC values ranging from −1.3 to −1.86 (Figure 15).

GeneFuncster (GF) functional enrichment analysis and Ingenuity® pathway analysis (IPA) were used to identify significant biological functions, networks and upstream regulators related to the differentially expressed genes in dexmedetomidine-stimulated vs. vehicle-

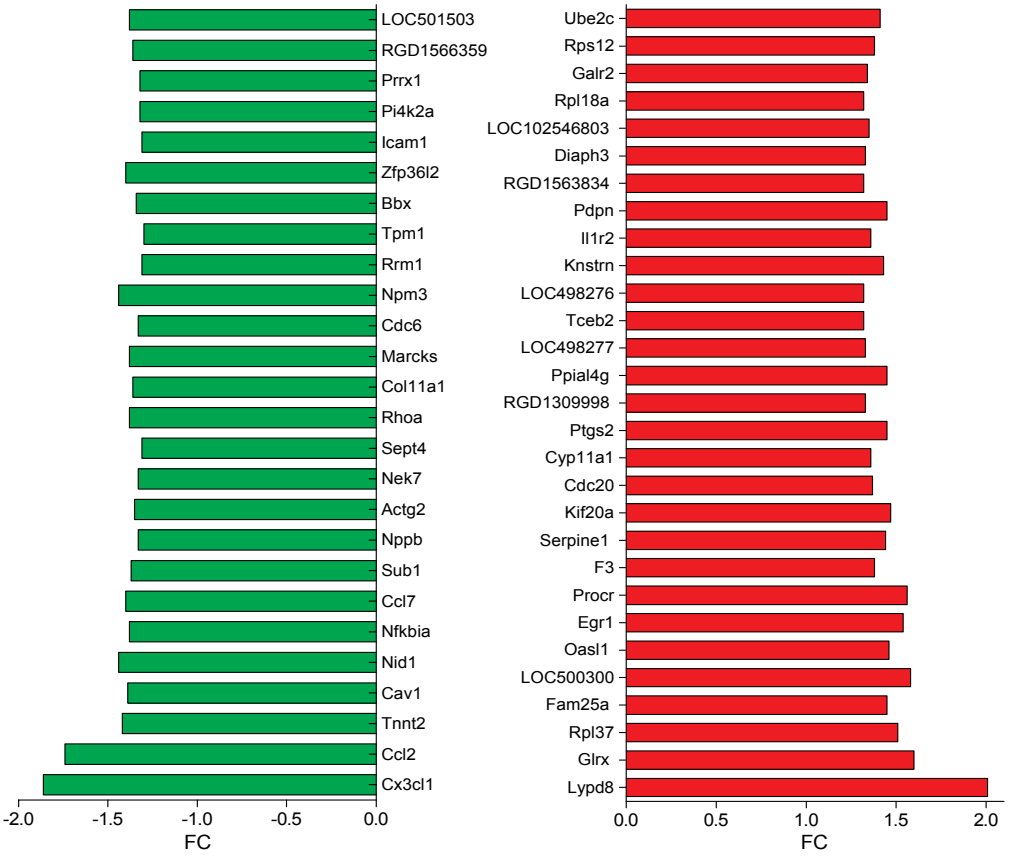

Figure 15. Fold change (FC) values of upregulated (red) and downregulated (green) genes in dexmedetomidine-treated (100 nM) A7r5-α_{2B} VSMCs compared with vehicle-treated control cells in the Illumina DNA microarray. The genes are ordered based on the associated adjusted P values, in decreasing order. Explanations of gene symbols are provided in Supplemental file 2 of Paper III. The figure is from Study III.

treated A7r5-α_{2B} cells. GF identified a high number (> 200) of significantly ($p < 0.01$) enriched biological process Gene Ontology (GO) terms among the differentially expressed genes induced by α_{2B}-AR activation. Out of the first 200 enriched biological process GO terms, 49 terms were related to cardiovascular system development, blood circulation, cell migration and motility, cell proliferation, cell adhesion, vasoconstriction, mitotic cell cycle, cytoskeleton organization and regulation of cell shape (Figure 16). Notably, GO terms such as “regulation of smooth muscle cell proliferation” (GO: 0042127), “positive regulation of cell proliferation” (GO: 0048660) and “positive regulation of vasculature development” (GO: 1904018) were among the enriched biological process GO terms. Some of the differentially expressed genes were involved in several aspects of cell function, including cardiovascular system development, regulation of proliferation, migration and adhesion, vasoconstriction and mitotic cell cycle. Upregulated genes, which had the largest number of annotations within the selected 49 biological process GO terms, were prostaglandin-endoperoxide synthase 2 (Ptgs2 or COX2; 38 annotations), early growth response 1 (Egr1; 29 annotations), serpin peptidase inhibitor (Serpine1 or plasminogen activator inhibitor type 1; PAI-1; 28 annotations), coagulation factor III, tissue factor (F3; 24 annotations), and podoplanin (Pdpn; 21 annotations). Downregulated genes with the most annotations per gene were chemokine (C-X3-C motif) ligand 1 (Cx3cl1 or fractalkine; 40 annotations), RhoA (34 annotations), caveolin 1 (Cav1; 33 annotations), chemokine (C-C motif) ligand 2 (ccl2; 29 annotations), intercellular adhesion molecule 1 (Icam1; 28 annotations) and tropomyosin 1 alpha (Tpm1; 23 annotations). There were many genes among those differentially expressed that are known to participate in the regulation of proliferation in different types of cells, such as Egr1, serpine1, Pdpn, Cx3cl1, glutaredoxin (Glrx), cell division cycle 20 (cdc20), kinesin family member 20a (Kif20a) and ubiquitin-conjugating enzyme E2C (ube2c).

Most important up- and down-regulated genes identified by IPA Core Functional Analysis included partly the same genes that were determined to be involved in many of the functionally enriched biological process GO terms in the GF functional enrichment analysis: Egr1, Glrx, Kif20a, Cx3cl1, RhoA, Cav1 and Ccl2. The most significant biological functions predicted by the IPA core analysis included (changes in) cellular movement, cell-to-cell signaling, cellular growth and proliferation, cellular development, skeletal and muscular system development and function and cell cycle. Egr1, Ptgs2, serpine1 and F3 were involved in migration and proliferation of smooth muscle cells. Ptgs2 was also involved in smooth muscle cell contraction. Downregulation of RhoA was associated with migration, adhesion, proliferation and contraction of smooth muscle cells. Downregulation of Cx3cl1 was associated with adhesion and proliferation. Downregulation of Cav1 was associated with VSMC proliferation and contraction. Other downregulated genes associated with cell proliferation were Nppb and Prrx1.

According to the IPA core analysis, upregulation of Egr1 (FC 1.54; $P = 0.005$), F3 (FC 1.38; $P = 0.008$) and Ptgs2 (FC 1.45; $P = 0.02$) was predicted to lead to activation of smooth muscle cell proliferation. Cav1 (FC 1.39; $P = 0.008$) and Nppb (FC -1.33; $P = 0.02$) were predicted to have inhibitory effects on smooth muscle cell proliferation, which was in line with the observed decreased expression of these genes. According to IPA, Cx3cl1, Prrx1 and RhoA had indirect activating effects on smooth muscle cell proliferation. However, Cx3cl1 (FC −1.86; $P = 0.00002$), Prrx1 (FC −1.32; $P = 0.04$) and RhoA (FC −1.38; $P = 0.02$) were downregulated, which was inconsistent with the predicted actions of these genes in the IPA core analysis. Serpine1

Figure 16. Selected significantly enriched ($p < 0.01$) biological process GO terms related to cardiovascular functions in upregulated (red) and downregulated (green) genes induced by α_{2B}-AR activation upon dexmedetomidine stimulation in A7r5-α_{2B} vascular smooth muscle cells. Ptgs2 = prostaglandin-endoperoxide synthase 2 (COX2); Egr1 = early growth response 1; serpine1 = serpin peptidase inhibitor, member 1 (PAI-1); F3 = coagulation factor III, tissue factor; Pdpn = podoplanin; Cdc20 = cell division cycle 20; Knstrn = kinetochore-localized astrin/SPAG5 binding protein; Kif20a = kinesin family member 20A; Ube2c = ubiquitin-conjugating enzyme E2C; Glrx = glutaredoxin; Diaph3 = diaphanous-related formin 3; Galr2 = galanin receptor 2; Cx3cl1 = chemokine (C-X3-C motif) ligand 1 (fractalkine); Cav1 = caveolin 1; Ccl2 = chemokine (C-C motif) ligand 2; Icam1 = intercellular adhesion molecule 1; Tpm1 = tropomyosin 1, alpha; Nppb = natriuretic peptide B; Ccl7 = chemokine (C-C motif) ligand 7; Tnnt2 = troponin T type 2; Prrx1 = paired related homeobox 1; Marcks = myristoylated alanine rich protein kinase C substrate; Cdc6 = cell division cycle 6; Col11a1 = collagen, type XI, alpha 1; Nid1 = nidogen 1; Zfp36l2 = zinc finger protein 36, C3H type-like 2; Nfkbia = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; Rrm1 = ribonucleotide reductase catalytic subunit M1; Pi4k2a = phosphatidylinositol 4-kinase type 2 alpha. The figure is from Study III.

(FC 1.44; $P = 0.008$) was indicated to be involved in the proliferation of smooth muscle cells, but IPA did not predict the direction of the effect. GF identified the biological process GO terms "regulation of smooth muscle cell proliferation" and "regulation of cell proliferation" as significantly enriched, with P-values of 2.1×10^{-5} and 1.2×10^{-4}, respectively. The differentially expressed genes linked to these GO terms were mainly the same as those identified by IPA. Egr1, Ptgs2, Cx3cl1, Cav1 and Nppb were linked to both GO terms (Figure 16). In GF, F3, serpine1, RhoA and Prrx1 were linked only to general regulation of cell proliferation, although IPA identified them as genes regulating smooth muscle cell proliferation.

The IPA upstream regulator analysis identified six upstream regulators with |z-score| > 2: platelet-activating factor (PAF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF/FGF2), cAMP and aryl hydrocarbon receptor (AHR) were predicted to be activated and NF-kappa-B inhibitor α (NFKBIA) was predicted to be inhibited. The activation z-score is used to predict activation or inhibition of regulators based on relationships with dataset genes and direction of change of dataset genes and a prediction of effect on the function, increased or decreased, is given for |z-score| > 2. The predicted effects of these upstream regulators on differentially expressed genes in our dataset are illustrated in Figure 17A. There were 9 genes (proteins) in the dataset showing regulatory effects by NFKBIA. Among them, 8 genes had expression change directions consistent with the inhibition of NFKBIA (z-score = −2.482, overlap P-value = 1.9×10^{-7}). Seven of nine genes and ten of eleven genes showed expression change directions consistent with the activation of FGF2 (z-score = 2.140, overlap P-value = $3.6 \cdot 10^{-5}$) and HGF (z-score = 2.203, overlap P-value = $4.9 \cdot 10^{-7}$), respectively. Five genes indicated regulatory effects by cAMP (z-score = 2.141, overlap P-value = $5.1 \cdot 10^{-4}$), six genes by PAF (z-score = 2.159, overlap P-value = $1.4 \cdot 10^{-7}$) and seven genes by AHR (z-score = 2.183, overlap P-value = $1.5 \cdot 10^{-3}$), and all genes had expression change directions consistent with the activation of these upstream regulators.

PKC (different isoforms), PKA and PDGF obtained z-scores of 1.788, 1.980 and 1.988, respectively. Because they did not reach a z-score of 2, IPA did not give a prediction on their activation state. However, based on their relatively high z-scores, activation may be assumed. PKC, PKA and PDGF are known to regulate the proliferation of many cell types, including VSMCs; therefore, their roles as upstream regulators of the genes in our dataset were inspected (Figure 17B). Six genes demonstrated regulatory effects by PKCs and among them, five genes had expression change directions consistent with the assumed activation of PKCs (overlap P-value = $1.7 \cdot 10^{-5}$). Six of seven genes and four of four genes showed changes consistent with the assumed activation of PDGF (overlap P-value = $6.0 \cdot 10^{-9}$) and PKA (overlap P-value = $3.9 \cdot 10^{-4}$), respectively.

To validate the microarray results, the expression of selected genes was analyzed by means of quantitative RT-PCR, using the same samples as in the microarray experiment. These genes were chosen to represent a spectrum of significant expression level changes in the microarray. Out of the differentially expressed genes that were annotated within the selected 49 biological process GO terms, Glrx was the most upregulated gene (FC 1.60) and Cx3cl1 was the most downregulated gene (FC −1.86). Cav1 (FC −1.39) and Prrx1 (FC −1.32) represent genes with smaller expression level changes. Consistent with the microarray results, the expression differences (FC) for Glrx and Cx3cl1 were 1.75 and −3.02, respectively. Cav1 and Prrx1 represented genes with smaller FC-values in the microarray; they did not show differential expression compared to control samples in the RT-PCR experiments.

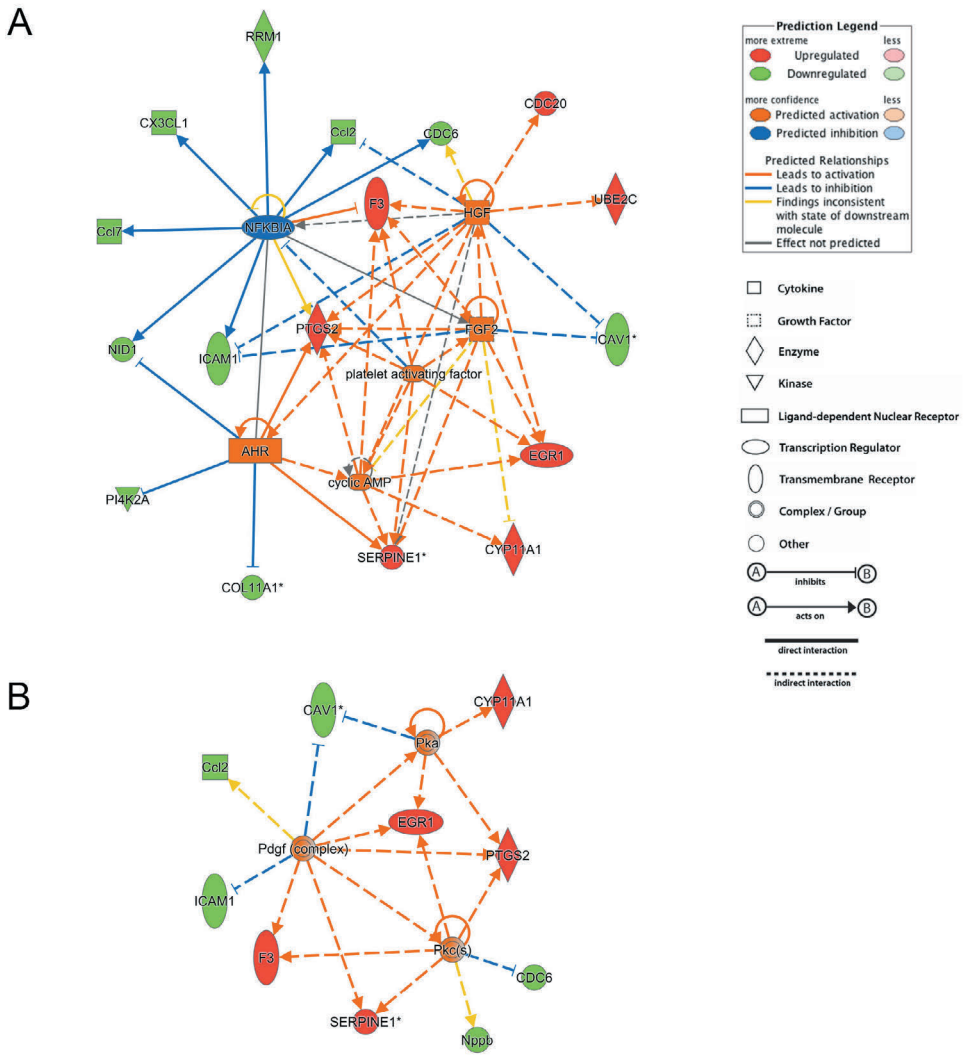

Figure 17. Upstream regulators identified by Ingenuity Pathway Analysis, predicted to participate in the regulation of gene expression upon α_{2B}-AR activation in A7r5-α_{2B} vascular smooth muscle cells. The network of upstream regulators with |z-score| > 2 is illustrated in panel A. The network of PKC, PKA and Pdgf targets is presented in panel B. Red and green colors indicate upregulation and downregulation, respectively. The figure is from Study III.

5.3.2 Identification of kinases participating in the α_{2B}-adrenoceptor-evoked proliferation response

An 84-compound library consisting of 70 kinase inhibitors and 14 phosphatase inhibitors was screened in order to investigate their effects on the α_{2B}-AR-evoked proliferation in A7r5-α_{2B} VSMCs, and possibly identify specific signaling pathways involved in the proliferation response. The inhibitor effects showed rather large variation between the replicates, and thus a statistical analysis was employed to evaluate the significance of the effects. Altogether 15 compounds were detected to inhibit the dexmedetomidine-induced proliferation response in A7r5-α_{2B} cells with statistical significance ($p < 0.01$). Another 18 inhibitors were associated with trend-level effects with $0.01 < p < 0.05$. Statistically significant ($p < 0.01$) inhibitors repressed the

Table 4. Significant (p < 0.01) inhibitors, their potential target kinases and phosphatases and their effects on VSMC proliferation.

Inhibitor	Inhibition of proliferation [% of control]	Potential target kinases and phosphatases	Effect of target kinase/ phosphatase on VSMC proliferation	Reference
Fenvalerate	32	calcineurin	↑	Lipskaia *et al.*, 2003; Li and Sun, 2005; Pang and Sun, 2009; Kudryavtseva *et al.*, 2013
5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole	31	CK2	↑	Pinna and Meggio, 1997; Dumler *et al.*, 1999; Guerra and Issinger, 1999; Ahmed *et al.*, 2002; Kristof *et al.*, 2005
		Cdk9	↑	Romano and Giordano, 2008; Wang *et al.*, 2014
PP2	31	Src family kinases	↑	Jeremy, 1999; Waltenberger *et al.*, 1999; Kawabe *et al.*, 2004; Walcher *et al.*, 2006; Kim *et al.*, 2009a; Li *et al.*, 2010; Davis *et al.*, 2012
PP1	18			
SP600125	30	JNKs	↑	Nagayama *et al.*, 2015; Yang *et al.*, 2015
SB-202190	30	p38 MAPKs	↑	Chen *et al.*, 2014; Shen *et al.*, 2014; Yu *et al.*, 2015
Olomoucine	29	Cdk1, Cdk2	↑	Terano *et al.*, 1999; Chen *et al.*, 2010; Schad *et al.*, 2011; Zhang *et al.*, 2013; Zhang *et al.*, 2014
Benzylphosphonic acid	27	tyrosine phosphatases	↑/↓	Chang *et al.*, 2004; Chang *et al.*, 2006; Kandadi *et al.*, 2010
L-p-bromotetramisole	25	alkaline phosphatase	n.d.	Buchet *et al.*, 2013; Liu *et al.*, 2016
		tyrosine phosphatases	↑/↓	Chang *et al.*, 2004; Chang *et al.*, 2006; Kandadi *et al.*, 2010
LFM-A13	24	BTK, Plk, Jak2	↑	Satterthwaite *et al.*, 1998; Zhao *et al.*, 2013; Sur *et al.*, 2016; Wang *et al.*, 2016
Daidzein	24	*arrests cell cycle at G1*	↓	Dubey *et al.*, 1999; Pan *et al.*, 2001; Liu *et al.*, 2006
LY294002	23	PI3-kinases	↑	Saward and Zahradka, 1997; Goncharova *et al.*, 2002; Silfani and Freeman, 2002; Lipskaia *et al.*, 2003; Kristof *et al.*, 2005; Walcher *et al.*, 2006
Tyrphostin AG 1478	21	EGFR	↑	Kim *et al.*, 2009a; Freeman *et al.*, 2010; Robinson and Pitcher, 2013; Rodríguez-Moyano *et al.*, 2013; Savikko *et al.*, 2015
2-Aminopurine	17	PKR	↓	García *et al.*, 2007; Handy and Patel, 2013
GW5074	16	Raf-1	↑	Watanabe *et al.*, 2001; Zhang and Liu, 2002; Davis *et al.*, 2012

CK2 = protein kinase CK2 (casein kinase), Cdks 1,2 and 9 = cyclin-dependent kinases 1,2 and 9, Src family kinases = Src, Fyn, Hck and Lck, JNKs = c-Jun N-terminal kinases, p38 MAPKs = p38 mitogen-activated protein kinases, BTK = Bruton's tyrosine kinase, Plk = Polo-like kinase, Jak2 = Janus kinase 2, PI3 kinase = phosphoinositide 3-kinase, EGFR = epidermal growth factor receptor, PKR = ds-RNA-activated protein kinase, Raf-1 = RAF proto-oncogene serine/threonine-protein kinase. In cases when little data are available in VSMCs, the table was augmented with information of kinase/phosphatase effects on cell proliferation in other cell types. The table is from Study III.

dexmedetomidine-induced proliferation response by 16 to 32 % when compared to vehicle-treated control cells. Their potential target kinases and phosphatases are listed in Table 4, and include receptor (EGFR) and cytosolic (Src, BTK) tyrosine kinases, serine/threonine (Ser/Thr) kinases (p38 MAPK, CK2, JNK, Cdks, Raf-1) as well as phosphatases (calcineurin, protein

tyrosine phosphatases) known for their important functions in cell signaling and regulation of cell proliferation, differentiation, migration and apoptosis.

A kinase activity profiling study using PamChip microarrays was performed as a broad screen of the kinases participating in the regulation of VSMC proliferation,. The aim was to investigate the activity profiles of protein tyrosine kinases (PTK) and serine/threonine kinases (STK) in A7r5-α_{2B} VSMCs after 5 min, 30 min, 2 h or 24 h stimulation with the agonist dexmedetomidine, and to further identify signaling proteins/pathways involved in the generation of the α_{2B}-AR-evoked proliferation response. Preliminary Western blot experiments were performed to evaluate the early time course of the phosphorylation of the MAP kinases ERK1/2 and p38 following dexmedetomidine stimulation (Figure 18). Strong phosphorylation was detected already at 5 min and the response lasted up to 30 min. Based on these results, 5 min and 30 min time points were selected for the evaluation of primary kinase activities. The later time points were selected based on stimulation/ treatment times that had been reported in the literature.

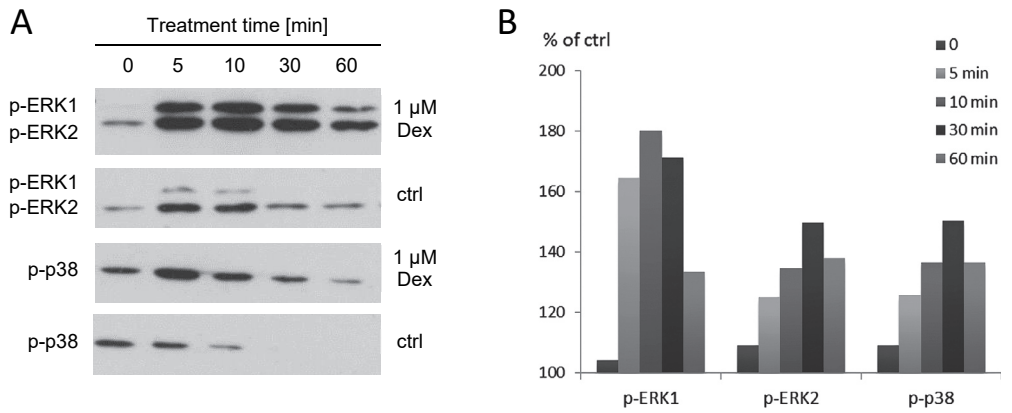

Figure 18. Detection of ERK1/2 and p38 phosphorylation upon dexmedetomidine stimulation in A7r5-α_{2B} VSMCs. (A) Western blot membranes showing p-ERK1/2 and p-p38 immunoreactivity in A7r5-α_{2B} cells treated with vehicle or 1 µM dexmedetomidine (Dex) for 0, 5, 10, 30 or 60 min; n = 1. (B) Western blot densitometry analysis of p-ERK1/2 and p-p38 expressed as percentage of vehicle controls.

Each chip included 144 peptides representing known phosphorylation sites of PTKs and STKs. Kinase activity profiling of dexmedetomidine- vs. vehicle-treated A7r5-α_{2B} cells resulted in clear activity signals; out of the 144 peptides, 96 peptides on the PTK chip and 98 peptides on the STK chip were detected above the threshold level in one or more of the samples. Figure 19 shows statistically significant ($p < 0.05$) effects of dexmedetomidine exposure at different time points on the PTK (A) and STK (B) chips, being either significantly increased (green) or decreased (blue) compared with vehicle-treated control samples. Altered kinase signaling was most pronounced at 30 min, where decreased phosphorylation (compared to vehicle-treated controls) of altogether 40 peptides (36 and 4 on the PTK and STK chips, respectively) was detected. The generated PTK and STK activity profiles showed that dexmedetomidine stimulation induced transient decreases of kinase signaling at the early time points, 5 min and 30 min, which then recovered at the later time points resulting

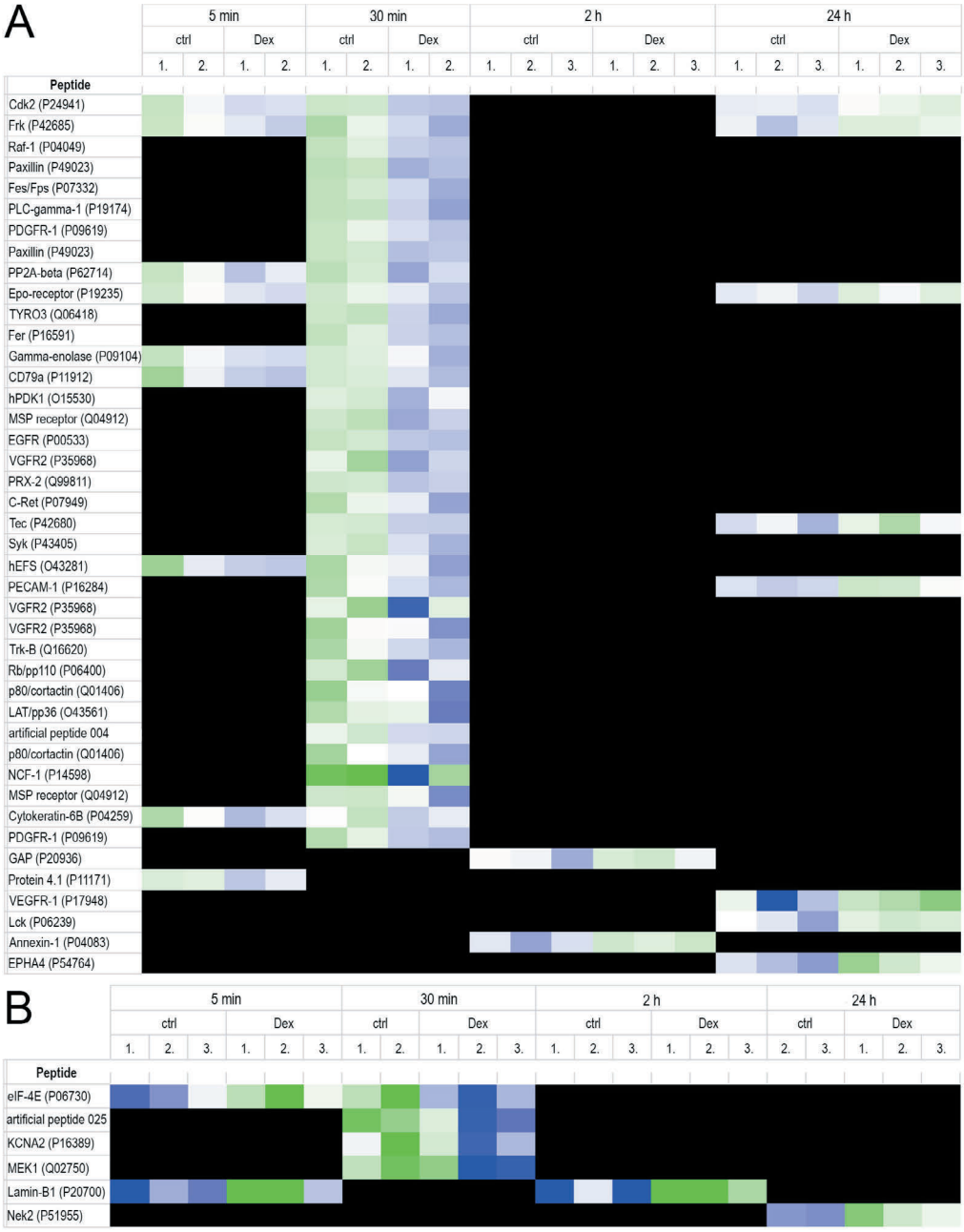

Figure 19. Protein tyrosine kinase (PTK, panel A) and serine/threonine kinase (STK, panel B) activity profiles at 5 min, 30 min, 2 h and 24 h in A7r5-α_{2B} VSMCs treated with 100 nM dexmedetomidine (DEX) or vehicle (ctrl). Green and blue colors indicate increased or decreased kinase activity, respectively, in dexmedetomidine-treated cells when compared with vehicle-treated controls. Black color indicates no differences between dexmedetomidine-treated cells and vehicle-treated controls. The replication CV of high signal spots on the PTK and STK chips was < 20%, indicating good experimental quality. *n* = 3 per time point; except for t = 5 min in the PTK experiment, t = 30 min (ctrl) and t = 24 h (ctrl) in the STK experiment, where *n* = 2. The figure is from Study III.

in slight activation of kinase signaling at 24 h. Changes in kinase activities were more substantial on the PTK chip: decreased phosphorylation of 9 peptides or 36 peptides was detected after 5 min or 30 min of dexmedetomidine exposure, respectively. On the STK chip, increased or decreased phosphorylation was detected in 2 (5 min) and 4 peptides (30 min), respectively. After 24 h of dexmedetomidine exposure, slightly increased kinase activities could be detected; increased phosphorylation of 8 peptides on the PTK chip and 1 peptide on the STK chip was seen. The effects of dexmedetomidine treatment on the STK chip were rather limited, with very few individually significant peptides, which can be explained by the fact that tyrosine kinase signaling, as seen on the PTK chip, is more typical for neurotransmitter receptors than Ser/Thr signaling (Hunter, 2009).

The kinase phosphorylation activities measured on peptides showing different responses on the PTK and STK chips were further evaluated by canonical pathway analysis (GeneGo MetaCore™) at 30 min, where kinase signaling was clearly decreased, and at 24 h, where kinase activity was slightly increased. At the 30 min time point, significant decreases of Raf-1, MEK1 and MEK2 phosphorylation were detected following dexmedetomidine-induced α_{2B}-AR activation. The Raf-1−MEK−ERK pathway is one of the best characterized MAP kinase signaling pathways, known to regulate cell proliferation (Zhang and Liu, 2002). Furthermore, phosphorylation of EGFR, known to provoke the activation of MAPKs, was significantly decreased. In addition, decreased phosphorylation of linker for activated T cells (LAT), platelet-derived growth factor receptor (PDGFR), erythropoietin receptor (Epo-receptor), spleen tyrosine kinase (Syk), PLC isoform γ (PLCγ), phosphoinositide-dependent kinase (PDK) and the tyrosine protein kinase Fer were detected. More than 10 pathways were identified as significant (−log(p) < 7), such as “Signal transduction_ IP3 signaling”, “Development of EPO-induced PI3K/AKT pathway and Ca^{2+} influx” and “Development of EPO-induced MAPK pathway”. The Raf−1−MEK1−MEK2 signaling cascade appeared in most of these pathways; Raf-1 phosphorylates MEK1 and MEK2, and dexmedetomidine treatment (30 min) decreased the activity of these kinases. Also interactions of PLCγ with LAT, PDGFR or Syk and interactions of the Epo-receptor with Syk and PLCγ were suggested by the pathway maps. In addition, EGFR, PDK and Fer appeared in many of the significant pathways. At the 24 h time point, increased phosphorylation by a total of 9 kinases was observed (Figure 19), indicating delayed α_{2B}-AR-evoked increases in kinase activity. However, all pathways identified by the canonical pathway analysis at this time point had low statistical significance (−log(p) < 4). Receptor tyrosine kinases and cytosolic kinases showing increased phosphorylation activity in the identified pathways included Epo-receptor, lymphocyte-specific protein tyrosine kinase (Lck), cell division protein kinase 2 (Cdk2), serine/threonine (Ser/Thr) kinase Nek2 and vascular endothelial growth factor receptor 1 (VEGFR1). The phosphorylation of target peptides by Lck, Nek2 and VEGFR1 was clearly increased, whereas Cdk2 and Epo-receptor showed lesser extents of activation.

The canonical pathway analysis disregarded different phosphorylation sites in the same protein. Therefore, an additional putative upstream kinase analysis was performed using a computational tool that was able to link differentially phosphorylated peptides to the upstream kinases that might be responsible for their phosphorylation. At 30 min, an overall decrease in kinase signaling due to dexmedetomidine-evoked α_{2B}-AR activation was detected. The most specific putative upstream PTKs included C-terminal Src kinase (CSK),

Src family kinase Fgr, hepatocyte growth factor receptor (HGFR; c-MET), EGFR, tyrosine kinase Abl2 (also known as Arg) and receptor tyrosine kinase ErbB-2. In addition to Fgr, all other eight members of the Src family kinases were among the putative upstream kinases (Lyn, Blk, Hck, Src, Fyn, Yes, Frk and Lck). Many receptor tyrosine kinases were included: ErbB-3 and ErbB-4, fibroblast growth factor receptors 1-4 (FGFR1−4), vascular endothelial growth factor receptors 2 and 3 (VEGFR-2 = KDR; VEGFR-3 = Flt4) and tropomyosin receptor kinases A, B and C (TrkA−C). In addition, tyrosine kinase Abl1 and all Tec family kinases (Tec, Btk, Itk, Bmx, Txk) appeared on the list of putative upstream tyrosine kinases. The most specific putative upstream STKs included guanylyl cyclase/natriuretic peptide receptors A and B (GCA/NPRA, GC-B/NPRB), serum/glucocorticoid regulated kinases (Sgk, Sgk2, Sgk3), G protein-coupled receptor kinase 1 (GRK1, RHOK), inhibitor of nuclear factor K-B kinases β and ε and extracellular-signal-regulated kinase 5 (ERK5). Nine isozymes of the PKC family (α, β1, γ, δ, ε, η, θ, ι, ζ) and five cyclin-dependent kinases (Cdk1, Cdk2, Cdk3, Cdk6, Cdk7) were among the putative upstream Ser/Thr kinases. In addition, many members of the MAPK signaling pathways, including Raf-1, MEK1/2, ERK1/2, p38 kinases and JNK kinases, appeared on the list of putative upstream Ser/Thr kinases. After 24 h of dexmedetomidine stimulation, slight activation of kinase signaling was observed. At this later time point, putative upstream tyrosine kinases included many kinases that showed decreased activity at the earlier time points: Abl1 and Abl2, Tec family kinases Bmx and Ltk, receptor tyrosine kinases HGFR, EGFR and VEGFR-2 and several Src kinase family members (Frk, Hck, Yes, Src). Similar changes in kinase activity from decreased activity at earlier time points to increased activity at later time points was also observed for certain putative upstream Ser/Thr kinases, such as several PKC isozymes (α, β1, γ, δ, η, θ, ι, ζ), p38 kinases and JNK kinases. In addition to several PKC isoforms, the most specific putative upstream Ser/Thr kinases included Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMK4), serine/threonine-protein kinase H1 (PSKH1), mitogen- and stress-activated protein kinase-1 (MSK1), cyclin-dependent kinase family member CRK7, cyclin-dependent kinase-like 1, 2 and 4 (Cdkl1, Cdkl2, Cdkl4) and the β-adrenergic receptor kinases 1 and 2 (BARK1/GRK2, BARK2/GRK3).

It was concluded that the cellular mechanisms participating in the α_{2B}-AR-regulated proliferation response of A7r5-α_{2B} VSMCs appeared to be complex and included redundancy. Several genes and kinases, previously implicated in the regulation of cell growth and proliferation, were identified, giving biological significance to the study. The employed screening assays and their respective data analysis approaches were found to be useful as tools to map the activation of cellular signaling networks in a situation where the exact mechanisms still remain unknown. From the screening results, also weaker signals could be identified by combining pathway analysis and integrated approaches. However, no direct mechanistic conclusions about the observed effects were considered warranted, as these changes could have been either causes or consequences of the proliferation response.

6 DISCUSSION

6.1 Considerations of recombinant cell models

6.1.1 A7r5 cell line as a VSMC model

Based on the number of published studies (in the PubMed database), the A7r5 cell line is by far the most commonly exploited VSMC line. Indeed, the A7r5 cell line has several features that make it a good model of VSMCs in culture. It is a clonal smooth muscle cell line originally derived from embryonic rat aorta (Kimes and Brandt, 1976). Although the cells are of embryonic origin and proliferate as myoblasts, once they reach a stationary phase, they differentiate into a phenotype similar to adult VSMCs, and show expression and promoter activity of several highly restricted smooth muscle cell markers (Firulli *et al.*, 1998). A7r5 cells are able to contract in culture by both Ca^{2+}-dependent and -independent mechanisms (Nakajima *et al.*, 1993; Fultz *et al.*, 2000) and they have been demonstrated to display contractile responses to different agonists, such as phenylephrine and vasopressin, and K^{+}-evoked depolarization. Even after prolonged cultivation, A7r5 cells express a set of smooth muscle marker proteins, including α-SMA, SM-MHC and tropomyosins. A7r5 cells also express calponin and SM22α, which are highly specific markers of the differentiated phenotype and necessary for smooth muscle contraction; however, their expression levels are reduced compared with cells derived from primary VSMC cultures (Gimona *et al.*, 2003). In many applications, it is important to differentiate A7r5 cells from the proliferating to the contractile phenotype before performing experiments. It has been shown that serum deprivation induces quiescence of SMCs and promotes differentiation with increased expression of α-SMA, SM-MHC and calponin (Su *et al.*, 2001). Furthermore, in the case of A7r5 cells, placing the cells for 24 h in serum-poor medium is a common procedure to ensure differentiation (Vetterkind *et al.*, 2010). The actin network of proliferating A7r5 cells shows many structural similarities to fibroblasts, much like other smooth muscle cell types that revert to a less differentiated phenotype when cultured. Hence, results obtained from studies with such VSMC lines should be evaluated carefully, as the functionality and regulatory mechanisms of cell lines may be altered compared with highly differentiated VSMCs in the blood vessel wall (Chamley-Campbell *et al.*, 1979; Firulli *et al.*, 1998). Evidently, A7r5 cells have many advantages compared to other VSMC lines, but most importantly, their transfectability is better compared to many other VSMC lines (Firulli *et al.*, 1998). Therefore, A7r5 cells were chosen as the VSMC model system to be employed in Study I, the aim of which was to develop a heterologous VSMC line stably expressing the human α_{2B}-AR. Initially, also a human coronary artery smooth muscle cell line (hCASMC) was tested as a VSMC model system, but due to more challenging cell culture conditions, slow growth rates and very poor transfectability, hCASMCs were considered a non-ideal VSM model system.

6.1.2 Transfection techniques

Recombinant cell expression systems are a convenient way of obtaining cell models where one's receptor of interest is present and functional in a standardized and well defined host cell environment. Such systems have been quite useful for defining the functional and second messenger-coupling behaviors e.g. characterizing the nine subtypes of ARs.

Stable or transient transfection is used to achieve heterologous protein expression in host cell lines, and the choice of expression system depends on the nature and purpose of the study. In stable expression systems, the transfected DNA is integrated into the host cell's genome, and typically employs selection of single-cell clones for further propagation. This technique is time-consuming but has distinct advantages compared to transient transfection for the pharmacological and functional characterization of receptor subtypes (Marjamäki *et al.*, 1992; Daunt *et al.*, 1997; Peltonen *et al.*, 1998; Björk *et al.*, 2005).

The choice of a suitable transfection technique and expression system always depends on the cell type and the molecule to be transfected. Especially for hard-to-transfect cells, the advantages and disadvantages of different transfection techniques should be considered. Maximization of transfection efficiency is critical for many transient transfection experiments. In a comparison of several VSMC lines, the transfectability of A7r5 cells was much better than could be achieved with the other VSMC lines but still remained less than 5 % (Firulli *et al.*, 1998). Transfection efficiencies of 5−20 % have been reported for A7r5 cells using a variety of transfection methods. Consistent with the findings of Study I, lipid transfection reagents have been reported to be inefficient in the transient transfection of A7r5 cells (Lee *et al.*, 2003). Among different lipid transfection reagents, FuGene 6 has been reported to produce the highest transfection efficiencies in hard-to-transfect cells (Kovala *et al.*, 2000). This is in line with the observations of Study I, where FuGene 6 was the least toxic transfection reagent and produced transfection efficiencies of approximately 10 % in A7r5 cells. Subsequently, it has been demonstrated that targeted gene delivery and higher transfection efficiency of smooth muscle cells may be achieved by using a specific peptide (HMGB1) as a gene carrier in the expression vector (Han *et al.*, 2009). Nevertheless, transfection efficiencies less than 50 % are insufficient for many types of experimental applications, such as those involving the expression of signaling molecules mediating signal transduction events following the activation of membrane receptors by extracellular ligands (Kovala *et al.*, 2000).

If one can acquire a stably transfected cell line, this may help to circumvent the low transfection efficiencies encountered with transient transfection protocols. Since adult rat aortic SMCs can be cultured for >100 passages without alterations in their properties, they are generally suitable for the generation of stably transfected cell lines. However, it has been anticipated that the relatively slow growth rate of A7r5 cells may represent an obstacle for the derivation of stably transfected cell lines (Firulli *et al.*, 1998). This observation was made also in Study I. According to preliminary antibiotic selection tests, the growth of non-transfected A7r5 cells was inhibited by antibiotic (G418) concentrations of 200−400 µg/ml, while A7r5 cells transfected with a plasmid containing the neomycin resistance gene tolerated relatively high concentrations of G418 (800 µg/ml), indicating that the generation of stably transfected A7r5 cells by antibiotic selection was possible. A7r5 cells were transfected by nucleofection and heterogeneous cell populations could be successfully propagated under antibiotic selection (Study I). Isolation of clones by limiting dilution was also tried, but no surviving clones could be expanded.

6.1.3 Aspects influencing the use of recombinant cell models

Despite their wide-spread use, certain limitations pertain to the use of recombinant cell models; thus careful validation of such cell models and interpretation of results obtained

from experiments with recombinant cell models are necessary. Endogenous ARs are usually expressed in tissues at relatively low expression levels. For example, the following AR expression levels have been determined from cell homogenates: < 100 fmol receptor / mg protein in the heart (Rohrer and Kobilka, 1998), 450−700 fmol receptor / mg protein in human adipose tissue (Castan *et al.*, 1993) and 60−300 fmol receptor / mg protein in adult rat brain (Happe *et al.*, 1999). Compared to their low endogenous expression levels, recombinant expression systems can achieve expression levels many hundreds of times greater. Thus, the potential exists under these circumstances to saturate preferred G proteins and effector systems, and cross-over into non-preferred signaling pathways becomes possible (Rohrer and Kobilka, 1998). Studies on recombinant α_{2B}-ARs expressed in different cell lines have demonstrated that the receptor is capable of coupling to several signaling pathways (Regan and Cotecchia, 1992). It has been shown that α_{2B}-ARs have the potential to couple physically and functionally not only to G_i but also to G_s proteins (Eason *et al.*, 1992; Näsman *et al.*, 1997; Pohjanoksa *et al.*, 1997). However, as the receptor expression in the generated A7r5-α_{2B} cell line was relatively close to endogenous expression levels, depletion of preferred G proteins and effector systems and crossing over into non-preferred signaling pathways were not considered likely to occur and affect receptor coupling in A7r5-α_{2B} cells. In Study I, possible coupling of α_{2B}-ARs to PTX-insensitive G_s proteins was not directly addressed.

Another aspect possibly affecting the signal transduction of receptors in intact cells is that some sets of receptors, G proteins and effectors may be organized into microdomains and not have access to other sets. This organization may be missing or perturbed in isolated cell membranes that are used to study receptor-evoked signal transduction. However, even transfected whole-cell models might not faithfully reflect the organization of wild-type cells because overexpression of a certain receptor may saturate the receptor's normal compartments and place signaling components into abnormal places (Neer, 1995).

Heterogeneous expression of receptors limits the applicability of a recombinant cell line. Individual cells of a cell population express different receptor densities, and therefore single cell experiments are likely to exhibit extensive variation between repeated measurements. Another example of a situation, where a heterogeneous cell population can produce misleading results is the monitoring of an inhibitory effect, in this case G_i-mediated inhibition of AC activity. Björk *et al.* (2005) demonstrated that a homogeneous cell population stably transfected to express α_{2A}-ARs exhibited concentration-dependent inhibition of forskolin-stimulated AC activity upon receptor activation, as expected. However, a heterogeneous cell population with uneven α_{2A}-AR expression between individual members of the transfected cell population displayed no detectable AC inhibition; 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 (Björk *et al.*, 2005). Such observations underline the importance of careful characterization and validation of recombinant cell models. The generated A7r5-α_{2B} VSMC line was found to be best suited for applications where large numbers (thousands) of cells per sample were used.

In transfected cell lines, such as COS-7, Rat-1, MDCK II, CHO and HEK-293, α_{2B}-AR immunoreactivity has been detected mainly on plasma membranes, and endocytosis

and desensitization of the receptors have also been observed (von Zastrow *et al.*, 1993; Wozniak and Limbird, 1996; Daunt *et al.*, 1997; Hurt *et al.*, 2000). In transfected PC12 cells, α_{2B}-ARs are localized on the plasma membrane and are rapidly internalized following agonist exposure (Olli-Lähdesmäki *et al.*, 1999). It has been reported that α_{2B}-ARs are more prone to internalization than α_{2A}-ARs, and α_{2C}-ARs are predominantly located in intracellular compartments (Philipp *et al.*, 2002a). The subcellular localization of α_{2B}-ARs has also been studied in Semliki Forest virus (SFV)-transduced CHO and BHK-21 cells. Receptors were widely distributed in intracellular compartments, and only a minority of the receptors was detected in the plasma membrane. It was anticipated that due to the great overexpression of the receptor protein, the cellular machinery was likely not capable of delivering all receptors to the plasma membrane (Sen *et al.*, 2003).

Richman and Regan (1998) indicated by means of immunofluorescent microscopy that all three α_2-AR subtypes were present in primary rat aortic SMCs. Interestingly, there was a difference in immunofluorescent reactivity among the receptor subtypes. Although all three receptor subtypes localized diffusely along the plasma membrane, α_{2A}- and α_{2C}-AR subtypes exhibited also nuclear and perinuclear staining patterns, respectively. For the α_{2B}-AR subtype, no subcellular staining was reported (Richman and Regan, 1998). It has been shown in heterologous cell systems that neuronal cells are able to target α_{2C}-ARs to the plasma membrane, whereas this is not the case in non-neuronal cells (Hurt *et al.*, 2000). Differential subcellular localization of receptors may be explained in a number of ways; differential subcellular localization may also be a function of the host cell type (Keefer *et al.*, 1994; Daunt *et al.*, 1997). Therefore, when generating a recombinant cell line, it should be carefully considered whether the host cell line has the appropriate machinery that allows efficient targeting of receptors in the plasma membrane. In the generated A7r5-α_{2B} cell line, α_{2B}-ARs appeared to localize mainly to the plasma membrane, but due to limitations of the applied methods, the exact localization of the receptors remained uncertain. Co-localization staining using a fluorescent cell membrane dye simultaneously with the anti-HA antibody targeting the recombinant α_{2B}-ARs could have been applied, but also in this case, the fact that A7r5 cells are very thin (2−6 µm) and flat could have made it difficult to distinguish between staining of plasma membrane and the cytosol. Electron microscopy or subcellular fractionation might have been useful for the determination of the exact localization of recombinant α_{2B}-ARs in A7r5 cells.

6.2 Measurement of vascular smooth muscle contraction

6.2.1 Methods for the measurement of vascular smooth muscle contraction

Traditionally, VSM contraction has been studied by tension measurements on blood vessels, and also most studies on α_2-AR-mediated smooth muscle contraction have been performed using isometric tension measurements on intact or endothelium-denuded blood vessels. Typically, ring segments of arteries or veins are suspended in an isolated tissue bath, and changes in vascular tension are determined by isometric force measurements (Waen-Safranchik and Deth, 1994; Roberts, 2004; Yamboliev and Mutafova-Yambolieva, 2005; Kim *et al.*, 2009b; Ok *et al.*, 2011; Ok *et al.*, 2014). Similar isometric force measurements on isolated blood vessels can also be recorded with a wire myograph (Nielsen *et al.*, 1989;

Parkinson and Hughes, 1995; Schmidt and Carrillo-Sepulveda, 2015; Nong *et al.*, 2016). An alternative way to measure vasoconstriction is a perfusion cuvette system or perfusion myograph, in which blood vessels are mounted and perfused and vasoconstriction is monitored as increases in perfusion pressure, measured with a pressure transducer (Chotani *et al.*, 2000; Spitzbarth-Régrigny *et al.*, 2000; Flavahan, 2005). Traction force microscopy is a novel method for the measurement of the contractile strength of isolated VSMCs. In this method, cells are cultured on a soft substrate and the force of contraction of isolated VSMCs is quantified by computing the traction force and strain energy applied by the cell on the soft substrate. Characterization of both absolute and relative changes in contractile strength of isolated VSMCs in response to external stimuli can be achieved with this method (Ye *et al.*, 2014). In research practice, most studies on cultured VSMCs have focused on the monitoring of intracellular Ca^{2+} levels (Mironneau and Macrez-Leprêtre, 1995; Hughes *et al.*, 1996; Chotani *et al.*, 2004). Monitoring of MLC_{20} phosphorylation by Western blotting has been applied to investigate the contractile responses and signaling pathways in isolated VSMCs (Aburto *et al.*, 1993; Shimokawa *et al.*, 1999; Sathishkumar *et al.*, 2010). However, the above mentioned methods are in general time consuming, labor-intensive and low-throughput in nature, and are therefore not suitable for screening of large numbers of receptor ligands or signaling pathway inhibitors.

6.2.2 Validity of myosin light chain phosphorylation as an indicator of vascular smooth muscle contraction

In vascular smooth muscle, force of contraction is produced through an interaction between actin and myosin filaments. The primary signal to initiate smooth muscle contraction is myosin regulatory light chain (MLC_{20}) phosphorylation at Ser^{19} ($pMLC_{20}$) by myosin light chain kinase (MLCK) (Kamm and Stull, 1985; Khalil, 2010). $pMLC_{20}$ can be further phosphorylated at Thr^{18} ($ppMLC_{20}$), requiring either very high (unphysiological) concentrations of MLCK or the involvement of other kinases (Ikebe and Hartshorne, 1985; Ikebe *et al.*, 1986; Deng *et al.*, 2001; Niiro and Ikebe, 2001; Wilson *et al.*, 2005; Moffat *et al.*, 2011). However, phosphorylation at Thr^{18} does not increase the force elicited by Ser^{19} phosphorylation, but only slows relaxation of vascular smooth muscle (Sutherland and Walsh, 2012). A linear correlation has been established between the extent of MLC_{20} phosphorylation and smooth muscle contractility, and therefore, phosphorylation of MLC_{20} at Ser^{19} is considered a valid biochemical read-out of SMC contraction. In SMCs, the concentrations of MLCK and its substrate MLC_{20} are approximately 4 µM and 30−40 µM, respectively, and the K_M and V_{max} of MLCK are 3−5 µM and 10−20/s, respectively. With these kinetics and the assumption that MLCK and MLC_{20} are located close enough to interact *in vivo*, MLC_{20} is completely phosphorylated within 1−2 s. This short delay is well matched to the time taken from agonist stimulation to MLC_{20} phosphorylation. In contrast, in skeletal and cardiac muscles such a linear correlation between MLC_{20} phosphorylation and contractility does not exist, because these cells use troponin as a $[Ca^{2+}]_i$ sensor instead of MLCK (Takashima, 2009).

6.2.3 Evaluation of the $pMLC_{20}$ assay

In Study II, a cell-based functional assay was optimized and validated in order to biochemically quantify SMC contraction, using phosphorylation of myosin light chains at Ser^{19} as readout. The assay was based on approaches already validated in earlier

studies (Schröter *et al.*, 2008; Aguilar *et al.*, 2010) and validation of the developed assay was performed with Western blotting, immunostaining and analysis of single-cell measurements of intracellular Ca^{2+} responses. The maximal MLC_{20} phosphorylation response was typically reached at 20−45 s after agonist addition, and $pMLC_{20}$ levels returned to baseline levels by 120 s, highlighting the importance of quick and accurate sample handling. The maximal response evoked by dexmedetomidine was approximately 60 % over the control value measured in vehicle-treated control cells, and the endogenous agonist arginine vasopressin evoked responses of similar magnitude. Thus, the magnitude of the agonist-evoked MLC_{20} phosphorylation response was not very large, which is due to many contributing factors. (1) A7r5 VSMCs exhibited rather high basal levels of MLC_{20} phosphorylation and different external stimuli, such as pipetting or shaking the plate, further increased the basal level of $pMLC_{20}$. When one considers the VSMCs in their natural environment, the blood vessel wall, it is easily understood that various stimuli, such as temperature, pressure and flow, can evoke contraction (Shepherd *et al.*, 1983; Bevan *et al.*, 1990; Bevan and Joyce, 1990). In order to keep the basal level of $pMLC_{20}$ as low as possible, handling of the assay plates was kept to a minimum. Nonetheless, results had to be normalized to vehicle-treated controls, thus masking a significant proportion of the actual response. Only simultaneously treated samples were truly comparable, and therefore, the layout of an assay plate had to be designed in such way that each sample had all necessary controls in the same row. (2) DMSO, used to dissolve some inhibitors used in the study, also attenuated the maximal response $pMLC_{20}$ induced by dexmedetomidine. Inhibitors are commonly employed to deduce the role of specific proteins in the intracellular signaling pathways. Nevertheless, inhibitors may give inconsistent results because of the complexity of the involved pathways and signaling networks, toxicity, limited solubility and lack of specificity (Davies *et al.*, 2000). It was important to use freshly prepared inhibitor solutions in all experiments, and the cells had to be visually inspected to confirm their viability after inhibitor treatments. Even although the use of inhibitors is not problem-free, in many cases, it is still the most feasible approach to investigate the involvement of specific proteins in the intracellular signaling cascades of receptors. An alternative approach could be to apply siRNA technology, but this is more laborious and most likely would require considerable optimization efforts because of the fact that A7r5 VSMCs are not easily transfected. Moreover, the ability of VSMCs to contract is crucial for the assay. It is well known that confluency is important for the contractile function of VSMCs. *In vitro* VSMCs with a high number of cell−cell contacts exhibit a more contractile, *in vivo*-like behavior than sparsely seeded VSMCs (Cao *et al.*, 2010), and VSMCs with an *in vivo*-like alignment express a more contractile phenotype than disorganized but confluent cells (Williams *et al.*, 2011). It has also been reported that confluent and serum-deprived VSMCs show marked contraction upon agonist stimulation, whereas serum-deprived subconfluent cells or cells (whether subconfluent or confluent) under normal serum-containing culture conditions do not respond to agonist stimulation (Han *et al.*, 2006). In line with these findings, also in this study, it was observed that cell confluency and serum deprivation were of great importance in order to have contractile A7r5 cells in the assay. In particular, confluent A7r5 cells forming hill-and-valley patterns (i.e. *in vivo*-like alignment) typically had good contractile responses to dexmedetomidine stimulation. Therefore confluency was always assured by visual inspection before performing the experiments.

6.3 General aspects of screening methods

Cell-based screening assays are commonly used tools in research and drug discovery. With screening methods, it is possible to conduct a vast number of genetic, chemical or pharmacological tests in a relatively short period of time, and to quickly identify active compounds, antibodies, kinases or genes that modulate a particular biomolecular pathway. Screening results provide a starting point for drug design and for gaining better understanding of the roles or interactions of particular signaling pathways and biochemical processes in cells and organisms. The combination of biochemical and cell-based screening assays to perform hit identification and lead optimization has been a growing trend in drug discovery, because appropriate cellular assays are more physiologically relevant than isolated target approaches. In relatively recent surveys, 80−90 % of high-throughput screening (HTS) laboratories reported running both biochemical and cellular assays, with 50−60 % of all primary screens being cellular or pathway assays (Macarron *et al.*, 2011). However, the selection of the cell line for expression of the target molecule has often been based more on habit and technical feasibility (e.g. HEK-293 and CHO) than physiological or disease relevance, and presumably, this has contributed to the lack of efficacy for some of the compounds in subsequent *in vivo* testing. Primary cells and human stem cells have emerged as alternative tools to remove this bias and present the target molecules in a more physiological context. Indeed, the use of primary cell systems for drug development and discovery has become more and more popular, and these are being adapted to the field of high-content screening (HCS) to deliver more physiological data sets and provide access to new disease models. Unlike standard HTS, where one readout is used to monitor one target/effect, HCS can address multiple readouts simultaneously. For instance, GPCR drug discovery has benefited from the addition of HCS to an already comprehensive assay collection; in recognition of native GPCR signaling and cell-dependent signal trafficking, HCS allows for the assessment of ligand activation to various key pathways in parallel, thus aiding in the selection of compounds for further optimization (Möller and Slack, 2010).

Independent of the applied screening assay, it should be kept in mind for any cell model system that the cells have been isolated from their natural environment, and may therefore be lacking extracellular regulators that under normal conditions impact the function of these cells. For example, VSMCs are in their natural environment in close interaction with endothelial cells of the intima and fibroblasts of the adventitia. The endothelial layer in particular can influence the function of VSMCs by releasing vasodilating factors (NO and prostacyclin), contraction-inducing factors (e.g. endothelin and AT-II) and growth inhibitors or promoters (e.g. heparin and PDGF) (Rajendran *et al.*, 2013). Such interactions are missing when VSMC function is studied using established cell lines or isolated primary cells.

The aim of Study III was to broadly explore changes in gene expression, signaling pathways and kinase activation profiles related to α_{2B}-AR-evoked proliferation of A7r5 VSMCs by making use of three different cell-based screening approaches, namely DNA microarray, kinase/phosphatase inhibitor small compound library screening and peptide microarray. All of these screening methods have their own advantages and drawbacks, and they are aimed at different levels of the cellular signaling mechanisms. A combination of these diverse and potentially complementary approaches was expected to generate novel

hypotheses for testing, and to provide mechanistic insights into how α_{2B}-AR-evoked VSMC proliferation takes place.

6.3.1 DNA microarray

Gene microarrays enable the analysis of mRNA expression levels and are of significant value for the elucidation of molecular mechanisms that govern cellular physiology. The use of DNA/RNA microarrays in genomics has led to a fast and reliable way to screen the transcriptome of an organism; it may be automated, analysis tools are readily available and the approach is widely used (Peppelenbosch, 2012). The predominate application of DNA microarrays has been to measure gene expression levels, but microarrays have also been widely used as single-nucleotide-polymorphism genotyping platforms. However, some limitations pertain to the use of DNA microarrays. They provide an indirect measure of relative concentrations, meaning that at high concentrations, the array will become saturated and at low concentrations, equilibrium favors no binding. Therefore, the signal is linear only over a limited range of concentrations in the solution. In addition, particularly for complex mammalian genomes, it is challenging to design arrays in which genes that exhibit significant sequence homology will not bind to the same probe on the array. Moreover, a DNA array can only detect those sequences that the array was designed to detect; typically separate DNA microarrays are designed for different animal species, which usually means that genes that have not yet been annotated in a genome will not be represented in the array (Bumgarner, 2013). Moreover, unlike the genome, which is quite fixed for a given cell line, the transcriptome can vary with external environmental conditions. The transcriptome includes all mRNA transcripts in the cell, and therefore, it reflects the genes that are being actively expressed at any given time. However, only a small fraction of all transcripts present in the cell determines the identity of the cell, and these critical transcripts may be expressed at low levels. Therefore, small changes in the expression profiles in the transcriptome can lead to large changes in the cell phenotype, e.g. leading to significant differences in cell function (Velculescu, 1999; Velculescu *et al.*, 1999). Improved sequencing technologies have emerged adopting a more direct and relatively unbiased approach for measuring all DNA/RNA species present in a solution. While sample preparation or different enzymes may bias sequencing counts, sequencing, unlike DNA arrays, is not dependent on prior knowledge of which nucleic acids may be present. Consequently, DNA arrays have been rapidly replaced by sequencing for nearly every assay that has been previously performed on microarrays. It has even been predicted that DNA arrays could be fully replaced by sequencing methods within the next decade (Bumgarner, 2013).

6.3.2 Inhibitor library screening

Protein kinases represent a large group of structurally and evolutionarily related enzymes that regulate the function of other proteins by phosphorylation of serine, threonine, or tyrosine residues in the target protein. Protein kinases play a crucial role in the regulation of virtually all cellular processes, including cell growth, proliferation and differentiation. In particular, knowing which kinase signaling pathways are being utilized in specific cells is of major importance for understanding the mechanisms behind different biological/physiological and pathological events (Manning *et al.*, 2002; Noble *et al.*, 2004; Schutkowski *et al.*, 2005; Peppelenbosch, 2012). Indeed, kinases have become attractive therapeutic

targets − in fact, they have become the second most important group of drug targets after GPCRs (Cohen, 2002). Currently, 40 small-molecule kinase inhibitors have been approved for clinical use by the FDA, more than half of which have been approved in the past five years (Rudolf *et al.*, 2014; Wu *et al.*, 2015b; Baharani *et al.*, 2017). An additional 500 inhibitors are under investigation in more than 3000 clinical trials, and thousands of other inhibitors are under investigation in various stages of preclinical development (Baharani *et al.*, 2017).

Small-molecule inhibitors enable the probing of protein function in a highly controlled and pharmacologically relevant way and are, therefore, valuable tools for investigating the biology and therapeutic potential of specific kinases. Nevertheless, a major disadvantage is typically their lack of specificity, as highly specific inhibitors are available for only a very small fraction of all protein kinases. Apart from their intended target proteins, kinase inhibitors often interact with multiple other members of the protein kinase family, and therefore, an approach purely based on inhibitor compounds is often unable to define the specific function of an individual target. A thorough characterization of the full spectrum of action of inhibitor molecules would be required before their effects on a complex biological system could be reliably interpreted, as systematic profiling of well-known inhibitors has revealed diverse interaction patterns across the kinome, including undescribed activities and other previously unknown targets. In fact, the biological activity of such inhibitors may be a result of a complex balance of inhibition of multiple targets, and therefore, it is not a straightforward task to use kinase inhibitors to unravel signaling pathways with high selectivity. It is recognized that more specific inhibitors are required in order to dissect signaling pathways and to validate drug targets. For the time being, there is not enough comprehensive data available to explain biological findings, especially those related to the effects of inhibitors used in different screening assays. This lack of data is hampering the interpretation of biological observations and has also often lead to incorrect conclusions (Shokat and Velleca, 2002; Knight and Shokat, 2005; Davis *et al.*, 2011).

The pharmaceutical industry has made great efforts to design curated compound libraries, including specific kinase-focused sub-libraries with thousands, even tens of thousands of compounds. These libraries are the foundation for kinase inhibitor discovery efforts, but, although they are chemically well characterized, their functional annotation and understanding of kinome-wide inhibition potential is generally limited and is being enhanced only slowly as screens against additional individual targets are performed over time (Goldstein *et al.*, 2008). High-throughput profiling of such kinase-focused libraries may be used to systematically determine the chemical structure−kinase activity relationships against the kinome, thus enabling the identification of novel inhibitor−target interactions. Fully annotated (small-sized) compound libraries, on the other hand, may be used to dissect the signaling pathways related to a specific cellular response, but even with such targeted use, the possibility of novel, uncharacterized biological findings should be kept in mind.

Commercial inhibitor libraries are applicable to different types of studies, as any desirable readout can be selected to evaluate the effects of the inhibitors, and in general data analysis of the primary results is very straightforward. In Study III, an inhibitor library

screening was used to investigate the signaling pathways participating in the α_{2B}-AR-evoked proliferation response in A7r5 VSMCs using BrdU incorporation as readout of the proliferation response. In another study, the same 84-compound library consisting of kinase and phosphatase inhibitors was used to identify unreported inhibitors and activators of phosphoinositide 3-kinases (PI3K), since abnormal regulation of PI3K activity has been implicated in diseases such as cancer and diabetes (Stankewicz and Rininsland, 2006). In yet another study, screening of this library was performed as a starting point in the search of new inhibitors for PRK1 (protein kinase C-related kinase 1) which is considered to be a promising target for the treatment of prostate cancer (Köhler *et al.*, 2012). Thus it is clear that very different applications are possible; for instance, a similar small library of commercial protein kinase inhibitors was evaluated for effects on pairing and egg production by specific parasitic flatworms as well as their toxicity towards flatworm larvae (Morel *et al.*, 2014).

The selectivity of kinase inhibitors may be characterized by screening the compounds against a panel of kinases, but it has been demonstrated that small assay panels (tens of kinases) do not provide a robust measure of inhibitor selectivity. It is rather common that kinase inhibitors exhibit diverse interaction patterns, and inhibitors may have other potential and therapeutically useful targets that remain unidentified if screening is performed with small-sized compound libraries. In some cases, the discovery of such novel, potentially clinically important targets has been made only after a drug candidate has already entered clinical trials for other indications (Karaman *et al.*, 2008). A study evaluating the specificity of 156 validated kinase inhibitors (including inhibitors used in clinical trials) against 60 human Ser/Thr kinases revealed many unexpected interactions for inhibitors thought to be specific for certain targets. Novel inhibitor targets possibly leading to alternative clinical applications were also uncovered, e.g. a PKCβ inhibitor (LY333531), that had already entered phase III clinical trials was found to efficiently inhibit PIM1 kinase, a suggested target for the treatment of some forms of leukemia (Fedorov *et al.*, 2007). In another study, 158 compounds initially identified as potent and specific inhibitors of kinases regarded as important therapeutic targets and/or signal transduction regulators were screened against 234 human kinases, representing all branches of the kinome tree. The results revealed several inhibitors that inhibited with high potency previously unidentified targets, and these off-target effects could be seen across the kinome for both Ser/Thr and tyrosine kinases. This has major implications for drug discovery and so-called polypharmacology strategies which seek to target multiple pathways or multiple targets within a pathway. Moreover, the interpretation of results obtained with inhibitors to define signaling mechanisms needs to be considered cautiously when the activity profiles of the employed inhibitors have not been thoroughly investigated (Gao *et al.*, 2013). Recently, an integrated experimental and computational method was reported to be able to clarify the roles of specific kinases in the drug response of a given cell line. The method incorporates profiled libraries and *in vitro* measurements to predict the response of cells to previously untested drugs. Apart from making predictions about the cellular responses to drugs, the method may be used to identify critical kinase targets and pathways that are statistically associated with drug sensitivity in a given cell line, thus facilitating the design of new kinase inhibitors and the development of therapeutic interventions with combinations of many inhibitors (Tran *et al.*, 2014).

6.3.3 Peptide microarray

Traditional genetic and biochemical approaches that are used to investigate kinase signaling are for technical and practical reasons typically pursued one gene or pathway at a time. However, a comprehensive description of cellular metabolism and definition of those proteins that participate in signaling pathways that are active in cells could be a more useful and informative approach. To some extent, this can be achieved by monitoring the activation of kinases or phosphorylation of their intracellular protein substrates. A protein microarray should be appropriate for this purpose, but proteins are generally difficult to handle due to their poor stability and the laborious preparation required. Peptides, on the other hand, are chemically more stable and relatively easy to design, prepare and handle, and in many cases the biological activities of proteins can be substituted by shorter peptides that are extracted from the primary sequence. Thus, the peptide microarray has become a readily available, promising tool for analyzing protein functions and the activation of kinases (Shigaki *et al.*, 2007; Hilhorst *et al.*, 2009; Hilhorst *et al.*, 2013; Li *et al.*, 2013; Grötzinger, 2016; Lin *et al.*, 2016).

Peptide arrays detecting kinase activity from cell lysates have proven to be convenient and suitable for a dynamic and comprehensive analysis of protein phosphorylation. Their simplicity and high throughput make peptide microarrays especially advantageous for practical applications, e.g. monitoring of drug effects on intracellular protein kinase activity (as a tool in drug development), and for the diagnosis and follow-up of diseases associated with altered kinomics. In addition, peptide arrays with cell lysates are useful for drug screening purposes, because the target protein kinases can be assayed without any isolation or purification of the enzymes (Schutkowski *et al.*, 2005; Shigaki *et al.*, 2007; Yamamoto *et al.*, 2014). The peptide array technology can also be used for the analysis of the kinome. Spotting consensus substrate peptides for kinases on a solid support, incubation with cell lysates and detection with radioactive or fluorescent peptides makes it possible to determine the kinases that are active in the assay system. Multiplexed treatment of peptide microarrays with cell lysates generates snapshots of the actual phosphorylation equilibrium within cells and reflects the activity of kinases and phosphatases. This should facilitate novel diagnostic concepts based on phosphorylation fingerprints (Schutkowski *et al.*, 2005). Moreover, peptide arrays with cell lysates allow analysis of cellular signaling without *a priori* assumptions of possibly influenced pathways (Peppelenbosch *et al.*, 2016). Eukaryotic protein kinases form a large superfamily of homologous proteins and their kinase domains (catalytic domains) are rather well conserved, making peptide arrays suitable for the analysis of cell lysates independent of their origin in terms of tissues and species (Hanks and Hunter, 1995; Peppelenbosch, 2012). This is a great advantage compared to DNA microarrays, where arrays are performed on species-specific chips.

Overall, there is growing interest in cellular kinase activity analysis, because it provides valuable possibilities to gain insights into complex biological and pathological events. Kinomics has become an efficient approach for understanding basic biology, such as elucidating active signaling pathways, identifying novel substrates, understanding the molecular mechanisms of complex phenotypes and pathological states and characterizing host-pathogen interactions. In addition, kinomics has made important contributions toward clinical applications, including the discovery of prognostic, therapy-predictive and

pharmacodynamic biomarkers. Moreover, with the growing use of kinase inhibitors as therapeutic agents, the application of kinomics for identifying therapeutic targets and understanding the mode of action of therapeutic compounds has become increasingly important. This has fueled efforts to develop and optimize technologies that enable characterization of phosphorylation-mediated signaling events in a cost-effective, high-throughput manner (Baharani *et al.*, 2017).

The PamChip system is an alternative to previous peptide microarray approaches. In this system, peptides are immobilized onto a porous matrix and cell lysates are repeatedly pumped through the matrix, providing active kinases with the opportunity to phosphorylate their peptide substrates in a manner analogous to the peptide arrays. Each round of pumping results in further phosphorylation of the peptides that can be detected and quantified in real time using fluorescently labeled phosphospecific antibodies. This system has been postulated to provide sensitive and accurate measurement of kinase activity, but the PamChip arrays include only 144 peptides, whereas previously employed peptide arrays typically include more than 1000 peptides (Hilhorst *et al.*, 2013; Baharani *et al.*, 2017). As with any experimental approach, also kinomics has the potential to introduce bias or experimental artifacts that could obscure the insight into true biology. Therefore, kinomics should best be used to suggest biological events for subsequent validation through independent techniques, including phosphorylation-specific antibodies, kinase inhibitors, and functional assays. In Study III, PamChip microarrays were applied to explore kinase activation profiles in VSMCs evoked by activation of α_{2B}-ARs exposed to dexmedetomidine for varying periods of time in order to identify kinases and/or signaling cascades involved in the α_{2B}-AR-evoked proliferation of VSMCs. Based on the published literature, this is not a very typical application of this array system. PamChip microarrays have been used in different kinds of studies, e.g. in the evaluation of the inhibitory activities of kinase inhibitors (Mori *et al.*, 2008), in kinase activity profiling in zebrafish embryos (Lemeer *et al.*, 2007) and in substrate identification and kinetic characterization of protein kinases (Hilhorst *et al.*, 2009). In recent years, one of the most popular applications of these arrays has been the identification of potential drug therapy targets and altered kinase activity in different diseases, such as many types of cancers, schizophrenia and Alzheimer's disease (Hoozeman *et al.*, 2014; McGuire *et al.*, 2014; Ree *et al.*, 2015; Stoltz *et al.*, 2015; Risberg *et al.*, 2016).

6.3.4 Aspects related to signaling mechanisms and biological networks

Signal transduction has traditionally been described by canonical signaling pathways consisting of a specific set of proteins, compartmentalized, hierarchical and independent from the rest of the proteome, and it has been applied to explain the properties and functions of biological systems. It has long been recognized that connections between some pathway components and other distinct pathways may exist. Indeed, in the past two decades, hundreds of potential regulators of known signaling pathways have been identified. Based on the wealth of genetic and biochemical data, it has become evident that cellular/biological processes are coordinated by a much larger network of proteins than previously thought. Two major improvements to the traditional canonical view of signaling pathways have been proposed: (1) a remarkable increase in the number of proteins linked to a particular pathway, and (2) an appreciation for the variable

quantitative contribution of each of these components to dynamic signal propagation. The composition and feedback control of a cellular network determine how extracellular signals are transmitted through a dense network of proteins and how this network responds to perturbations. For example, removal of a protein through which 20 % of the input signal propagates when measured locally, may lead to compensation by other proteins, resulting in only a miniscule change when measured more distantly. Therefore, signal transduction should rather be re-contextualized within network biology than kept confined by the traditional canonical pathways (Friedman and Perrimon, 2007).

Due to the complexity of cellular signaling networks and the inherent robustness of biological systems, perturbation of a single protein may lead to only minor, if any, alterations in cellular function. In many cases, cellular mechanisms and signaling pathways regulating a specific function, such as proliferation or differentiation, include redundancy and compensatory mechanisms. Signaling networks involved in animal development are well-known examples of biological complexity and robustness, since the development of a normal individual is highly resistant to both environmental influences and intrinsic perturbations (e.g. genetic mutations). For example, Drosophila embryos from females with several extra copies of a maternal effect gene have disproportionate body parts but still develop into rather normal adults. Another example is the number of mutated genes found in cancerous tumors; on the average, 90 mutated genes are found in a cancer but only less than 20 % of these are causative, suggesting that a combination of many mutations is required to overwhelm the robust cell signaling network (Friedman and Perrimon, 2007). Studies on kinase inhibitors have demonstrated that, apart from the complex interaction patterns related to many inhibitors as such, intracellular signaling pathways contain considerable redundancy such that single drugs might not be able to overcome the robustness of biological networks (Feala *et al.*, 2012; Tran *et al.*, 2014). If one pathway is blocked with an inhibitor, the cell may find several compensating mechanisms to bypass the inhibited route. For instance, a given receptor may mediate its effects through several different signaling mechanisms, and parallel mechanisms may compensate for the effects of a blocked signaling route.

Also in Study III, it was observed that no single inhibitor compound (of the 84-compound library) could completely block the proliferation response; even with the most effective inhibitors, proliferation was inhibited by less than 30 %. Indeed, for many kinase inhibitors it is anticipated that their optimal effect, e.g. to affect a disease, will require combination with drugs acting on other targets or other types of therapy. Therefore, there has been increasing interest in combining different inhibitors that selectively target individual kinases, as well as in compounds that inhibit multiple kinases. Simultaneous inhibition of multiple targets may prevent genetically unstable tumors from developing resistance, or may allow the same compound to be used for more than one indication with different relevant targets (Goldstein *et al.*, 2008). Moreover, it should be kept in mind that a protein's activity can be perturbed in different ways depending of its biological functions. For example, JNK2 knockout cells exhibit normal c-Jun phosphorylation and increased proliferation, whereas chemical inhibition of JNK2 blocks c-Jun phosphorylation and proliferation. A cellular compensation mechanism is suggested to explain this; the JNK2 gene deletion is suggested to lead to a compensatory increase in JNK1 activity and c-Jun expression (Knight and Shokat, 2007).

Another aspect complicating the investigation and understanding of intracellular signaling mechanism is the fact that many drug molecules elicit their effects by modulating multiple cellular targets (Hopkins, 2008). The efficacy and toxicity of drugs, whether designed as single- or multi-target therapeutics, often result from complex interplay between different signaling cascades, initiated by multiple drug-target interactions and then propagated throughout the whole cellular network. Pharmacodynamic, pharmacokinetic, genetic, epigenetic, and environmental factors may be involved. An observed drug response is not only based on the intended primary targets, which produce the "on-target" therapeutic effect, but also on unintended secondary targets, which may be responsible for unwanted "off-target" effects that may either cause adverse effects or contribute to the therapeutic effect (Xie *et al.*, 2012; Futamura *et al.*, 2013). Network models have recently gained much popularity in exploring the system-level mechanisms behind drug action, resistance, and potential side-effects by connecting chemical compounds and their targets in the context of biological networks and interconnected pathways (Hopkins, 2008; Zhao and Iyengar, 2012). Improved understanding and prediction of such polypharmacological effects is crucial for the development of more effective and less toxic drug therapy either by multi-target drugs or targeted drug combinations. Multi-target treatments are regarded as a promising approach to tackle the compensatory mechanisms and robustness of cellular systems (Kitano, 2007; Knight *et al.*, 2010).

6.4 Signaling pathways involved in α_{2B}-adrenoceptor-evoked responses in VSMCs

6.4.1 Contraction

In addition to developing an assay allowing the assessment of the capacity of α_2-AR ligands to evoke or inhibit VSMC contraction, the aim of Study II was to investigate the intracellular pathways activated upon α_{2B}-AR stimulation and ultimately leading to MLC_{20} phosphorylation and VSMC contraction. Even though α_2-ARs have been demonstrated to regulate a multitude of signaling pathways in different cell types and tissues (Cotecchia *et al.*, 1990; Eason *et al.*, 1992; Leprêtre and Mironneau, 1994; Mironneau and Macrez-Leprêtre, 1995; Wright *et al.*, 1995b; Hughes *et al.*, 1996; Dorn *et al.*, 1997), and the intracellular mechanisms of many vasoactive compounds, such as AT-II, endothelin-1 and vasopressin, have been characterized (Holmes *et al.*, 2003; Wynne *et al.*, 2009), the intracellular events involved in α_{2B}-AR-evoked VSMC contraction have remained largely unresolved.

Traditionally, G_i protein-dependent inhibition of AC activity has been described as the mechanism of action of α_2-ARs, but this alone cannot account for all of the mechanisms involved in VSMC contraction. Elevated $[Ca^{2+}]_i$ is necessary for the MLC_{20} phosphorylation evoked by stimulation of α_2-ARs (Aburto *et al.*, 1993). It has been suggested that most of the agonist-induced Ca^{2+} influx is likely to occur through L-type Ca^{2+} channels (Wynne *et al.*, 2009), and it has also been demonstrated in VSMCs that α_2-AR activation increases $[Ca^{2+}]_i$ (Chotani *et al.*, 2004). PTX-sensitive, G_i protein-mediated activation of L-type Ca^{2+} channels has been proposed as a mechanism (Leprêtre and Mironneau, 1994; Mironneau and Macrez-Leprêtre, 1995; Hughes *et al.*, 1996), and this pathway has been confirmed in studies using isometric

tension measurements on blood vessels (Parkinson and Hughes, 1995; Roberts, 2001). The results of Study II are in line with these proposed mechanisms, since inhibition of G_i proteins with PTX almost completely abolished the $pMLC_{20}$ response evoked by α_{2B}-AR activation, and the L-type Ca^{2+} channel blocker nifedipine also efficiently inhibited the $pMLC_{20}$ response. Stimulation of PLC activity by PTX-sensitive G proteins in fibroblasts has been reported for α_{2A}- and α_{2C}-ARs (Cotecchia *et al.*, 1990; Chabre *et al.*, 1994), and the activation of PLC has been suggested to be mediated by Gβγ subunits, followed by an IP_3-induced increase in $[Ca^{2+}]_i$ (Dorn *et al.*, 1997). The role of Gβγ-mediated signaling was evaluated by employing the βγ inhibitor gallein, which almost completely abolished the dexmedetomidine-evoked $pMLC_{20}$ response, strongly indicating involvement of Gβγ signaling. Moreover, involvement of Gβγ-mediated activation of PLC was also suggested, since the PLC inhibitor U73122 completely abolished the $pMLC_{20}$ response. Thus, it appeared that both extracellular and intracellular sources of Ca^{2+} ions were essential for this process.

Activation of PKC is associated with numerous cellular functions, and it may also exhibit negative feedback control on intracellular signaling through different mechanisms (Kanashiro and Khalil, 1998). Many studies suggest that PKC is involved in the regulation of VSM contraction (Horowitz *et al.*, 1996; Kanashiro and Khalil, 1998), and PKC inhibitors have been demonstrated to cause significant inhibition of agonist-induced VSMC contraction (Dallas and Khalil, 2003; McNair *et al.*, 2004). PKC is activated by DAG, which is formed together with IP_3 as a result of PLC activation (Berridge and Irvine, 1984). Earlier studies on α_2-AR-mediated vasoconstriction in isolated, endothelium-denuded blood vessels have proposed that α_2-AR agonists induce contraction by mechanisms involving PKC activation and opening of L-type Ca^{2+} channels (Suenaga and Kamata, 2000; Yamboliev and Mutafova-Yambolieva, 2005). Furthermore, in isolated VSMCs, α_2-ARs promoted sustained Ca^{2+} influx through voltage-gated Ca^{2+} channels by a mechanism involving activation of PKC by DAG (Mironneau and Macrez-Leprêtre, 1995), and accordingly, inhibition of PKC led to decreased Ca^{2+} influx through Ca^{2+} channels (Leprêtre *et al.*, 1994). However, in contrast to these previously reported results, the PKC inhibitors employed in Study II induced a major potentiation of the dexmedetomidine-evoked $pMLC_{20}$ response. This observation was unexpected and possible explanations were sought. It has been reported for other SMCs that inhibition of PKC results in increased responsiveness of the inositol-lipid signaling pathways, possibly because of a negative feedback control on phosphoinositide (PI) turnover and intracellular Ca^{2+} release (Pfeilschifter *et al.*, 1989; Zhong *et al.*, 2008). Indeed, several studies have described the inhibition of GPCR-mediated PI turnover or intracellular Ca^{2+} release by PKC (Ali *et al.*, 1997; Cunningham *et al.*, 1999; Woodruff *et al.*, 1999). Moreover, PKC has been reported to inhibit receptor-coupled, inositol phospholipid-specific PLC activity (Ryu *et al.*, 1990), which could also explain the potentiation of the dexmedetomidine-evoked $pMLC_{20}$ response when PKC inhibitors were employed. Yue et al. (2000) suggested that direct inhibition of PLC by PKC may play a role in the regulation of receptor-mediated PI turnover and intracellular Ca^{2+} release. They demonstrated that PKC inhibited both $G\alpha_q$-coupled and $G\alpha_i$-coupled receptor-stimulated PI turnover in four different cell lines expressing PLC. They showed that PKC, and also PKA, inhibited $G\alpha_q$-stimulated PLC activity as a result of phosphorylation of a serine residue (Ser^{1105}) of PLC. Furthermore, PKC and PKA also inhibited Gβγ-stimulated PLC activity, but by mechanisms that did not involve Ser^{1105} (Yue *et al.*, 2000). This could be a possible

explanation also for the observed potentiation of the dexmedetomidine-evoked $pMLC_{20}$ response when the PKA inhibitor H-89 was employed.

Based on the results of Study II, it can be stated that the signaling pathways involved in α_{2B}-AR-mediated contraction of A7r5 VSMCs appear to be complex and probably interconnected, forming a network of participating pathways involving many mediators. Such mediators include G_i proteins, Gβγ subunits, PLC, PKC and L-type Ca^{2+} channels (Figure 20). In Study II, a limited selection of inhibitors was employed and it is acknowledged that the results of this study may therefore reflect only a small part of the actual signaling pathways involved. Considering other studies on the signaling mechanisms related to α_2-AR-mediated vascular contraction, it appears obvious that additional mediators are also involved. In the porcine palmar lateral vein, α_2-AR-mediated vasoconstriction has been proposed to be dependent on the stimulation of PI3-kinase, leading to an influx of Ca^{2+} and subsequent activation of EGFR and finally activation of the ERK signal transduction cascade (Roberts, 2001; Roberts, 2003). Activation of ROCK has also been shown to be involved in α_2-AR-mediated vasoconstriction by increasing phosphorylation of MLC_{20} through inhibition of MLCP. Furthermore, the ROCK- and ERK-mediated signaling pathways appeared to be interconnected, because inhibition of either one of these pathways prevented vasoconstriction mediated through the other pathway (Roberts, 2004). In rat aortic SMCs, dexmedetomidine-induced contraction has been demonstrated to involve JNK- and p38 MAPK-mediated pathways downstream of α_2-AR activation, and to be primarily dependent on Ca^{2+} influx through L-type Ca^{2+} channels (Ok *et al.*, 2011; Ok *et al.*, 2014).

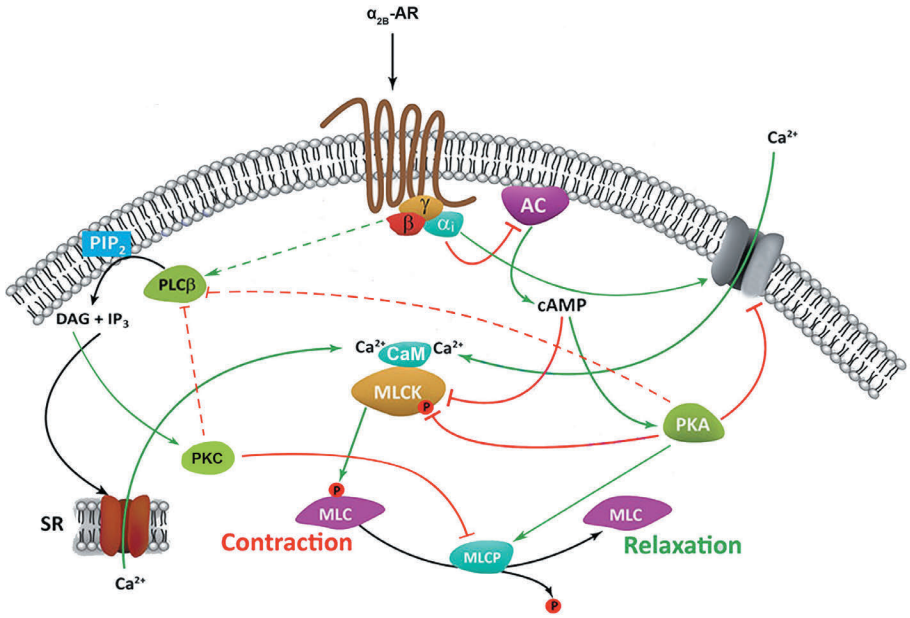

Figure 20. α_{2B}-Adrenoceptor-mediated regulation of vascular smooth muscle cell contraction, modified to include the results of Study II. Green arrows indicate stimulation, red T-shaped lines indicate inhibition, and dashed lines indicate hypothetical regulation based on the results of Study II. AC = adenylyl cyclase; cAMP = cyclic AMP; PLCβ = phospholipase C, β-isoform; PKA = protein kinase A; PKC = protein kinase C; MLCK = myosin light chain kinase; CaM = calmodulin; MLCP = myosin light chain phosphatase; DAG = diacylglycerol; IP_3 = inositol-1,4,5-trisphosphate; PIP_2 = phosphatidylinositol-4,5-bisphosphate [modified from (King, 2017)].

However, it should be borne in mind that the results of Study II were generated in an isolated VSMC culture system, and cannot be directly used to explain the functions of α_2-ARs in intact blood vessels. The capability of activated α_2-ARs to induce vasoconstriction has varied greatly depending on the luminal diameter of the investigated blood vessel and its anatomical origin, with α_2-AR-mediated contraction being more prominent in small arteries and veins compared to large arteries (Nielsen *et al.*, 1989; Parkinson and Hughes, 1995; Roberts, 2001; Roberts, 2003). It appears that the role of G_i-mediated signaling in the regulation of VSM tone varies between different types of blood vessels; PTX was shown to have an inhibitory effect on noradrenaline-induced contraction in the rat tail artery (Spitzbarth-Régrigny *et al.*, 2000; Petitcolin *et al.*, 2001a), but not in the rat aorta (Petitcolin *et al.*, 2001b). Furthermore, the density of α_2-ARs has been suggested to affect the contractile responses mediated by these receptors in different types of blood vessels (Wright *et al.*, 1995a). In particular, the regulation of the vascular responses stimulated by dexmedetomidine appears to be very complex. Dexmedetomidine induced contraction in endothelium-denuded arteries, while in intact blood vessels the relaxing and contracting effects of the drug appeared to oppose each other (Kim *et al.*, 2009b; Wong *et al.*, 2010; Ok *et al.*, 2014; Nong *et al.*, 2016). Moreover, depending on the employed concentration and the presence of functional endothelium, dexmedetomidine has induced both relaxation (small concentrations) and contraction (higher concentrations) in isolated arteries (Wong *et al.*, 2010). In the intact blood vessel, VSMCs are undergoing a constant interaction with endothelial cells, and the endothelium plays an important role in the regulation of vascular responses. It is obvious that the cell-based assay presented in Study II is a simplified model system lacking the influence of such important cell−cell interactions and the results are thus to be interpreted accordingly.

6.4.2 Proliferation

Although α_2-AR-mediated cellular functions have been the topic of numerous studies, regulation of cell proliferation is a less well investigated consequence of α_2-AR activation. It has been reported that α_2-AR activation may enhance (Seuwen *et al.*, 1990; Kribben *et al.*, 1997; Cussac *et al.*, 2002a; Vázquez *et al.*, 2006; Bruzzone *et al.*, 2008; Pérez Piñero *et al.*, 2012; Xia *et al.*, 2016b) or inhibit (Kanno *et al.*, 2002; Karkoulias *et al.*, 2006a) the proliferation of different types of cells. Nevertheless, in many of these studies, the investigated cells expressed all three α_2-AR subtypes, making it impossible to specify the subtype(s) involved in the regulation of cell proliferation. In general, α_{2A}-ARs (Seuwen *et al.*, 1990) or α_{2B}-ARs (Cussac *et al.*, 2002a; Vázquez *et al.*, 2006) appeared to mediate enhanced proliferation responses, whereas inhibition of proliferation was ascribed to α_{2C}-ARs (Karkoulias *et al.*, 2006a). Moreover, the differential effects of α_2-AR activation on cell proliferation may depend on the cell type and how their signal transduction pathways are regulated. Furthermore, different cell culture conditions may alter the effects of α_2-AR activation on cell proliferation. In human intestinal epithelial cells, recombinant α_2-ARs increased proliferation when assays were carried out in culture medium containing 0.5 % FBS, but had no effect on proliferation when assays were carried out in serum-free culture medium or in culture medium containing 10 % FBS (Schaak *et al.*, 2000). However, little is known about the effects of α_2-AR activation on the proliferation of VSMCs. The results of Study I demonstrated that activation of α_{2B}-ARs with dexmedetomidine potently increased the proliferation of cultured A7r5 VSMCs. Study III was then designed in order

to investigate the genetic regulation and intracellular signaling mechanisms involved in this α_{2B}-AR-evoked proliferation response in A7r5 cells by exploiting DNA and kinase activity profiling microarrays and kinase/phosphatase inhibitor library screening.

6.4.2.1 Genetic regulation of α_{2B}-adrenoceptor-evoked A7r5 VSMC proliferation

DNA microarray analysis identified altogether 55 genes that were differentially expressed upon α_{2B}-AR activation. It was expected that α_{2B}-AR activation would modulate gene expression in A7r5 cells, but there were not many previously published studies on α_2-AR-mediated regulation of gene expression in cultured cells, let alone VSMCs. In experimental rat models, activation of α_2-AR-related signaling pathways has been shown to up-regulate the expression of some inflammatory cytokines (Zhou *et al.*, 2005) and cytochrome CYP1A1 expression in the liver (Konstandi *et al.*, 2005). In addition, inhibitory effects of α_2-AR activation on gene expression have been reported: α_2-AR activation attenuated the dedifferentiation of chicken retinal glial (Müller) cells by inhibiting the expression of transitin and retinal progenitor cell genes, and synergistically upregulating several negative ERK-signal feedback regulators (Harun-Or-Rashid *et al.*, 2015). Dexmedetomidine inhibited the expression of the immediate early gene c-fos and 70 kDa heat shock protein (hsp70) and enhanced the expression of nerve growth factor-induced gene A (NGFI-A) in the post-ischemic hippocampi of gerbils, possibly reflecting mechanisms mediating the neuroprotective effects of α_2-AR agonists (Wittner *et al.*, 1997). Another study revealed that the effect of dexmedetomidine to reduce inflammation in sepsis involved a decrease in the expression of tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein (MCP-1) and increased expression of bone morphogenetic protein 7 (Hsing *et al.*, 2012).

There are only a few studies on the effects of AR activation on gene expression in VSMCs. Wang *et al.* (2004) investigated the downstream gene targets of endogenously expressed α_1- and β-ARs in A7r5 cells. Activation of β-ARs and α_1-ARs leads to altered gene expression profiles in A7r5 cells; 75 % and 85 % of the altered genes displayed decreased expression, respectively. Wang *et al.* also observed that both α_1- and β-AR activation inhibited the proliferation of A7r5 cells, but their microarray results pointed to differences in the mechanisms involved: α_1-AR activation induced the expression of metabolic genes, resulting in inhibition of proliferation, whereas β-AR activation changed the expression of genes encoding signaling and structural proteins to inhibit cell proliferation (Wang *et al.*, 2004). In contrast, in primary rat aortic SMCs, activation of α_1-ARs by phenylephrine increased VSMC proliferation and cell number (Pang and Sun, 2009). However, phenylephrine is not a subtype-selective α_1-AR agonist and the observed opposing effects may have been caused by different α_1-AR subtypes. Another potential explanation for the opposing effects is that the same receptor may have different functions depending on its cellular environment.

Functional annotation tools (GeneFuncster and Ingenuity® pathway analysis) were applied to identify enriched biological processes, significant networks and upstream regulators related to the differentially expressed genes in dexmedetomidine-exposed vs. vehicle-treated A7r5-α_{2B} cells. The use of such functional annotation tools may provide additional proof for the success of the experiment if expected and relevant functional categories are identified, and, in addition, these tools may enable the detection of weaker signals from a given dataset. Both functional analyses identified functional enrichment in categories

related to cardiovascular system development/smooth muscle cells, cell proliferation, migration, adhesion and contraction. Both analyses identified essentially the same genes as being involved in these functions, either through up- or downregulated expression, including Egr1, F3, Ptgs2, Serpine1, Cav1, Nppb, Rhoa, Cx3cl1 and Prrx1. However, for Cx3cl1 and Prrx1, the observed change in expression and the associated effect on proliferation differed from previously reported effects.

Interestingly, the most highly upregulated gene in the dataset, Ly6/PLAUR domain containing 8 (Lypd8; FC = 2.01, $p = 4.2 \times 10^{-6}$) did not receive any annotations in the functional analyses. Lypd8 is a member of the Ly6/PLAUR family of glycophosphatidylinositol-anchored cell surface proteins with immunity-related roles (LeClair *et al.*, 1986). Other members of the Ly6/PLAUR family have been shown to be involved with proliferation of neutrophils (Stroncek *et al.*, 2004) and to be able to activate transcription factors like activator protein 1 (AP-1) (Ni *et al.*, 2009), which regulates gene expression and controls a number of cellular processes including differentiation, proliferation, and apoptosis (Ameyar *et al.*, 2003). However, no studies on the possible functions of Lypd8 existed, until very recently, when Okumura *et al.* showed that Lypd8 is selectively expressed in intestinal epithelial cells, and that its product is capable of preventing flagellated bacteria from invading the colonic epithelium in mice (Okumura *et al.*, 2016). The present microarray results clearly indicated that Lypd8 expression was upregulated in A7r5 VSMCs upon activation of α_{2B}-ARs, suggesting that the functions of this gene would not be restricted solely to the intestine. However, the possible role of Lypd8 in the regulation of A7r5 VSMC proliferation remains to be clarified.

In line with the results of Study III, Egr1 appears to be essential for proliferation in many cell types, including VSMCs (Tarcic *et al.*, 2012; Kimura *et al.*, 2014), and its expression levels often closely correlate with cell proliferation (Thiel and Cibelli, 2002; Khachigian, 2006; Liu *et al.*, 2013). Tissue factor F3 is constitutively expressed in VSMCs (Schecter *et al.*, 1997), and it has been shown to induce proliferation and migration of VSMCs (Pyo *et al.*, 2004; Steffel *et al.*, 2006; Giannarelli *et al.*, 2014). VSMC proliferation has been associated with upregulated F3 expression (Yisireyili *et al.*, 2014), whereas F3 knockdown has led to inhibition of proliferation and increased apoptosis (Wan *et al.*, 2015). Ptgs2 promotes VSMC proliferation directly through upregulated gene expression (Tzeng *et al.*, 2007) or by mediating the growth promoting responses of compounds such as AT-II and TNFα (Hu *et al.*, 2002; Haider *et al.*, 2003; Aguado *et al.*, 2013). Consistently, inhibition or decreased expression of Ptgs2 is associated with decreased VSMC proliferation and inhibition of cell migration (Choi *et al.*, 2008; Chang *et al.*, 2014; Yang *et al.*, 2014). In the literature, a rather uniform impression exists that serpine1 expression promotes the proliferation of VSMCs (Knudsen *et al.*, 1987; Beauchamp *et al.*, 2003; Chen *et al.*, 2006; Balsara and Ploplis, 2008; Samarakoon and Higgins, 2008; Jeong *et al.*, 2011), which is in good agreement with the results of Study III.

The proliferation of VSMCs is not only enhanced by upregulation of growth-promoting genes but also by downregulation of genes with antiproliferative effects. Cav1, Nppb and RhoA represented such genes in Study III; the decreased expression of these genes was associated with increased proliferation of A7r5-α_{2B} cells. Decreased Cav1 expression has been reported to be associated with proliferating VSMCs (Williams and Lisanti,

2004; Schwencke *et al.*, 2005; Luo *et al.*, 2010; Carrillo-Sepulveda and Matsumoto, 2014; Mulas *et al.*, 2014). In addition, natriuretic peptides, including Nppb, have potent antiproliferative and antimigratory effects on VSMCs (Schirger *et al.*, 2000; Berk, 2001; Casco *et al.*, 2002; Nakayama, 2005), which supports the observation of downregulated Nppb in response to α_{2B}-AR activation and the concomitant increase in A7r5-α_{2B} cell proliferation. In line with the observation of decreased RhoA expression correlating with increased proliferation of A7r5-α_{2B} cells are the findings of Tseliou *et al.* who showed that knockdown of the RhoA protein in human cytomegalovirus infected cell lines restored their proliferation rate (Tseliou *et al.*, 2016). However, contrasting with the results of Study III there are many studies reporting that suppression or downregulation of RhoA leads to inhibited proliferation of VSMCs (Croft and Olson, 2006; Yu *et al.*, 2011; Hung *et al.*, 2013; Renteria *et al.*, 2013; Du *et al.*, 2015; Rigassi *et al.*, 2015). These differences may perhaps be attributed to the existence of many different environmental signals affecting RhoA signaling and to inherent differences in RhoA signaling in different cell types (Mack and Hinson, 2005). Moreover, a common feature of the Cav1 and RhoA genes is that their expression has been related to the differentiated, contractile phenotype of VSMCs rather than the proliferative, noncontractile VSMC phenotype (Williams and Lisanti, 2004; Sedding and Braun-Dullaeus, 2006; Gosens *et al.*, 2011; Maeng *et al.*, 2014; Shimokawa *et al.*, 2016). Therefore, it was not surprising that these genes were downregulated in proliferating VSMCs as observed in Study III.

For the majority of these differentially regulated genes, that may participate in the regulation of α_{2B}-AR-evoked proliferation of A7r5 VSMCs, there is strong evidence in the published literature supporting the results of the present study. However, for Cx3cl1 and Prrx1, the observations differed from the previous reports. In the present results, downregulation of Cx3cl1 and Prrx1 was associated with increased proliferation. In contrast, there is solid evidence indicating that Cx3cl1 induces proliferation of VSMCs of different origin (Porreca *et al.*, 1997; Viedt *et al.*, 2002; Chandrasekar *et al.*, 2003; Perros *et al.*, 2007; White *et al.*, 2010; Bhardwaj *et al.*, 2011; Qi *et al.*, 2012; Girona *et al.*, 2013; Kundumani-Sridharan *et al.*, 2013; van der Vorst *et al.*, 2013; Yang *et al.*, 2013; Liu *et al.*, 2016b). Similarly, overexpression of Prrx1 has a pro-proliferative effect on (A10) VSMCs (Jones *et al.*, 2001; Jones *et al.*, 2002). Cx3cl1 is induced by inflammatory cytokines (Chandrasekar *et al.*, 2003). The pro-proliferative effects of this gene/protein may be mediated through a pro-inflammatory signaling pathway, whereas the α_{2B}-AR-evoked proliferation response, as seen in Study III, could be mediated through another mechanism not requiring Cx3cl1. Furthermore, Prrx1 has been shown to regulate SMC differentiation marker genes in fully differentiated VSMCs (Hautmann *et al.*, 1997; Yoshida *et al.*, 2004; Mack and Hinson, 2005; Shang *et al.*, 2008), which is in line with this gene being downregulated in proliferating VSMCs, as seen in the present study.

Another factor possibly influencing the differential gene expression in dexmedetomidine-stimulated A7r5-α_{2B} cells may be the intracellular signaling molecule cAMP. α_{2B}-ARs are capable of coupling to both inhibitory G_i proteins and stimulatory G_s proteins leading to decreased or increased cAMP levels, respectively, through regulation of AC activity. cAMP, in turn, can activate or inhibit kinases and protein expression, and in such ways regulate cellular processes like proliferation. The expression of some of the genes that were found to be differentially regulated in Study III, can be affected by cAMP. cAMP can

inhibit the proliferation of VSMCs by inhibiting Egr1 expression (Kimura *et al.*, 2014) or the expression of other pro-mitogenic genes by inhibiting RhoA activity (Kimura *et al.*, 2016). Ptgs2 induction is known to regulate VSMC proliferation but the effect depends on several factors including the coupling of cAMP to either growth-inhibitory or growth-promoting pathways (Ohnaka *et al.*, 2000). Decreased cAMP levels could provide an explanation for the upregulation of Egr1 and Ptgs2 and the concomitant increase in A7r5 VSMC proliferation but, on the other hand, this explanation would not be applicable for RhoA. However, whether and how cAMP regulates gene expression may be a cell type-specific response, as shown for Egr1 expression, which was inhibited by cAMP in VSMCs but not in endothelial cells (Kimura *et al.*, 2014). Moreover, it remains unclear how other cellular mechanisms determine the coupling of cAMP to either growth-promoting or growth-inhibitory pathways. Consistent with the observed upregulation of Ptgs2 in response to dexmedetomidine exposure (100 nM) in Study III, Ptgs2 has been reported to be upregulated by dexmedetomidine (1 μM) in isolated, endothelium-denuded rat aorta (Ok *et al.*, 2014). In murine macrophages, 1 and 10 μM dexmedetomidine significantly inhibited LPS-induced Ptgs2 expression, whereas 100 μM dexmedetomidine significantly enhanced LPS-induced Ptgs2 expression (Lai *et al.*, 2009).

6.4.2.2 Signaling mechanisms involved in α_{2B}-adrenoceptor-evoked A7r5 VSMC proliferation

Protein kinases form a large group of structurally related enzymes that participate in mediation of signal transduction in virtually all cellular processes, including cell proliferation (Manning *et al.*, 2002). The intracellular signaling mechanisms involved in the α_{2B}-AR-evoked proliferation response in A7r5 cells were investigated both by evaluating the role of individual kinases by making use of inhibitors and by broadly screening for kinase activity profiles at different time points after dexmedetomidine stimulation. Among the targets of the screened inhibitors, several kinases and phosphatases were identified that have been implicated in the promotion of VSMC proliferation, thus increasing the biological significance of these findings. Kinases and phosphatases possibly promoting the mechanisms by which α_{2B}-AR activation leads to increased proliferation of A7r5 VSMCs included calcineurin (Lipskaia *et al.*, 2003; Li and Sun, 2005; Pang and Sun, 2009; Kudryavtseva *et al.*, 2013), protein kinase CK2 (casein kinase) (Dumler *et al.*, 1999), Src kinases (Jeremy, 1999; Waltenberger *et al.*, 1999; Walcher *et al.*, 2006; Kim *et al.*, 2009a; Li *et al.*, 2010; Davis *et al.*, 2012), JNK (Nagayama *et al.*, 2015; Yang *et al.*, 2015), p38 MAPK (Chen *et al.*, 2014; Shen *et al.*, 2014; Yu *et al.*, 2015), Cdk1/2 (Terano *et al.*, 1999; Chen *et al.*, 2010; Schad *et al.*, 2011; Zhang *et al.*, 2013; Zhang *et al.*, 2014), PI3-kinases (Saward and Zahradka, 1997; Goncharova *et al.*, 2002; Silfani and Freeman, 2002; Lipskaia *et al.*, 2003; Kristof *et al.*, 2005), EGFR (Kim *et al.*, 2009a; Freeman *et al.*, 2010; Robinson and Pitcher, 2013; Rodríguez-Moyano *et al.*, 2013; Savikko *et al.*, 2015) and Raf-1 (Watanabe *et al.*, 2001; Zhang and Liu, 2002; Davis *et al.*, 2012).

Although inhibitors are valuable tools with which to study the biology and therapeutic potential of specific kinases, significant limitations in the use of inhibitors may arise because of their poor solubility, general toxicity and lack of specificity (Davies *et al.*, 2000). Apart from the complex interaction patterns related to many kinase inhibitors, intracellular signaling pathways are so multifaceted that single inhibitors might not be

able to overcome the robustness of biological networks (Feala *et al.*, 2012; Tran *et al.*, 2014). If one pathway is blocked with an inhibitor, the cell may find several compensating mechanisms to bypass the inhibited route; e.g. a receptor may mediate its effects through several different signaling mechanisms and parallel mechanisms may compensate for the effects of a blocked signaling route. This might provide an explanation also for the results of Study III, where no single inhibitor could completely block the α_{2B}-AR-evoked proliferation response; even the most effective inhibitors included in the screen inhibited the proliferation response by less than 30 %.

Because little is known about the mechanisms by which α_2-ARs regulate cell proliferation, especially with regard to the α_{2B}-AR subtype in the regulation of VSMC proliferation, it would have been unlikely that attempting to elucidate the signaling mechanisms involved in the regulation of cell proliferation by investigating the effects of individual kinases one at a time would have been successful. Therefore, to broadly screen for kinases participating in the regulation of proliferation (both primary kinase activities and secondary kinase responses), kinase activity profiles were generated after 5 min, 30 min, 2 h and 24 h of dexmedetomidine stimulation by making use of PamChip PTK and STK microarrays. The resulting kinase activity profiles indicated that dexmedetomidine stimulation induced transient decreases of kinase signaling at the early time points ($t \leq 30$ min), which then recovered at the later time points and in many cases, even resulted in slight increases in kinase activity at 24 h. Altered kinase signaling was most pronounced at 30 min, where decreased phosphorylation of altogether 40 peptides (36 and 4 on the PTK and STK chips, respectively) was detected. Based on the results of Study II, it was postulated that at the 30 min time point the A7r5-α_{2B} cells may have been in a state of metabolic perturbation, still recovering from the contraction caused by dexmedetomidine-induced activation of α_{2B}-ARs. Therefore, cellular activity may have been directed towards relaxation and MLC dephosphorylation, and the metabolic state of the cells would not have favored proliferation. It appeared as if there was a general inhibition of proliferative activity at the earlier time points. However, the observed effects and overall inhibition of kinase activity may not have been directly evoked by α_{2B}-AR activation, but they might have represented indirect effects caused by other signaling mechanisms. By two hours, no differences in kinase activities between dexmedetomidine-stimulated and vehicle-treated cells could be observed, suggesting that by this time the cells had completely recovered from the contraction and returned to a basal state. After 24 hours of dexmedetomidine exposure, a slight increase in overall kinase activities was detected. These small increases in kinase activities could have represented either the tail or the beginning of a kinase activity peak, and by assaying additional time points e.g. at 12 h and 36 h, it might have been possible to answer to this question. In general, dexmedetomidine stimulation had much more pronounced effects on the PTK chip compared with the rather limited effects on the STK chip. There is a logical biological explanation for the detected differences in tyrosine kinase versus Ser/Thr kinase activities; tyrosine kinase signaling, as detected on the PTK chip, is more typical for receptors than serine/threonine signaling, and tyrosine phosphorylation, as opposed to serine/threonine phosphorylation, which has evolved as a mechanism of intracellular communication. Protein tyrosine phosphorylation has been recognized as a fundamentally important mechanism of signal transduction and regulation in all eukaryotic

cells, governing cellular processes such as proliferation, migration, differentiation and survival (Hunter, 1998; Waltenberger *et al.*, 1999; Hunter, 2009).

For the above mentioned reasons, the subsequent canonical pathway and upstream kinase analyses were mostly driven by the kinase activity results obtained from the PTK chips. Canonical pathway analysis revealed that the Raf-1−MEK1−MEK2 signaling cascade appeared in most of the statistically significant pathways; Raf-1 phosphorylates MEK1 and MEK2, and dexmedetomidine treatment (30 min) decreased the activity of these kinases. The Raf-1−MEK−ERK pathway is one of the best characterized MAPK signaling pathways known to regulate cell proliferation (Zhang and Liu, 2002). Inhibition of this pathway after 30 min of dexmedetomidine exposure supported the hypothesis of the cells being in a state of metabolic perturbation after the initial α_{2B}-AR-evoked contraction.

Although the PTK chip did not contain a peptide substrate for Lyn kinase, it appears that Lyn kinase activity was also modulated by dexmedetomidine, because three successive downstream targets (Syk, LAT and PLCγ) in the Lyn signaling cascade showed decreased activity after dexmedetomidine treatment (Additional file 11 of Study III). Moreover, Lyn was also identified as a putative upstream kinase in the upstream kinase analysis, which utilizes different databases than the canonical pathway analysis; it includes known phosphorylation sites and is altogether more specific. The finding that different databases point in the same direction confers more confidence on the validity of the results, and inhibition of Lyn kinase activity appears to affect many downstream kinases. Lyn is known to be expressed in VSMCs (Dumler *et al.*, 1998; Hollenbeck *et al.*, 2004) and to be regulated by PTX-insensitive GPCR signaling (Pertel *et al.*, 2005), and has been identified as an important regulator of GPCR trafficking and how this influences cell proliferation (Luttrell and Luttrell, 2004; Ingley, 2012).

To look further into the possible signaling mechanisms involved in the α_{2B}-AR-evoked proliferation response of A7r5-α_{2B} VSMCs, a putative upstream kinase analysis was performed i.e. linking differentially phosphorylated peptides to the upstream kinases possibly causing the phosphorylation. The putative upstream tyrosine kinases identified included cytosolic kinases Abl2 and Bmx, and several growth factors and growth factor receptors, such as bFGF (FGF2), PDGF, EGFR, HGFR, VEGFR-1 (Flt1) and VEGFR-2 (KDR). These growth factors/growth factor receptors have been implicated in the regulation of VSMC proliferation, which increases the biological significance of these results. Abl2 has been reported to promote the proliferation of breast cancer cells and recombinant 293T cells (Mitra *et al.*, 2008; Srinivasan *et al.*, 2008), and Bmx is known to be involved in angiogenic events (Pan *et al.*, 2002) and in the regulation of actin cytoskeleton and cell motility (Abassi *et al.*, 2003). These kinases could exert similar effects on observed proliferation responses also in the present study. A multifaceted interplay may exist between different receptors and growth factors; receptors on one hand are capable of inducing growth factor expression whereas on the other hand, growth factors are capable of desensitizing receptors, thereby affecting receptor-mediated responses. It has long been known that bFGF and PDGF can induce VSMC proliferation (Grotendorst *et al.*, 1982; Lindner and Reidy, 1991; Jawien *et al.*, 1992; Olson *et al.*, 1992); however, little is known about the interaction of α_{2B}-ARs with bFGF or PDGF. β-AR activation increased the expression of bFGF and PDGF in VSMCs derived from spontaneously hypertensive

rats (Satoh *et al.*, 2001). AT-II also stimulated VSMC hypertrophy and bFGF expression, but increased bFGF expression alone was not sufficient to stimulate VSMC growth; PKC activation also was required (Ali *et al.*, 1994). Conversely, PDGF has been implicated in the desensitization of β- and α_1-ARs (Ikenouchi *et al.*, 2008; Rodríguez-Pérez *et al.*, 2009). It is well established that agonist activation of diverse GPCRs can lead to the transactivation of EGFR, triggering the activation of multiple mitogenic pathways; activation of the EGFR–MAPK pathway is commonly involved in regulation of gene expression and cell proliferation (New and Wong, 2007; Lappano and Maggiolini, 2011). The intensity and duration of EGFR transactivation and subsequent MAPK activation may represent a decision point between cell proliferation and alternative fates such as differentiation (Marshall, 1995; Zwick *et al.*, 1999). α_2-ARs are capable of activating MAPKs through transactivation of EGFR in different cell types (Cussac *et al.*, 2002b; Karkoulias *et al.*, 2006b; Buffin-Meyer *et al.*, 2007; Li *et al.*, 2008; Harun-Or-Rashid *et al.*, 2015), and activation of MAPK pathways by the α_{2B}-AR subtype, in particular, has been reported to be dependent on EGFR transactivation (Cussac *et al.*, 2002b; Karkoulias *et al.*, 2006b). HGF/HGFR control essential cellular responses including cell proliferation, motility and differentiation (Zarnegar, 1995; Birchmeier *et al.*, 2003; You and McDonald, 2008; Salgia, 2009). HGFR may be transactivated by both EGFR and a wide diversity of GPCRs (Fischer *et al.*, 2004). HGF/HGFR signaling is activated in angiogenesis (You and McDonald, 2008), and it has possibly also a role in the pathogenesis of atherosclerosis and restenosis (Taher *et al.*, 2002); HGFR is expressed on VSMCs isolated from atherosclerotic plaques and it triggers signaling cascades (involving PI3-kinases, Akt, MEK and ERK1/2) mediating migration of VSMCs (Taher *et al.*, 2002; Ma *et al.*, 2003). The VEGF/VEGFR signaling pathway, and especially VEGFR-2, is vital for the induction of angiogenesis, and it drives both endothelial cell proliferation and migration (Meyer and Rahimi, 2003; Nakatsu *et al.*, 2003; Greenberg *et al.*, 2008). However, contrasting reports exist about the effects of VEGF/VEGFR signaling on VSMC proliferation: on one hand, a VEGF-mediated pathway has been implicated in the promotion of VSMC proliferation (Parenti *et al.*, 2002; Cardús *et al.*, 2006), and on the other hand, VEGF/VEGFR-2 has been identified as a negative regulator of VSMCs (Greenberg *et al.*, 2008). VEGF has also been reported to be a potent stimulator of VSMC migration while having no effect on VSMC proliferation (Chandra and Angle, 2005). Furthermore, the VEGF/VEGFR signaling pathway has been reported to act on angiogenesis synergistically with the HGF/HGFR signaling pathway (You and McDonald, 2008). Based on the results of this upstream regulator analysis, many growth factors and growth factor receptors appeared to be activated upon sustained (24 h) exposure to dexmedetomidine. These findings suggested that α_{2B}-ARs may mediate their pro-proliferative effects in A7r5 VSMCs by promoting the activity of endogenous bFGF and PDGF and the growth factor receptors EGFR, HGFR and VEGFR-1/2.

Even though the upstream kinase analysis found weaker evidence for Ser/Thr kinase involvement in the regulation of VSMC proliferation by α_{2B}-ARs than for tyrosine kinases, the putative identified Ser/Thr kinases included relevant kinases like PKC and the β-adrenergic receptor kinases 1 (GRK2) and 2 (GRK3). These may also be involved in the observed activation of kinase signaling after 24 h of exposure to dexmedetomidine. As discussed earlier, PKC has been implicated in the modulation of several VSM processes, including smooth muscle contraction and modulation of cell growth and proliferation (Lee and Severson, 1994; Khalil, 2010; Khalil, 2013). PKC has been shown to exert both

proliferative and antiproliferative effects on cultured VSMCs (Lee and Severson, 1994). Activation of PKC and, concomitantly, ERK1/2 have been implicated in VSMC proliferation induced by different stimuli (Liou *et al.*, 2004; Wang *et al.*, 2008; Yang *et al.*, 2011; Molnar *et al.*, 2014; Ma *et al.*, 2016). PKCδ, a ubiquitously expressed isoform in VSMCs, appears to have a dual, critical role in VSMC proliferation: overexpression of PKCδ in A10 or A7r5 VSMCs resulted in a suppression of proliferation (Fukumoto *et al.*, 1997; Liu *et al.*, 2007), but selective PKCδ gene deletion also decreased VSMC proliferation (Liu *et al.*, 2007). This dual role of PKCδ and the opposing effects of different PKC isoforms emphasize the complexity of regulation of VSMC proliferation. Since many PKC isoforms were among the identified putative upstream kinases, it appeared possible that PKC actually was an upstream kinase regulating downstream kinase activities detected in the present kinase activity profiling experiments. A possible mechanism, related to the proliferation response caused by α_{2B}-AR activation, could be mediated through a pathway involving PKC activation. Moreover, IPA identified PKC as a potential upstream regulator participating in the regulation of gene expression upon α_{2B}-AR activation.

It is known that signaling of many GPCRs is diminished by GRK2. In fact, GRK2 has been identified as a key regulator in the desensitization of GPCRs; for example, TGFβ-induced GRK2 expression attenuated AT-II-regulated proliferation and migration of VSMCs (Guo *et al.*, 2009). Overall, GRK2 appears to have an inhibitory effect on cell proliferation; GRK2 overexpression in VSMCs decreases mitogenic signaling and proliferation in response not only to many GPCR agonists, but also to PDGF. However, GRK2 does not affect EGFR-mediated VSMC proliferation (Peppel *et al.*, 2000; Peppel *et al.*, 2002). Furthermore, in fibroblast-like synoviocytes suppression of GRK2 has been associated with inhibition of proliferation (Chen *et al.*, 2012). Like most GPCRs, prolonged exposure of α_{2B}-ARs to agonists results in decreased responsiveness primarily caused by downregulation of the receptors (Heck and Bylund, 1997). The literature provides evidence for and against the role of GRKs in agonist-induced downregulation of α_2-ARs, however, there is strong evidence for a critical role of both GRK2 and GRK3 in the downregulation of α_{2B}-ARs (Desai *et al.*, 2006). Although little is known of the effects of GRK3 on VSMC proliferation, it has been established that GRK3 plays an important role in the survival and proliferation of metastatic prostate cancer cells and in stimulation of tumor angiogenesis (Li *et al.*, 2014; Sang *et al.*, 2016). Based on this, it was speculated that the signaling mechanisms of the α_{2B}-AR-evoked proliferation response in A7r5 VSMCs could involve transactivation of EGFR, which is not affected by the inhibitory effects of GRK2, and that increased activities of GRK2 and GRK3 after 24 h of dexmedetomidine treatment could be a counteracting mechanism, leading to α_{2B}-AR downregulation and attenuation of the α_{2B}-AR-evoked proliferation response.

At the beginning of Study III, the mechanisms involved in the enhanced VSMC proliferation evoked by α_{2B}-AR activation had not been extensively investigated. Study III employed an integrated screening approach to identify already described regulatory genes and active signaling cascades participating in the α_{2B}-AR-evoked proliferation of VSMCs. Pathway analysis and upstream kinase analysis proved to be useful tools because also weaker signals could be identified, and they provided a broader overview of the signaling events within the cell compared with experiments on individual genes or kinases. Based on the results of Study III, the cellular mechanisms participating in this proliferation response appeared

to be complex and to include redundancy. Functional enrichment analysis and pathway analysis identified differentially expressed genes associated with α_{2B}-AR-regulated VSMC proliferation. They included the upregulated genes Egr1, F3, Ptgs2 and serpine1 and the downregulated genes Cx3cl1, Cav1, RhoA, Nppb and Prrx1. The most highly upregulated gene, Lypd8, represented a novel finding in the VSMC context. Inhibitor library screening and kinase activity profiling were applied to identify kinases in the involved signaling pathways. Putative upstream kinases identified by two different screens included PKC, Raf-1, Src, the MAPKs p38 and JNK and the receptor tyrosine kinases EGFR and HGF/HGFR. As a novel finding, the Src family kinase Lyn was also identified as a putative upstream kinase. It was concluded that α_{2B}-ARs may mediate their pro-proliferative effects in A7r5 VSMCs by promoting the activity of bFGF and PDGF and the growth factor receptors EGFR, HGFR and VEGFR-1/2. The identified Ser/Thr kinases included several PKC isoforms and the β-adrenoceptor kinases 1 and 2. Cross-talk between the signaling mechanisms participating in α_{2B}-AR-evoked VSMC proliferation thus appeared to involve PKC activation, subsequent changes in gene expression, transactivation of EGFR, and modulation of kinase activities and growth factor-mediated signaling. However, it should be borne in mind that a literature-based approach was used for validation, and the results of Study III should, therefore, be regarded as exploratory, offering starting points for further studies. Recombinant overexpression cell models, gene silencing with siRNA and Western blot analysis, among others, could be suitable approaches for further investigation and validation of the identified genes and kinases.

7 CONCLUSIONS

α_2-ARs are involved in the regulation of important vascular functions, including contraction and proliferation of VSMCs. Until now, α_2-AR-evoked VSM contraction had mainly been investigated in isolated blood vessels and α_2-AR-evoked cell proliferation had been studied in other cell types, but not in VSMCs; thus the intracellular signaling mechanisms related to these functions have remained largely unknown. This thesis investigated the role of the α_{2B}-AR subtype in the regulation of VSMC contraction and proliferation, and strived to gain an increased understanding of the signaling mechanisms related to these α_{2B}-AR-regulated functions. The main results and conclusions were:

1. A VSMC line stably expressing the human α_{2B}-AR was generated by transfection of rat A7r5 cells. Conventional transfection techniques resulted in very low transfection efficiencies, and therefore, nucleofection combined with antibiotic selection was applied for the generation of the stably transfected A7r5-α_{2B} VSMC line. The localization and ligand binding properties of the receptors were in line with previous studies of α_{2B}-ARs in different host cell types. Functional characterization by [^{35}S]GTPγS binding experiments indicated that the expressed receptors had the expected pharmacological characteristics. An unexpected finding was the significant induction of VSMC proliferation evoked by activation of α_{2B}-ARs. The generated A7r5-α_{2B} cell line was considered to be a useful tool for studying the function of α_{2B}-ARs in VSMCs, a cellular environment relevant for cardiovascular diseases.

2. A functional cell-based assay for the quantitative determination of VSMC contraction by measuring myosin light chain phosphorylation was developed. The α_2-AR agonist dexmedetomidine induced rapid and concentration-dependent myosin light chain phosphorylation in A7r5-α_{2B} cells, indicating that α_{2B}-ARs were capable of mediating VSMC contraction. The developed assay proved to be suitable for the assessment of the capacity of ligands to evoke or inhibit VSMC contraction, and for investigating the intracellular pathways involved in this process. The signaling pathways involved in α_{2B}-AR-mediated contraction of A7r5 VSMCs appeared to be complex and to involve many mediators, such as G_i proteins, Gβγ subunits, PLC, PKC and L-type Ca^{2+} channels, but the complete network of mediators involved in this contractile response remained unsolved.

3. The cellular mechanisms and signal transduction pathways participating in the α_{2B}-AR-evoked proliferation response of A7r5 VSMCs appeared to be complex and to include redundancy. Identified genes associated with α_{2B}-AR-regulated VSMC proliferation included the upregulated genes Egr1, F3, Ptgs2 and serpine1 and the downregulated genes Cx3cl1, Cav1, Rhoa, Nppb and Prrx1. The most highly upregulated gene, Lypd8, represented a novel finding in the VSMC context. α_{2B}-ARs may mediate their pro-proliferative effects in VSMCs by promoting the activity of bFGF and PDGF and the growth factor receptors EGFR, HGFR and VEGFR-1/2. The Src family kinase Lyn was also identified as a putative upstream kinase. Lyn is known to be expressed in VSMCs, and has been considered to be an important

regulator of GPCR trafficking and GPCR effects on cell proliferation. The identified Ser/Thr kinases included several PKC isoforms and the β-adrenoceptor kinases 1 and 2. Cross-talk between the signaling mechanisms involved in α_{2B}-AR-evoked VSMC proliferation thus appears to involve PKC activation, subsequent changes in gene expression, transactivation of EGFR, and modulation of kinase activities and growth factor-mediated signaling. The employed screening assays, i.e. DNA microarray, small compound library screening and kinase activity profiling, and their respective data analysis approaches, were found to be useful as tools to map the activation of cellular signaling networks in a situation where the exact mechanisms still remain unknown. From the screening results, also weaker signals could be identified by combining pathway analysis and integrated approaches. However, no direct mechanistic conclusions about an observed effect could be made, as the observed change might be either a cause or a consequence of the proliferation response. It was concluded that the employed screening tools are useful for hypothesis generation, but hypothesis testing will require additional approaches, such as overexpression cell models or gene silencing e.g. with siRNA technology.

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