2003

CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF THE á1-ADRENOCEPTOR SUBTYPES

Dan Chalothorn
University of Kentucky, chalotd@uky.edu

ABSTRACT OF DISSERTATION

Dan Chalothorn

The Graduate School

University of Kentucky

2003

CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF THE α_1-ADRENOCEPTOR SUBTYPES

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy
in the College of Medicine at the University of Kentucky

By

Dan Chalothorn

Lexington, Kentucky

Director: Dr. Michael T. Piascik, Professor of Pharmacology

Lexington, KY

2003

ABSTRACT OF DISSERTATION

CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF THE α_1-ADRENOCEPTOR SUBTYPES

The α_1-adrenoceptors (α_1-ARs) serve as an interface between the sympathetic nervous system and the cardiovascular system where they are mediators of systemic arterial blood pressure, initiators of positive inotropy, and regulators of cellular growth responses. There are three subtypes: α_{1A}-, α_{1B}-, and α_{1D}-ARs.

This dissertation research investigated the trafficking properties of the α_1-ARs at the cellular level as well as physiological relevance of the α_1-ARs at the tissue level. *In vitro* studies using transiently transfected α_1-AR/GFP subtypes revealed distinct basal localization patterns and different agonist-mediated activation and desensitization properties. The α_{1A}- and the α_{1B}-AR/GFP subtypes displayed agonist-mediated receptor redistribution, in which rate and degree of redistribution differed. Additionally, redistribution of either of these two receptor subtypes required β arrestin-1, a protein often associated with receptor internalization. In contrast, the α_{1D}-AR/GFP did not require β arrestin-1 for maintaining the basal receptor orientation pattern.

Although these data increase our knowledge of trafficking properties of the α_1-AR subtypes, it is of equal importance to determine the role(s) that each subtype contributes

to cardiovascular function. The lack of subtype-selective α_1-AR pharmacological agents prompted the use of genetically manipulated mouse models with a systemic over-expression of a constitutively active α_{1B}-AR. Echocardiographic analysis of transgenic hearts indicated both an enlarged left ventricular chamber in the absence of hypertrophy and a depressed cardiac function. From isolated transgenic hearts, experimental results suggested a role for the α_{1B}-AR in attenuating the inotropic responses. However, experiments using isolated thoracic aortae from transgenic animals suggested that the α_{1B}-AR does not participate in vascular smooth muscle contractile responses. Additional studies investigated the role of α_{1D}-AR in cardiovascular function by using animals systemically lacking the α_{1D}-AR subtype. Experimental data suggested an α_{1D}-AR participation in vascular smooth muscle function since the deficiency of the α_{1D}-AR subtype affected vasoconstriction in the coronary arteries but not inotropy in the heart.

The data presented in this dissertation research suggest subtype specific differences of α_1-ARs in cellular localization, signal regulation, and trafficking. Additionally, the data provide an investigation into the physiologic significance of both the α_{1B}- and the α_{1D}-ARs in cardiovascular tissue.

Key Words: α_1-Adrenoceptors (α_1-ARs), Receptor Localization and Trafficking, Transgenic Animals, Vascular Smooth Muscle, Heart

_____

_____

CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF THE α_1-ADRENOCEPTOR SUBTYPES

By

Dan Chalothorn

_____
Director of Dissertation

_____
Director of Graduate Studies

_____

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name	Date

DISSERTATION

Dan Chalothorn

The Graduate School

University of Kentucky

2003

CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF THE α_1-ADRENOCEPTOR SUBTYPES

DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy
in the College of Medicine at the University of Kentucky

By

Dan Chalothorn

Lexington, Kentucky

Director: Dr. Michael T. Piascik, Professor of Pharmacology

Lexington, KY

2003

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude for those individuals involved in my dissertation work. First and foremost, I want to thank my Dissertation Chair Dr. Piascik, who I am indebted since he has provided me with an opportunity to conduct some interesting research in "Mike's Play Land." His invaluable guidance and "comments" through the years have helped me develop as a better student and person. Thus this experience should serve me well in my scientific endeavors. In addition, I would like to thank Drs. Lisa Cassis, Hollie Swanson, Robert Lasley, and Robert Hadley for agreeing to serve as members on my dissertation committee. The entire committee has been beneficial in shaping the goals of this dissertation project as well as in making me realize the practicality of my proposal. Also I appreciate Dr. Eric Smart's commitment to serve as an outside examiner for this dissertation defense.

Many of the projects reported in this dissertation would not have been possible without gifts from several investigators. Therefore, I want to thank Dr. Gozoh Tsujimoto for providing us with the α_1-AR/GFP constructs and the α_{1D}-AR knockout mice. In addition, I am grateful to Dr. Jeffrey Benovic for supplying us with β-arrestin 1 and 2, β-arrestin 1 (319-418) constructs, and the β-arrestin antibodies. I am also indebted to Dr. Dianne Perez for providing this laboratory with the α_{1B}-AR transgenic animals, in which my pre-doctoral grant is based; in addition, I am grateful to the Ohio Valley Affilate of the American Heart Association for funding my proposal.

As far as members of the Piascik laboratory, I would like to thank Stephanie Edelmann for her assistance in maintaining and plating numerous dishes of HEK-293 cells for transfection studies, passing on the technique of arterial ring contraction, serving

as a role a model for “effective” use of time. I would like to thank Mary Lolis Garcia and Dr. Dan McCune for their experimental contributions to the numerous abstracts and manuscripts submitted. Although never acknowledged in our communications, I deeply feel that Dr. Mary Piascik played a significant role in making our presentations comphrendable to the public, so I wanted to thank her for her contributions to the success of our abstracts and manuscripts. All members of the Piascik lab, including Jennifer Olges-Murphy, have been great for discussing experimental data, critiquing thoughts, and discussing non-lab-related issues.

In addition, I would like to acknowledge the core imaging facility personnel (Mary Gail Engle, Mary Jennes, Dr. Bruce Maley, and Richard Watson) for teaching me how to use the confocal microscope. I appreciate Ruth Oremus’s effort to teach me how to measure the mean arterial pressure in small rodents. Other individuals deserving thanks are those involved in teaching me the isolated-perfused heart and the myocyte isolation and contraction techniques (Drs. Robert Lasley, Parkash Narayan, and Eric Kilpatrick). I would like to thank Dr. Bradley Keller, Dr. Kimimasa Tobita, and Joseph Tinney for performing echocardiographic analyses on transgenic animals and discussing the results in a way that I could comprehend.

Finally, I want to thank the faculty, staff, and students of the Department of Molecular and Biomedical Pharmacology for their continued support over the years. In particular, I want to express my gratitude to Deborah Turner for taking care of classes and room schedulings, paper work, and numerous things associated with getting though the semester to attending scientific conferences. I would also like to thank both Deborah and Mary Pat Mertz for providing a place to talk about things other than science.

TABLE OF CONTENTS

LISTS OF TABLES

LIST OF FIGURES

LIST OF FILE

Chalothorn Dissertation.pdf

Chapter One

Background

Receptor Concept and History

The term given to a foreign molecule with the potential to elicit physiological effect(s) within the biological milieu is a "drug". A drug uses its intrinsic structural features to interact with a functionally relevant biological molecule. The term for the biological entity that undergoes a physiocochemical interaction to elicit a response in the presence of a drug molecule is a "receptor".

In the late 19th and early 20th century, the receptor concept developed from two distinct lines of experimentation, and John Newton Langley developed the first line. In his studies, Langley discovered that atropine, a muscarinic antagonist, inhibits feline salivary flow induced by the alkaloid pilocarpine, a muscarinic agonist. From his observations, he surmised that atropine and pilocarpine form complexes with a substance present on the nerve terminals that ultimately contributes to an overall response (Langley, 1878). However, he did not coin the term "receptive substance" until 1905 after performing a follow-up study on Claude Bernard's observations of the South American arrow poison, curare. Bernard illustrated that curare inhibits impulse transmissions from the motor nerves to the skeletal muscle and that the drug localizes its effects to the nerve terminal (Bernard, 1864). Langley observed that even with a severed motor nerve, a muscle fiber retains the ability to contract in the presence of the cholinergic agonist nicotine. Additional experiments with curare led to the observation that direct electrical stimulation is capable of eliciting a contractile response in either the innervated or the denervated muscle fiber. From these results, Langley suggested that both nicotine and

curare interact with an entity other than the nerve or the muscle directly. So he termed this biological entity as a "receptive substance" (Langley, 1905).

Paul Erlich's work on antigen-antibody interactions contributed to the development of the second line of experimentation in the receptor concept. His fascination with antibody specificity for antigenic substances led to his belief that there are precise interactions between the antibody and the antigenic molecule. From his work with organic dyes and parasitic protozoa, Ehrlich observed that changes in the functional group(s) on a drug would affect the anti-parasitic potency. Therefore, he postulated that drugs interact with side chains on the cell to evoke specific actions. In 1913, Ehrlich coined the term "receptor" to describe the side chains that bind drugs (Ehrlich, 1913).

Adrenoceptor History

Many investigators used naturally occurring alkaloids and sympathomimetic amines to study the physiological responses on the autonomic nervous system, and the adrenoceptor family would emerge from this line of study. Using ergot derivatives and adrenaline, Sir Henry Dale made the observation that ergot alone raises blood pressure in cats. However, a combination of ergot and adrenaline results in the fall of blood pressure, from which he concluded that the ergot alkaloids could antagonize the stimulatory but not the inhibitory effects of adrenaline (Dale, 1906). Dale's documentation of feline physiological responses to ergot alkaloids and adrenaline is likely the first evidence of multiple receptors.

R.P. Ahlquist later provided more definitive evidence for the existence of multiple adrenoceptors. Using sympathomimetic amines in animals, Ahlquist demonstrated that

there are distinct differences in the order of agonists (epinephrine, norepinephrine, and isoproterenol) potency to induce physiological responses (blood pressure, heart rate, and myocardial contractility) (Ahlquist, 1948). He defined sympathomimetic amine-induced vasoconstrictive responses as being α-adrenotropic receptor-mediated, while the sympathomimetic amine-induced vasodilatory and myocardial contractile responses as being β-adrenotropic receptor-regulated.

α-Adrenoceptor Subtypes

While the work by Ahlquist documented the α-adrenoceptors (α-ARs) as mediators of vasoconstriction, later studies proved that this receptor group could also participate in synaptic transmission and myocardial function (Wenzel and Su, 1966; Govier *et al*., 1966; Benfey and Varma, 1967). Studies using the non-selective α-AR antagonists, phenoxybenzamine and phentolamine, illustrated increases of norepinephrine release following nerve stimulation (Brown and Gillespie, 1957). This finding indicated that the α-AR can modulate the release of neurotransmitters from the synaptic nerve terminals. Thus, activation of the pre-synaptic receptor would result in the inhibition of the neurotransmitter release, whereas the blockade of the pre-synaptic receptor would augment the neurotransmitter release.

Studies conducted by Starke documented that the use of imidazoline derivatives and phenylephrine increases while the use of dihydroergotamine decreases norepinephrine release in the isolated heart. However, use of the imidazoline derivative oxymetazoline antagonizes the phentolamine-mediated norepinephrine release from the pre-synaptic junction (for review, see Starke, 1977). From these observations as well as

the realization that the imidazoline agonists do not increase the myocardial contractile force as phenylephrine does, Starke proposed that different α-ARs are responsible for mediating different physiological responses such as myocardial contraction and neurotransmitter release.

In conjunction with the emerging evidence of multiple α-ARs, Dubocovich and Langer observed that low concentrations of phenoxybenzamine blocks the increase in perfusion pressure (mediated by the later identified post-synaptic vascular receptors) without altering norepinephrine release (Dubocovich and Langer, 1974). On the contrary, they reported that a 10- to 100-fold dose increase of this irreversible non-selective α-AR antagonist would enhance the norepinephrine release. The differences noted from this study confirmed the existence of distinct pre- and post-synaptic receptors.

In 1974, Langer termed the pre-junctional α-ARs as α_2 while the post-junctional α-ARs as α_1. Unlike the previous pharmacological classification that distinguished the α- from the β-ARs, anatomical location was the basis of the early α-AR subdivision scheme. Following the discovery of a population of pre-synaptic α_2-ARs and the emerging data implicating α_2-AR participation in more than inhibition of synaptic transmissions, the criteria for α-AR subclassification would evolve to better define the groups of α-ARs. In 1977, Berthelsen and Pettinger suggested that physiological functions should serve as the basis for α-AR subclassification (Berthelsen and Pettinger, 1977). With this newer classification scheme, the α_1-ARs defined the receptors associated with vascular smooth muscle contraction while the α_2-ARs included receptors associated with functional inhibition. Redefining the α-AR classification would continue with the advent of more selective ligands. Drew and Whiting investigated the pressor responses of the agonists,

phenylephrine and norepinephrine, in the presence of either prazosin or yohimbine. They observed that yohimbine antagonizes both agonists, but only prazosin antagonizes the phenylephrine-induced pressor response (Drew and Whiting, 1979). Thus, they deduced that α_1-AR is prazosin sensitive and α_2-AR is prazosin insensitive. This pharmacological classification also defined the α_1-ARs as the group responsive to the agonists as phenylephrine, methoxamine, or cirazoline and the antagonists as prazosin or WB 4101; the α_2-ARs as the group receptive to the agonists as BHT-933, UK 14304, or BHT-920 and the antagonists as yohimbine, rauwolsine, or idazoxan. Additional support entertaining the notion of two α-ARs demonstrated that the Ca^{2+}-channel blocker verapamil antagonizes the clonidine-induced constriction in the rabbit pulmonary artery, but minimally affects the methoxamine-induced response (Holck *et al*., 1983). Emerging studies embraced this pharmacological classification that discerned the receptor subtypes according to ligand binding specificities (Timmermans and Van Zwieten, 1980; Ruffolo *et al*., 1991).

Heterogeneity of the α_1-Adrenoceptors

Following the pharmacological classification of the α-ARs into α_1- and α_2-ARs, several studies using various vascular smooth muscle preparations generated results inconsistent with the notion of a single post-junctional α_1-AR. Emerging data favored the notion of α_1-AR heterogeneity. In 1977, Ruffolo reported that oxymetazoline could desensitize the α_1-AR imidazoline-mediated response in the vas deferens; however, the phenylethylamines (phenylephrine, norepinephrine, and methoxamine) could still elicit a response, which suggested the possibility of distinct interactions between α_1-ARs and

structurally unrelated agonists (Ruffolo *et al*., 1977). Later, Bevan generated biphasic dose-response curves in a series of experiments with phenylethylamine agonists, and again the results from this study suggested the existence of more than one receptor subtype (Bevan, 1981). In other studies, Coates *et al*. (1982) and Coates and Weetman (1983) demonstrated the existence of two types of α_1-ARs in the rat anococcygeal muscle using the α_1-AR selective imidazoline agonist, SGD 101/75. They showed that phenoxybenzamine preferentially blocks what would be termed the α_{1S}-AR-mediated SGD 101/75 response but not the other α_1-AR-mediated norepinephrine response.

From data such as those discussed above, McGrath proposed two types of post-junctional α_1-ARs: α_{1A}- and α_{1B}-ARs. Under this classification scheme, the α_{1A}-AR defined the group mediating the effects of both phenylethylamines at low concentrations and non-phenylethylamines while the α_{1B}-AR defined the group mediating the effects of both phenylethylamines at a high concentration and non-phenylethylamines at low concentrations (McGrath, 1982). Additional evidence supporting the notion of two α_1-ARs came from a study observing differences in the extracellular Ca^{2+}-sensitivity to the two agonists: clonidine and methoxamine (Holck *et al*. 1983). More evidence of α_1-AR heterogeneity emerged with the finding that Ca^{2+}-channel blockers inhibit the SGD 101/75-mediated increases in blood pressure in the pithed rat (Timmermans *et al*., 1983). A study investigating vasoconstriction in the rat superfused tail artery revealed a population of α_1-ARs with a high affinity and another population with a low affinity for prazosin (Medgett and Langer, 1984).

Although physiological studies indicated α_1-AR heterogeneity, early binding studies were unable to confirm this notion (Hughes *et al*., 1982; Tsujimoto *et al*., 1984).

Later, several laboratories including our own generated Scatchard plots displaying high and low affinity binding sites for [^{3}H]-prazosin, suggestive of the existence of two types of α_1-ARs (Drew, 1985; Flavahan and Vanhoutte, 1986; Babich *et al*., 1987; Piascik *et* al., 1988). In binding studies using [^{3}H]-prazosin with a series of agonists and antagonists, Morrow and Creese (1986) pharmacologically identified the high affinity binding α_1-AR as the α_{1A}-AR and the α_{1B}-AR as the low affinity receptor. In support of this notion, several studies indicated that the α_{1A}-AR possesses higher binding affinities than the α_{1B}-AR for the following ligands: 5-methylurapidil (Gross *et al*., 1988; Hanft and Gross, 1989), niguldipine (Boer *et al*., 1989), oxymetazoline and citrazoline (Horie *et al*., 1995), and Abbott 61603 (Knepper *et al*., 1995).

Further pharmacological characterization of the α_1-ARs indicated that both dibenamine and benextramine could eliminate the [^{125}IBE] α_1-AR binding population completely in the rat cerebral cortex (Johnson and Minneman, 1987). However, these investigators noted that the alkylating analog of clonidine, chloroethylclonidine (CEC), could reduce the [^{125}IBE] α_1-AR binding population by approximately 35 to 40%, which suggested that one α_1-AR subtype is sensitive to CEC inactivation. Additional experiments revealed that CEC does not inactivate the receptor subtype population possessing a high affinity for WB 4101, 5-methylurapidil, phentolamine, or niguldipine (Han *et al*., 1987a, 1987b; Minneman *et al*., 1988). In *in vivo* experiments, the alkylating analog of prazosin, SZL-49, selectively blocks the α_{1A}-AR population without affecting the α_{1B}-AR population (Kusiak *et al*., 1989; Piascik *et al*., 1988; Piascik *et al*., 1990). However, subsequent work using SZL-49 revealed that this selectivity does not apply during *in vitro* conditions (Piascik *et al*., 1991).

Molecular Cloning of the α_1-Adrenoceptor Subtypes

Although the pharmacological characterization of the α_1-ARs identified two subtypes, the molecular cloning of the α_1-ARs found three receptor subtypes. From the hamster smooth muscle cell line, Cotecchia *et al*. (1988) isolated and cloned the first α_1-AR. Experiments with the cloned α_1-AR revealed that the complementary DNA (cDNA) encodes a polypeptide of 515 amino acids with an approximate molecular weight of 56 kDa. The pharmacological characterization of the cloned receptor showed similarities to the α_{1B}-AR pharmacology because of the ability to bind the antagonist 2-{β-(4-hydroxy-3-[^{125}I] iodophenyl)ethylaminomethyl]-tetralone ([^{125}I]HEAT) with high affinity and the α_{1A}-AR selective antagonists phentolamine and WB 4101 with low affinity. The cloned receptor possessed low affinity for many α_1-AR ligands and demonstrated sensitivity to CEC inactivation. Because of the similarities with the pharmacological profile of the α_{1B}-AR defined by Morrow and Creese (1986), Cotecchia *et al*. (1988) identified the cloned hamster α_1-AR as the α_{1b}-AR.

Schwinn *et al*. (1990) isolated and cloned a different α_1-AR from the bovine brain. These investigators showed that the cDNA for this receptor encodes a polypeptide of 466 amino acids with an approximate molecular weight of 51 kDa and shares about a 72% homology of the membrane spanning domains with the α_{1B}-AR. The pharmacological studies revealed that this cloned α_1-AR receptor possesses binding affinities higher than the α_{1B}-AR but lower than the defined α_{1A}-AR. Schwinn *et al*. (1990) found no correlations between the properties of this cloned receptor and the previous α_1-AR pharmacological binding profiles; in addition, the investigators could not locate the mRNA transcripts for the cloned receptor in tissues known to express the α_{1A}-

AR. Further experiments revealed partial receptor sensitivity to CEC inactivation, which the α_{1A}-AR is resistant. Because experiments with the cloned receptor revealed distinct properties from both the pharmacologically defined α_{1A}- and α_{1B}-ARs, these investigators concluded the discovery of a new receptor subtype; thus, they termed the novel cloned receptor as the α_{1c}-AR. However, other studies using more sensitive ribonuclease protection assays and Northern blots revealed the presence of the mRNA for the α_{1c}-AR in tissues expressing the α_{1A}-AR (Forray *et al*., 1994; Laz *et al*., 1994; Perez *et al*., 1994; Price *et al*., 1994a; Rokosh *et al*., 1994). Increasing evidence correlated the similarities of the pharmacological properties of the α_{1c}-AR to the α_{1A}-AR; consequently, researchers agreed that the α_{1c}-AR was the α_{1a}-AR (Hieble *et al*., 1995).

Similar to the confusion involved in identifying the α_{1a}-AR, the identification of the third cloned α_1-AR subtype also entailed controversy. Lomasney *et al*. (1991) isolated and cloned an α_1-AR from the rat cerebral cortex. The investigators showed that the cDNA for this isolated receptor encodes a polypeptide of 560 amino acids that is approximately 73% homologous to the α_{1B}-AR membrane spanning domains. In addition, they noted that the cloned receptor lacked a consensus site in the 3rd cytosolic loop for protein kinase C (PKC) phosphorylation. The pharmacological studies on the receptor revealed a similar profile to the α_{1A}-AR because of the higher binding affinities for α_1-AR ligands. Additional experiments demonstrated receptor insensitivity to CEC inactivation like the α_{1A}-AR, thus the investigators termed the cloned α_1-AR as the α_{1a}-AR. On the contrary, a different study reported a cloned receptor from the rat hippocampal tissue of nearly identical nucleic acid sequence (98% homologous) with binding properties unlike the pharmacologically defined α_{1A}-AR (Perez *et al*., 1991). The

pharmacological experiments performed by these investigators revealed the binding affinity of this novel receptor for (+)-niguldipine to be lower than the α_{1A}-AR yet higher than the α_{1B}-AR. In addition, these investigators provided further evidence that the cloned receptor from the rat cerebral cortex could not be the α_{1A}-AR by demonstrating that the cloned receptor was susceptible to CEC inactivation (70% inactivated). So owning to the different pharmacological profiles previously documented, Perez *et al*. (1991) designated this cloned receptor as the α_{1d}-AR. **Table 1** lists the accepted nomenclature and the pharmacological profiles associated with the α_1-ARs.

Structure of the α_1-Adrenoceptors

From the cloned α_{1a}-, α_{1b}-, and α_{1d}-AR amino acid sequences, the hydrophobicity analyses revealed stretches of hydrophilic residues linking seven clusters of 20 to 25 hydrophobic residues (Cotecchia *et al*., 1988; Schwinn *et al*., 1991; Lomasney *et al*., 1991; Perez *et al*., 1991). The characteristics of these amino acid sequences are similar to the family of guanine nucleotide binding-protein-coupled receptors (GPCRs). The structural arrangement of the GPCR consists of seven hydrophobic domains spanning the cellular membrane with the hydrophilic domains making up the amino-terminus, the alternating loops between hydrophobic domains, and the carboxyl-terminus (reviewed in O'Dowd *et al*., 1989; Dohlman *et al*., 1991; Caron and Lefkowitz, 1993).

Similar to all GPCRs, modification may occur on specific segments of the protein. For example, N-linked glycosylation can occur on the amino-terminus and palmitylation may occur on the carboxyl-terminus, which could potentially affect receptor signal transduction (O'Dowd *et al*., 1989; Kennedy and Limbird, 1993). Receptor

Table 1. Nomenclature and pharmacology of the α_1-Adrenoceptors.

			Selective		Non-selective		
						Antagonists	
	Previous Name(s)	**CEC Sensitivity (% inactivated)**	**Agonist**	**Antagonist(s)**	**Agonists**	**Reversible**	**Irreversible**
α_{1A}-AR	α_{1C}-AR	Low (30)	A 61603	SNAP 6991, 5-MU, Niguldipine, Tamulosin, WB 4104, SZL-49(irrev)	Phenylephrine, Methoxamine, Epinephrine, Norepinephrine, Oxymetazoline, Citrazoline, Imidazolines	Prazosin, Doxazosin, Phentolamine,	Phenoxybenzamine, Dibenamine, Benextramine
α_{1B}-AR	α_{1B}-AR	High (90)	___	___			
α_{1D}-AR	α_{1A}-AR & $\alpha_{1A/D}$-AR	Moderate to High (70)	___	BMY 7378			

glycosylation potentially affects the number of receptors on the cellular surface while receptor palmitylation potentially affects the anchoring of the receptor to the plasma membrane (for review, see Chen and Manning, 2001). For discussion pertaining to the phosphorylation of the intracellular loops and the carboxyl-terminus, see the section on regulation of receptor signaling.

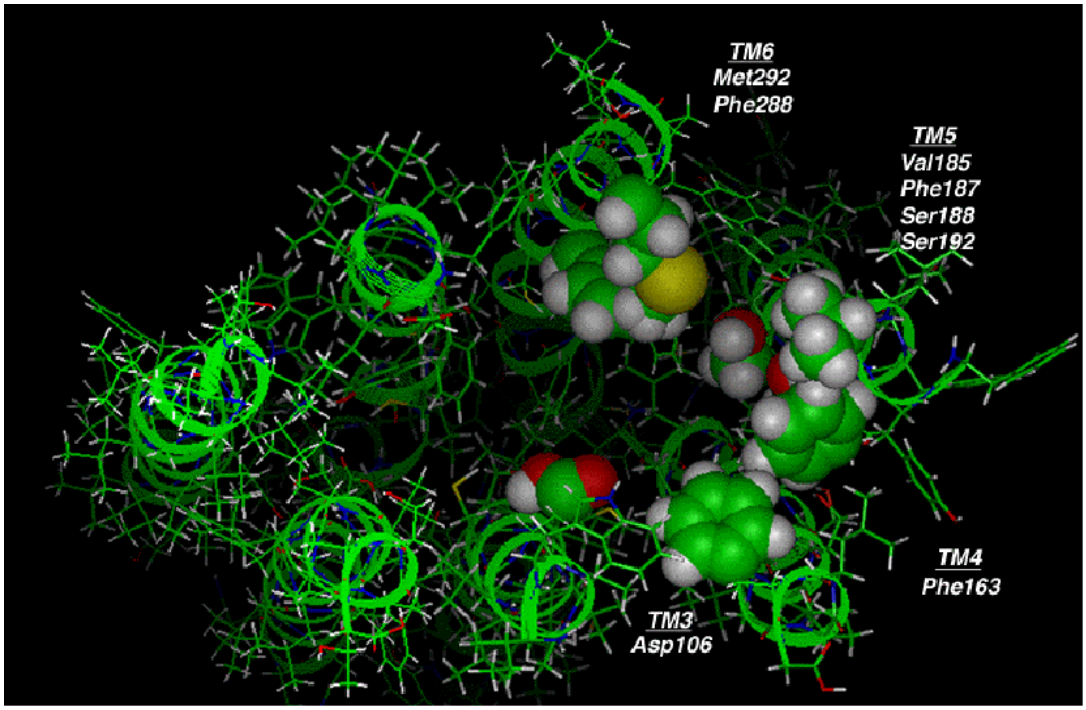
Ligand Binding Pocket of the α_1-Adrenoceptor

As members of the GPCR superfamily, the ARs use one or a combination of the following three domains for ligand binding: the amino terminus, the extracellular loops, and the membrane-spanning sections. Based on the residues in the membrane-spanning domains of the β_2-AR, site-directed mutagenesis studies of the aminergic receptors favored the notion that amino acids in the membrane-spanning domains participate in interactions with key functional groups on the ligand (Strader *et al*., 1987). The binding domains form a binding pocket where sets of highly conserved amino acid residues interact with specific structural features of the endogenous catecholamines. For a time, investigators believed that endogenous catecholamines bind all ARs in a similar fashion; however, a later study revealed distinct differences within the ligand bind pocket that would confer the ability to discriminate among different ligands (Hwa *et al*., 1995). These investigators confirmed that two amino acid residues in the binding pocket are responsible for a specific pharmacological profile between two different but related α_1-AR subtypes.

Interactions between catecholamines and ARs are likely to involve the use of the following key structural features: the phenyl ring, the hydroxyls attached at the para- and

the meta-positions, and the positive charge on the basic amine moiety (for review, see Strader *et al*., 1994). A study investigating the molecular structure of the α_{1B}-AR showed that Phe^{310} is relevant in the formation of an aromatic bond between the receptor and the phenyl ring since loss of the interaction results in reduction of binding affinity for antagonists such as phentolamine and prazosin (Chen *et al*., 1999). Additionally, these investigators demonstrated a decrease in agonist potency and efficacy with the loss of the aromatic bond. With regard to interactions of the catechol hydroxyls, Strader *et al*. (1989) revealed that the β_2-AR uses both Ser^{204} and Ser^{207}; however, the α_{1A}-AR requires only Ser^{188} for *meta*-hydroxyl binding (Hwa and Perez, 1996) (see **Figure 1**). In addition, there is a difference in the number of residues separating the serines required for H-bonding to hydroxyls, so there is approximately a 120° rotation of the catechol ring. This difference in orientation may contribute to the observed differences in agonist binding specificity between the α_1- and the β_2-ARs. Similar to the Asp^{113} of β_2-AR described by Strader *et al*. (1988), the α_{1B}-AR uses the Asp^{125} to interact with the protonated amine of the catecholamine molecule. In the absence of the catecholamine, a salt bridge exists between Asp^{125} and Lys^{331} that constrains the receptor in an inactive state; however, in the presence of the catecholamine there is a competition between the protonated amine and Lys^{331} residue for the Asp^{125} residue (Porter *et al*., 1996). Ultimately, binding of the catecholamine results in the formation of a new salt bridge between the protonated amine of the ligand and the Asp^{125} residue. Thus, the Asp^{125} residue serves as a counter ion relevant for agonist binding and activation of the receptor.

Figure 1. A space-filled representation of the α_{1A}-AR binding pocket. The model shows the relevant amino acid residues believed to participate in the ligand binding (Figure from Piascik and Perez, 2001).



Receptor Activation

In the unbound state, the receptor has a salt bridge that links two amino acid residues together to constrain the receptor to the ground state or the inactivated conformation. In the presence of an agonist, this salt bridge breaks and a new salt bridge forms between an aspartic acid residue and an aliphatic nitrogen atom common to all sympathomimetic amines (Porter *et al*., 1996). Consequently, this disruption of the salt bridge between the two amino acid residues results in an altered receptor conformation and receptor activation.

A study using site-directed mutagenesis on the α_{1B}-AR mRNA revealed that altering the Asp^{293} to any other amino acid results in receptors that are active in the absence of an agonist (a characteristic of a constitutively active receptor, which is the topic of the following section) (Kjelsberg *et al*., 1992). This study proposed that there is an element within the 3rd intracellular loop that constrains the receptor to the basal state until an agonist binds. In a similar study, replacing Lys^{331} with Ala in the α_{1B}-AR eliminates a positive charge that results in the disruption of a salt bridge (Porter *et al*., 1996). This mutagenesis study also produced a receptor possessing the ability to initiate signaling without agonist binding. Additionally, site-directed mutagenesis of the Asp^{125} to either a neutral Ala or a positively charged Lys resulted in an activated receptor and the postulate: the positively charged Lys^{331} is responsible for stabilizing the negatively charged Asp^{125} via salt bridge formation.

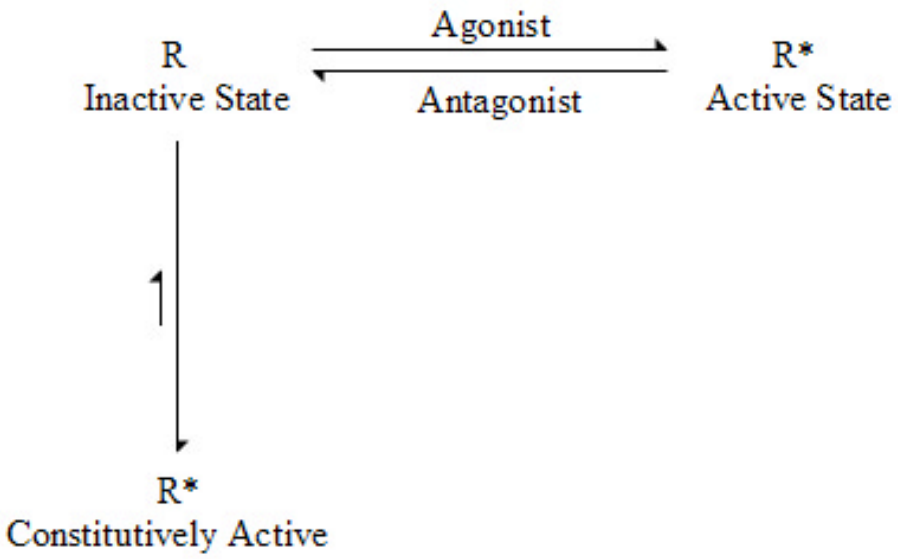
Constitutively Active Receptors

In traditional signaling paradigms, receptor activation occurs subsequent to

agonist-induced 3-dimensional conformational changes of the receptor. Thus, the classical notion views that an unbound receptor lacks the capacity to initiate signaling or coupling to effector pathways. However, the more recent consensus on receptor activation favors the notion that receptors may exist in two conformations: an inactive state (R) and an active state (R*) (Milligan *et al*., 1995; Gether and Kobilka, 1998). Because the receptor can exist in one of two states at any given time, the state of the receptor in the absence of agonists determines the basal activity level. At baseline, the receptor resides mainly in the R state whereas in the presence of an agonist the R* state predominates. The current notion postulates that that agonists have a higher binding affinity for the R* and will stabilize the receptor in the active form, which promotes signal transduction. On the other hand, competitive antagonists can interact with either the R or the R* with similar affinity to prevent conversion of the receptor to the active state and to inhibit signal transduction (See **Figure 2**).

Under basal conditions, certain receptors have the majority of their population in the R* state, and the term for describing these types of receptors is constitutively active. With the ability of site-directed mutagenesis to produce such receptors, constitutively active receptors are proving to be useful for investigating receptors that do not have highly selective agonists available. Using the α_{1B}-AR, Kjelsberg *et al.* (1992) documented an increase in signaling associated with the mutation of the Asp^{293} to any other amino acid residue. These investigators postulated that the constitutive activity is the result of relieving the structural constraint associated with a salt bridge formation. Another study arriving at the same conclusion used site-directed mutagenesis to eliminate the positive charge associated with the Lys^{331} residue, which disrupts the salt bridge

Figure 2. Diagram of the equilibrium existing between an inactive R state and either an active R* state or a constitutively active R* state. In the presence of an agonist, the equilibrium shifts from the R to the R* state. In the presence of an antagonist, the equilibrium tends to shift from the R* to the R state. The majority of the constitutively active receptor population resides in the R* state, in which an inverse agonists can shift the equilibrium to the R state.



formation and results in enhanced ligand binding affinity and increased signal transduction both in the absence and the presence of an agonist (Porter *et al*., 1996).

In a different study, Perez *et al*. (1996) showed that site-directed mutagenesis at approximately one helical turn away from the Asp^{125} results in different degrees of constitutive activity depending on the intrinsic properties of the amino acid replacing the Cys^{128} residue. These investigators noted that the greater the size and the more hydrophobic the residue, the greater the receptor activity. The residue likely interferes with the formation of the salt bridge, which would cause the receptor to adopt the R* state. Unlike the constitutively active mutants described thus far, Hwa *et al*. (1996) generated a constitutively active receptor by altering the helical packing between transmembrane domains. These investigators found that replacing the Ala^{204} with Val in the α_{1B}-AR would alter the 3-dimensional arrangement of the transmembrane domains; consequently, both the Ser^{207} and the Ser^{211} residues are positioned closer together. The result of this 3-dimensional alteration results in increased agonist binding affinity, potency, and signal transduction.

Other constitutively active mutants of the α_{1B}-AR exist, but these receptors have mutations unrelated to the formation of the salt bridge. Cotecchia *et al*. (1990) generated several constitutively active α_{1B}-ARs with a combination of mutations on the 3rd cytoplasmic loop. These mutations resulted in the generation of receptors possessing increased basal level of inositol phosphates and increased potency to agonists.

Signal Transduction Pathways and Coupling to Guanine Nucleotide Proteins

Once activated, the α_1-ARs are capable of participating in ionic transport of

molecules across the plasma membrane by modulating Na^+/H^+-pumps, Na^+/Ca^{2+}-exchange, Ca^{2+}-, and K^+-channels (reviewed in Terzic *et al*., 1993; Graham *et al*., 1996). In cells that endogenously express the α_1-ARs, the postulated function of these receptors is to modulate the increases of intracellular Ca^{2+} concentrations (for review, see Van Zwieten and Timmermans, 1987; Graham *et al*., 1996).

These receptors may participate in complex signal transduction cascades via their coupling to different G-proteins. Similar to other GPCRs, the α_1-AR family uses the 3rd intracellular loop and the carboxyl-terminus to couple to a variety of G-proteins that may interact with a multitude of effector pathways (reviewed in Gilman, 1987; Savarese and Fraser, 1992). The heterotrimeric G-protein has three subunits (α, β, and γ) in which a guanosine diphosphate (GDP) molecule binds the α subunit. Once activated, the heterotrimeric G-protein exchanges the GDP for a guanosine triphosphate (GTP) molecule, which leads to the dissociation of the GTP-bound α subunit from the βγ complex. The dissociated complexes activate specific downstream effectors until the α subunit hydrolyzes GTP to GDP, which results in the inactivation of the subunit and the reassociation of the heterotrimeric complexes (reviewed by Carman and Benovic, 1998).

Of the many heterotrimeric G-proteins available, the α_1-ARs have a proclivity for coupling to the pertussis toxin (PTx)-insensitive G-proteins of the $G_{q/11}$ family (Braum *et al*., 1990; Wu *et al*., 1992a). Additionally, these receptors couple to the PTx-sensitive ($G_{i/o}$) proteins (Steinberg *et al*., 1985, 1987; Bohm *et al*., 1987; Shah *et al*., 1988; Perez *et al*., 1993). Other investigators demonstrated that the α_{1B}-AR is capable of coupling to the G_h, a different form of the heterotrimeric G-protein that still retains the ability to mediate intracellular signaling in the absence of GTP, but this protein has a Ca^{2+}-dependent

transglutaminase activity antagonizable by GTP for cross-linking proteins (Nakaoka *et al*., 1994).

Phospholipase Pathways and the Generation of 2nd Messengers

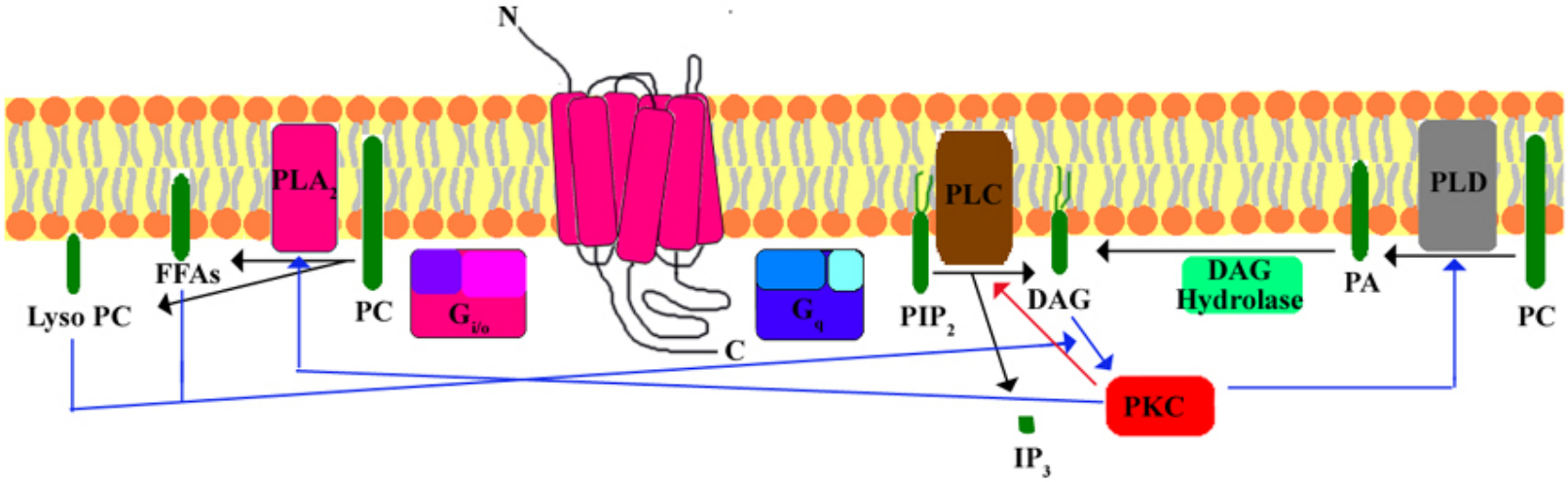
The α_1-AR-activated signal transduction scheme is complex because the α_1-ARs can couple to different G-proteins that activate three distinct phospholipase pathways: phospholipase D (PLD), phospholipase A_2 (PLA_2), and phospholipase C (PLC) (for review, see Terzic *et al*., 1993; Graham *et al*., 1996; Varma and Deng, 2000).

In the rat cerebral cortex, Llahi and Fain (1992) reported that α_1-AR stimulation results in the activation of the PLD signaling pathway. Activation of this pathway results in the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (reviewed in Billah and Anthes, 1990; Exton, 1990; Shukla and Halenda, 1991). DAG hydrolase converts phosphatidic acid to the 2nd messenger 1,2-diacylglycerol (DAG), which is an activator of protein kinase C (PKC) (see **Figure 3**).

The ability of the α_1-AR to couple to the PTx-sensitive G-proteins permits the receptor to activate the PLA_2 pathway (Slivka and Insel, 1987; Weiss and Insel, 1991; for review, see Insel *et al*., 1991). Activation of this signaling pathway results in the hydrolysis of PC to arachidonic acid. Moreover, the second messengers generated by the hydrolysis of glycerolphospholipids are fatty acids and lysophospholipids, both of which augment the DAG-induced activation of PKC (for review, see Bell and Burns, 1991) (see **Figure 3**).

The phospholipase pathway prevalently associated with α_1-AR activation is the PLC pathway. Three isoforms make up the phospholipase C group: PLC-β, PLC-γ, and

Figure 3. Diagram of the phospholipase pathways coupled to α_1-Adrenoceptor activation. PLC, phospholipase C; PIP_2, phosphoinositide-4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; PLD, phospholipase D; PC, phosphocholine, PA, phosphatidic acid; PLA_2, phospholipase A_2; FFAs, cis-unsaturated fatty acids; LysoPC, lysophosphatidylcholine. The black lines represent the enzymatic processes, the blue lines represent stimulation, and the red line represents inhibition.



PLC-δ. The α_1-ARs mainly associate with the PLC-β isoform (Im *et al*., 1990; Blank *et al*., 1991; Berstein *et al*., 1992). Either the GTP-bound $G_{q\alpha}$ subunit or the $G_{q\beta\gamma}$ complex can promote PLC-β activation (Wu *et al*., 1992b). Substrates for activated PLC-β enzyme are phosphatidylinositol (PI), PI-4-phosphate (PIP), and PI-4,5-bisphosphate (PIP_2). The activation of the PLC-β enzyme results in the cleavage of PIP_2 to two relevant 2nd messengers, DAG and inositol-1,4,5-trisphosphate (IP_3) (reviewed by Terzic *et al*., 1993; Woodcock 1995; Graham *et al*., 1996) (see **Figure 3**). As mentioned previously, DAG activates PKC while IP_3 is responsible for evoking changes in intracellular Ca^{2+} concentrations. IP_3 increases the intracellular Ca^{2+} concentration upon binding to the IP_3 receptors located on intracellular organelles that store Ca^{2+}; thus, resulting in the release of Ca^{2+}, a relevant biological ion.

2nd Messenger-Induced Effector Activation

The 2nd messenger product of both the PLD and the PLC pathways is DAG. This molecule stimulates PKC, which is a serine/threonine kinase. PKCs belong to a family of enzymes that consists of 11 isoforms categorized into three groups based on primary structure and function: Ca^{2+}-dependent PKC, Ca^{2+}-independent PKC, and atypical PKC (for review, see Nishizuka 1992, 1995). The α_1-AR-G_q-PLC-β signaling pathway mainly results in the activation of the Ca^{2+}-independent PKC group (Clerk *et al*., 1994; Puceat *et al*., 1994). Activated PKC will translocate from the cytosol to specific regions of the cell in order to phosphorylate cellular substrates, ion channels, and/or receptors (Kraft and Anderson, 1983; Clerk *et al*., 1994; Puceat *et al*., 1994).

Regulation of Receptor Signaling

Cellular signaling commences upon receptor conformational changes induced by specific agonist interactions within the ligand binding pocket. In many receptor systems, the stimulus-induced signal transduction pathway is a stringently regulated event that ensures appropriate cellular and physiological responses. The agonist-induced response becomes attenuated under conditions of chronic or excessive receptor stimulation. In studies investigating α_1-AR-induced inositol phosphate turnover, investigators demonstrated that under prolonged agonist stimulation, there is a remarkable reduction in both receptor responsiveness and the population of receptors on the cellular surface (Wikberg *et al*., 1983; Leeb-Lundberg *et al*., 1987; Fonseca *et al*., 1995). For the GPCR superfamily, the diminished response is typically the result of three distinct mechanisms: desensitization, internalization, or down-regulation.

Desensitization is a rapid process occurring within seconds to minutes of receptor activation that involves the covalent modification (phosphorylation) that renders the receptor incapable of coupling to the heterotrimeric G-proteins (Lurie *et al*., 1985; Brown *et al*., 1986; Lee-Lundberg *et al*., 1987). Depending on the causative stimulus, desensitization proceeds in one of two ways: heterologous (agonist-non-specific) and homologous (agonist-specific) desensitization (for review, see García-Sáinz *et al*., 2000). Heterologous receptor desensitization involves an exposure to agonists of different receptor systems. This process involves the activation of either PKC or cAMP-dependent protein kinase (PKA), which subsequently phosphorylates the 2nd and the 3rd intracellular loops and/or the carboxyl terminus of the GPCR. On the other hand, homologous desensitization involves receptor activation that leads to the activation of a set of kinases

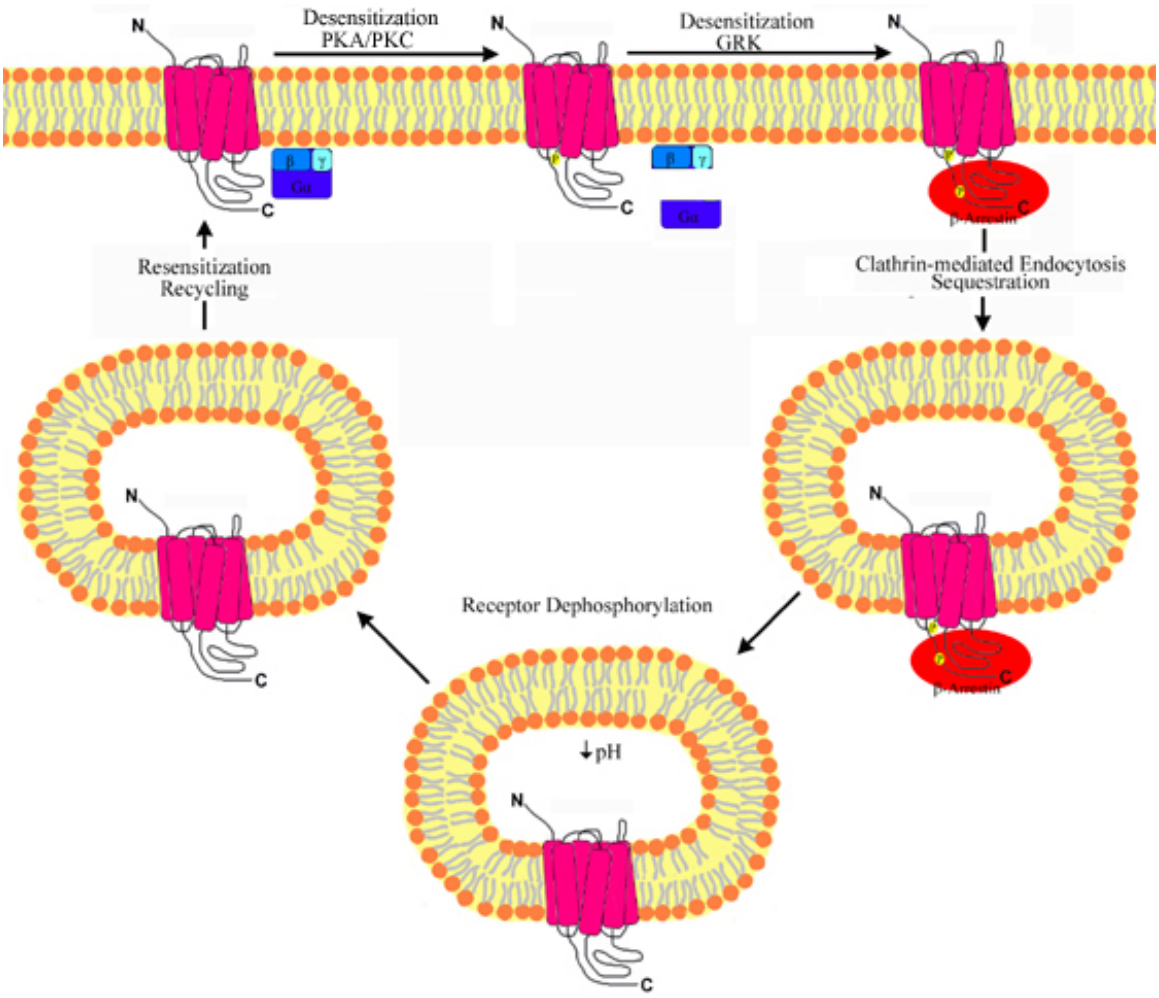
termed G-protein receptor kinases (GRKs). Some studies demonstrated that the GRKs can be activated by PKC phosphorylation (Chuang *et al*., 1995; Winstel *et al*., 1996). There are currently six mammalian GRKs: GRK1 (rhodopsin kinase), GRK2 (β-AR kinase 1 or βARK1), GRK3 (β-AR kinase 2), GRK4, GRK5, and GRK6 (for review, see Premont *et al*., 1995; Pitcher *et al*., 1998). These GRKs co-localize to the plasma membrane where they have a unique ability to recognize and to phosphorylate the activated GPCRs (Benovic *et al*., 1986).

The current model of GRK action postulates that GRK-phosphorylated GPCRs have an increased binding affinity for a family of cytoplasmic inhibitory proteins known as arrestins. The arrestin family includes visual arrestin (arrestin-1), β-arrestin 1 (arrestin-2), β-arrestin 2 (arrestin-3), and cone arrestin (arrestin-4) (reviewed by Krupnick and Benovic, 1998). The β-arrestin 1 and β-arrestin 2 molecules are the non-visual arrestins that contain binding domains for GPCRs (Gurevich *et al*., 1995). The binding of arrestin to the phosphorylated receptor quenches signal transduction by disrupting the receptor/heterotrimeric G-protein interactions (Benovic *et al*., 1987; Lohse *et al*., 1990).

The arrestin molecule also acts as an "adaptor" protein that serves as an intermediary between the GRK-phosphorylated GPCR carboxyl terminus and the clathrin molecule (Goodman *et al*., 1996, 1997; Krupnick *et al*., 1997). This protein-protein association promotes receptor endocytosis into endosomal vesicles, so arrestin participates in both desensitization and agonist-induced internalization (see **Figure 4**) (Ferguson *et al*., 1995; Goodman *et al*., 1998).

Another mechanism the cell uses to reduce the response(s) invoked by an endogenous or an exogenous stimulus is receptor internalization (Fratelli and DeBlasi,

Figure 4. Diagram depicting the processes associated with receptor internalization and cycling of activated G-Protein-Coupled Receptors.



1987; Cowlen and Toews, 1988) (see **Figure 4** for a diagram concerning the process of receptor cycling). The study by Fonseca *et al*. (1995) showed that prolonged stimulation of the hamster α_{1B}-AR in HEK-293 cells would lead to a concurrent decrease in receptor responsiveness of inositol phosphate production as well as an increase in co-localization of receptor and transferrin, an endosomal marker (via immunocytochemistry). These investigators observed that the translocation of receptors from the cellular surface to the endosomes upon PKC activation is antagonizable by staurosporine, a non-selective kinase inhibitor. However, others suggest that GRKs are responsible for mediating the receptor internalization process (Lattion *et al*., 1994; Diviani *et al*., 1996). In a different study using the A293E hamster α_{1B}-AR mutant, Mhaouty-Kodja *et al*. (1999) observed that this constitutively active receptor assumes a conformation that makes it a substrate for GRK2 phosphorylation, and subsequently increases the β-arrestin binding and the degree of receptor internalization. In addition, these investigators generated constitutively active receptors with mutations in different domains. The results of these mutations were functional receptors that could not undergo either phosphorylation or internalization. Therefore, this study demonstrated that mutations in different domains may induce constitutive activity but may have divergent results on the regulatory properties of the receptor.

Internalization of a GPCR results in one of two processes: down-regulation (degradation) or resensitization. Down-regulation of the receptor results in a reduction of the total receptor population, thereby reducing the overall stimuli-induced effect(s) (Hughes and Insel, 1986; Wickberg *et al*., 1983). The long-term event of down-regulation lasts for hours to days depending on turnover rate of the receptor protein, and

replacing the degraded receptor population requires time. Resensitization is a relatively simple process involving the dephosphorylation of the receptor in vesicular endosomes. Subsequently this process returns a functional receptor to the plasma membrane (Fonseca *et al*., 1995).

α_1-Adrenoceptor Localization and Trafficking

The internalization of the receptors into endosomal vesicles for down-regulation or resensitization is a relevant process in controlling signal transduction, so it should be of no surprise that studies investigated the localization and the trafficking properties of the receptors. A study by Hirasawa *et al*. (1997) visualized cellular localization of the α_{1A}- and the α_{1B}-ARs in COS-7 cells using FLAG-tagged epitopes and green fluorescent proteins (GFPs) conjugated to the amino termini and to the carboxyl termini, respectively. These investigators reported a dense α_{1A}-AR localization mainly in the intracellular space in the perinuclear region whereas a diffuse α_{1B}-AR localization was observed predominantly on the plasma membrane. Additionally, they showed that CEC, a highly hydrophilic alkylating antagonist, inactivates approximately the same proportion of α_{1A}- as α_{1B}-ARs on membrane incubated with CEC; thus suggesting that receptors on the membrane are more susceptible to CEC inactivation because of ligand accessibility. Also Awaji *et al*. (1998) investigated the internalization properties for the α_{1B}-AR/GFP in stably transfected mouse αT3 cells using real-time conditions, and they found that agonist stimulation promotes the redistribution of the receptor from the cellular surface to the intracellular region. This group provided additional evidence that the receptor internalizes to the endosomal compartment. McCune *et al*. (2000) used

immunocytochemistry and laser-scanning confocal microscopy to demonstrate in stably transfected fibroblasts that the α_{1B}-AR predominantly localizes to the cellular surface whereas the α_{1D}-AR localizes to the intracellular region.

Distribution of the α_1-Adrenoceptors

Many studies reported a wide distribution of the α_1-AR mRNA transcripts in several tissues (Ping and Faber, 1993; Piascik *et al*., 1994; Rokosh *et al*., 1994; Scofield *et al*., 1995). More notably α_1-AR mRNA transcripts co-localize in the peripheral vasculature where the translated products participate in the regulation of vascular smooth muscle contraction (Piascik *et al*., 1995, 1997; Hrometz *et al*., 1999). With respect to mRNA expression, the rank order of distribution in many arteries studied so far is $\alpha_{1A} > \alpha_{1B} > \alpha_{1D}$ with the α_{1A}-AR making up approximately 90% of the total α_1-AR mRNA (Piascik *et al*., 1995). Due to the paucity of highly selective ligands that can distinguish between the α_{1B}- and the α_{1D}-ARs in radioligand-binding experiments, determining the protein expression level for each subtype is difficult. However, many studies generated estimates for the α_{1A}-AR subtype population in the rat aorta, caudal artery, mesenteric artery, heart, and brain using α_{1A}-AR subtype selective ligands (Morrow and Creese, 1986; Hanft and Gross, 1989; Piascik *et al*., 1990; Han and Minneman, 1991). These studies estimated approximately 5 to 30% of the α_1-AR population is the α_{1A}-AR, indicating that there is no correlation between mRNA transcript levels and protein expression. In the human heart, the α_1-ARs account for up to 15% of the cardiac adrenoceptors (Delhaye *et al*., 1983).

In addition to the vasculature, the α_1-ARs reside in several other tissues where

they maintain homeostasis. In brain, liver, and heart, these receptors participate in physiological functions such as sympathetic neurotransmission, regulation of hepatic metabolism, and mediation of myocardial inotropy, respectively. Studies using rat hearts demonstrated that mRNA transcripts for all three α_1-AR subtypes exist (Price *et al*., 1994; Rokosh *et al*., 1994; Scofield *et al*., 1995). Although the transcripts for the α_1-ARs exist in the rat heart, other studies examining the translated products confirmed that the α_{1A}- and the α_{1B}-AR make up the α_1-AR population in the heart (Deng *et al*., 1996a; Deng and Varma, 1997). These studies revealed that the α_{1D}-AR is nearly undetectable in neonatal rat hearts and virtually non-existent in the adult rat hearts. These findings suggest that the α_{1D}-AR may play a minor role in mediating physiological responses in the heart.

Functions of the α_1-Adrenoceptors in the Vascular Smooth Muscle

In the vasculature, the α_1-ARs mediate two responses: vasoconstriction and vascular growth. From a physiological standpoint, the α_1-ARs are important in their contribution to regulation of blood flow and maintenance of vascular resistance (for review, see Vargas and Gorman, 1995). The stimulation of α_1-ARs in vascular smooth muscle results in phosphoinositide turnover as well as PKC activation, both of which lead to an increase in Ca^{2+} sensitivity of contractile proteins and an increase in intracellular Ca^{2+} concentration via the opening of Ca^{2+} channels on the sacroplasmic reticulum or the cellular membrane (reviewed by Minneman, 1988). The released Ca^{2+} forms a complex with the Ca^{2+} binding protein, calmodulin, that promotes the activation of the myosin light chain kinase (MLCK), which phosphorylates the light chains of myosin (for review, see Gao *et al*., 2001). This phosphorylation subsequently leads to cross-bridge formation

and cycling during which the adenosine triphosphate molecule provides the energy for tension development and shortening of the muscle fiber (reviewed in Adelstein and Eisenberg, 1980). In addition, one notion postulates that PKC phosphorylation of calmodulin will increase the affinity of calmodulin for Ca^{2+}, indicating the possibility for vascular smooth muscle contraction under lower intracellular Ca^{2+} concentrations (Nishimura *et al*., 1990).

Since vascular smooth muscle expresses a combination of all three α_1-AR subtypes, there is a difficulty associated with determining which α_1-AR subtype predominates in the contractile response because all may participate to some degree (for review, see Nichols and Ruffolo, 1991; Vargas and Gorman, 1995; Piascik *et al*., 1996; Docherty, 1998). With both the lack of selective pharmacological tools and the uncertainty associated in the identification of the α_{1D}-AR, many early studies were unable to establish a correlation between subtype activity and vasoconstrictive response. In addition, concerns surrounded the interpretations from early studies that used CEC to distinguish between the α_{1A}- and the α_{1B}-ARs in contractile responses since later studies revealed that CEC is capable of inactivating the α_{1A}- and the α_{1D}-ARs to a low and moderate to high degree, respectively (Johnson and Minneman, 1987; Han *et al*., 1987). Although lacking a selective α_{1D}-AR antagonist at the time, several studies that sought to define the vasoconstrictive responses for the α_{1A}- and the α_{1B}-ARs in arterial vessels ended up generating results inconsistent with other studies (Tian *et al*., 1990; Piascik *et al*., 1991; Aboud *et al*., 1993; Oshita *et al*., 1993). However, Piascik *et al*. (1991) and Aboud *et al*. (1993) implicated the potential for a third binding site that could explain the discrepant observations.

Functional studies using isolated rat arterial vessels revealed that the α_{1A}-AR is the prominent receptor participating in contraction of the renal and the caudal arteries (Lachnit *et al*., 1997; Piascik *et al*., 1997; Hrometz *et al*., 1999). For a time, studies using CEC implicated a role for α_{1B}-AR participation in contraction of the rat thoracic aorta. However, with the discovery of the α_{1D}-AR selective antagonist BMY 7378, experiments using this antagonist disproved this notion (Kenny *et al*., 1995; Testa *et al*., 1995; Piascik *et al*., 1995, 1997; Buckner *et al*., 1996; Deng *et al*., 1996a; Saussy *et al*., 1996). With a more selective antagonist, Piascik *et al*. (1995, 1997) showed in functional studies that aside from the rat thoracic aorta, the α_{1D}-AR is the main mediator of contraction in the rat femoral, iliac, and superior mesenteric arteries. Presently, little evidence exists that conclusively links the α_{1B}-AR to vessel constriction, and data indicating that the α_{1B}-AR participates in the mesenteric resistance artery resulted from exclusion of the other two α_1-ARs (Piascik *et al*., 1994). Use of anti-sense nucleotides targeted against specific receptor subtypes in the rat vessels confirmed the results from earlier studies (Hrometz *et al*., 1999).

The cardiovascular system is a dynamic system able to respond to many stimuli; for instance, the vasculature maintains blood pressure and flow through the regulation of vascular tone by vasoconstriction or vasodilation. In some instances, regulation of vascular tone may occur through wall remodeling. With chronic changes in hemodynamics or humoral factors, the blood vessel wall alters its physical structure as an adaptive response (Dzau and Gibbons, 1993). In the cardiovascular disease of hypertension, increased sensitivity to vasopressors usually accompanies the structural changes in the blood vessel walls. The thickening of the layers in the vessel walls

attenuates the physical stimuli (stretch and tension) resulting from an increased blood pressure; in addition, hypersensitivity to vasopressors develops to modulate the effects of the ever changing levels of catecholamines. As a consequence of this process, the increase in vessel wall thickness results in a decrease of the lumen diameter which contributes to increased peripheral vascular resistance and increased after-loading on the heart.

Functions of the α_1-Adrenoceptors in the Myocardium

The α_1-ARs have a multi-functional role in the cardiovascular system. In addition to participating in vascular smooth muscle contraction, they serve a role in myocardial inotropy and hypertrophic growth responses. Wenzel and Su (1966) reported the first evidence that the α_1-ARs induce a positive inotropic effect in response to sympathomimetic amines on rat ventricular strips. The characteristics of positive inotropy include an augmentation of the myocardial contraction amplitude without a significant alteration in the contraction-relaxation cycle or the time to achieve peak tension and relaxation (reviewed in Terzic *et al*., 1993). The characteristics associated with the α_1-AR-mediated inotropic effects are the subject of several reviews (see Fedida *et al*., 1993; Terzic *et al*., 1993; Li *et al*., 1997).

The α_1-AR-mediated inotropic effect is usually a tri-phasic response and may include a negative inotropic component (Skomedal *et al*., 1983). The first two phases of the triphasic response are transient and subtle. In brief, the triphasic response first has a rapid increase in contractile force followed by a decline in contractile force below baseline then a sustained contractile force greater than the initial positive contractile

force. Although the mechanism of the triphasic response is not fully understood, this response has been seen in rat papilliary muscle (Otani *et al.,* 1988), mouse cardiac trabeculae (McCloskey *et al*., 2002), and isolated mouse hearts (Turnbull *et al*., 2003). This α_1-AR-mediated positive inotropic effect wanes with age in the rat heart and is likely the consequence of a reduction in the receptor density (Inayatulla *et al*., 1994; Deng *et al.*, 1996b).

Three proposed mechanisms associated with α_1-AR-mediated myocardial positive inotropy exist: increased myofibril sensitivity to Ca^{2+}, increased sarcolemmal Ca^{2+} influx, and alkalinization. In the first mechanism, the activation of PKC leads to the phosphorylation of MLCK that subsequently phosphorylates MLC-2, leading to increased myofibril sensitivity to Ca^{2+} (Morano *et al*., 1985; Venema *et al*., 1993). In the other mechanism, α_1-AR activation inhibits the K^+ current believed to prolong the action potential duration, resulting in the increase of the intracellular Ca^{2+} concentration (Apkon and Nerbonne, 1988; for review, see Fedida *et al*., 1993; Terzic *et al*., 1993). A third mechanism uses the Na^+/H^+ exchanger to regulate the intracellular Ca^{2+} (Fliegel and Wang, 1997). Studies have found a correlation between the magnitude of α_1-AR-mediated inotropic response and extent of alkalinization by α_1-AR agonists (Terzic *et al*., 1992; Gambassi *et al*., 1992).

To support the finding that the α_1-ARs use a different mechanism than the β_1-AR in mediating positive inotropy, studies reported that the presence of either a phosphodiesterase or an adenylyl cyclase inhibitor does not affect the α_1-AR-stimulated response (Endoh and Motomura, 1979; Endoh and Yamashita, 1980; Christiansen *et al*., 1987). Thus the α_1-AR response is independent of cAMP generation. Early studies

showed that the phenylephrine-mediated positive inotropy could be antagonized with prazosin in the adult rat heart (Skomedal *et al*., 1980). Skomedal *et al*. (1988) found that the α_1-ARs contribute approximately 25% of the norepinephrine-mediated inotropic response. At the cellular level, several studies documented an α_1-AR-regulated positive inotropic effect in rat cardiomyocytes (Capogrossi *et al*., 1991; Fedida and Bouchard, 1992; O'Rourke *et al*., 1992; Terzic *et al*., 1992; Gambassi *et al*., 1998).

As mentioned earlier, expression of the α_{1D}-AR subtype is not common in the myocardium; therefore, there is little data supporting a role for this receptor in the inotropic response (Deng and Varma, 1997; Wenham *et al*., 1997). Other studies have implicated the α_{1A}- and the α_{1B}-AR in the regulation of positive inotropy (Williamson *et a*., 1994; Deng *et al*., 1996b). Michel *et al*. (1990, 1994) and Yu and Han (1994) reported that the α_{1B}-AR is the more relevant subtype for myocardial inotropy in rat hearts. However, other studies have shown that SZL-49 and WB4101 but not CEC would antagonize the norepinephrine-induced response in the rat heart, which suggests α_{1A}-AR participation (Rokosh and Sulakhe, 1991; Gambassi *et al*., 1991).

As discussed above, β-AR mediates the majority of the catecholamine driven inotropic responses in the normal heart. Therefore, there are many questions pertaining to the relevance of the α_1-AR inotropic responses in the myocardium. However, there are suggestions that during heart failure the α_1-ARs play a greater role in inotropic responses to endogenous catecholamines (for review, see Terzic *et al*., 1993; Li *et al*., 1997; Varma and Deng, 2000). Heart failure is a condition characterized by the inability of the heart to provide adequate blood flow to tissues. As a consequence of this situation, the body attempts to adapt by increasing the levels of circulating catecholamines to stimulate the

β_1-AR-G_s-adenylyl cyclase signaling pathway which subsequently results in increased cardiac output. This activated signaling cascade increases positive inotropy, and with prolonged stimulation, the regulation of the β_1-AR-mediated response ensues. Consequently, this adaptive reflex becomes a maladaptive process because of the down-regulation of the β_1-AR population, which may cause ventricular dysfunction. Many studies suggested that activation of the α_1-ARs serves as a reserve mechanism to maintain myocardial responsiveness (Bristow *et al*., 1982; reviewed in Brodde *et al*., 1995). To support this notion, Bristow *et al*. (1988) showed that there are no differences between the failing and the non-failing hearts in terms of α_1-AR densities or α_1-AR-mediated PI turnover. Therefore, the question of whether the α_1-ARs can sustain myocardial function in the long-run still exists.

<u>α_1-Adrenoceptor Signaling in Growth-Related Responses</u>

The α_1-ARs are versatile receptors, and there is a growing interest concerning the roles these receptors play in cellular growth and proliferation. Growth of mammalian cells resulting in increases of cell size (hypertrophy) and/or number (hyperplasia) is attributable to a wide range of factors such as catecholamines, growth factors, physical stress, etc. The precise mechanism(s) of α_1-AR-mediated growth and proliferation is(are) poorly understood because of the multiple stimuli.

The activation of the heterotrimeric G-protein, $G_{\alpha q}$, by α_1-ARs leads to the up-regulation of transcriptional factors that are associated with increases in the activities of two effectors: Ras and PKC (for review, see Widmann *et al*., 1999; Hoffman and Hu, 2000; Varma and Deng, 2000; Michelotti *et al*., 2000). Signaling associated with the

activation of these effectors converge on a family of serine/threonine kinases known as the mitogen-activated protein kinases (MAPKs) (reviewed in Gutkind, 1998). Three types comprise this MAPK family: extracellular signal-regulated protein kinase (ERK or p42/p44), c-Jun N-terminal kinase (JNK), and p38.

A link between effector and MAPK activation has been difficult to establish due to the involvement of numerous elements in the signaling cascade and the potential for interaction among other MAPK signaling pathways. In a study using an animal model over-expressing the Ras effector, Ramirez *et al*. (1997) made the observation that enhanced MAPK kinase kinase (MEKK1) activity leads to the stimulation of JNK; thus, establishing the Ras-MEKK1-JNK pathway for cellular growth and proliferation. In addition, these investigators showed that a dominant negative mutant of Raf, a MAPK kinase kinase, does not impact the JNK pathway. This investigation supported the notion that MEKK1 preferentially stimulates the JNK pathway while Raf preferentially regulates the ERK pathway (reviewed in Minden and Karin, 1997). A controversial issue is that both PKC and Ras have the ability to activate both MAPK kinase kinases (MEKK1 and Raf) (Lazou *et al*., 1994; Bogoyevitch *et al*., 1995). Adding to the confusion, a study implicated Raf in the activation of a MEK6, a MAPK kinase that leads to the activation of p38 (Zechner *et al*., 1997). Despite the controversy, a degree of separation exists within the MAPK family that may clarify the role each member has in the growth and the proliferation response. Under stressful conditions, there is an enhancement in both the JNK and the p38 activities. Due to circumstances surrounding the activation of JNK and p38, researchers termed these proteins as the stress-activated protein kinases (SAPKs). Depending on the type of stimulus, activation of a specific MAPK pathway utilizes the

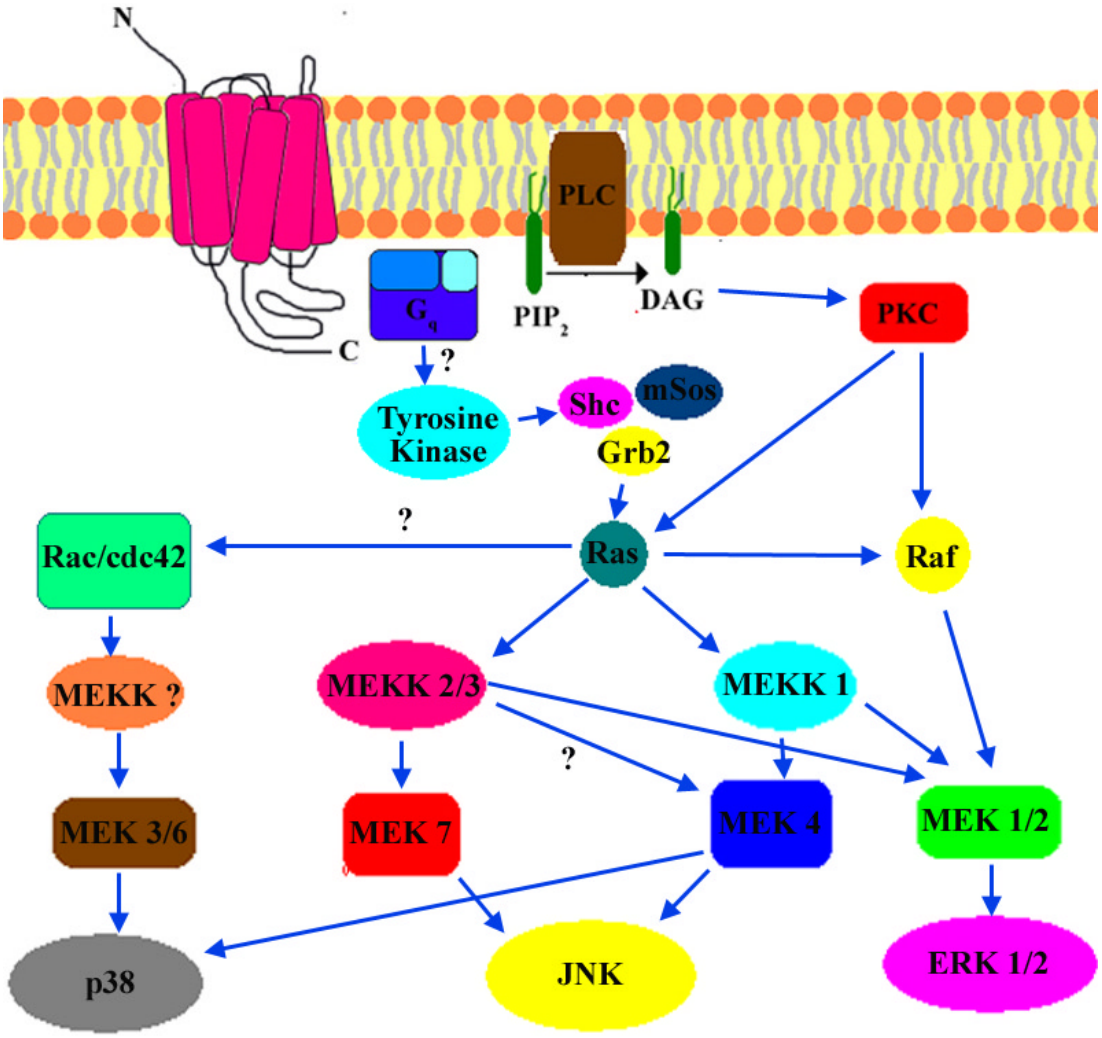
same effectors and proteins, but for unknown reasons the mechanisms and the results are different. **Figure 5** depicts portions of the α_1-AR-MAPK signaling cascade.

Many studies correlated the effects of catecholamines to cellular growth and proliferation in the cardiovascular system. Evidence stems from the observation that in the presence of catecholamines rat smooth muscle cells proliferate (Blaes and Boissel, 1983). Other work demonstrated that inhibiting the sympathetic neurotransmission in the rat smooth muscle tissue stunts the cellular proliferation (Fronek, 1983). Therefore, besides mediating vasoconstriction, the α_1-ARs regulate the sympathetic effects on vascular proliferation (reviewed in Jackson and Schwartz, 1992; Hoffman and Hu, 2000).

Nakaki *et al*. (1990) demonstrated that stimulation of α_1-ARs promotes DNA synthesis in quiescent rat smooth muscle cells. Additionally, stimulation of α_1-ARs in rat aortic smooth muscle cells markedly increases the gene expression of the transcriptional factor *c-fos* (Majesky *et al*., 1990). Other studies demonstrated that activation of α_1-ARs results in an increase of protein synthesis (Chen *et al*., 1995; Xin *et al*., 1997). These studies demonstrated that the activation of the α_{1B}- and α_{1D}-AR can mediate certain aspects of cellular proliferation in rat vascular smooth muscle tissue. However, Siwik and Brown (1996) demonstrated that unlike the response associated with prolonged α_{1B}-AR activation, the α_{1A}-AR results in attenuation of the α_{1B}-AR-mediated growth response.

The α_1-ARs are also capable of mediating events associated with cellular growth and proliferation in neonatal rat myocardial cells (Simpson, 1983, 1985; Meidell *et al*., 1986; reviewed by Simpson, 1988). Studies examining cellular growth and proliferation revealed two requirements: the up-regulation of the proto-oncogenes and the increased

Figure 5. A depiction of the α_1-AR mitogen-activated protein kinase (MAPK) signaling pathway. MEKK, MAPK Kinase Kinase; MEK, MAPK Kinase.



production of cellular proteins prior to cellular division (reviewed in Schluter and Piper, 1999). Indicators of cellular proliferation in cardiomyocytes are increases in α-actin mRNA and total cellular proteins (Meidell *et al*., 1986). Mitogenic growth is similar to hypertrophic growth with the exception that hypertrophic growth is a response for the terminally differentiated cells such as the cardiac myocytes.

Other than the expression of immediate early genes, there is an up-regulation of embryonic genes such as atrial naturetic peptide (ANP), skeletal α-actin, and β-myosin heavy chain (β-MHC). The activation of immediate early and embryonic genes leads to the up-regulation of the constitutively expressed contractile protein genes MLC-2 and cardiac α-actin (Long *et al*., 1989). All of these events culminate into a physically larger cell, which characteristically fulfills the hypertrophic phenotype.

In rat ventricular myocytes, Knowlton *et al*. (1993) linked the α_{1A}-AR subtype to cellular hypertrophy. In a study using transgenic mice harboring a constitutively active α_{1B}-AR, Milano *et al*. (1994) reported that targeting the mutant receptor to the heart induces cardiac hypertrophy. A study by D'Angelo *et al*. (1997) found that cardiac-targeted over-expression of the $G_{\alpha q}$ (a protein activated as part of the α_1-AR signaling cascade) induces cardiac hypertrophy along with cardiac dysfunction. These $G_{\alpha q}$ over-expressing mice showed increased heart weight, increased myocyte size, and increased levels of the genetic markers of cardiac hypertrophy: ANP, β-MHC, and α-skeletal actin. On the contrary, one study indicated that the α_{1B}-AR antagonizes the hypertrophic actions of the α_{1A}-AR in the rat myocardium (Deng *et al*., 1998). Despite these conflicting observations, many studies favor the notion that the α_1-ARs mediate the hypertrophic response through activation of different effector isoforms of PKC, Ras, and Raf

(reviewed in Gutkind, 1998). In one transgenic study using mice with cardiac targeted over-expression of the Ras gene, the investigators noted morphological changes in the atrial and ventricular chambers and also increases in the genetic markers of cardiac hypertrophy (Hunter *et al*., 1995). Thus, the activators that the α_1-AR subtypes can couple with may be the deciding factor in the regulation of the hypertrophic signaling pathways.

Chapter Two

Statement of the Problem

The α_1-ARs regulate systemic arterial blood pressure, provide a second source of positive inotropy during heart failure, and modulate growth responses. Additionally other studies indicate that alterations in the normal signaling patterns of these receptors may lead to hypertension and/or heart failure. There are three known subtypes: α_{1A}-, α_{1B}-, and α_{1D}-ARs. Currently many laboratories are focusing on addressing the relevance of multiple receptor subtypes with similar signaling properties expressed in the same tissue. Establishing the physiological relevance of each α_1-AR subtype is challenging due to the lack of selective agonists and antagonists available. This dissertation utilized novel tools and models to examine the signaling and the trafficking properties of these receptors at the cellular level and their physiological functions at the tissue and the whole animal level. In addition, this work advances the hypothesis that each receptor subtype subserves a different physiological function.

Specific Aims

Many investigators believe that the divergence of the amino acid sequence in the intracellular loops and the carboxyl-terminus can account for the unique properties inherent to each α_1-AR subtype. Thus, the first specific aim of this dissertation examined the hypothesis that there are differences in the trafficking properties by investigating the basal localization patterns, agonist-mediated internalization, and desensitization of each receptor subtype. Using a laser scanning confocal microscope, experiments investigated the basal localization patterns for each α_1-AR/green fluorescent protein (GFP) subtype.

Additionally, an immunocytochemistry technique assessed the ability of each receptor construct to activate extracellular signal-regulated kinase 1/2, as well as the ability of prolonged agonist incubation to desensitize this response. The other studies examined the agonist-mediated internalization properties of each α_1-AR/GFP subtype in real-time. In addition, experiments assessed the participation of the receptor trafficking protein, β-arrestin 1, in mediating α_1-AR internalization.

Since differences exist among the receptor subtypes at the cellular level, there is great interest to examine the contribution that each subtype has in physiological function(s). The second specific aim of this dissertation investigated the hypothesis that the α_1-AR subtypes participate in different regulatory activities in the cardiovascular system. Animals with the constitutively active α_{1B}-AR under the control of the endogenous promoter were used to study the role of the α_{1B}-AR participation in aortic smooth muscle contraction. Other experiments used echocardiographic techniques to determine the effect(s) of α_{1B}-AR on cardiac dimensions and functions and the potential for cardiac pathophysiology development. Another series of experiments investigated the left ventricular contractile function using the isolated-perfused heart technique. The differences in function response prompted a study to examine a possible mechanism underlying the depressed cardiac function in the animals over-expressing the constitutively active α_{1B}-AR. In a different series of experiment using mice deficient in the α_{1D}-AR, the study focused on the role of the α_{1D}-AR in regulating cardiac function and mediating coronary vasoconstriction.

In summary, the specific aims of this dissertation are as follows:

1) **Examine the hypothesis that there are differences in the cellular trafficking properties of the α_1-AR subtypes.**
2) **Examine the hypothesis that the α_1-AR subtypes modulate different physiological responses in the cardiovascular system.**

Chapter Three

Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the α_1-Adrenoceptor Subtypes

Dan Chalothorn, Dan F. McCune, Stephanie E. Edelmann, Mary L. García-Cazarín, Gozoh Tsujimoto, and Michael T. Piascik

The Department of Molecular and Biomedical Pharmacology, The University of Kentucky College of Medicine, Lexington, Kentucky (DC, DFM, SEE, MLG, MTP) and The Department of Molecular and Cell Pharmacology, National Children's Medical Research Center, Tokyo, Japan (GT).

Introduction

The α_1-ARs are members of the G-protein-coupled receptor (GPCR) family of receptors and are utilized by the sympathetic nervous system to regulate systemic arterial blood pressure and blood flow. The α_1-ARs also play a major role in mediating growth responses in cardiac and vascular smooth muscle cells (for reviews on all aspects of the α_1-ARs, see Graham *et al*., 1996; García-Sáinz *et al.,* 1999; Schwinn and Price, 1999; Zhong and Minneman, 1999; Piascik and Perez, 2001). Three genes encoding unique receptor subtypes, the α_{1A}-, α_{1B}-, and α_{1D}-ARs, have been cloned and characterized. These subtypes utilize a variety of second messengers and G-proteins to modulate cellular processes. Alterations in normal α_1-AR function may contribute to the pathophysiology of diseases such as hypertension, congestive heart failure, and benign prostatic hyperplasia.

GPCR signaling is also tightly regulated by a series of cellular proteins that promote receptor desensitization and internalization (Krupnick and Benovic, 1998; Lefkowitz, 1998). Agonist occupation promotes receptor phosphorylation by a series of GPCR kinases (Hausdorff *et al.,* 1990; Inglese *et al.,* 1993; Premont *et al.,* 1995). The phosphorylated receptor exhibits high affinity for the arrestins, which, in turn, prevent further interaction between the receptor and G-proteins (Wilden *et al.,* 1986; Benovic *et al.,* 1987). There are currently four known members of the arrestin family: visual arrestin (arrestin 1), β-arrestin 1 (arrestin 2), β-arrestin 2 (arrestin 3) and cone arrestin (arrestin 4) (Ferguson *et al*., 1996; Krupnick and Benovic, 1998). The β-arrestins promote internalization by binding to both the receptor and clathrin, thus, directing the receptor to coated pits (von Zastrow and Kobilka, 1992; Krupnick *et al.,* 1997a; Gagnon *et al.*,

1998). Oakley *et al.* (2000) demonstrated recently that GPCRs have different affinities for the different arrestins. Class A GPCRs, which include the β_2-AR, α_{1B}-AR, and μ-opioid receptor, have high affinity for β-arrestin 2 whereas Class B GPCRs, such as the angiotensin II type 1A receptor, neurotensin receptor 1, and vasopressin V2 receptor, exhibit high affinity for both β-arrestin 1 and 2 isoforms.

With regard to the α_1-AR subtypes, the desensitization, down-regulation, and internalization characteristics of the α_{1B}-AR have been most extensively examined. For example, agonist-mediated phosphorylation and internalization of the α_{1B}-AR have been demonstrated, and the domains of the receptor involved in internalization have been identified (Fonseca *et al.,* 1995; Mhaouty-Kodja *et al.,* 1999; Wang *et al.,* 1997, 2000). We know much less regarding the molecular determinants of desensitization, down-regulation, and internalization for the α_{1A}- and α_{1D}-ARs. Vázquez-Prado *et al.* (2000) showed that the α_{1A}-AR could undergo agonist-mediated phosphorylation, albeit not to the same extent as the α_{1B}-AR. Yang and co-workers (1999) used fibroblasts stably transfected with each of the α_1-ARs to show that the increase in inositol phosphates mediated by the α_{1A}- and α_{1B}-ARs could be desensitized, whereas the increase mediated by the α_{1D}-AR was refractory to agonist-mediated desensitization. In contrast to this, García-Sáinz *et al.* (2001) showed that the α_{1D}-AR could be phosphorylated and desensitized.

In this report, we have examined subcellular distribution, agonist-mediated internalization and desensitization characteristics of green fluorescent protein (GFP)-tagged α_1-ARs using real-time imaging in transiently transfected human embryonic kidney (HEK) 293 cells. We show that there are significant differences in these

parameters that could account for differences in the cellular signaling properties of the α_1-ARs.

Experimental Procedures

Materials

α_1-AR/green fluorescent protein (α_1-AR/GFP) vectors were constructed by ligating the coding region of the human α_{1A}-, α_{1B}-, and α_{1D}-AR into the *Eco*RI-*Kpn*I site of the basic pEGFP-N3 protein fusion vector (Clontech, Palo Alto, CA) as previously described (Hirasawa *et al.,* 1997; Awaji, *et al.*, 1998). The generation of wild-type β-arrestin 1 and a dominant negative β-arrestin 1 (319-418) in pcDNA3 has previously been reported (Krupnick *et al.*, 1997b). Rabbit polyclonal antibodies targeted against β-arrestin 1 were generated as described by Orsini and Benovic (1998).

Cell Culture and Transient Transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic cocktail [10,000 units/ml penicillin G sodium, 10,000 mg/ml streptomycin sulfate, and 25 mg/ml amphotericin B in 0.85% saline (Invitrogen, Carlsbad, CA)]. The cells were grown in T75 flasks in a 37°C cell culture incubator with a humidified atmosphere of 95% air and 5% CO_2 and were fed every 2 to 3 days. HEK cells used in immunocytochemistry protocols were grown on gelatin/laminin treated coverslips in 35-mm tissue culture dishes (Corning Glassworks, Corning, NY) whereas cells for real-time studies were grown in culture dishes with a glass coverslip bottom (MatTek Co., Ashland, MA) that were also gelatin/laminin treated. Cells were grown to approximately 80% confluence and used for experimentation 3 days after being plated. HEK cells were transfected with cDNA encoding either α_{1A}-, α_{1B}-, or α_{1D}-AR/GFP fusion protein using calcium phosphate

precipitation. In certain studies, the receptor/GFP constructs were co-transfected with a cDNA encoding wild-type β-arrestin 1 or β-arrestin 1 (319-418). β-arrestin 1 over-expression was confirmed using specific antibodies in immunocytochemistry protocols as we have described previously (Hrometz *et al.*, 1999; McCune *et al.*, 2000).

Activation of ERK1/2 Phosphorylation and Agonist-Mediated Desensitization

The coupling of the α_1-AR/GFP constructs to functional responses and agonist-mediated desensitization was assessed by measuring the phosphorylation of ERK1/2. Cells were challenged with 100 μM phenylephrine for a period of 2 h. After the appropriate time, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min, and immunocytochemistry was performed as described previously by Hrometz *et al.* (1999) and McCune *et al.* (2000). In brief, cells were treated with mouse monoclonal IgG pERK (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution and then incubated with Rhodamine Red-X-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:100. The degree of ERK1/2 phosphorylation was assessed using laser scanning confocal microscopy as described below. Desensitization experiments were conducted on HEK-293 cells 72 h after transient transfection with cDNA encoding α_{1A}-, α_{1B}-, or α_{1D}-AR/GFP. Cells were treated with 100 μM phenylephrine for 15 h. Vehicle-treated cells served as controls. After the incubation, cells were washed three times (30 min intervals between each wash) with Dulbecco's modified Eagle's medium, after which cells were rechallenged with phenylephrine for 2 h, and the effect on ERK1/2 phosphorylation was assessed.

Laser Scanning Confocal Microscopy

Transfected HEK-293 cells were imaged with a Spectra-Physics laser scanning confocal microscope attached to a TCS DM RXE microscope with a Plan-Apo 100x oil immersion objective lens (Leica, Wetzler, Germany). The software used to collect the images was the Leica TCS NT version 1.6.587. The images were transferred to a computer for reduction and analysis with Adobe Photoshop version 4.0 (Adobe Systems, Mountain View, CA). The setting on the laser was constant for all experiments. However, both GFP and rhodamine signals were digitally enhanced by adjusting the photomultiplier tube (PMT). Initial adjustment of the PMT allowed us to minimize the background signal while maximizing the fluorescent signal(s) of interest. Because individual cells required a different PMT setting, the differences in intensity should not be construed as a measure of receptor expression levels.

Data and Image Analysis

The rate and extent to which the α_1-AR/GFP constructs were internalized after exposure to agonist were analyzed using the image analysis software NIH ImageJ 1.18x (http://rsb.info.nih.gov/ij/). The change in fluorescence intensity was measured in a rectangular area just below the cell surface before and during the internalization process. Data were normalized to the fluorescence obtained before agonist treatment. The increase in fluorescence intensity above that observed in untreated cells is a measure of receptor internalization. A plot of the relative change in fluorescence intensity versus time after agonist treatment was then generated. The average phospho-ERK1/2 signal

per determined area was quantitated using the same image analysis software. Only images acquired using exactly the same PMT settings were compared with each other. Treated cells were normalized to the control phospho-ERK1/2 activation signal. All data are reported as the mean ± S.E. Data were analyzed by analysis of variance followed by Student- Newman-Kuels analysis to determine where statistically significant differences existed. A *P* value of less than 0.05 was considered significant.

Results

Basal Cellular Localization

HEK-293 cells were transiently transfected with expression plasmids encoding α_1-AR/GFP fusion proteins and the living cells were visualized 72 h later. Transfection with a cDNA encoding the α_{1B}-AR/GFP resulted in a specific fluorescence that was detected predominantly on the margin of the cell, indicative of a cell surface localization (**Figure 6**). Although there was cell surface expression, the majority of the α_{1D}-AR/GFP fluorescence was detected intracellularly in a perinuclear orientation (**Figure 6**). Exhibiting localization properties of each of these subtypes, α_{1A}-AR/GFP fluorescence was observed both on the cell surface and in a perinuclear orientation (**Figure 6**).

Functional Responses Mediated by the α_1-AR/GFP Fusion Proteins

To demonstrate that the expressed α_1-AR/GFP fusion proteins were functional, transfected cells were stimulated with phenylephrine, and, after fixing of the cells, the effect on ERK1/2 phosphorylation was determined with a monoclonal antibody specific for phospho-ERK1/2. Treatment with phenylephrine resulted in a statistically significant increase in phospho-ERK1/2 immunoreactivity in cells transfected with either the α_{1A} - or the α_{1B}-AR/GFP constructs (**Figures 7**, **a**, **b**, and **d**). This indicates that these GFP modified α_1-ARs are functional when expressed in HEK-293 cells. Phenylephrine treatment of cells transfected with the α_{1D}-AR also resulted in an increase in the level of ERK1/2 phosphorylation (**Figures 7**, **c** and **d**). However, this increase was not significantly different compared with the untreated control (**Figure 7d**). These findings could indicate that, although functional, the α_{1D}-AR is poorly coupled to second

Figure 6. Cellular localization of α_1-AR/GFP constructs in transiently transfected HEK-293 cells. Transient transfection of α_1-AR/GFP expression plasmids and laser scanning confocal microscopy were performed as described under *Experimental Procedures*. The images are representative of five to eight independent transfections. Data are from Chalothorn *et al*. (2002).

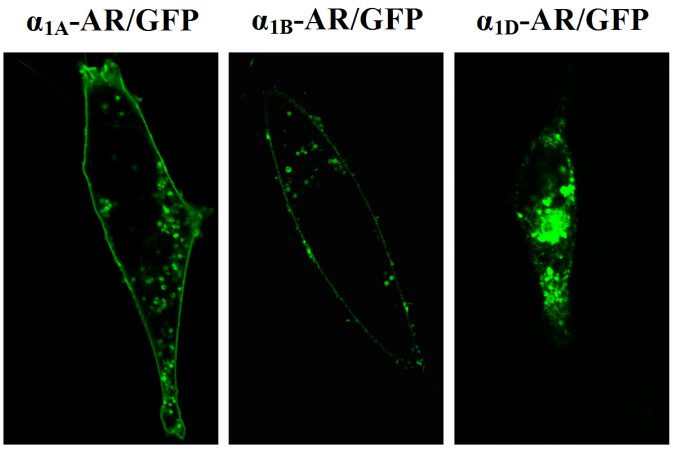
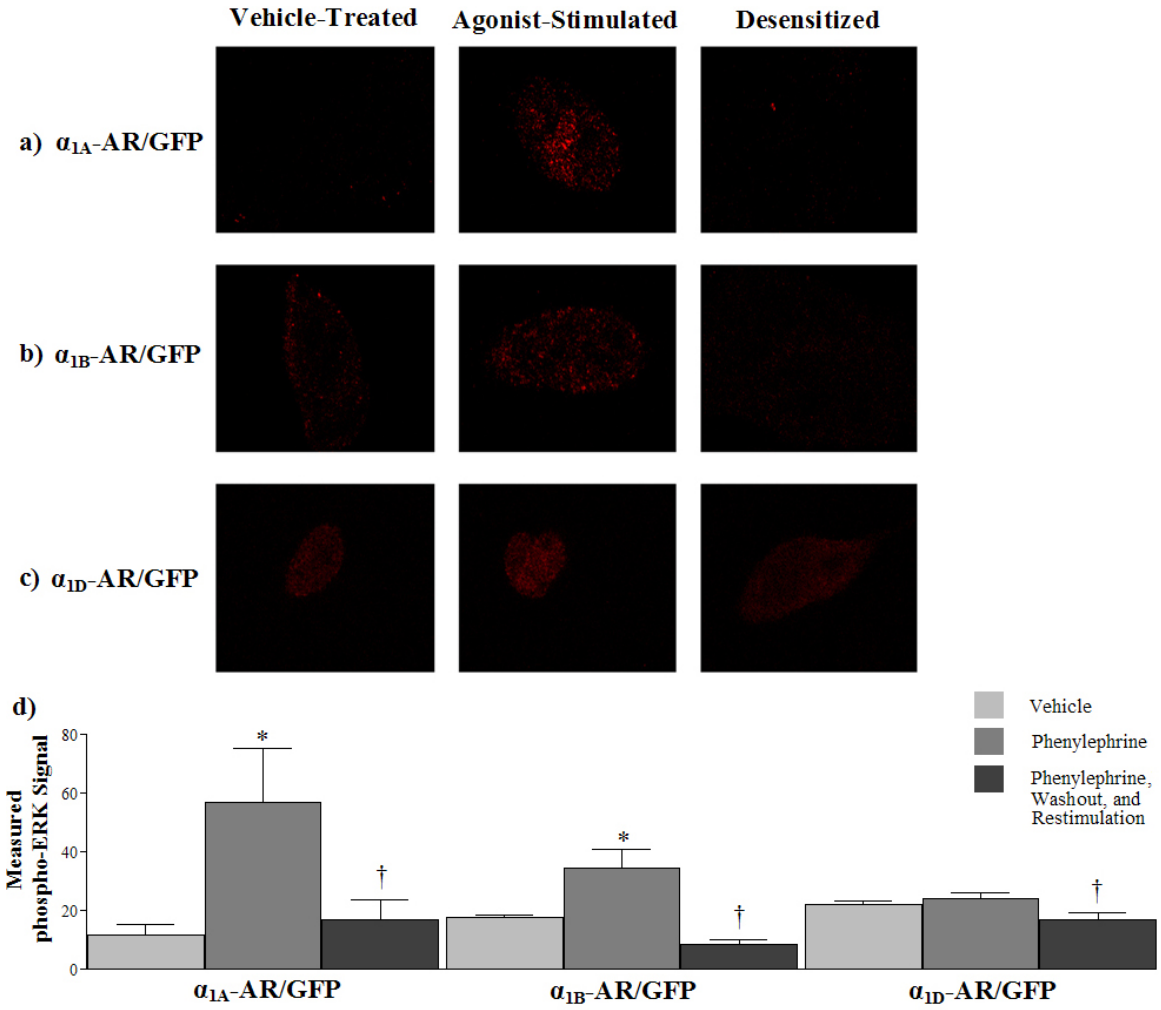


Figure 7. The activation of ERK1/2 phosphorylation and agonist-mediated desensitization. Immunocytochemistry demonstrating receptor functionality and agonist-induced desensitization were performed as described in *Experimental Procedures*. Images displayed are for the phospho-ERK1/2 signals. Data presented are for the vehicle-treated, the phenylephrine-stimulated ERK1/2 phosphorylation in naïve cells, and the effect of 15 h of phenylephrine treatment on the subsequent ability of phenylephrine to activate ERK1/2 phosphorylation. Data are for **a)** α_{1A}-AR/GFP, **b)** α_{1B}-AR/GFP, and **c)** α_{1D}-AR/GFP. The images are representative of three to seven independent transfections. **d**, bar graphs show the relative changes in the phospho-ERK1/2 signals for each receptor. *, significantly greater than the control level of ERK1/2 phosphorylation. †, statistically less than the phenylephrine stimulation of ERK 1/2 phosphorylation. Data generated by Mary L. García-Cazarín and reported in Chalothorn *et al*. (2002).



messenger pathways such that only a modest increase in ERK1/2 phosphorylation could be observed.

Effect of Agonist Stimulation on Receptor Localization

In addition to activating ERK1/2 phosphorylation, the ability of phenylephrine to promote changes in receptor localization was assessed in real-time. Addition of 100 μM phenylephrine to cells expressing the α_{1B}-AR/GFP resulted in a rapid translocation of the receptor from the cell surface to intracellular compartments (**Figure 8**). The α_1-AR antagonist, 1 μM prazosin, blocked this internalization (not shown).

The increase in intracellular fluorescence signal intensity, quantitated with image analysis software (as described under *Experimental Procedures*), was used to gain a measure of the rate of receptor internalization. A plot of the increase in intracellular fluorescence intensity versus time after phenylephrine administration is presented in **Figure 9b** and shows that α_{1B}-AR internalization occurred in a very rapid fashion.

Receptor activation with phenylephrine also promoted the internalization of the cell surface population of α_{1A}-ARs (**Figures 8** and **9a**). However, a significant increase in intracellular fluorescence was not detected until 50 minutes after agonist exposure. A plot of the increase in intracellular fluorescence intensity versus time revealed that the α_{1A}-AR internalization occurred at a slower rate than that seen for the α_{1B}-AR. Treatment of HEK cells expressing the α_{1D}-AR/GFP fusion protein with phenylephrine did not cause a translocation of the cell surface population of α_{1D}-ARs (**Figure 10**).

Agonist-Mediated Receptor Desensitization

Transfected HEK-293 cells were incubated for 15 h with 100 μM phenylephrine,

Figure 8. Effects of 100 μM phenylephrine on the cellular localization of either the α_{1A}- or α_{1B}-ARs transiently transfected into HEK-293 cells. Real-time images were captured before and at the specified time points after phenylephrine addition as described under *Experimental Procedures*. The images are representative of five to eight independent transfections. Data are taken from Chalothorn *et al*. (2002). The images from 20 mins through 60 mins appear on the following page.

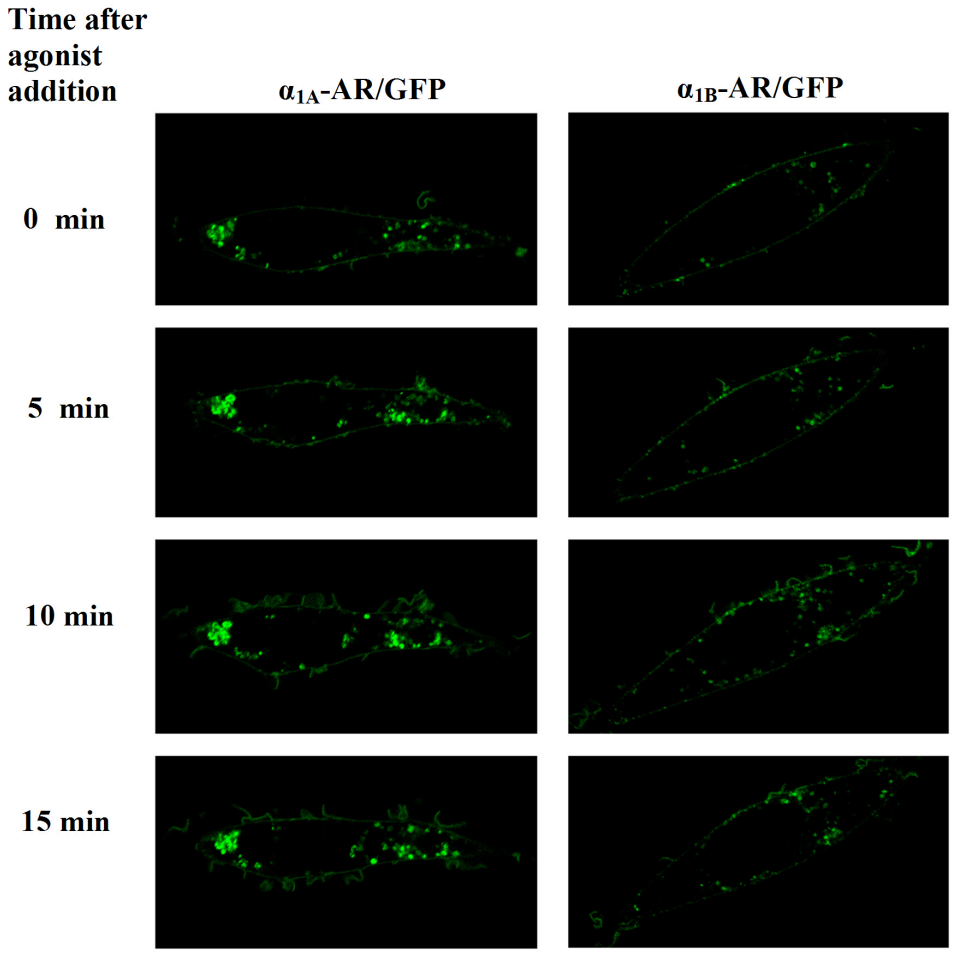


Figure 8. (continued)

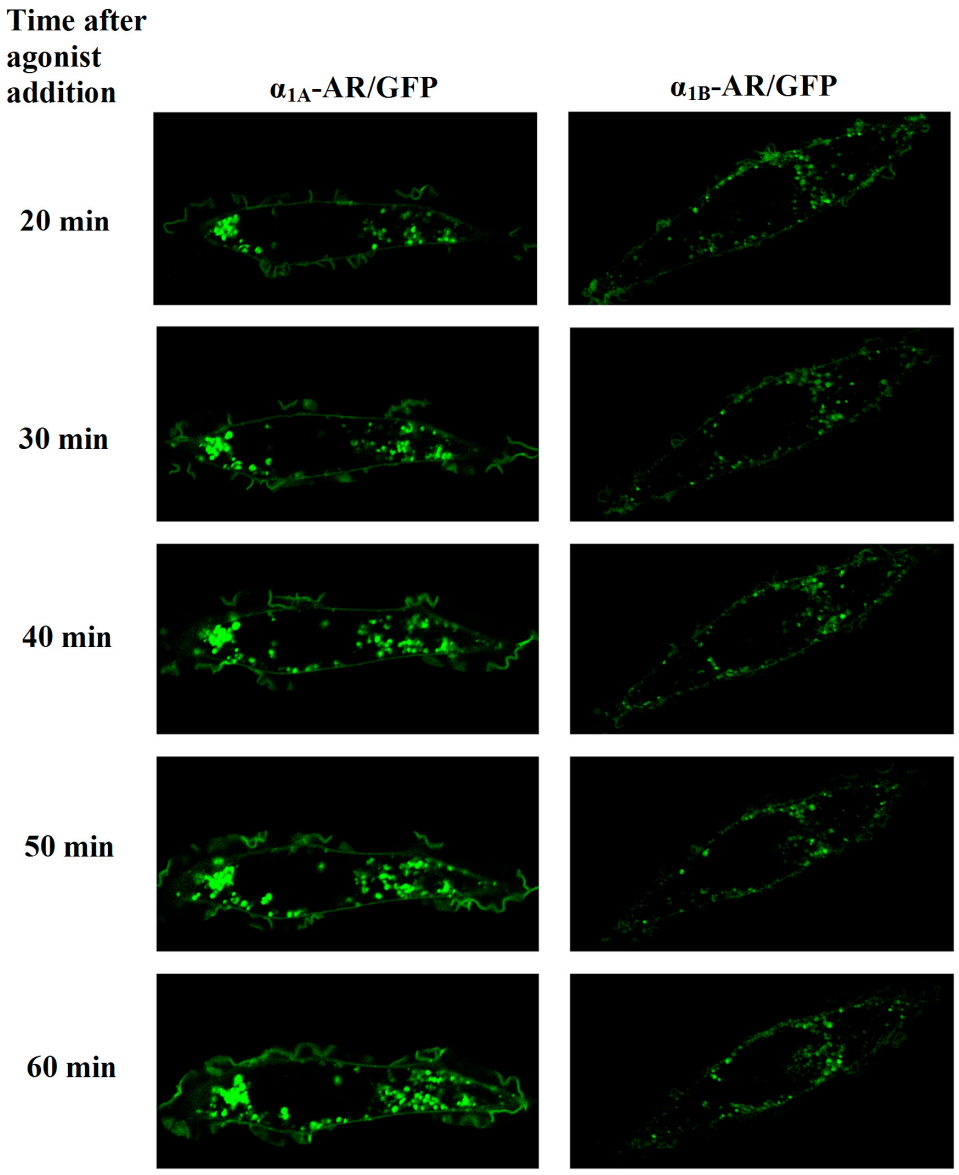
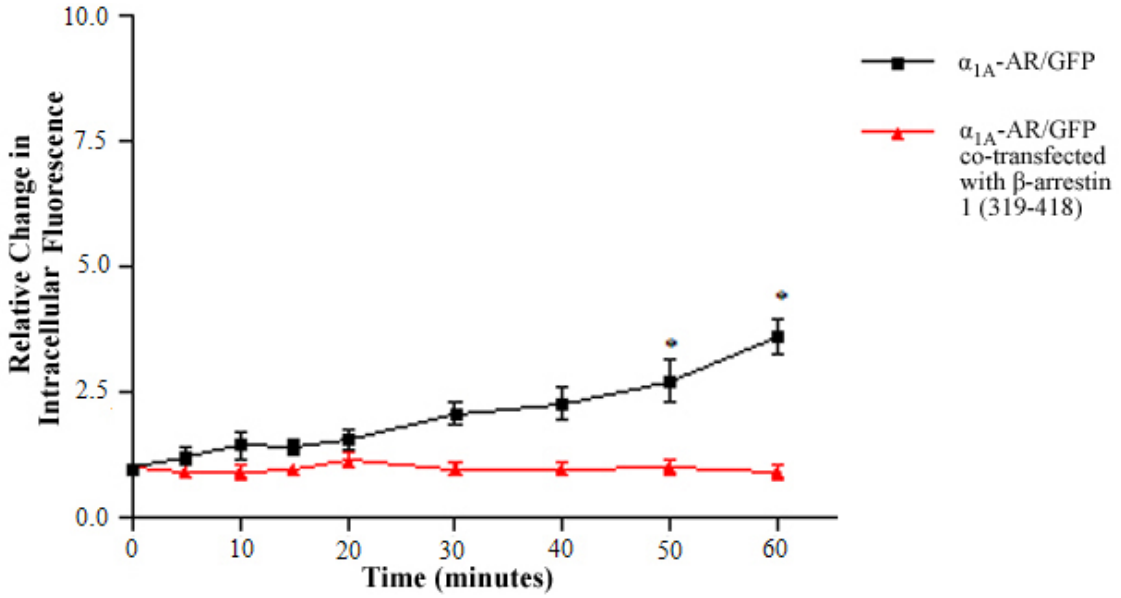


Figure 9. Comparison of the effect of 100 μM phenylephrine on changes in intracellular fluorescence intensity in cells transfected with the **a)** α_{1A}- or **b)** α_{1B}-AR/GFP in the absence or presence of β-arrestin 1 (319-418). Relative intensity assessment were performed as described under *Experimental Procedures*. Data represent the mean and standard error of the mean values of four to eight independent transfections. *, values are significantly greater than the unstimulated control or cells co-transfected with β-arrestin 1 (319-418). Data are from Chalothorn *et al*. (2002).

a)



b)

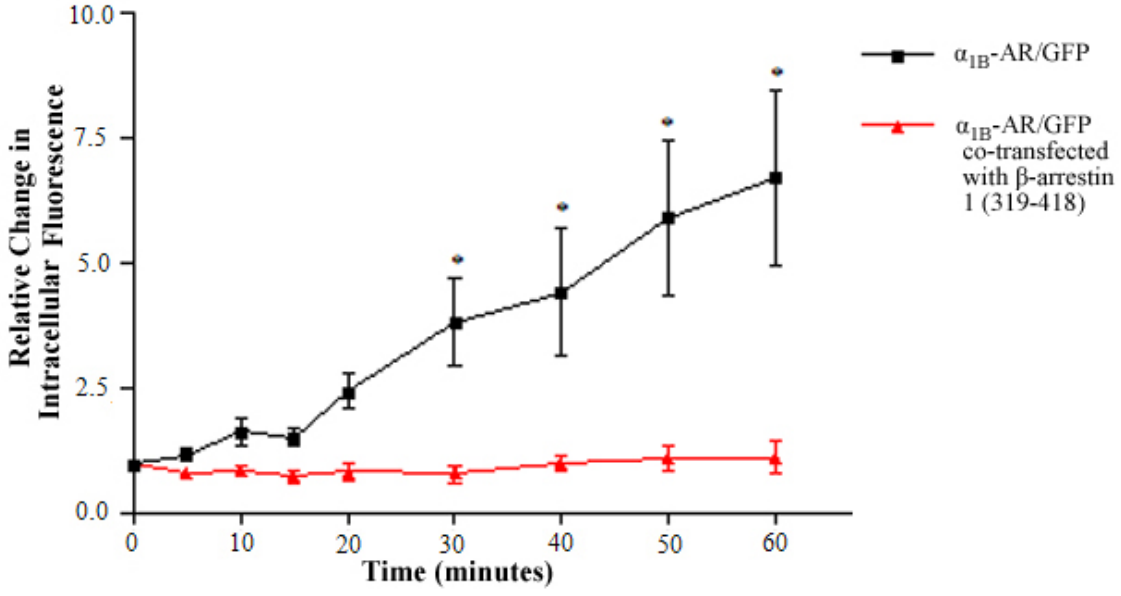
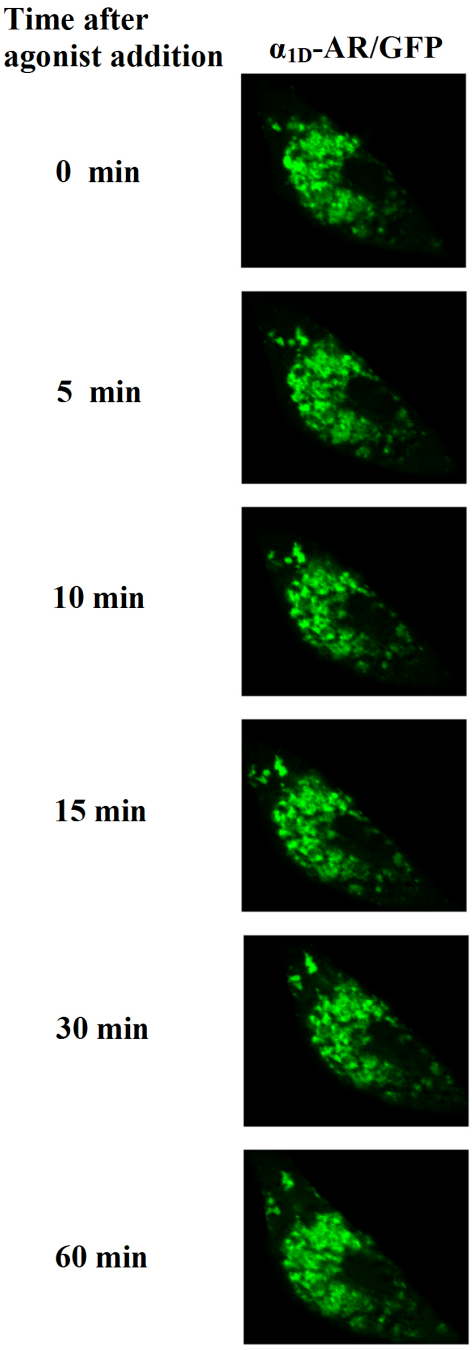


Figure 10. Effects of 100 μM phenylephrine on the cellular localization of the α_{1D}-AR/GFP transiently transfected into HEK-293 cells. Real-time images were taken at specific time points after phenylephrine treatment. Experiments were performed as described under *Experimental Procedures*. The images are representative of four independent transfections. Images taken from Chalothorn *et al*. (2002).



and the effect on α_1-AR/GFP localization and the ability of the α_1-ARs to stimulate ERK1/2 phosphorylation was assessed. Prolonged incubation with phenylephrine (followed by extensive washout) resulted in an internalization of α_{1A}- and α_{1B}-ARs (see **Appendix A**) in a fashion similar to that seen in untreated cells. Phenylephrine treatment for 15 h significantly decreased the ability of either the α_{1A}- or α_{1B} -AR to activate ERK1/2 phosphorylation in response to a second addition of phenylephrine (**Figure 7d**). The long exposure to phenylephrine had no effect on the cellular localization of the α_{1D}-AR. Long-term exposure to phenylephrine significantly reduced the level of phospho-ERK1/2 seen following rechallenge with agonist in α_{1D}-AR expressing cells (**Figure 7d**).

Effect of Arrestins on Agonist-Activated Receptor Internalization

HEK cells were co-transfected with α_1-AR/GFP constructs and an expression plasmid encoding β-arrestin 1. The over-expression of β-arrestin 1 was confirmed using immunocytochemical protocols with an antibody against β-arrestin1 (**Figure 11**). β-arrestin 1 over-expression did not increase the rate or extent of α_{1A}- or α_{1B}-AR internalization after stimulation with phenylephrine (see **Appendix B**). In a similar fashion, co-transfection with β-arrestin 2 had no effect on agonist-mediated internalization (see **Appendix B**).

HEK-293 cells were co-transfected with a cDNA encoding the α_{1B}-AR/GFP construct and a dominant-negative form of β-arrestin 1, β-arrestin 1 (319-418). β-arrestin 1 (319-418) had no effect on basal α_{1B}-AR cellular localization (**Figure 12**). However, the dominant-negative arrestin markedly decreased the ability of phenylephrine to promote α_{1B}-AR internalization (**Figure 12**). Analysis of these data revealed that the

Figure 11. Immunolocalization of endogenous β-arrestin 1 in native HEK-293 cells and cells transiently transfected with a cDNA encoding β-arrestin 1. The β-arrestin 1 immunofluorescence was detected with a specific antibody and a rhodamine-labeled secondary antibody as described under *Experimental Procedures*. Images selected from Chalothorn *et al*. (2002).

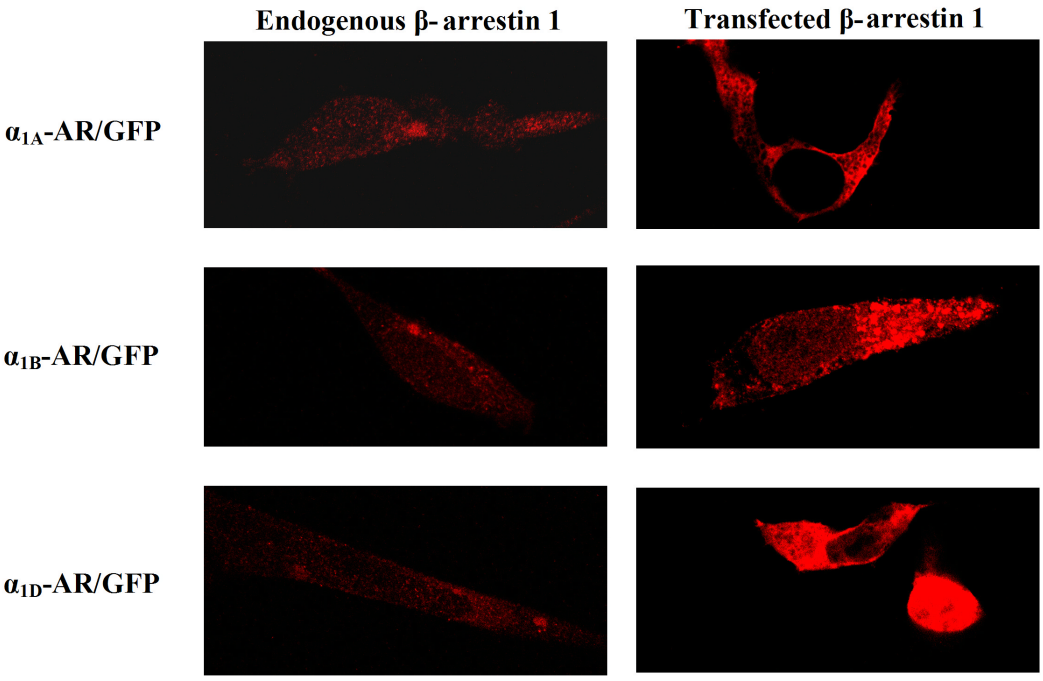
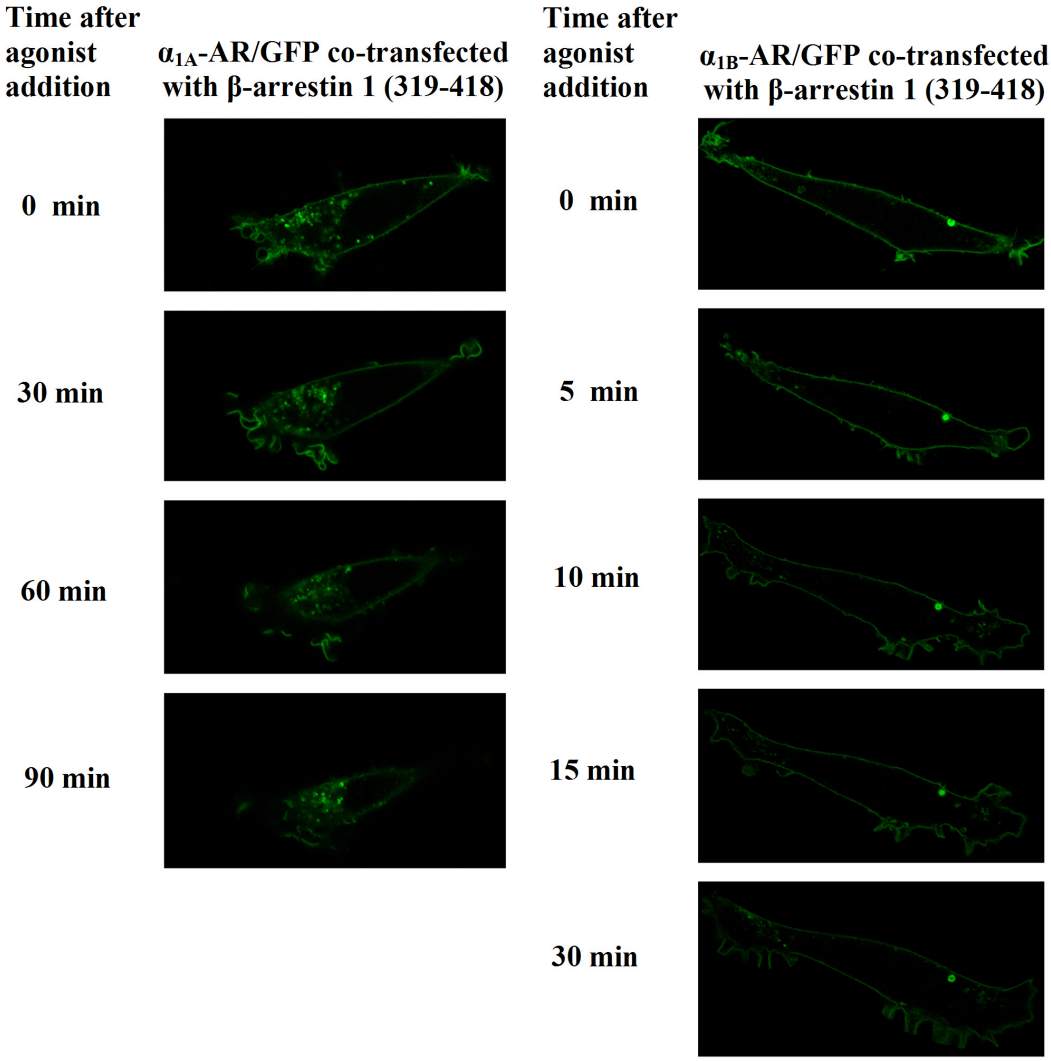


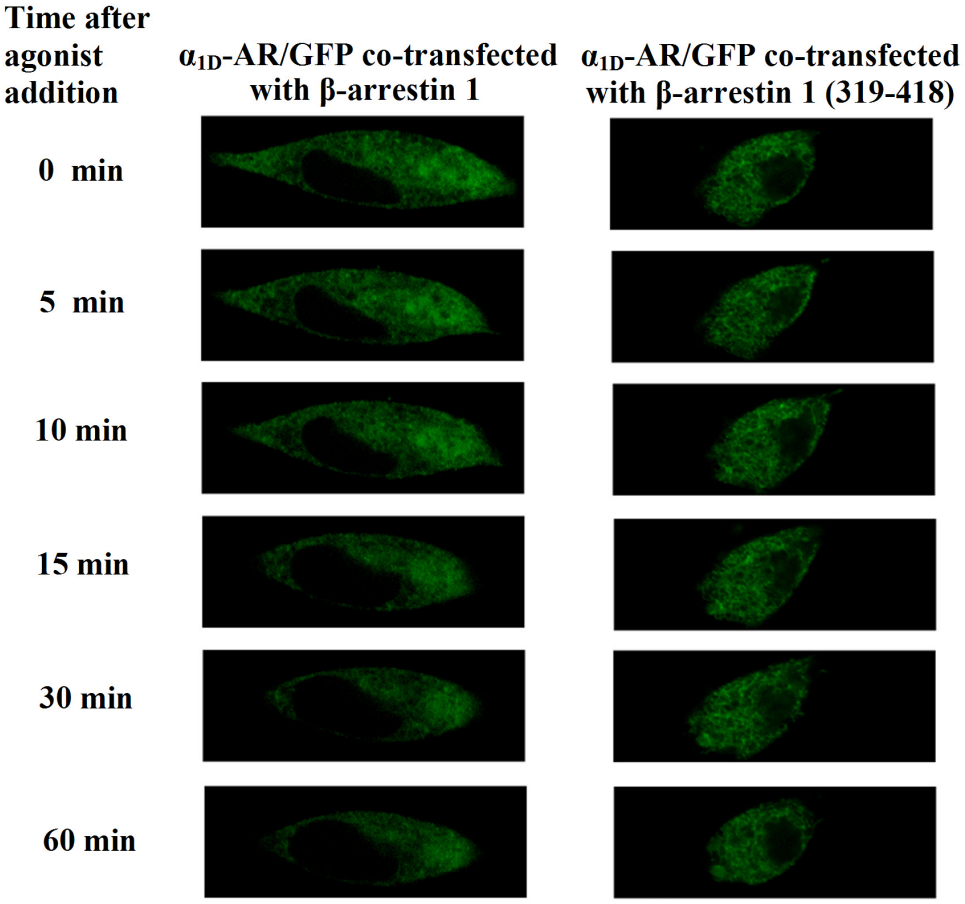
Figure 12. The effect of co-transfection of β-arrestin 1 (319-418) on the ability of 100 μM phenylephrine to promote internalization of the α_{1A}- and the α_{1B}-AR/GFP in HEK-293 cells. Drug administration and real-time visualization were performed as described under *Experimental Procedures*. Representative real-time images up to 90 and 30 mins after agonist addition for α_{1A}- and α_{1B}-AR/GFP, respectively. The images are representative of four (α_{1A}-AR/GFP) and seven (α_{1B}-AR/GFP) independent transfections. Images from Chalothorn *et al*. (2002).



dominant-negative arrestin significantly reduced the rate of increase in intracellular fluorescence intensity seen after the addition of phenylephrine to HEK-293 cells (**Figure 9b**). Similar to effects seen with the α_{1B}-AR, β-arrestin 1 (319-418) decreased the magnitude of the phenylephrine-induced α_{1A}-AR internalization (**Figures 9a** and **12**).

Wild-type β-arrestin 1 did not affect the basal cellular localization or the ability of phenylephrine to promote α_{1D}-AR internalization (**Figure 13**). Similarly, β-arrestin 1 (319-418) had no effect on the cellular localization of the α_{1D}-AR/GFP (**Figure 13**).

Figure 13. The effect of co-transfection of β-arrestin 1 or β-arrestin 1 (319-418) on the ability of 100 μM phenylephrine to promote internalization of the α_{1D}-AR/GFP in HEK-293 cells. Drug administration and real-time visualization were performed as described under *Experimental Procedures*. Each transfection was repeated three to four times. Representative images are shown for the cell co-transfected with the wild-type β-arrestin 1 and cell co-transfected with β-arrestin 1 (319-418). Images taken from Chalothorn *et al*. (2002).



Discussion

In this communication, we have examined the cellular localization, agonist-mediated internalization, and desensitization properties of α_1-AR/GFP fusion proteins in transiently transfected HEK-293 cells. Previous studies with the α_{1B}-AR/GFP construct demonstrated that it is fully functional and internalizes in the same manner as a non-GFP tagged α_{1B}-AR construct (Awaji *et al.,* 1998). In a similar fashion, previous studies showed that both the α_{1A}- and α_{1B}-ARs are coupled to the activation of ERK (see reviews by García-Sáinz *et al.,* 1999; Zhong and Minneman, 1999; Varma and Deng, 2000; Piascik and Perez 2001). In this report, we show that both the α_{1A}- and α_{1B}-ARs when coupled to GFP can promote an increase in ERK1/2 phosphorylation (**Figure 7**). The phosphorylation of ERK1/2 is thought to mediate growth responses, at least in part. Demonstration of ERK1/2 phosphorylation in these studies is evidence that the α_1-ARs are functional and retain their ability to activate cellular signaling when conjugated to the GFP.

In this report, we also noted that, although phenylephrine could increase the level of phospho-ERK1/2 in α_{1D}-AR expressing cells, this increase was not statistically significant. This could indicate that the small population of cell surface α_{1D}-ARs is not efficiently coupled to ERK1/2 phosphorylation. This result is consistent with the observations of Theroux *et al.* (1996) who noted that the α_{1D}-AR was the most poorly coupled of the α_1-AR subtypes. In previous work with stably transfected fibroblasts, we showed that the α_{1D}-AR was constitutively active with regard to ERK activation (McCune *et al*., 2000). We also noted that there was a high basal level of ERK activity in these cells and that phenylephrine could not promote a further enhancement of kinase

activity (McCune *et al*., 2000). Thus, the inability to detect a significant increase in ERK1/2 phosphorylation in this present study could also be due to a constitutively active α_{1D}-AR. Nonetheless, we must also accept the possibility that the α_{1D}-AR/GFP construct may not be functionally active (however, see the additional discussion below).

Laser scanning confocal microscopy revealed that the α_{1B}-AR was expressed predominantly on the cell surface (**Figure 6**). Although there is some cell surface expression of the α_{1D}-AR, the majority of this receptor is expressed in intracellular compartments. The α_{1A}-AR has localization characteristics of both the α_{1B}- and α_{1D}-ARs, being expressed not only on the cellular surface but also intracellularly. Using immunocytochemistry with subtype selective antibodies, we previously observed a similar distribution pattern for the α_1-ARs in stably transfected fibroblasts and vascular smooth muscle cells (Hrometz *et al.,* 1999; McCune *et al.,* 2000). However, studies of the cellular localization of the α_1-ARs have been hampered by the low affinity of the commercially available antibodies. The present studies confirm and extend our initial findings with techniques that do not involve antibodies. Therefore, it seems likely that our observation of differential cellular localization of the α_1-AR accurately portrays the distribution pattern in vascular smooth muscle cells that normally express all three α_1-ARs. Indeed, an elegant series of studies using prazosin labeled with BODIPY-FL to image the α_1-AR subtypes noted an intracellular expression of these receptors in cultured prostate smooth muscle cells and stably transfected fibroblasts (MacKenzie *et al.*, 2000). These authors estimated that in smooth muscle cells, 40% of the total α_1-AR population is expressed intracellularly.

Using real-time imaging of living cells, we observed differences in the agonist-

mediated internalization properties of the α_1-ARs (**Figures 8**, **9**, and **10**). In agreement with previous work (Fonseca *et al.,* 1995; Wang *et al.,* 1997, 2000), we observed that the α_{1B}-AR undergoes rapid agonist-mediated internalization. However, there has been little investigation of the effect of agonist activation on the translocation of the other α_1-AR subtypes. We noted that the α_{1A}-AR also undergoes agonist-mediated internalization. Interestingly, this internalization occurs at a slower rate than for the α_{1B}-AR (**Figures 9**, compare **a** and **b**). We were unable to detect any agonist-mediated internalization of the α_{1D}-AR (**Figure 10**). We cannot discount the possibility that a small amount of receptor internalization did take place. However, this small increase in intracellular fluorescence could not be detected because of the considerable fluorescence produced by the intracellular population of α_{1D}-ARs.

We then assessed the extent to which the receptors could be desensitized after prolonged exposure to phenylephrine. Transfected HEK-293 cells were incubated with phenylephrine for 15 h and then extensively washed. This long incubation period resulted in redistribution of each of the α_1-AR/GFPs similar to that seen in non-desensitized cells (data not shown). After the washout period the cells were rechallenged with phenylephrine. Using this protocol, we demonstrated that prolonged exposure to agonist desensitizes the ability of the α_{1A}- and α_{1B}-ARs to promote ERK1/2 phosphorylation (**Figure 7**). Interestingly, after a 15 h exposure to phenylephrine in α_{1D}-AR expressing cells, rechallenge with agonist could not promote any increase in the level of phospho-ERK1/2. Indeed, there was a statistically significant difference in the level of agonist-induced ERK1/2 phosphorylation in control HEK-293 cells and that seen following desensitization (**Figure 7**). Thus even though phenylephrine could only promote a

modest, non-significant increase in the level of phospho-ERK1/2 in control cells, this could be reduced by prolonged expose to agonist and supports the notion that the α_{1D}-AR/GFP construct is functional.

Our results are consistent with other studies that have examined the phosphorylation and desensitization of the α_1-ARs. Yang *et al.* (1999) noted that both the α_{1A}- and α_{1B}-ARs undergo agonist-mediated desensitization. However, these authors noted that the α_{1B}-AR was desensitized by lower concentrations of agonist. Similarly, Vázquez-Prado *et al.* (2000) found that the α_{1B}-AR underwent more extensive agonist-activated phosphorylation than did the α_{1A}-AR. The more rapid rate of α_{1B}-AR internalization noted here is also consistent with the observation that this receptor is more extensively phosphorylated and readily desensitized than the α_{1A}-AR. Yang *et al.* (1999) also observed that functional responses mediated by the α_{1D}-AR were not subject to desensitization. In contrast to the work of Yang *et al*. (1999), García-Sáinz *et al.* (2001) noted that in stably transfected fibroblasts, the α_{1D}-AR could be phosphorylated and desensitized. Therefore, a definitive answer regarding the desensitization characteristics of the α_{1D}-AR requires additional studies.

Arrestins have been implicated in mediating the internalization of a variety of GPCRs. There has been little work performed on the role of arrestins in agonist-mediated α_1-AR internalization. We were unable to observe any demonstrable effects of β-arrestin 1 over-expression to the degree to which agonist activation promotes the internalization of the α_{1A}- or α_{1B}-ARs. This probably reflects the fact that HEK-293 cells possess large amounts of β-arrestin 1 (**Figure 11**). Therefore, over-expression of additional arrestin molecules would not be expected to have an effect on agonist-

mediated receptor internalization. Similarly, over-expression of β-arrestin 2 had no effect on the degree of agonist-stimulated internalization of either the α_{1A}- or α_{1B}-ARs (data not shown). A dominant-negative arrestin, β-arrestin 1 (319-418), completely blocked agonist-mediated internalization of both the α_{1A}- and the α_{1B}-ARs (**Figures 9** and **12**). These data argue that agonist-activated internalization of the α_1-AR subtypes is mediated by arrestins. Although the dominant-negative arrestin confirms the role of arrestins in α_1-AR internalization, this reagent cannot be used to determine the specific role of β-arrestin 1 or 2 in the internalization process. This is because the dominant-negative arrestin binds to clathrin, thus preventing the binding of wild-type arrestin species. Therefore, the dominant-negative arrestin would be expected to block the actions of any wild-type arrestin.

The intracellular localization of α_{1D}-AR was not affected by over-expression of either wild-type β-arrestin 1 or β-arrestin 1 (319-418), arguing that the intracellular distribution of the α_{1D}-AR is not likely to be maintained by arrestin molecules (**Figure 13**). The significance of the predominantly intracellular localization of the α_{1D}-AR is not clear. We do not know which of the α_{1D}-ARs, the small population of cell surface receptors or the large population of intracellularly expressed receptors, are signaling competent and responsible for the regulatory activity of this subtype. As noted above, data from several labs including ours show that the α_{1D}-AR is constitutively active (Noguera *et al.,* 1993; García-Sáinz and Torres-Padilla, 1999; McCune *et al.,* 2000). The observation of constitutive activity may shed some light on the relationship between cellular localization and functional responses. A constitutively active receptor assumes an activated conformation in the absence of agonist. The large degree of intracellular

localization of the α_{1D}-AR may be due to continuous internalization of the receptor due to its constitutively active nature.

The three α_1-ARs are co-expressed on tissues and organs involved in cardiovascular regulation, yet these receptors modulate different physiological processes. We hypothesize that the observed differences in the cellular localization could contribute to the differences in the functional responses mediated by these receptors. We also propose that the α_{1B}-AR most approximates a prototypic GPCR in terms of cellular localization, agonist-mediated internalization, desensitization, and coupling to cellular signaling. In contrast, we postulate that the α_{1D}-AR is an atypical GPCR. Although the α_{1A}-AR is expressed intracellularly, it appears to have signaling properties expected of a GPCR.

Chapter Four

Differential Cardiovascular Regulatory Activities of the α_{1B}- and α_{1D}-Adrenoceptor Subtypes

Dan Chalothorn*, Dan F. McCune*, Stephanie E. Edelmann, Kimimasa Tobita, Bradley B. Keller, Robert D. Lasley, Dianne M. Perez, Akito Tanoue, Gozoh Tsujimoto, Ginell R. Post, and Michael T. Piascik

The Department of Molecular and Biomedical Pharmacology, The University of Kentucky College of Medicine, Lexington, Kentucky (DC, DFM, SEE, MTP), The Cardiovascular Development Research Program, Department of Pediatrics, University of Kentucky (KT, BBK), Department of Cardiothoracic Surgery, The University of Kentucky, College of Medicine, Lexington, Kentucky (RDL), The Department of Molecular Cardiology, The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH (DMP), Department of Molecular, Cell Pharmacology, National Center for Child Health and Development Research Institute, Tokyo, Japan (AK,GT), and The Division of Pharmaceutical Sciences, The University of Kentucky College of Pharmacy, Lexington, Kentucky (GRP).

* indicates equal contributions

Introduction

G-protein-coupled receptors (GPCR) comprise about 1% of the human genome and perform vital and diverse roles in the regulation of physiologic processes. One of the members of the GPCR family is the α_1-adrenergic receptor (α_1-AR). Three subtypes, the α_{1A}-, α_{1B}-, and α_{1D}-ARs, have been isolated, cloned, and characterized. These receptors are intimately involved in the regulation of peripheral vascular resistance, cardiac function, and vascular and myocardial cell growth (for recent reviews on all aspects of the α_1-ARs see García-Sáinz *et al.,* 1999; Varma and Deng, 2000; Piascik and Perez, 2001).

Data from heterologous expression systems have shown that all three α_1-ARs can couple to a variety of G-proteins and second messenger systems. The α_1-ARs signal through both pertussis toxin sensitive G-proteins (Perez *et al*., 1993) as well as G-proteins of the G_q family (Wu *et al*., 1992). Studies in both transiently and stably transfected cells have demonstrated that all α_1-ARs activate phospholipases C and A_2 (Perez *et al*., 1993; Schwinn *et al*., 1991). In addition to mobilizing intracellular calcium (which would occur subsequent to activation of phospholipase C), the α_1-ARs have also been shown to activate calcium influx via voltage-dependent and -independent calcium channels (Minneman and Esbenshade, 1994; Lazou *et al*., 1994; Sayet *et al*., 1993).

While these studies have increased our understanding of α_1-AR regulatory biology, certain caveats must be established. Data from heterologous expression systems indicate the potential properties and regulatory activities of a given receptor. However, these data do not necessarily confirm that these regulatory events have a correlation in mammalian tissues that natively express these receptors. High density expression of non-

native receptors into cells could promote promiscuous coupling to pathways that may not normally be involved in *in vivo* receptor function.

Progress on the integrated regulatory activities of the α_1-ARs has been slowed by the availability of selective agonists and antagonists for these receptors. This is especially true for the α_{1B}-AR. In this report we have taken advantage of a unique line of transgenic mice systemically over-expressing a constitutively active α_{1B}-AR (see Zuscik *et al*., 2000, 2001) (see **Appendix C**), to examine the cardiovascular regulatory activities of the α_{1B}-AR. A constitutively active receptor is tonically active, thus eliminating the need for agonists that non-selectively activate all α_1-ARs. We have also examined regulatory activities in an α_{1D}-AR knockout line of mice (see Tanoue *et al*., 2002). Transgenic mouse models also have inherent shortcomings (see Discussion). Nonetheless, we can still use these models to propose and test hypotheses. In this communication, we test the hypothesis that the α_{1B}- and α_{1D}-ARs perform distinctly different regulatory activities. We postulate that the α_{1B}-AR is involved in the regulation of cardiac function and that the α_{1D}-AR is responsible for regulating systemic arterial blood pressure.

Experimental Procedures

Animal Use and Care

All animal protocols were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee. Tissues from two transgenic mouse lines were used in all aspects of this work. In one line, mice over-expressed a constitutively active mutation of the α_{1B}-AR, α_{1B}-AR_{C128F}. The over-expression of the constitutively active α_{1B}-AR was driven by the endogenous promoter, and the initial characterization of this mouse line has been described (Zuscik *et al.,* 2000, 2001). The other mouse line was a recently described α_{1D}-AR knockout (Tanoue *et al.,* 2002).

Assessment of MAP Kinase Activity

Tissue Preparation: Transgenic mouse hearts were removed, quick frozen, and stored in liquid nitrogen. The frozen tissue was homogenized (Dremel, Racine, WI) and incubated on ice for 1 h in 400 µl of the lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, 20 mM β-glycerophosphate, 0.5% NP-40, 100 µM Na_3VO_4, 5 µM AEBSF, 1.5 nM aprotinin, 10 nM E-64, 10 nM leupeptin, pH 7.4). After incubation on ice for 1 h, the lysate was centrifuged for 15 min at 15,000 g at 4°C. The total protein content in the supernatant was determined by Lowry assay.

Assay of Extracellular Signal-Regulated Kinase Activity: Extracellular signal-regulated kinase (ERK) activity was determined using an in-gel kinase assay. Equal amounts of protein were resolved on 10% SDS-polyacrylamide gels containing 0.5 mg/ml myelin basic protein (MBP) substrate that is polymerized together with acryalamide thereby

immobilizing it in the gel. Activated ERK kinase (Calbiochem) was used as a positive control. After electrophoresis, gels were washed with 20% 2-propanol in 50 mM HEPES, pH 7.6 and then with 5 mM -mercaptoethanol in HEPES buffer. Proteins were denatured by washing the gels in 6 M Urea and then renatured with an overnight incubation in HEPES buffer containing 0.05% (v/v) Tween-20 (renaturation buffer) at 4°C. Following incubation in renaturation buffer, gels were pre-incubated in 25 ml cold kinase buffer (20 mM HEPES, 20 mM $MgCl_2$, 2 mM DTT, 5 mM γ-glycerophosphate, 0.1 mM Na_3VO_4) for 30 min. Phosphorylation of MBP was performed *in situ* by incubating the gels in kinase buffer containing 20 μM ATP and 150-160 μCi [γ^{32}P]ATP for 90-120 min at 30°C. Gels were washed extensively in 5% trichloroacetic acid/1% sodium pyrophosphate to remove unbound ATP, dried and exposed to a phosphor screen. Incorporation of [^{32}P] into MBP was quantified with a phosphoimager (Molecular Dynamics), using ImageQuant software. Enzyme activity from each sample was normalized to the total amount of ERK present. This value was determined from immunoblotting as described below. Activity is reported as integrated optical density units, and is normalized to a percentage of enzyme activity detected in untreated tissues.

Assay for c-Jun N-Terminal Kinase Activity: c-Jun N-terminal kinase (JNK) activity was determined using an in-gel kinase assay as described above. In this case, protein was resolved on 10% SDS-polyacrylamide gels containing 0.1 mg/ml GST-cJun(1-135). Anisomycin is a known activator of the stress activated MAPKs; therefore, C6 Anisomycin extracts (Cell Signaling, Beverly, MA) were used as a positive control.

Immunoblotting: Equal amounts of protein samples were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene fluoride) membranes (Biorad). The amount of total ERK was detected by immunoblotting using a 1:1,000 dilution of Goat (c-16) anti-ERK polyclonal IgG (Santa Cruz Biotech, Santa Cruz, CA) with horseradish peroxidase conjugated anti-goat IgG at 1:10,000 (Jackson ImmunoResearch Labs, West Grove, PA). The total JNK was detected by immunoblotting using a 1:1,000 dilution of Rabbit (c-17) anti-JNK1 polyclonal IgG (Santa Cruz Biotech, Santa Cruz, CA) with horseradish peroxidase conjugated donkey anti-rabbit IgG at 1:2,000 (Amersham, Buckinghamshire, U.K.). Following exposure of the membranes to ECL + reagent (Amersham, Buckinghamshire, U.K.), the chemiluminescent signal was detected with a phosphoimager (Molecular Dynamics). Quantitation was performed using ImageQuant software.

Experiments in the Isolated-Perfused Heart

The Isolated-Perfused Heart Preparation: Mice were heparinized (200 U) and anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). The chest cavity was opened and the heart was quickly excised and submersed in ice-cold saline. The aorta was dissected and the ascending aortic stump was cannulated with a 22-guage plastic cannula primed with ice cold modified-Krebs-Hensleit buffer (KHB) (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM Dextrose, 1.5 mM $CaCl_2$, and 1 mM pyruvate). The aorta was sutured into position and the cannula placed on a perfusion apparatus (Radnoti, Monrovia, CA). Retrograde (Langendorff) perfusion was immediately performed with oxygenated (95% O_2 and 5%

CO_2) modified KHB at 37.5°C. The hearts were allowed to beat spontaneously. The perfusion pressure was monitored with a pressure transducer (COBE, Lakewood, CO) connected to a Grass polygraph (Grass Instruments, Quincy, MA), and the coronary perfusion pressure was maintained at 75 mm Hg by adjusting the flow of the perfusion pump that was calibrated before each experiment by measuring volume perfused per min (Control Company, Friendswood, TX). A fluid-filled balloon catheter was inserted into the left ventricle, and the balloon was filled to attain a diastolic pressure of 5-10 mm Hg. The balloon catheter line was connected to a second pressure transducer and an amplifier module designated to measure the developed pressure, which was linked to a differentiator. The parameters measured were heart rate, left ventricular (LV) systolic and end diastolic pressure, and the rise and the fall in LV developed pressure as a function of time (+*dP/dt* and -*dP/dt*, respectively).

Drug-Induced Increases in Inotropy: In both lines of transgenic animals and their respective controls, hearts were perfused at a constant pressure of 75 mm Hg to assess the effects of α_{1B}-AR modulation on β_1-AR-induced positive inotropy. Following a 25 min equilibration period, an isoproterenol dose-response curve was generated by infusing a stock solution of 100 nM at increasing rates (.037 to 2.9 ml/min) into the aortic cannula. Measurements of coronary flow, heart rate, and ventricular function were collected at baseline (0 min) and 1 min after drug administration.

Drug-Induced Coronary Vasoconstriction: The effects of phenylephrine on coronary perfusion pressure were determined in the myocardium. Once a perfusion pressure of 80-

85 mm Hg was reached, experiments were performed at a constant flow. The protocol was conducted in the presence of 100 nM of propranolol to limit the effect of β-AR stimulation on coronary perfusion pressure. Following a 25 min equilibration, a stock solution of 1 mM phenylephrine was infused via an infusion pump to attain a final concentration of 100 μM. The effect of phenylephrine on coronary pressure was recorded, and constriction was assessed by determining the relative change in the coronary perfusion pressures from baseline at specified time points following phenylephrine infusion.

Echocardiography

Echocardiographic studies were performed on mice of 5-6 months of age (12 with the α_{1B}-AR_{C128F} and 10 NTs). Before determination of body weight, the mouse was anesthetized with 1.25% isoflurane, and the animal was placed on a custom-designed heated water-filled glass chamber that maintained an euthermic body temperature of 37°C. The thorax hair was shaved and warm ultrasonic coupling jelly was applied to cover the thorax. Transthoracic echocardiography was performed using the Acuson Sequoia C256 system with a 13 MHz linear ultrasonic transducer (15L8, Acuson, Mountain View, CA, USA) in a phased array format. This system offers 0.35 mm lateral resolution and 0.25 mm axial resolution and is capable of acquiring and storing real-time digital images simultaneously. M-mode measurements on the LV short axis view (papillary muscle level) was performed (see Gardin *et al*., 1995). The M-mode tracings were used to measure the end-diastolic and end-systolic LV internal chamber dimensions (LVID) as well as the posterior wall thickness (PWT). The maximum end-diastolic (ED)

LV internal chamber dimensions (LVIDd) and PWTd were measured when the LV chamber cavity reached end-diastole, and the LV end-systolic (ES) internal chamber dimensions (LVIDs) were measured at the time corresponding to maximum motion of the LV posterior wall. The cycle length (CL) and ejection time (ET) were measured from aortic flow waveforms. The LV fractional shortening (%FS), LV mass, and the heart rate corrected mean velocity of circumferential fiber shortening (mVcfc) were estimated as follows: $\%FS = [(LVIDd-LVIDs)/LVIDd]\cdot 100$; $LV\ mass = 1.055[(LVIDd + 2\cdot PWTd)^3 - LVIDd^3]$; and $mVcfc = [(LVIDd-LVIDs)/LVIDd]/(ET\cdot CL^{.5})$. The LV mass was calculated by using the uncorrected cube assumption (Pombo *et al*., 1971) without the use of the interventricular septal wall thickness since it was difficult to detect the endocardial border between the right ventricular cavity and the interventricular septum. Three beats were averaged for each measurement. The stroke volume (SV) was calculated from the dimensions as follows: SV = (ED volume - ES volume) and cardiac output (CO) was calculated from $SV\cdot HR$.

Assessment of Aortic Contractile Function

Isolated blood vessels were prepared by techniques routinely used in our laboratory (Piascik *et al.*, 1994, 1995, 1997). Briefly, aortic segments were removed from transgenic mice and placed in cold physiologic salt solution (PSS). Stainless steel or platinum wires were threaded through the lumen of each vessel. One wire was connected to a fixed base and the other to a micrometer clamp to adjust the passive force on the tissue. The tissues were mounted in water-jacketed muscle baths filled with PSS maintained at 37°C under constant oxygenation (95% O_2, 5% CO_2; pH 7.4). A passive

force of 1.0 g was placed on the aorta. Previous studies have shown that this passive force gives optimal agonist responses. Changes in the force generation were recorded using Grass FT .03 force transducers connected to a Grass model 7 polygraph. The muscle rings were equilibrated in oxygenated PSS and then challenged with KCl at 80 mM for 1 min. The muscles were then washed with oxygenated PSS every 15 min until the contraction returned to baseline. Arterial segments were exposed to phenylephrine and the contractile effects were recorded. Contractile responses to phenylephrine were also measured following a 20 min incubation with 30 nM BMY 7378, a selective α_{1D}-AR antagonist. The equilibrium dissociation constant for BMY 7378 was calculated as described by Besse and Furchgott (1976).

Cyclic AMP Assay in the Mouse Myocardium

Tissue Preparation and Treatment: Mouse hearts were quickly removed and cleaned in non-supplemented DMEM. The ventricles were sliced and placed in a fresh non-supplemented DMEM with 100 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemicals, St. Louis, MO) in a 37°C incubator with a 5% CO_2 atmosphere. At the appropriate time, the tissue was treated with vehicle, isoproterenol alone or isoproterenol in the presence of propranolol. Forskolin was used as a positive control. Following drug treatment, the slices were quick frozen in liquid nitrogen and stored at -80°C. The tissue samples were powdered and incubated in 250 μl of lysis solution (0.1 M HCl) for 1 h on ice. The lysate was centrifuged for 5 sec at 11,750 g. The supernatant was collected for the determination of cAMP levels and total protein content (determined by Lowry assay).

Assaying for cAMP Levels: After the total protein content was adjusted to 100 µg/ml with 0.1 M HCl, the lysate was assayed for cAMP levels (non-acetylated) using a commercial cAMP assay kit (BioMol, Plymouth Meeting, PA). Samples were performed in duplicates. The optical densities of the samples were read at 405 nm. The quality control parameters and the mean and the standard errors of the mean are listed below for four curves: Total activity (maximum colormetric enzymatic reaction with substrate) added = 11.02 ± 0.35 Optical Density; % Non-specific binding = 0.0008 ± 0.0003 %; % Maximum Binding/Total Activity = 2.92 ± 0.07 %. From cAMP standards, the curves for cAMP concentration of the unknown had a 20 % Intercept = 35.00 ± 5.85 pmol/ml, 50 % Intercept = 7.65 ± 0.59 pmol/ml, and 80 % Intercept = 1.60 ± 0.28 pmol/ml. The line obtained has a slope of -32.85 ± 1.54 with a correlation coefficient of 0.942 ± 0.012.

Statistical Analysis

In all figures, the data are expressed as the mean and standard error of the mean (S.E.). When appropriate, statistical significance was assessed with either the unpaired two-tailed Student's *t* test or the two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls analysis. A value of $P<0.05$ was considered statistically significant.

Results

Experiments in Mice Over-Expressing the α_{1B}-AR_{C128F}

Activation of Mouse Myocardial MAPKs

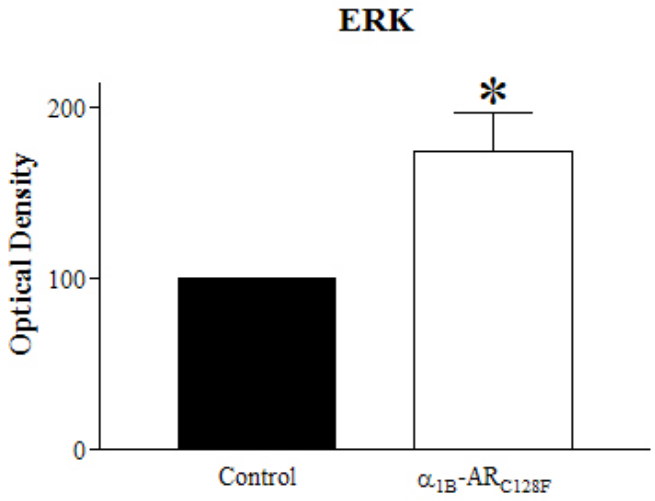
The hearts from α_{1B}-AR_{C128F} mice exhibited significantly elevated levels of ERK and JNK activity when compared to the non-transgenic controls (**Figure 14**, **a** and **b**). These results support the idea that the over-expressed α_{1B}-AR_{C128F} is functional and can couple to signaling pathways in the absence of agonists. ERK activity was not altered in hearts from α_{1D}-AR knockout mice (data not shown).

Echocardiographic Analysis

Activation of MAPKs has been proposed to link the α_1-ARs to growth responses. Echocardiography was performed as a non-invasive method of assessing the effect(s) of constitutive activation of the α_{1B}-AR on left ventricular (LV) dimensions and cardiac function (**Table 2**, **a** and **b**). The LV dimensions were normalized to the body weight. The transgenic animals showed significantly increased LV internal dimensions during either diastole or systole (**Table 2a**). Chamber dimensions were increased in the transgenic animals without a change in the wall thickness (this is indicated by no change in the posterior wall thickness in either diastole or systole in **Table 2a**). In **Table 2b**, LV dimensional analysis reveals that there is a significant reduction in the percent fractional shortening for mice over-expressing the α_{1B}-AR_{C128F} when compared to the non-transgenic controls. Fractional shortening value, an index of LV function, indicates reduced cardiac performance in the transgenic line. The ejection time, heart rate, and mean velocity for circumferential fiber shortening corrected for heart rate were

Figure 14. MAPK activity in transgenic mouse hearts. **a)** ERK activity and **b)** JNK activity measured by in-gel kinase assays, where each bar represents the mean and the S.E. of 7 independent determinations. The asterisk (*) indicates significantly different values from non-transgenic control values. Data are taken from Chalothorn *et al*., 2003. Data generated by Dr. Dan F. McCune.

a)



b)

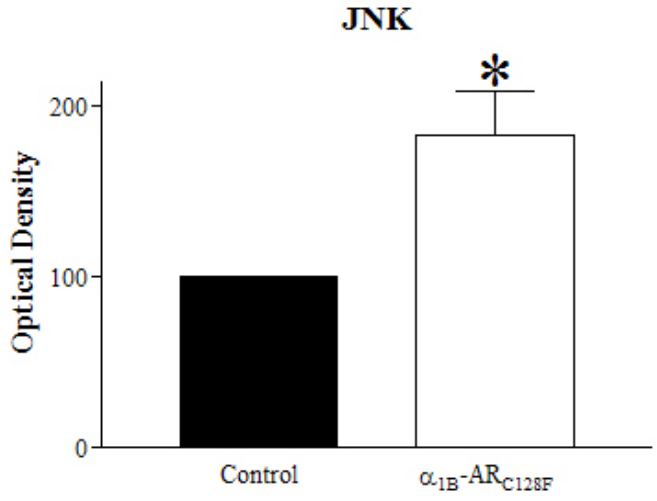


Table 2. Echocardiographic assessment of the murine left ventricular **a)** dimensions and **b)** function in mice over-expressing the α_{1B}-AR_{C128F}. The values are the mean ± S.E., and * indicates statistical difference from the non-transgenic value ($P<0.05$). Data from Chalothorn *et al*., 2003. Data generated with the help of Dr. Kimimasa Tobita.

a)

	N	Body Weight (g)	Left Ventricular Internal Dimension (mm/g)		Posterior Wall Thickness (mm/g)		Left Ventricular Mass (g)	Left Ventricular Mass/ Body Weight (10^{-3})
			Diastole	Systole	Diastole	Systole		
Control	10	30.03 ± 1.43	0.1364 ± 0.0046	0.0770 ± 0.0055	0.0197 ± 0.0022	0.0358 ± 0.0041	0.0782 ± 0.0074	2.659 ± 0.302
α_{1B}-AR_{C128F}	12	27.99 ± 1.16	0.1523* ± 0.0065	0.1038* ± 0.0066	0.0171 ± 0.0009	0.0313 ± 0.0032	0.0683 ± 0.0051	2.441 ± 0.149

b)

	n	Ejection Time (msec)	Mean velocity for circumferential fiber shortening ($1/\sqrt{sec}$)	Heart Rate (beats/min)	Stroke Volume (mm^3)	Cardiac Output (mm^3/min)	Fractional Shortening (%)
Control	10	57.23 ± 3.83	0.6935 ± 0.1195	454.1 ± 22.7	0.0273 ± 0.0018	12.31 ± 0.83	43.10 ± 4.11
α_{1B}-AR_{C128F}	12	51.76 ± 2.00	0.5550 ± 0.0984	484.6 ± 20.9	0.0274 ± 0.0023	13.21 ± 1.22	32.05* ± 2.44

reduced in the animals with the α_{1B}-AR_{C128F} mutation. However these reductions were not statistically significant. Neither the stroke volume nor the cardiac output was found to be statistically different between groups. Therefore persistent, unregulated activation of the α_{1B}-AR results in a decrease in cardiac function and chamber dilation.

Responses in the Isolated-Perfused Heart

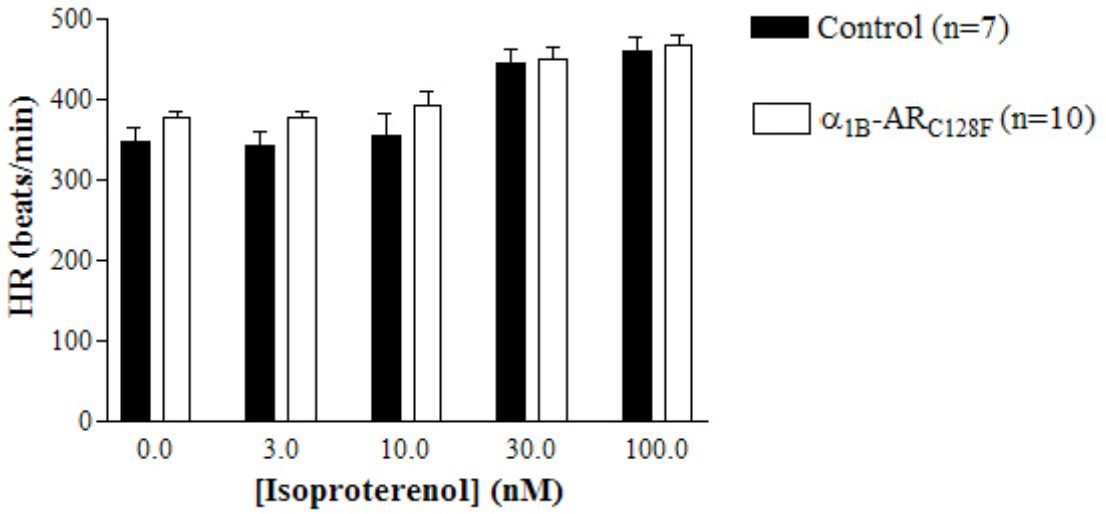
To more completely assess the effect of constitutive activation of the α_{1B}-AR on contractile responses, experiments were performed in the isolated-perfused heart. Resting heart rates were 348 ± 18 bpm and 384 ± 12 bpm in control and transgenic mouse hearts, respectively. This difference was not statistically significant and is consistent with the echocardiographic analysis of heart rate. We did not observe any significant change in basal coronary flow rate in these hearts (see **Appendix D**). Isoproterenol infusion produced similar increases in heart rate in both groups (**Figure 15a**). The ability of isoproterenol (30 and 100 nM) to increase contractile force was significantly decreased in hearts from mice over-expressing the α_{1B}-AR_{C128F} mutation (LVDP and +dP/dt in **Figures 15**, **b** and **c**). The -dP/dt curves were not significantly different (**Figure 15d**).

cAMP Production

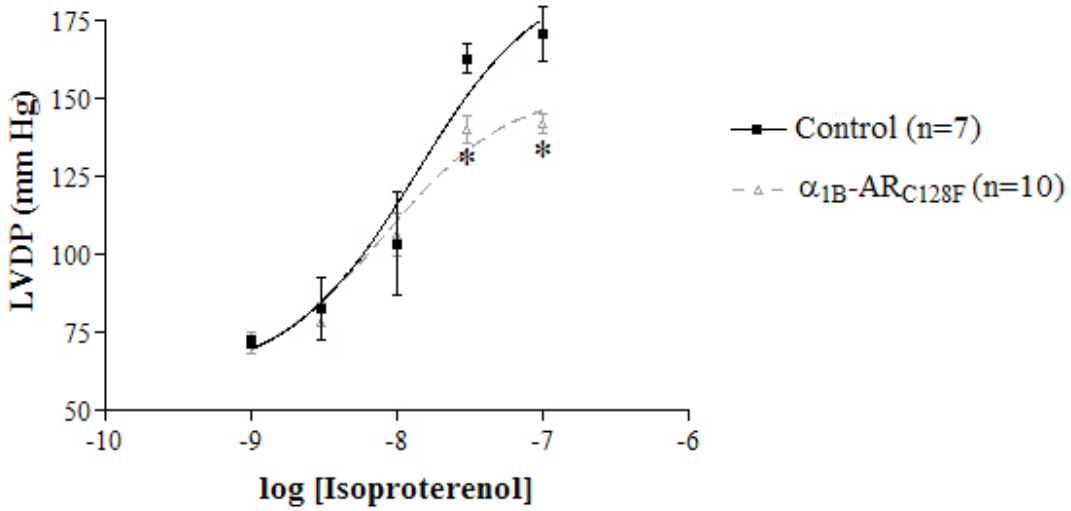
The blunted isoproterenol-induced response prompted additional experiments to determine if there were changes in the β_1-AR signaling pathway that resulted from α_{1B}-AR over-activity. We therefore assessed the ability of isoproterenol to increase cAMP levels in ventricular slices from control and transgenic animals. The positive control,

Figure 15. Functional responses of mouse hearts to 3, 10, 30, and 100 nM of isoproterenol. **a)** Heart rate (HR), **b)** left ventricular developed pressure (LVDP), **c)** positive change in the developed pressure as a function of time (+dP/dt), and **d)** negative change in the developed pressure as a function of time (-dP/dt). Each bar or point on the curve represents the mean and the S.E. of 7 and 10 independent experiments for the non-transgenic control and α_{1B}-AR_{C128F} hearts, respectively. The asterisk (*) indicates statistical differences from the non-transgenic control value at the respective isoproterenol concentration. Data from Chalothorn *et al*., 2003.

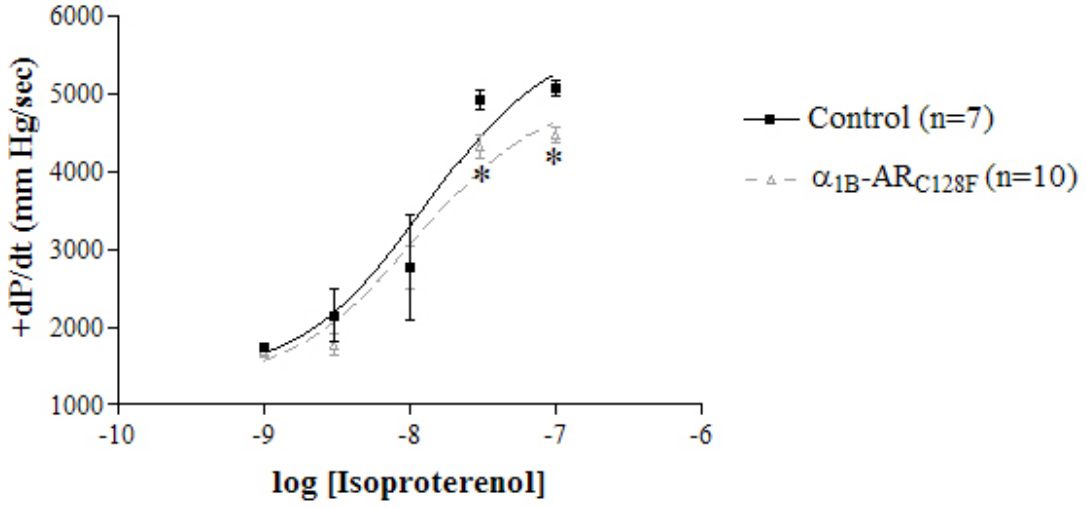
a)



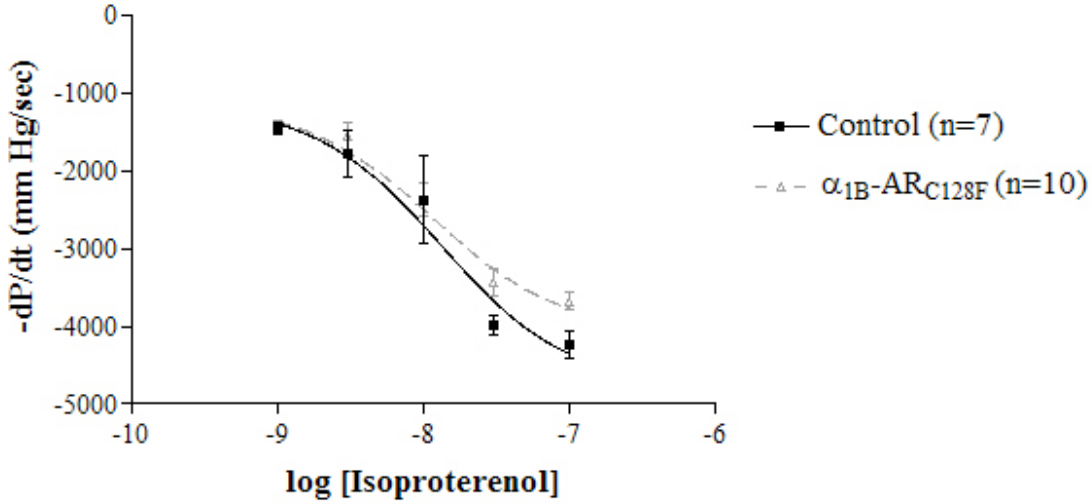
b)



c)


6000
5000
4000
3000
2000
1000
+dP/dt (mm Hg/sec)
-10
-9
-8
-7
-6
log [Isoproterenol]
*
*
Control (n=7)
α1B-AR C128F (n=10)

d)


0
-1000
-2000
-3000
-4000
-5000
-dP/dt (mm Hg/sec)
-10
-9
-8
-7
-6
log [Isoproterenol]
Control (n=7)
α1B-AR C128F (n=10)

sodium forskolin, produced similar increases in cAMP in both groups (**Figure 16**). In control ventricular slices, isoproterenol (1 and 10 μM) produced an increase in cAMP levels that was antagonized by 0.1 μM propranolol. In ventricular segments from α_{1B}-AR_{C128F} mice, the cAMP response to either 1 or 10 μM isoproterenol was reduced. This difference was statistically significant at a concentration of 10 μM.

Contractile Responses in the Mouse Aorta

In the aortae from non-transgenic control mice, phenylephrine produced concentration-dependent increases in developed tension (**Figure 17a**). The dose-response curve was shifted to the right by 30 nM of the α_{1D}-AR selective antagonist BMY 7378. From these data, we calculated the equilibrium dissociation constant for BMY to be 0.294 ± 0.149 nM. This value is in good agreement with that obtained from experiments with cloned α_{1D}-AR as well as the receptor expressed on rat blood vessels (2 nM, Piascik *et al*., 1995), indicating that, like the rat aorta, the phenylephrine contractile response in the mouse aorta is mediated by the α_{1D}-AR. Over-expression of a constitutively active α_{1B}-AR did not enhance the response of the mouse aortae to phenylephrine (**Figure 17b**). BMY 7378 was also a potent antagonist in the aorta from α_{1B}-AR_{C128F} expressing mice with an estimated equilibrium dissociation constant of 0.385 ± 0.401 nM (**Table 3**), indicating that the α_{1D}-AR still mediates contraction in this blood vessel. These data show that despite over-expression of a constitutively active and signaling competent form of the α_{1B}-AR, the response of the aorta is unaffected and remains under the control of the α_{1D}-AR (experiments with aorta from α_{1D}-AR knockout mice have previously been reported in Tanoue *et al*. (2002), see Discussion). Consistent

Figure 16. The ability of isoproterenol to increase cAMP levels in ventricular slices from non-transgenic control and α_{1B}-AR_{C128F} animals. cAMP levels are presented as pmol of cAMP/20 mg of protein. Data are the mean and the S.E. of 5 and 8 heart samples from experiments performed in duplicate from the non-transgenic control and the α_{1B}-AR_{C128F} hearts, respectively. *, indicate a significantly different cAMP level from the control at 10 μM isoproterenol. Data from Chalothorn *et al*., 2003.

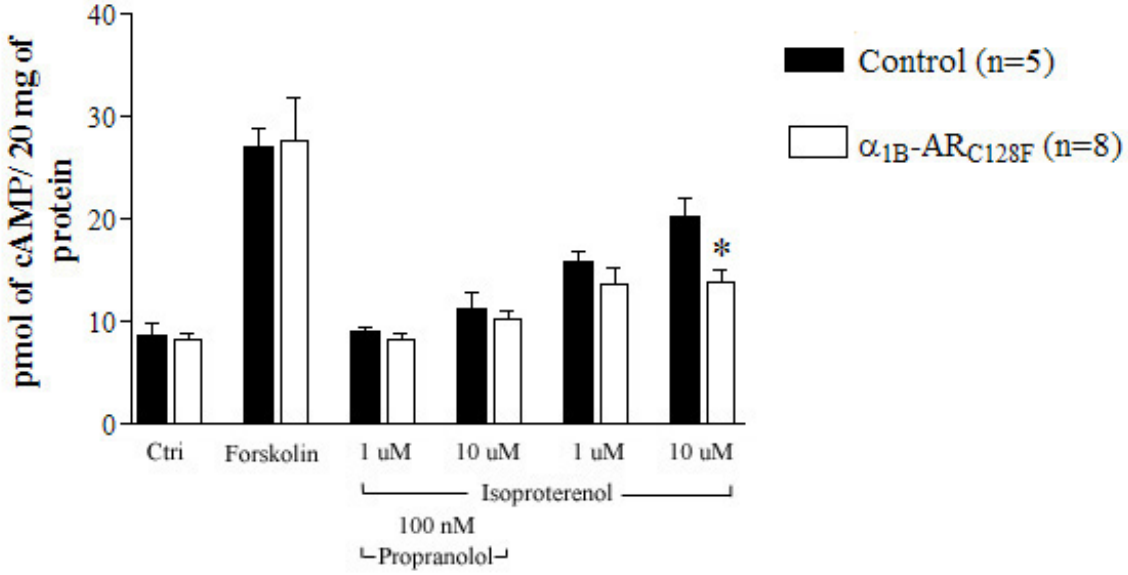
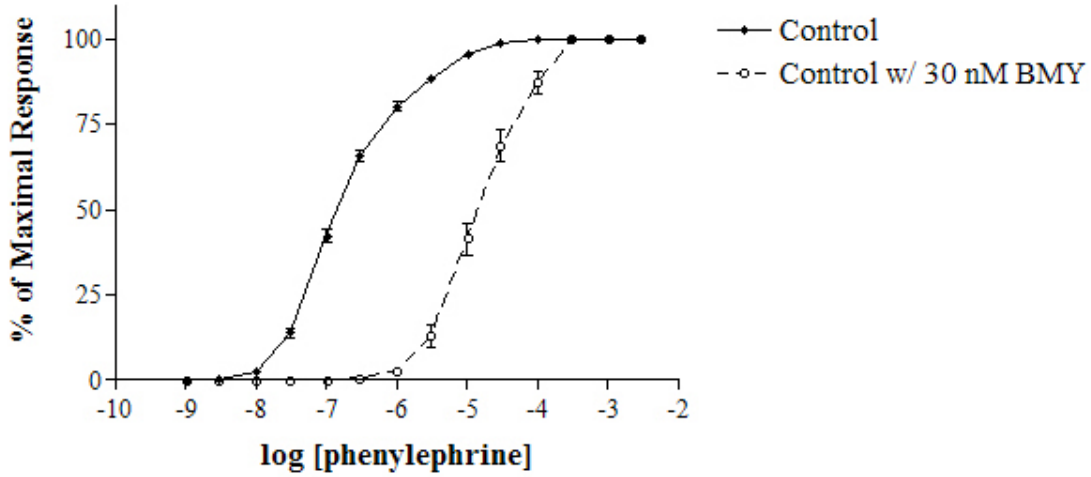


Figure 17. Log-dose response curves of the phenylephrine-induced contraction in mouse thoracic aortae in the absence and the presence of 30 nM BMY 7378. **a)** Non-transgenic control, where the curves in the absence and the presence of BMY 7378 are composed of the average and the S.E. of 52 and 23 independent experiments, respectively and **b)** α_{1B}-AR_{C128F}, where the curves in the absence and the presence of BMY 7378 are composed of the average and the S.E. of 39 and 10 independent experiments, respectively. Data taken from Chalothorn *et al*., 2003.

a)



b)

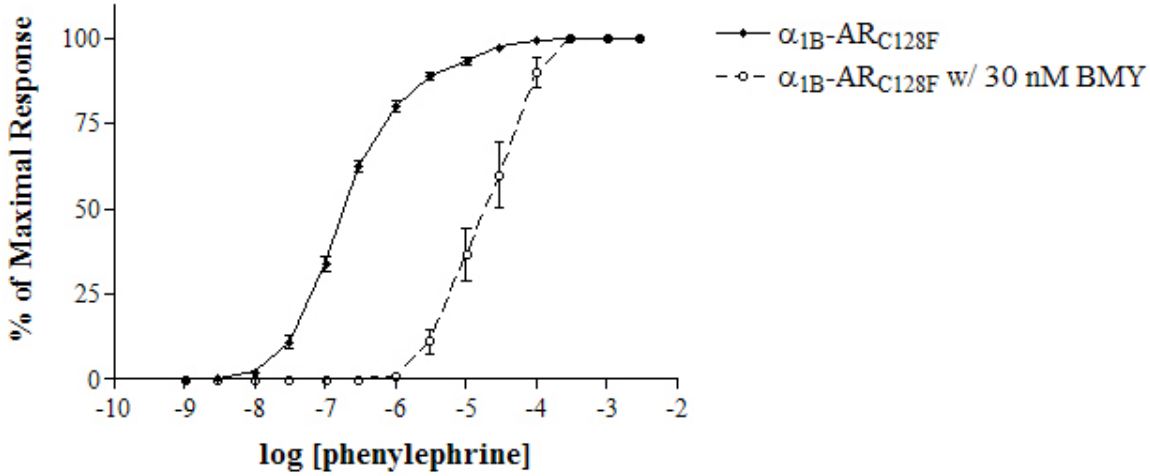


Table 3. Characteristics of the phenylephrine response in control and α_{1B}-AR_{C128F} over-expressing aortae. Calculated pA_2 values and their 95% confidence intervals (C.I.) are listed along with the dissociation constant (K_i). Data taken from Chalothorn *et al*., 2003.

	n	Phenylephrine		Characterization of BMY 7378 Antagonism			
		Log EC_{50}	S.E.	pA_2	95% C.I. of pA_2	K_i (nM)	95% C.I. of K_i (nM)
Control	52	-6.869	0.0662	9.531	0.219	0.294	0.149
α_{1B}-AR_{C128F}	39	-6.795	0.1036	9.414	0.452	0.385	0.401

with this lack of effect on the ability of phenylephrine to induce coronary vasoconstriction in hearts from mice expressing the constitutively active α_{1B}-AR.

Experiments in the α_{1D}-AR Knockout Mice

Responses in the Isolated-Perfused Heart

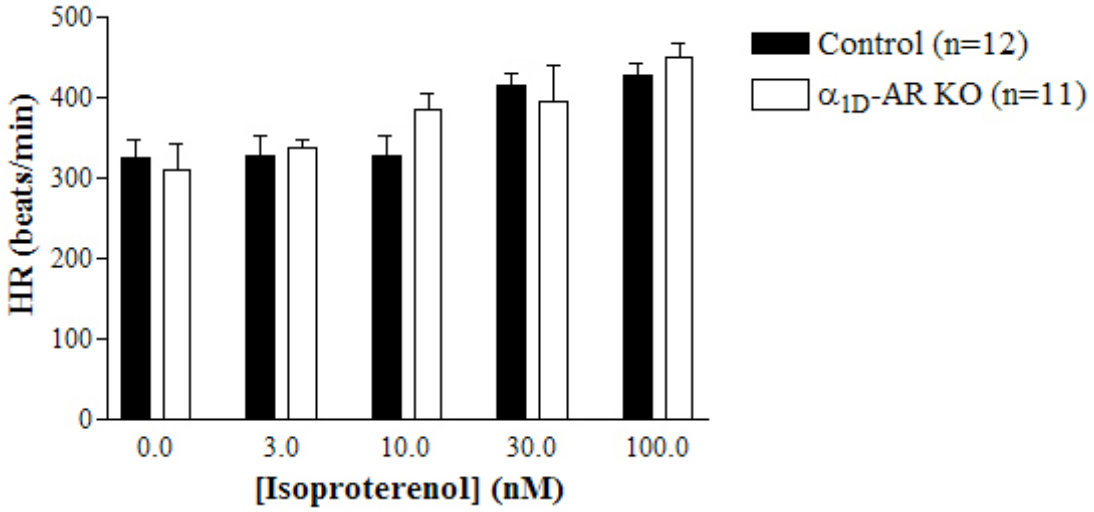
The effects of α_{1D}-AR deficiency on β-AR-induced responses were assessed in the isolated-perfused heart preparation. The ability of isoproterenol to induce positive chronotrophy or inotropy was not significantly different between the control and the mice lacking the α_{1D}-AR (**Figures 18**, **a** and **b**). (+) or (-) dP/dt curves were also not different in hearts from α_{1D}-AR deficient mice (**Figures 18**, **c** and **d**). Echocardiographic analysis also showed no differences in cardiac parameters between the experimental groups (see **Appendix E**).

Effects on Coronary Perfusion Pressure

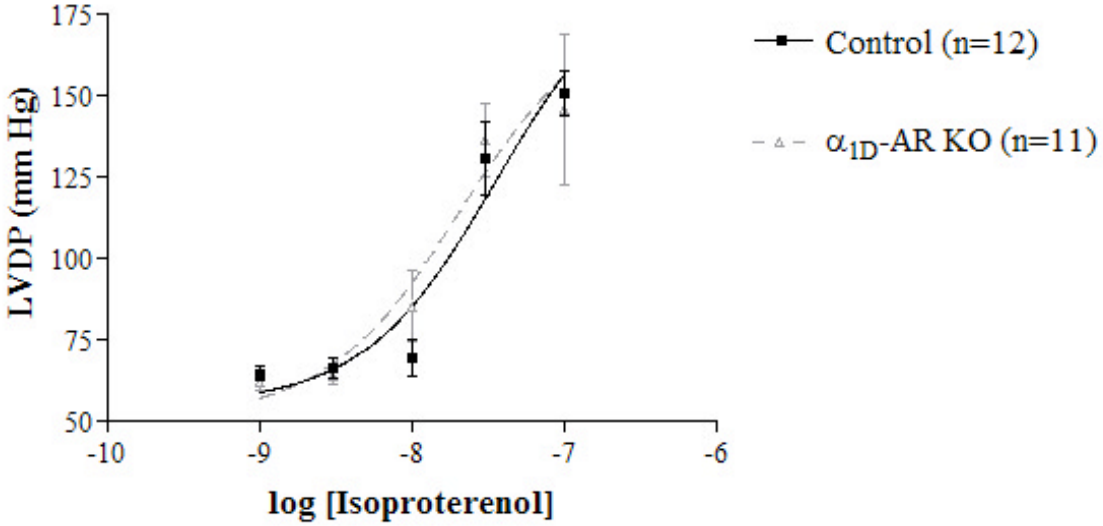
In contrast to having little effect on cardiac contractile responses, knockout of the α_{1D}-AR has prominent effects on coronary vascular responses. The basal coronary flow rate required to maintain the coronary perfusion pressure was found to be significantly greater in α_{1D}-AR knockout animals when compared to non-transgenic controls (**Figure 19**). In hearts from control mice, 100 μM phenylephrine infusion caused a significant increase in coronary perfusion pressure (**Figure 20**). Phenylephrine-induced increases in perfusion pressure were significantly reduced in hearts from α_{1D}-AR knockout mice.

Figure 18. Functional responses of α_{1D}-AR KO mouse hearts to 3, 10, 30, and 100 nM of isoproterenol. **a)** Heart rate (HR), **b)** left ventricular developed pressure (LVDP), **c)** positive change in the developed pressure as a function of time (+dP/dt), and **d)** negative change in the developed pressure as a function of time (-dP/dt). Each bar or point on the curve represents the mean and the S.E. of 12 and 11 independent experiments for the control and the α_{1D}-AR KO hearts, respectively. Data from Chalothorn *et al*., 2003.

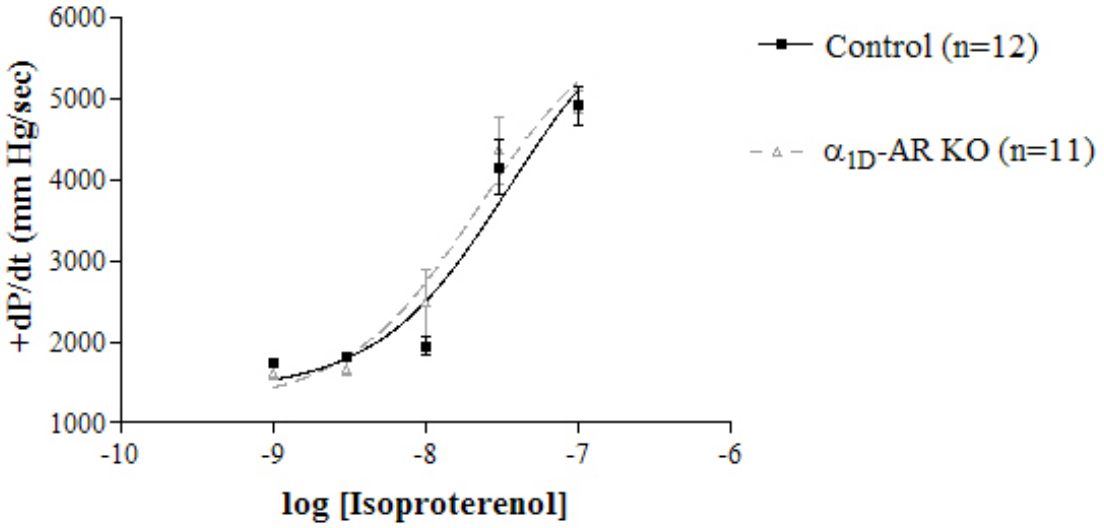
a)



b)



c)


6000
5000
4000
3000
2000
1000
+dP/dt (mm Hg/sec)
-10
-9
-8
-7
-6
log [Isoproterenol]
Control (n=12)
α1D-AR KO (n=11)

d)

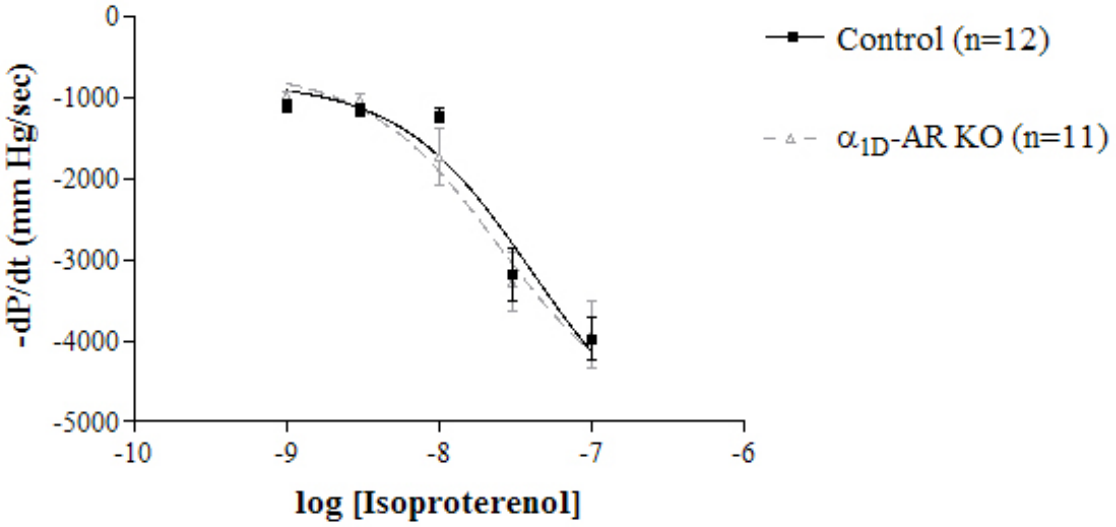

0
-1000
-2000
-3000
-4000
-5000
-dP/dt (mm Hg/sec)
-10
-9
-8
-7
-6
log [Isoproterenol]
Control (n=12)
α1D-AR KO (n=11)

Figure 19. Basal coronary flow rate required to maintain a constant perfusion pressure. Each bar represents the average and the S.E. of 7 independent experiments. The asterisk (*) indicates statistical significance from the control group. Data from Chalothorn *et al*., 2003.

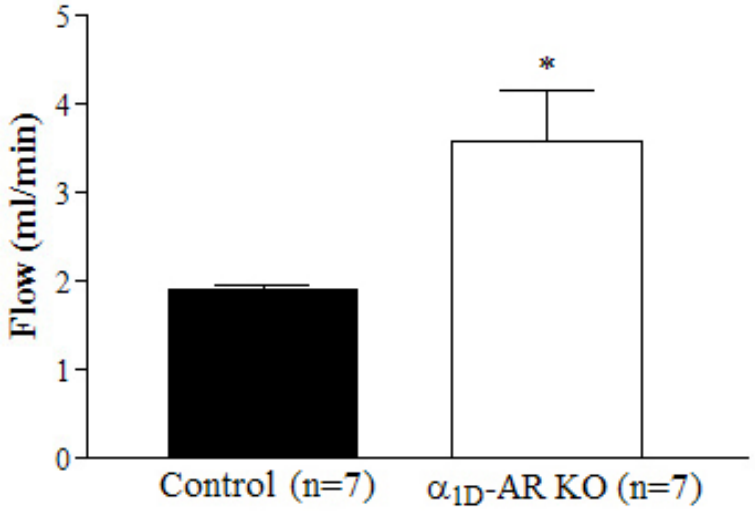
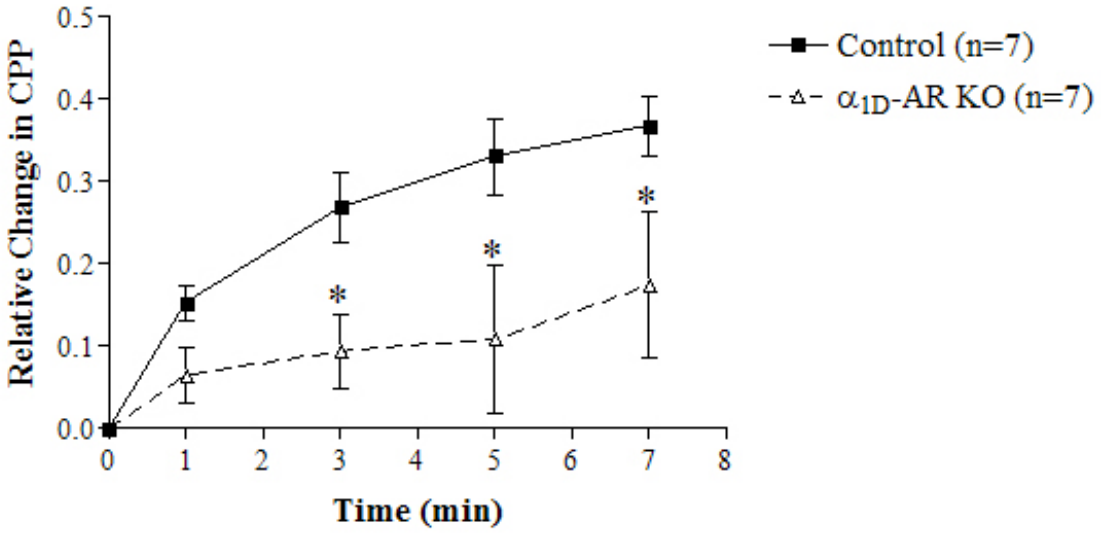


Figure 20. Effect of 100 μM of phenylephrine on relative changes in the coronary perfusion pressure (CPP) of hearts lacking the α_{1D}-AR. The initial CPPs were 83.3 ± 2.3 and 81.9 ± 2.2 mm Hg for the control and the α_{1D}-AR KO, respectively. The recordings were performed over a 7 minute period. Each curve is composed of the average and the S.E. for 7 different experiments where the asterisk (*) indicates statistical significance between the α_{1D}-AR KO and the control group at the respective time point. Data from Chalothorn *et al*., 2003.



Discussion

While it is clear that the α_1-AR family plays a prominent role in the regulation of cardiac and vascular function, the specific function of each subtype has been difficult to discern. Despite the fact that many tissues express multiple α_1-AR, we do not believe that there is redundancy in the regulatory activities of these receptors. Rather, we hypothesize that each subtype is coupled to distinct regulatory processes. We propose that the α_{1B}-AR plays a role in the modulation of cardiac function while the α_{1D}-AR is a specific regulator of vascular contractile function

These hypotheses were tested using two newly developed lines of transgenic mice. While transgenic models offer a unique and powerful approach to receptor research, they are not without shortcomings. The assumption is that the observed biochemical or physiologic alterations are a direct result of transgenic receptor expression or deletion. However, we must concede that any effects we observe could also be non-specific and occur as result of interference in the expression of vital signaling molecules unrelated to the α_1-ARs whose expression were altered.

To examine the regulatory activity of the α_{1B}-AR we chose a transgenic line of mice over-expressing a constitutively active mutant of this α_{1B}-AR. An α_{1B}-AR knockout line of mice is also available (Cavalli *et al.,* 1997). Studying these knockout animals would essentially be a loss of function protocol. However, by studying constitutively active receptors we are able to use the gain of function as a read out of receptor activity. The use of constitutively active receptors offers another advantage in studying receptor systems like the α_{1B}-AR for which there are no selective agonists. Without such selective ligands, wild-type receptor activation can only be achieved by administering non-

selective agonists such as phenylephrine that would activate all α_1-AR subtypes. Because constitutively active receptors engage signaling pathways in the absence of agonists, we can observe the results of α_{1B}-AR activation without the need to administer α_1-AR agonist compounds.

In previous work we showed that in the absence of agonist, the α_{1B}-AR_{C128F} can couple to inositol phosphate formation (Zuscik *et al.,* 2001). In this work we show that there is an increase in the activity of MAPKs (see Figure 1) in α_{1B}-AR_{C128F} animals. This would imply that this receptor is indeed constitutively coupled to signaling pathways. Coupling of the α_{1B}-AR to MAPKs would be in agreement with a great deal of data from non-transgenic sources (see reviews of García-Sáinz *et al.,* 1999; Varma and Deng, 2000; Piascik and Perez, 2001). However considering the uncertainties of experiments with transgenic animals we cannot be completely sanguine that the observed increases in kinase activity are a direct result of receptor expression as opposed to being non-specific and secondary to other pathophysiologic alterations.

Echocardiographic analysis of mice over-expressing the α_{1B}-AR_{C128F} revealed a statistically significant reduction in fractional shortening when compared to non-transgenic controls (**Table 2**). A decrease in fractional shortening is evidence for contractile dysfunction in these animals.

Further evidence that over-expression of the α_{1B}-AR_{C128F} interferes with myocardial contractility was obtained in the isolated-perfused heart experiments. The ability of isoproterenol to increase contractile force was significantly reduced in hearts from transgenic animals (see **Figures 15, b** and **c**). We also noted an impaired ability of isoproterenol to promote increases in cAMP levels (see **Figure 16**). This indicates the

possibility that tonic unregulated activation of the α_{1B}-AR impairs β_1-AR signaling and could be the underlying reason for the decrease in contractile function.

Activation of members of the α_1-AR subtype family has been associated with increases in myocardial contraction (see Varma and Deng, 2000 and references therein). This present work and that of others (Akhter *et al*., 1997; Lemire *et al*., 2001) clearly shows that the α_{1B}-AR is not the subtype coupled to this positive inotropic effect. In other work with the α_{1B}-AR_{C128F} over-expressing mice, we have shown that it is the α_{1A}-AR that mediates the positive inotropic actions of phenylephrine (Ross *et al.,* 2003 in revision; see **Appendix F**). We have further shown that constitutive activation of the α_{1B}-AR decreases the ability of the α_{1A}-AR to activate myocardial contraction (Ross *et al.,* 2003 in revision) as well as decreasing α_{1A}-AR mRNA levels. Taking into consideration the caveats raised above regarding the use of transgenic models, our data can also be used to argue that tonic unregulated activation of the α_{1B}-AR diminishes cardiac contractile activity by decreasing the positive inotropic signaling emanating from both the β_1- and the α_{1A}-ARs.

In addition to contractile dysfunction, echocardiographic analysis also revealed increases in the left ventricular internal dimensions of the α_{1B}-AR_{C128F} heart. This is evidence of an increase in chamber size. This phenotype of contractile dysfunction and increased chamber dimensions has also been seen in a distinctly different mouse model that uses cardiac targeting to over-express the wild-type α_{1B}-AR (Grupp *et al*., 1998; Lemire *et al*., 2001). In contrast to these results, other reports with a cardiac-targeted constitutively active α_{1B}-AR (Milano *et al*., 1994) or our systemic over-expression model provide evidence of contractile dysfunction and cardiac hypertrophy. It is not clear as to

why studies in the same mouse models reveal differences in cardiac phenotype. What is clear is that tonic unregulated activation of the α_{1B}-AR has significant and negative effects on cardiac function that can progress into hypertrophy or dilated cardiomyopathy. Factors that determine how biosignals emanating from the α_{1B}-AR lead to these pathophysiologies are being investigated.

Consistent with published works (see Piascik and Perez, 2001; García-Sáinz *et al*., 1999 and references therein) we propose that the α_{1B}-AR has minimal activity as a regulator of vascular function. Previously, we showed that over-expression of the α_{1B}-AR_{C128F} does not increase resting systemic arterial blood pressure (Zuscik *et al.,* 2001). Knockout of the α_{1B}-AR also had no effect on resting blood pressure (Cavalli *et al.,* 1997). Herein we show that over-expression of the α_{1B}-AR_{C128F} does not alter the response characteristics in the isolated aorta. Therefore in the same mouse line where over-expression of a constitutively active α_{1B}-AR has demonstrable effects on cardiac function, we are unable to detect any increases in systemic arterial blood pressure or contractility in the aorta. If over-expression of the constitutively active α_{1B}-AR produced non-specific effects on cardiovascular function then it would be reasonable to suppose that vascular function would also be impaired. These data support our hypothesis that there is specificity in coupling amongst the α_1-AR subtype family and that the α_{1B}-AR is coupled to regulatory events in the heart without participating in the contraction of vascular smooth muscle.

The α_{1D}-AR is an enigmatic and the least well-studied member of the α_1-AR subtype family. In previous work, it has been shown that this receptor is expressed mainly in intracellular compartments (McCune *et al.,* 2000; Chalothorn *et al.,* 2002). We

do not yet know the reason for this atypical localization pattern or if the regulatory activities of the α_{1D}-AR are accomplished by these intracellular receptors. Recently, it has been shown that the α_{1D}-AR is constitutively active (García-Sáinz and Torres-Padilla, 1999; Gisbert *et al*., 2000; McCune *et al.,* 2000). D'Ocon's group has shown that the constitutively active α_{1D}-ARs are capable of mediating vascular smooth muscle contraction. Other studies have demonstrated that the α_{1D}-AR is expressed throughout the cardiovascular system (Rudner *et al*., 1999; Hrometz *et al*., 1999). This includes being expressed on vascular beds such as the renal artery where the α_{1D}-AR has not been shown to have a function. We do not yet understand why members of the α_1-AR family are expressed on tissues in the cardiovascular system and do not participate in regulatory events. However, in keeping with this conundrum, we observed little effect of α_{1D}-AR gene detection on dimensions or contractility as assessed echocardiographically or in the isolated-perfused heart (see also Tanoue *et al.,* 2002).

We hypothesize that the major regulatory activity of the α_{1D}-AR is the regulation of vascular smooth muscle contraction in specific blood vessels (Piascik and Perez, 2001). Evidence supporting this postulate also comes from work with the α_{1D}-AR knockout line of mice (Tanoue *et al.,* 2002). Tanoue *et al.* (2002) showed that knockout of the α_{1D}-AR significantly decreased systemic arterial blood pressure as well as the pressor responses to norepinephrine. In this work we show that knockout of the α_{1D}-AR significantly impaired the ability of phenylephrine to promote increases in coronary perfusion pressure. Therefore, in the same mouse line, where we can demonstrate prominent effects on vascular function, we do not see measurable effects on the examined cardiac parameters. This adds support to our hypothesis that the α_{1D}-AR serves

predominately in vascular function.

Chapter 5

Conclusions

This dissertation work investigated the α_1-ARs at the cellular and at the *in vitro* and the *in vivo* response levels. The two papers comprising this dissertation each has a thorough discussion of the experimental data. This section highlights and integrates key points from each paper into a unified conclusionary statement. Discussion of the data in Chalothorn *et al*. (2002) will encompass the differences observed among the α_1-AR subtypes with respect to basal localization, functional responses, and agonist-mediated internalization, desensitization, and trafficking properties. The discussion of the results in Chalothorn *et al*. (2003) will focus on the roles of specific α_1-AR subtypes in physiological responses such as myocardial regulation and function, vascular smooth muscle contraction, and potential roles in cardiac pathophysiology development.

CHALOTHORN *ET AL*. (2002)

Basal localization of α_1-AR/GFPs

Our use of the α_1-AR/GFPs in living HEK-293 cells revealed distinct differences in the basal localization patterns of the α_1-ARs that were consistent within the subtypes regardless of cell shape or size. We observed a predominant surface orientation for the α_{1B}-AR/GFP and an intracellular distribution for the α_{1D}-AR/GFP (Chalothorn *et al*., 2002). Because of the receptor distribution, the α_{1B}-AR subtype has characteristics of a typical GPCR whereas the α_{1D}-AR has properties of an atypical GPCR. The α_{1A}-AR falls between the two classifications because the α_{1A}-AR/GFP displayed a combination of surface and intracellular receptor distribution. Reasons for the different localization

patterns are not clear; however, the patterns observed for the α_{1A}- and the α_{1B}-AR/GFPs are consistent with studies using antibodies targeted against these receptors in fixed cells (Fonseca *et al*., 1995) or studies using α_{1A}- and α_{1B}-AR/GFP constructs in different cell lines (Hirasawa *et al*., 1997; Awaji *et al*., 1998).

Functional responses of the α_{1A}- and the α_{1B}-AR/GFPs

The GFP conjugation to the carboxyl-terminus (portion of the receptor participating in signal transduction) raises a possibility that the receptor may not fold or function properly. An improperly folded protein may be a reason for differences in cellular distribution. A previous study from the laboratory generating these fusion proteins demonstrated that the α_{1B}-AR/GFP construct expression in mouse α-T3 cells yields a functional receptor capable of activating phosphatidylinositol/Ca^{2+} signaling (Awaji *et al*., 1998). In this work, we demonstrated that the α_{1A}- and α_{1B}-AR/GFP subtypes are coupled functionally to ERK1/2 activation with the α_{1A}-AR/GFP showing a greater efficiency of coupling than the α_{1B}-AR/GFP (Chalothorn *et al*., 2002). In our experimentation, we were unable to detect ERK activation as early as 5 minutes considering that the ERK response has been documented to be rapid yet sustained for at least 3 hours after stimulation for platlet-derived growth factor in Chinese hamster embryo fibroblasts (Weber *et al*., 1997) and lysophosphatidic acid in rat-1 cells (Cook and McCormick, 1996). Thus, we assessed for ERK activation after a 2 hour incubation.

Properties of the α_{1D}-AR/GFP

The small population of α_{1D}-AR/GFP on the cellular surface was found to be

coupled poorly to ERK1/2 activation. Our findings coincide with a study in PC-12 cells that observed greater ERK activation for both the α_{1A}- and the α_{1B}-ARs than the α_{1D}-AR (Zhong and Minneman, 1999). The reason(s) for this atypical behavior of the α_{1D}-AR/GFP is(are) not clear, but our observations agree with the work of Theroux *et al*. (1996) that showed the α_{1D}-AR to be inefficiently coupled to both inositol phosphate formation and intracellular Ca^{2+} release in HEK-293 cells. Another reason for our observations may be related to the predominant intracellular localization of the α_{1D}-AR/GFP. With an intracellular orientation, the ability of the receptor to activate signaling pathways may be limited by access to stimuli, substrates, and/or effectors. However, we noted in HEK-293 cells transfectd with the α_{1D}-AR/GFP that there was both a high basal ERK activity and an inefficient agonist-induced ERK1/2 activation response. The high basal ERK activity is evidence that this receptor/GFP construct is active. These characteristics are similar to those documented for the α_{1D}-AR in the stably transfected fibroblasts. From those studies, we proposed that the receptor was constitutively active with respect to both ERK activation and inositol phosphate formation (McCune *et al*., 2000). Other studies have implicated the α_{1D}-AR as a constitutively active receptor (Noguera *et al*., 1993; García-Sáinz and Torres-Padilla, 1999). A constitutively active receptor would offer a plausible explanation for the observed high basal ERK activity since the majority of the receptor population would be in the R* state, and addition of an agonist is not likely to shift the overall equilibrium from R to R* significantly (see **Figure 2**).

The α_{1D}-AR/GFP did not undergo agonist-mediated internalization (Chalothorn *et al*., 2002). However, we cannot exclude the possibility that some degree of α_{1D}-AR/GFP

internalization may occur and is indistinguishable with the image analysis software used. Constitutive activity would also explain the unique localization, signaling, and internalization properties we observed, since this type of receptor is likely to be continuously cycling between the cellular surface and the intracellular region in endosomes to undergo resensitization.

Following prolonged agonist exposure, the α_{1D}-AR/GFP exhibited a minor degree of agonist-mediated desensitization of ERK1/2 activation (Chalothorn *et al*., 2002). This is further evidence that the α_{1D}-AR/GFP is a functionally active protein. The Lomasney *et al*. (1991) work documented that the α_{1D}-AR lacks a consensus sequence for PKC phosphorylation on the 3rd cytosolic loop, and this sequence may affect the ability of the receptor to be desensitized. Desensitization of the α_{1D}-AR is controversial because a study by Yang *et al*. (1999) revealed that in the stably transfected rat-1 fibroblast, the α_{1D}-AR is not subject to phenylephrine- or phorbol ester-induced functional desensitization of inositol phosphate formation. However, the study by García-Sáinz *et al*. (2001) also using the α_{1D}-AR stably transfected into the rat-1 fibroblast demonstrated that both norepinephrine and phorbol ester could desensitize the functional intracellular Ca^{2+} release. The notion that a constitutively active receptor can be desensitized is supported by a study looking at a constitutively active β_2-AR caused by a point mutation on the 3rd intracellular loop (Pei *et al*., 1994). The investigators demonstrated that in the absence of an agonist, the constitutively active receptor was phosphorylated by β-ARK 1 in a manner similar to the agonist-mediated wild-type receptor phosphorylation. Therefore, desensitization of the constitutively activity of the α_{1D}-AR/GFP may further explain the poor response to agonist incubation.

Agonist-mediated internalization of the α_{1A}- and the α_{1B}-AR/GFP

Differences in the agonist-mediated internalization responses were noted between the α_{1A}- and the α_{1B}-AR/GFP subtypes. The α_{1B}-AR/GFP was observed to rapidly internalize after agonist stimulation (Chalothorn *et al*., 2002). Our finding is in good agreement with works using antibodies and radioligands to detect the α_{1B}-AR agonist-mediated redistribution (Fonseca *et al*., 1995; Wang *et al*., 1997, 2000). The internalization of the α_{1A}-AR has not been studied. We showed that the α_{1A}-AR/GFP internalizes slower and less extensively than the α_{1B}-AR/GFP (Chalothorn *et al*., 2002). Evidence supporting our observation comes from a study by Vázquez-Prado *et al*. (2000) that found more extensive degrees of carboxyl-terminus phosphorylation to PKC activation (by norepinephrine or phorbol esters) for the α_{1B}- than the α_{1A}-AR. This greater degree of α_{1B}-AR receptor phosphorylation is consistent with our finding that the α_{1B}-AR/GFP internalizes quicker since internalization proteins are more likely to bind to phosphorylated receptors.

Agonist-mediated desensitization of the α_{1A}- and the α_{1B}-AR/GFP

Investigation of the agonist-mediated desensitization of ERK1/2 activation revealed differences between the α_{1A}- and the α_{1B}-AR/GFP subtypes. Both the α_{1A}- and the α_{1B}-AR/GFPs demonstrated characteristics typical of desensitization, in which the α_{1B}-AR/GFP was more readily desensitized (Chalothorn *et al*., 2002). Our observations agree with a study documenting that both the α_{1A}- and the α_{1B}-ARs undergo functional desensitization of agonist-stimulated inositol phosphate formation in rat-1 fibroblasts

(Yang *et al*., 1999). Additionally, our observation that the α_{1B}-AR/GFP is desensitized more readily than the α_{1A}-AR/GFP is in good agreement with the Vázquez-Prado *et al*. (2000) study that found a greater degree of PKC phosphorylation for the α_{1B}-AR than the α_{1A}-AR.

β-arrestin 1 participation in α_1-AR/GFP internalization

The GPCR-mediated internalization and desensitization processes require arrestin/clathrin participation. Since little is known about the role that arrestins play in α_1-AR-mediated internalization, we performed studies to determine if these proteins are important for internalization. In experiments using the α_{1A}- and the α_{1B}-AR/GFPs co-transfected with either β-arrestin 1 or 2, we found no differences in the ability of either β-arrestins to influence the α_{1A}- or the α_{1B}-AR/GFP basal localization and internalization properties (Chalothorn *et al*., 2002). This suggested that either β-arrestins do not participate in α_1-AR internalization or there are already adequate levels of endogenous β-arrestins in HEK-293 cells. Since over-expression of the β-arrestins did not conclusively link the β-arrestins to α_1-AR/GFP internalization, additional experiments examined the relationship between arrestin/clathrin interaction and α_1-AR/GFP internalization. To this end, we used a dominant-negative β-arrestin 1 [β-arrestin 1 (319-418)]. Studies using this dominant negative protein revealed an effective disruption of native arrestin and clathrin interactions in HEK-293 cells, which prevented β_2-AR internalization (Krupnick *et al*., 1997; Orsini and Benovic, 1998). Expression of β-arrestin 1 (319-418) did not affect the basal localization patterns for any of the α_1-AR/GFPs, but it did antagonize the α_{1A}- and the α_{1B}-AR/GFPs agonist-mediated internalization responses (Chalothorn *et al*.,

2002). The β-arrestin 1 (319-418) did not produce a detectable change in the α_{1D}-AR/GFP localization or agonist-mediated internalization response. Our findings suggest that arrestin/clathrin interaction is irrelevant for maintaining basal localization patterns for the α_{1D}-AR/GFPs but relevant for α_{1A}- and α_{1B}-AR/GFP agonist-mediated internalization. Although the use of β-arrestin 1 (319-418) confirms the role of arrestin/clathrin interaction in α_{1A}- and α_{1B}-AR/GFP internalization, the specific role that either β-arrestin 1 or 2 plays in α_1-AR internalization is difficult to assess because β-arrestin 1 (319-418) protein binds to clathrin to prevent interactions with all wild-type arrestin subtypes.

CHALOTHORN *ET AL*. (2003)

Work from Chalothorn *et al*. (2002) shows differences in the α_1-AR subtype localization, activation, internalization, desensitization, and trafficking properties. Because of these differences, we propose that the α_1-AR subtypes subserve different biological responses by coupling to distinct regulatory processes. We believe that the α_{1B}-AR is involved in the modulation of cardiac function whereas the α_{1D}-AR is involved in the regulation of vascular contractile function.

Strengths and weaknesses of transgenic models

In order to test our hypothesis, we used transgenic animals to investigate the regulatory properties of the α_1-AR subtypes at the physiologic level. We used transgenic animals expressing a constitutively active form of the α_{1B}-AR (α_{1B}-AR_{C128F}) under the control of the isogenic promoter (Zuscik *et al*., 2000, 2001) to determine the activity of

the receptor. Use of this model offers two distinct advantages: activation of the α_{1B}-AR without use of agonists that would activate all α_1-ARs and investigation of the α_{1B}-AR function(s) in tissues endogenously expressing this subtype. These types of animals have limitations, and it is possible that the observed biochemical or physiologic alterations observed are not a direct result of transgenic receptor expression or deletion. These effects observed could be non-specific and a result of gene alteration unrelated to α_1-AR expression.

MAPK activity in α_{1B}-AR_{C128F} hearts

Work reported in Chalothorn *et al*. (2003) demonstrated that these transgenic mice of 5 to 6 months old have increased basal activity of two mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK) and *c-jun* N-terminal kinase (JNK) (Chalothorn *et al*., 2003). This enhanced signaling supports the constitutively active nature of the α_{1B}-AR_{C128F}, which has been demonstrated previously for inositol phosphate formation in the mouse kidney (Zuscik *et al*., 2001). Our study suggests that this receptor subtype couples to MAPK activation *in vivo.* This finding agrees with *in vitro* studies investigating the α_{1B}-AR mediated-ERK activation in heterologous expression systems (Zhong and Minneman, 1999; McCune *et al*., 2000; Waldrop *et al*., 2002; Chalothorn *et al*., 2002). However, we have made the assumption that the differences observed in our transgenic model are the result of α_{1B}-AR_{C128F} activity. It is possible that the increased MAPK activity is the result of a pathophysiology associated with stress evoked from a dilated chamber phenotype (see below), since many studies have revealed several different stimuli involved in MAPK activation (as discussed

in the background).

In vivo assessment of cardiac dimensions in α_{1B}-AR_{C128F} hearts

The echocardiographic study revealed distinct differences of the left ventricular internal dimensions in animals expressing the α_{1B}-AR_{C128F} relative to non-transgenic controls. The transgenic animal displayed increased left ventricular chamber size without any overt signs of hypertrophy, a symptom associated with dilated cardiomyopathy (Chalothorn *et al*., 2003). The dilated cardiomyopathy phenotype is a condition characterized by a dilation of the ventricular chamber and accompanied with reduced cardiac function (see below). This phenotype has been documented in distinctly different models using a cardiac-targeted over-expression of the wild-type α_{1B}-AR (Akhter *et al*., 1997; Grupp *et al*., 1998; Lemire *et al*., 2001). In contrast to these results, the Zuscik *et al*. (2001) study using systemic over-expression of either a wild-type or a constitutively active α_{1B}-AR provided evidence of cardiac hypertrophy. Similarly, the Milano *et al*. (1994) study using cardiac-targeted over-expression of a constitutively active α_{1B}-AR found evidence of cardiac hypertrophy, yet the Harrison *et al*. (1998) and the Wang *et al*. (2000) studies using the same line of mice were unable to observe signs of cardiac hypertrophy. It is not clear why there is a discrepancy in the cardiac phenotypes between studies using the same animal models. However, it is clear that tonic unregulated activation of α_{1B}-AR leads to a cardiac pathophysiology such as cardiac hypertrophy or dilated cardiomyopathy.

In vivo and *in vitro* assessment of cardiac function in α_{1B}-AR_{C128F} hearts

In the control and the α_{1B}-AR_{C128F} over-expressing groups, left ventricular functions/performances were assessed by echocardiography. The cardiac performance parameters (dependent on pre-load, contractility, after-load, and heart rate) measured were stroke volume, cardiac output, mean velocity of circumferential fiber shortening, and percent fractional shortening. Of these measured cardiac parameters, only the percent fractional shortening values were found to be significantly different between the transgenic and the control groups. The reduced percent fractional shortening is consistent with reduced cardiac performance associated with the dilated cardiomyopathy phenotype (Chalothorn *et al*., 2003). The blood pressure may affect the percent fractional shortening because this index of cardiac function is dependent on ventricular loading; however, we previously documented that these transgenic animals are hypotensive (Zuscik *et al*., 2001). Since cardiac output is approximately maintained, there is likely to be a reduction in the total peripheral resistance in animals over-expressing the α_{1B}-AR_{C128F}, which should contribute to an increase in percent fractional shortening, but the measured percent fractional shortening values were significantly less for animals over-expressing the α_{1B}-AR_{C128F} than control animals. To account for the decreased percent fractional shortening without significant changes to stroke volume but increased end-diastolic and end-systolic volumes, the left ventricular contractility is likely to be reduced in α_{1B}-AR_{C128F} over-expressing hearts. Additionally, we did observe decreased contractility (altered end-systolic pressure-volume relationship) for β_1-AR-mediated inotropic responses in isolated-perfused heart experiments in α_{1B}-AR_{C128F} over-expressing hearts than control hearts. The log-dose response curves for maximal

increases in left ventricular developed pressure as a function of β_1-AR stimulation were 171.0 ± 8.9 and 142.3 ± 3.0 mm Hg for control and α_{1B}-AR_{C128F} over-expressing hearts, respectively. In addition, the log EC_{50} calculated were -7.87 ± 0.20 and -8.09 ± 0.15 for control and α_{1B}-AR_{C128F} over-expressing hearts, respectively. These data show no significant change in the log EC_{50} value, but a significantly reduced contractile function as noted by reduced left ventricular developed pressure and positive rise in pressure as a function of time (+dP/dt). Our results and implications are in agreement with studies that found poor cardiac function in hearts over-expressing the wild-type α_{1B}-AR (Akhter *et al*., 1997; Grupp *et al*., 1998; Lemire *et al*., 2001). We found no differences in isoproterenol-mediated chronotrophic responses although it was a reduced for both groups when compared to echocardiographic analysis, which could be explained by the absence of autonomic influences (reflexes and catecholamines).

Agonist-induced cAMP generation in α_{1B}-AR_{C128F} ventricular slices

Since the β_1-AR inotropic response was impaired in hearts over-expressing the α_{1B}-AR_{C128F}, we examined the β_1-AR-mediated cAMP response in ventricular homogenates. At baseline, no differences were found in the cAMP levels between the control and the hearts with the α_{1B}-AR_{C128F}, indicating that the phosphodiesterase activity was not enhanced in ventricle slices as a result of α_{1B}-AR_{C128F} over-expression. We found attenuated cAMP generation to isoproterenol treatment of hearts with the over-expression of the α_{1B}-AR_{C128F}, which suggests that the tonic unregulated activation of the α_{1B}-AR impairs the β_1-AR signaling (Chalothorn *et al*., 2003). Because Perez *et al*. (1997) showed that the α_{1B}-AR_{C128F} is only able to couple to the PLC-β pathway, the

likely mechanism for the reduced isoproterenol-mediated cAMP generation is heterologous desensitization of the β_1-AR population by PKC phosphorylation. In support of this, Akhter *et al*. (1997) and Lemire *et al*. (2001) indicated that the enhanced α_{1B}-AR activity increases the PKC phosphorylation of the GRKs leading to heterologous desensitization of the β_1-AR without reducing the β_1-AR population. In addition, Akhter *et al*. (1997) observed lower cAMP levels in isoproterenol-treated transgenic heart homogenates, which was attributed to α_{1B}-AR coupling with $G_{i/o}$ protein to antagonize the β_1-AR-G_s pathway.

Inotropic response and α_{1B}-AR activation

Collaborative work with the Perez laboratory has shown that over-expression of the α_{1B}-AR_{C128F} reduces the mRNA levels of the α_{1A}-AR in the heart (Ross *et al*., in revision). This work agrees with data from the Harrison *et al*. (1998) study demonstrating cardiac-targeted over-expression of the constitutively active α_{1B}-AR results in α_{1A}-AR down-regulation. The mechanism the α_{1B}-AR uses to influence α_{1A}-AR mRNA levels is under investigation. Our work and that of others suggest that the increased activity of the α_{1B}-AR negatively modulates α_1-AR inotropic signaling in the mouse heart. The down-regulation of the α_{1A}-AR attenuates the α_1-AR positive inotropic response, which suggests that the α_{1A}-AR is coupled to positive inotropy (Ross *et al*., in revision). Our data are consistent with studies showing that the α_{1A}-AR is the mediator of positive inotropy in the mouse (Lin *et al*., 2001) and the rat (Rokosh and Sulakhe, 1991; Gambassi *et al*., 1991) hearts. In contrast to these studies, other groups found the α_{1B}-AR to be the mediator of positive inotropy in the rat heart (Michel *et al*., 1990, 1994; Yu and

Han, 1994). From our studies using α_{1B}-AR_{C128F} over-expressing mice, it is reasonable to believe that α_{1B}-AR is a cardiac specific receptor that has the potential to negatively modulate the positive inotropic effects of the α_{1A}- and the β_1-ARs.

Contractility assessment in α_{1B}-AR_{C128F} thoracic aortae

Since our laboratory hypothesizes that the α_{1B}-AR is a specific modulator of cardiac function with minimal activity in regulating vascular smooth muscle contraction, we tested the effect of over-expressing the α_{1B}-AR_{C128F} in the mouse thoracic aorta. In aortic contractility experiments, we found no notable differences in the phenylephrine-mediated contractile responses in the transgenic thoracic aorta (Chalothorn *et al*., 2003). In the same transgenic animals showing demonstratable effects of α_{1B}-AR_{C128F} over-expression on myocardial regulation and function, we were unable to note a difference in the ability of the aorta to contract to phenylephrine. We cannot exclude the possibility that the there maybe some additional growth in the aortic vessel caused by the constitutive activation of the α_{1B}-AR_{C128F} transgene; however, we did not detect a significant differences in the maximal contraction between the control aortae and the aortae over-expressing the α_{1B}-AR_{C128F} (0.441 ± 0.021g vs. 0.405 ± 0.035g, respectively). This agrees with our earlier finding that the over-expression of the α_{1B}-AR_{C128F} does not increase the basal mean arterial pressure (Zuscik *et al*., 2001). The knockout of the α_{1B}-AR in mice also has no effect on the resting mean arterial pressure (Cavalli *et al*., 1997). Other evidence supporting our observations comes from a different study also using mice lacking the α_{1B}-AR, and this study demonstrated that the α_{1B}-AR plays a minor role in vasoconstriction of the thoracic aorta (Daly *et al*., 2002). However, the Cavalli *et al*.

(1997) study suggested that the α_{1B}-AR plays a modest role in aortic contractility. The reason(s) for the different observations and conclusions between the two studies using the same type of mice is(are) not clear.

Assessment of cardiac function in α_{1D}-AR KO hearts

To further test our hypothesis of specificity in the regulatory activities of the α_1-ARs, we used 6 to 7 months old mice lacking the expression of the α_{1D}-AR (α_{1D}-AR KO). Echocardiographic data from these α_{1D}-AR KO animals did not indicate anomalies in cardiac dimensions or functions (Chalothorn *et al*., 2003). The finding that α_{1D}-AR deficiency does not have detrimental effects on the heart is in agreement with Tanoue *et al*. (2002) and further strengthens our hypothesis that the α_{1D}-AR has little or no effect on cardiac dimensions and/or function. In the isolated-perfused heart experiments, we found no evidence that the α_{1D}-AR plays a role in regulating cardiac function since no differences were noted in the ability of the α_{1D}-AR KO hearts to respond to isoproterenol when compared to the non-transgenic control responses (Chalothorn *et al*., 2003).

Role of α_{1B}- and α_{1D}-AR in vascular smooth muscle contraction

In contrast to the minor effect on cardiac function, the α_{1D}-AR plays a prominent role in regulating vascular smooth muscle contraction. Studies with the α_{1B}-AR_{C128F} aortae and the α_{1D}-AR antagonist, BMY 7378, indicate that the primary mediator of the α_1-AR-induced contraction is the α_{1D}-AR (Chalothorn *et al*., 2003). This finding further supports the idea that α_1-AR subtypes subserve different regulatory functions. In agreement with our data, the studies of Yamamoto and Koike (2001) and Tanoue *et al*.

(2002) demonstrated that the α_{1D}-AR is the subtype responsible for contraction of the mouse thoracic aorta. Many other studies support the conclusion that the α_{1D}-AR is a regulatory receptor involved in vascular smooth muscle contraction (see background).

Additional evidence that the α_{1D}-AR plays a role in vascular smooth muscle contraction comes from the isolated-perfused heart experiments examining the phenylephrine-induced effect on coronary vasoconstriction. We noted that the α_{1D}-AR KO hearts had higher coronary flow rates, which suggested a decreased coronary vascular tone (Chalothorn *et al*., 2003). The vasoconstrictive response to phenylephrine was found to be blunted in hearts lacking the α_{1D}-AR, which indicated that the α_{1D}-AR has a role in mediating coronary vasoconstriction. These results also implicate the relevance of this receptor in mediating coronary flow to cardiac tissue. Our data are in good agreement with the Tanoue *et al*. (2002) study demonstrating that the lack of this receptor subtype results in the decreased ability of the thoracic aortic rings to contract in response to α_1-AR agonists. Our study and the Tanoue *et al*. (2002) study demonstrate that the α_{1D}-AR is a relevant subtype responsible for mediating vascular smooth muscle contraction in arteries.

Concluding remarks

Work from this dissertation has shown that differences exist within the α_1-AR subtypes for cellular and physiological responses. Using the α_1-AR/GFP constructs, we demonstrate that the α_{1B}-AR subtype most approximates the prototypic GPCR with respect to cellular localization, agonist-mediated activation, internalization, desensitization, and trafficking properties. Although the α_{1A}-AR subtype displays both

surface and intracellular localization, this subtype possesses properties expected of a GPCR. However, the α_{1D}-AR subtype seems to be an atypical GPCR. The work done in transgenic animals reveals that the α_{1B}-AR is involved in the regulation of growth response, cardiac regulation and function, and possibly in pathophysiology development. In addition, we provide evidence that the α_{1D}-AR subtype is not involved in maintaining cardiac regulation or function, yet the α_{1D}-AR is more involved in the regulation of vascular smooth muscle contraction.

Future Directions

Studies from this dissertation work could potentially be a forerunner for future projects further characterizing the α_1-AR subtypes. At the cellular level, we noted that there are indeed differences in the α_1-AR subtype distribution, signaling, and trafficking properties. Although the reasons why each subtype adopts these differences are not clear, the amino acid sequence of each subtype has been documented; therefore, it is possible to exchange key areas of the intracellular loops and/or the segments of the carboxyl-terminus to generate chimeric receptors. The construction of these chimeric receptors with a GFP tag on the carboxyl-terminus would extend the investigation into which region(s) is(are) responsible for receptor subtype distribution. In addition, the characteristics of agonist-mediated internalization, signaling, and regulation may be uncovered as well. From these studies, site-directed mutagenesis on identified regions could be used to verify the relevance of specific amino acid sequences involved in these processes.

This study observed a very atypical behavior of the α_{1D}-AR/GFP, and the region(s) of the receptor responsible for these observations may be identified through chimeric and mutagenesis studies; however, it would be interesting to verify that the observed properties are due to a constitutively active nature of this subtype which has been proposed by a few studies. Use of the chimeric receptor may determine if the proposed constitutively active nature of the α_{1D}-AR is the result of structural relief in the transmembrane domains and/or a specific configuration of the intracellular loop and/or carboxyl-terminus. Additionally, use of the inverse agonists in real-time microscopy on the α_{1D}-AR/GFP and the chimeric versions may provide support to the notion that the α_{1D}-AR is a constitutively active subtype that undergoes constant receptor cycling.

At the tissue level, it would be interesting to determine if the many properties observed among the α_1-AR subtypes in a recombinant system mirror the receptors in the endogenous tissue. So additional experiments with a GFP-tagged α_1-AR subtype could be used to transfect (via adenovirus) a tissue that has a specific subtype knocked-out. These studies could test the receptor distribution, signaling efficiency, regulation, etc. Other lines of investigations that may ensue from this study are related to functional restoration of responses lost or attenuated by knocking out a specific α_1-AR/GFP subtype. These experiments are potentially useful for verifying which roles certain receptor subtype play in responses.

Because many studies indicate that there is an interplay among gene products when a gene gets over-expressed or knocked-out, it would be interesting to determine what changes in gene regulation occur in both of the transgenic models used in this dissertation. Using gene chip microarray, it may be possible to correlate a phenotype

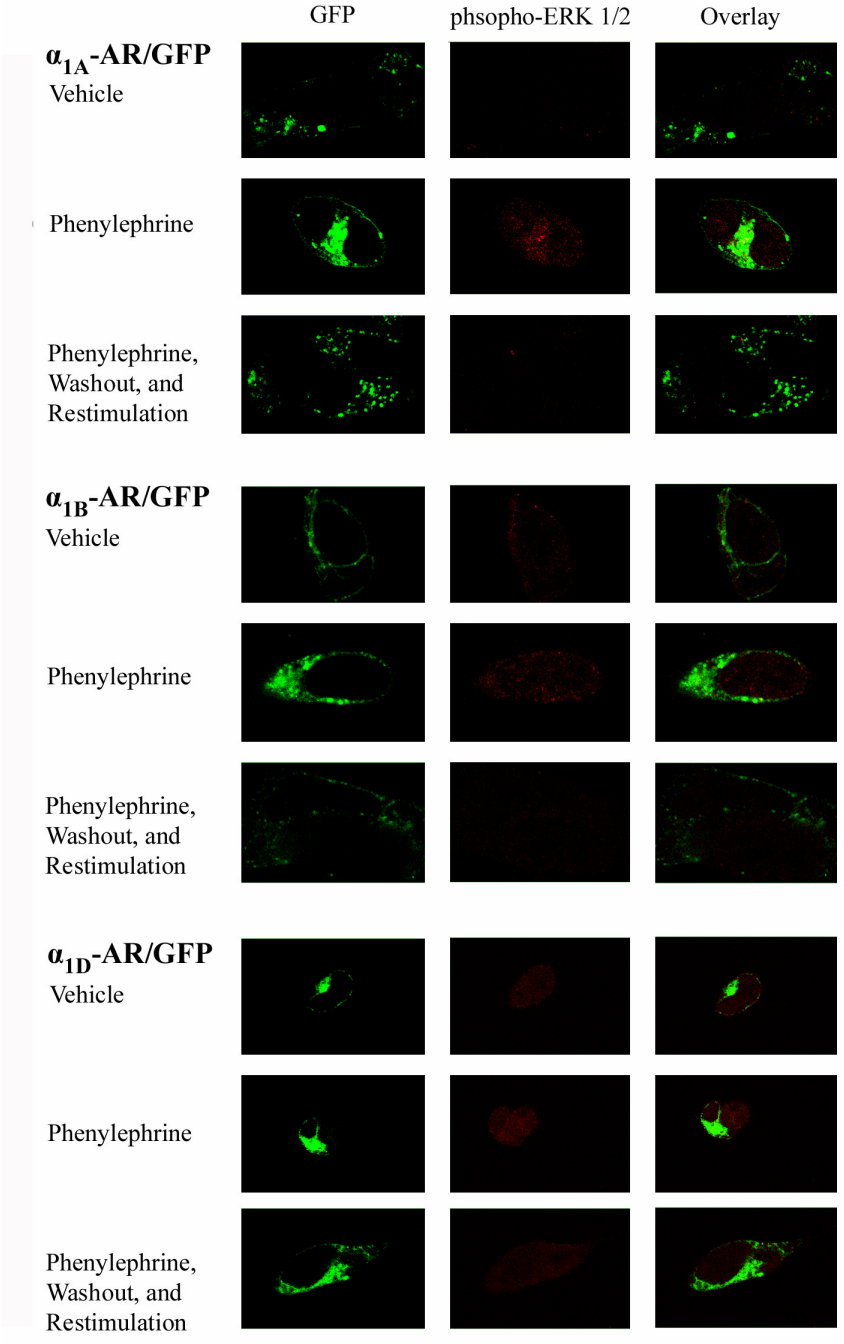
observed with an alteration in gene regulation. Other pursuits with genetic manipulation may be the utilization of the cre-lox system to determine the physiological effect(s) of over-expressing any one of the α_1-ARs at a certain time after the animal has reached adulthood. These studies may offer a better method of assessing the function of the α_1-ARs *in vivo*.

In addressing the reason why there is a decreased β_1-AR positive inotropic response in the α_{1B}-AR_{C128F} expressing mice, binding studies investigating the β_1-AR density in the heart may be of use. In addition, determining what effects the constitutive activation of the α_{1B}-AR has on β_1-AR mRNA stability and levels may increase our understanding of interplay between these two receptors. Also, in addressing the inotropic response issue, it would be worthwhile to use an inverse agonist such as prazosin to determine if both the constitutively active receptor can be suppressed and the inotropic response can be restored.

Although the α_{1D}-AR over-expressing transgenic model is not available yet, it would be interesting to perform the same series of experiments on this model to confirm that the α_{1D}-AR predominantly plays a role in vascular smooth muscle contraction.

Appendix A

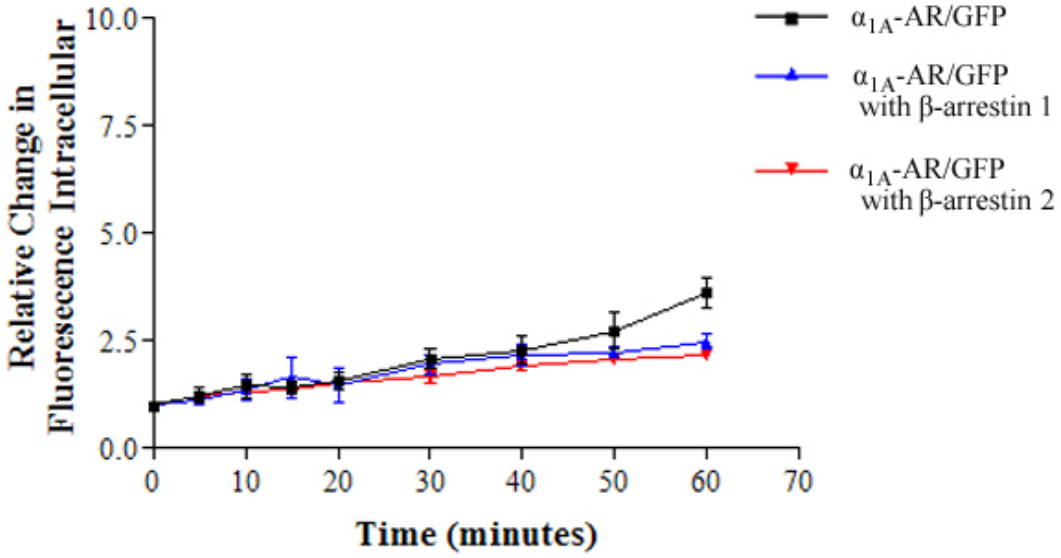
Representative images from at least 3 to 7 independent transfection experiments showing the effect of vehicle or agonist treatment on GFP localization and activation of ERK 1/2 in HEK-293 cells transiently transfected with an α_1-AR/GFP subtype (data generated by Mary L. García-Cazarín). See *Methods* for the description of α_1-AR/GFP transfection, immunocytochemistry of phospho-ERK, and laser-scanning confocal microscopy procedures. Data reported in Chalothorn *et al*. (2002).



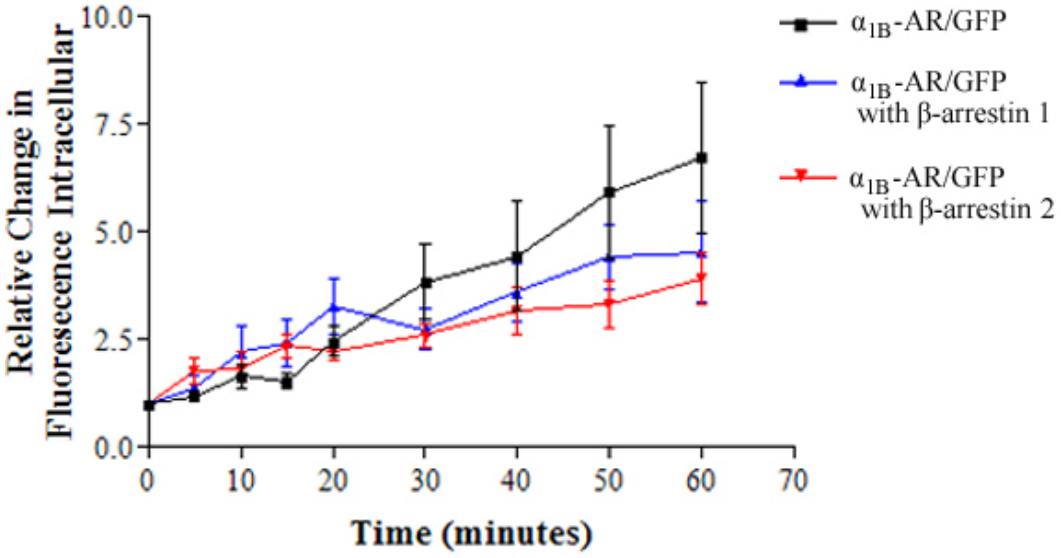
Appendix B

Comparison of the effect of 100 μM phenylephrine on changes in intracellular fluorescence intensity in cells transfected with **a**) α_{1A}- or **b**) α_{1B}-AR/GFP in the absence (retracing of **Figure 9**) and the presence of either β-arrestin 1 or 2. See *Methods* for the description on measuring and analyzing the signal intensities for receptor internalization. Points on the graph represent the mean and standard error of the mean values for at least 3 independent experiments. Data analysis used the two-way analysis of variance followed by the Student-Newman-Kuels post-test.

a)



b)



THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 17, Issue of April 27, pp. 13738–13743, 2001
Printed in U.S.A.

Hypotension, Autonomic Failure, and Cardiac Hypertrophy in Transgenic Mice Overexpressing the α_{1B}-Adrenergic Receptor*

Received for publication, September 22, 2000, and in revised form, January 30, 2001
Published, JBC Papers in Press, February 1, 2001, DOI 10.1074/jbc.M008693200

Michael J. Zuscik‡, Dan Chalothorn§, David Hellard‡, Clare Deighan¶‖, Ann McGee¶, Craig J. Daly¶, David J. J. Waugh‡, Sean A. Ross‡, Robert J. Gaivin‡, Annitta J. Morehead, James D. Thomas**, Edward F. Plow‡, John C. McGrath¶, Michael T. Piascik§, and Dianne M. Perez‡****

*From the ‡Department of Molecular Cardiology, The Lerner Research Institute, the **Department of Cardiology, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, the ¶Autonomic Physiology Unit, Division of Neuroscience and Biomedical Systems, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland G12 8QQ, United Kingdom, and the §Department of Pharmacology and the Vascular Biology Research Group, The University of Kentucky College of Medicine, Lexington, Kentucky 40536*

α_1-Adrenergic receptors (α_{1A}, α_{1B}, and α_{1D}) are regulators of systemic arterial blood pressure and blood flow. Whereas vasoconstrictory action of the α_{1A} and α_{1D} subtypes is thought to be mainly responsible for this activity, the role of the α_{1B}-adrenergic receptor (α_{1B}AR) in this process is controversial. We have generated transgenic mice that overexpress either wild type or constitutively active α_{1B}ARs. Transgenic expression was under the control of the isogenic promoter, thus assuring appropriate developmental and tissue-specific expression. Cardiovascular phenotypes displayed by transgenic mice included myocardial hypertrophy and hypotension. Indicative of cardiac hypertrophy, transgenic mice displayed an increased heart to body weight ratio, which was confirmed by the echocardiographic finding of an increased thickness of the interventricular septum and posterior wall. Functional deficits included an increased isovolumetric relaxation time, a decreased heart rate, and cardiac output. Transgenic mice were hypotensive and exhibited a decreased pressor response. Vasoconstrictory regulation by α_{1B}AR was absent as shown by the lack of phenylephrine-induced contractile differences between *ex vivo* mesenteric artery preparations. Plasma epinephrine, norepinephrine, and cortisol levels were also reduced in transgenic mice, suggesting a loss of sympathetic nerve activity. Reduced catecholamine levels together with basal hypotension, bradycardia, reproductive problems, and weight loss suggest autonomic failure, a phenotype that is consistent with the multiple system atrophy-like neurodegeneration that has been reported previously in these mice. These results also suggest that this receptor subtype is not involved in the classic vasoconstrictory action of α_1ARs that is important in systemic regulation of blood pressure.

The adrenergic receptor family, which includes 3 α_1, 3 α_2, and 3 β-receptor subtypes, is a group of heptahelical G protein-coupled receptors that mediate the effects of the sympathetic nervous system. Extensive effort has been spent in classifying the three known α_1-adrenergic receptor (α_1AR)[1] subtypes (α_{1A}, α_{1B}, and α_{1D}) via molecular cloning techniques (1–4) and pharmacological analyses (5). The most well characterized cardiovascular regulatory actions associated with α_1AR activation include the contraction, growth and proliferation of vascular smooth muscle cells (6–9), increased cardiac contractility (10), and regulation of the hypertrophic program in the myocardium (11, 12). In other α_1AR-expressing tissues such as liver and kidney, the function of these receptors is to regulate metabolic processes (13) and sodium and water reabsorption (14), respectively. These responses are transduced primarily via receptor coupling to the G_q/phospholipase C pathway (5), which leads to the subsequent activation of downstream signaling molecules including protein kinase C and inositol 1,4,5-trisphosphate.

The progress toward elucidating the distinct regulatory role of each α_1 subtype in the various physiologic responses mentioned above has been constrained by a limited number of subtype-selective agonists and antagonists. This is especially true in the α_{1B} system where there are no selective agonists or antagonists available. We have alleviated this constraint by examining the unique attributes of the α_{1B}AR in a transgenic mouse model that exhibits constitutive α_{1B}AR activity targeted only to tissues that normally express the receptor. The appropriate distribution of receptor overactivity was achieved by using the mouse isogenic α_{1B}AR promoter (15) to drive the overexpression of a transgene containing cDNAs of either the wild type (W) hamster α_{1B}AR (3) or the constitutively active mutant forms of the receptor. Two such mutants were employed, a C128F single mutant (S) and a C128F/A204V/A293E triple mutant (T), both of which spontaneously couple to G_q (16, 17). The systemic expression of constitutively active α_{1B}ARs in these transgenic mice has already led to the identification of a pathology similar to multiple system atrophy suggesting that overstimulation of these receptors leads to neurodegeneration (18). In the present study, we extend this examination of phenotype to the cardiovascular system. Discrete overexpression of

* This work was funded by National Institutes of Health Grants RO1HL61438 (to D.M.P.), RO1HL31820 (to M.T.P.), and F32HL10004 (to M.J.Z.), EC FP5 Program Grant QLG1-1999-00084 and Medical Research Council Innovation Award G0000042 (to J.C.M.), and an American Heart Established Investigator Award and an unrestricted research grant from Glaxo Wellcome (to D.M.P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Recipient of British Heart Foundation Postgraduate Scholarship FS/97053.

** To whom correspondence should be addressed: Dept. of Molecular Cardiology NB50, The Lerner Research Inst., The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-2058; Fax: 216-444-9263; E-mail: perezd@ccf.org.

[1] The abbreviations used are: α_1AR, α_1-adrenergic receptor; α_{1B}AR, α_{1B}-adrenergic receptor; W, wild type α_{1B}AR; S, C128F single mutant α_{1B}AR; T, C128F/A204V/A293E triple mutant α_{1B}AR; NT, non-transgenic; NK, non-knockout; KO, knockout; ANF, atrial natriuretic factor; MAP, mean arterial pressure; gBw, gram body weight.

constitutive α_{1B}AR activity in the cardiovascular system makes these mice well suited to address questions regarding α_{1B}AR regulation of cardiovascular homeostasis. Our findings not only confirm the involvement of the α_{1B}AR in cardiac hypertrophy but suggest that this subtype is not involved with blood pressure-related vasoconstriction. Rather, the hypotension seems to be a manifestation of autonomic failure and not the result of a direct action of the α_{1B} subtype in the peripheral vasculature. Understanding the α_{1B}AR control over these processes and the manifestation of disease will further define the therapeutic potential that would come from the development of α_{1B}AR-selective antagonists and will have an impact on the future development of novel gene therapies.

EXPERIMENTAL PROCEDURES

Mice—The generation and genotyping of transgenic mice possessing systemic α_{1B}AR overactivity has been described elsewhere (18). Tissue-specific distribution of systemic α_{1B}AR overactivity was achieved by using the mouse α_{1B}AR gene promoter (15) to drive the overexpression of a transgene containing a cDNA coding for the wild type (W) α_{1B}AR (3) or the constitutively active single mutant (S) C128F α_{1B}AR (16) or triple mutant (T) C128F/A204V/A293E α_{1B}AR (17). The Cleveland Clinic Foundation Transgenic Core Facility injected ~200 copies of each transgene into the pronuclei of one cell B_6/CBA mouse embryos, which were surgically implanted into pseudo-pregnant female mice. 3 W, 5 single mutant, and 3 triple mutant founder mice were identified, and subsequent generations were genotyped by Southern analysis of genomic DNA extracted from tail biopsies. All phenotypic studies detailed below are carried out using equal proportions of male and female mice.

Echocardiography—Echocardiographic measurements were performed on mice according to a previously published transthoracic echocardiographic method (19). The mice were anesthetized via intraperitoneal injection of 0.05 mg/g ketamine HCl and 0.1 mg/g thiobutabarbital. The chest area was shaved and ultrasonic gel was applied. Measurements were made as previously described using the Acuson Sequoia 512 system (Mountain View, CA) that employed a dynamically focused symmetrical annular array transducer (13 MHz) for two-dimensional and M-mode imaging. The parasternal long and short axes and apical four chamber views were visualized. Five consecutive cycles of each parameter were measured. Cardiac output was calculated from echocardiographic data using the following equation, $[\pi \times (PA)^2 \times VTI \times HR]/4$, where PA was the diameter of the pulmonary artery, VTI was the doppler velocity time integral in the pulmonary artery, and HR was the echocardiographically determined heart rate.

Mean Carotid Artery Pressure in the Conscious Mouse—The measurement of the mean carotid artery blood pressure in conscious mice was performed as described previously (20). The mice were anesthetized via intraperitoneal injection of 0.1 mg/g ketamine and 2 μg/g acepromazine maleate. A carotid catheter was inserted and connected to a low compliance COBE CDXIII pressure transducer (Cobe Cardiovascular, Arvada, CO). Blood pressure readings were collected on a Model 7D Polygraph (Grass Instrument Division, West Warwick, RI). The recording began immediately after surgery and continued for a 7-h period.

Mean Femoral Artery Pressure in the Anesthetized Mouse—The mean femoral artery pressure was determined in mice using a modified version of a previously described method (21). The mice were anesthetized with an initial intraperitoneal injection of ketamine (0.05 mg/g) followed 5 min later by an injection of thiobutabarbital (0.1 mg/g). Supplemental doses of thiobutabarbital were delivered only when necessary to maintain stable anesthesia. A femoral catheter was inserted and connected to a low compliance COBE CDXIII pressure transducer (Cobe Cardiovascular) interfaced with an AH 60-9315 universal oscillograph (Harvard Apparatus, Holliston, MA). The right femoral vein was cannulated similarly for intravenous administration of increasing amounts of phenylephrine at a delivery rate of 0.1 μl/g/min using a microinfusion pump.

Ex Vivo Arterial Contractile Studies—Four strains of mice were used for this experiment. The α_{1B} knockout mouse (KO, C57-black) and its non-knockout control (NK mice) were bred in Glasgow from breeding pairs supplied by Professor S. Cotecchia (University of Lausanne, Lausanne, Switzerland) (for review see Ref. 8). Tissues were also taken from the W2 α_{1B}AR overexpressor in this study and its appropriate non-transgenic control (NT mice). Mice weighing 25–35 g (KO and their age-matched controls) or 35–55 g (overexpressed and their age-matched controls) were killed by an overdose of CO_2. The mesentery was removed, and the branches of first order mesenteric artery were dissected and cleared of connective tissue before mounting on a wire myograph for isometric force recording. The arteries were bathed in Krebs solution, and the temperature was maintained at 37 °C at a pH of 7.4 with a gas mixture of 95% O_2, 5% CO_2 throughout the experiment. Preliminary studies employed the normalization technique of Mulvany and Halpern (22) in 1977 to obtain vessel internal diameter and normalized resting tension. Thereafter, the vessel segments were set at 0.17 mg of resting tension before construction of concentration response curves. After a "priming" protocol involving challenges of norepinephrine (10 μM) and/or KCl (50 mM), tissues were washed and allowed 30 min before beginning construction of a cumulative concentration response curve to phenylephrine (1–10 μM). The potency of the agonist was determined by comparing EC_{50} values (concentration required to produce 50% of maximum response) obtained in each tissue.

Catecholamine and Cortisol Determination—Mice were anesthetized via intraperitoneal injection of thiobutabarbital (0.125 mg/g). An abdominal incision was made, and blood samples were obtained via venipuncture of the vena cava either 5 min after application of the anesthetic or after 1 h of stable anesthesia. Total plasma epinephrine and norepinephrine levels were determined in 100 μl of plasma samples using the commercially available plasma catecholamines by high pressure liquid chromatography kit (Bio-Rad). Plasma cortisol levels were determined in parallel in 100 μl of plasma samples using the commercially available fluorescence polarization immunoassay kit (Abbott).

Statistical Analyses—All reported errors and error bars represent S.E., and significance was determined using either an unpaired two-tailed Student's t test ($p < 0.05$) or a one-way analysis of variance (see Figure and Table Legends).

RESULTS AND DISCUSSION

General Characterization of Mice Possessing α_{1B}AR Overactivity—We have previously described the genotypic and initial phenotypic analysis of systemic α_{1B}AR mice (18), confirming transgene integration. The tissue-specific overexpression of wild type and mutant α_{1B}ARs was confirmed via saturation binding analysis of various tissues from F1 and F2 generation of heterozygous mice. Of the seven transmitting founder lines, five exhibited significant transgene overexpression including two W lines (W1 and W2), one single mutant line (S1), and two triple mutant lines (T1 and T2). The distribution and magnitude of receptor overexpression were not significantly different among the various lines as expected for the housekeeping nature of the promoter. The level of α_{1B}AR overexpression was ~2-fold in the heart with greater overexpression seen in the liver, lung, brain, and spleen (18). Confirming constitutive signaling of these overexpressed receptors in the transgenic lines, inositol 1,4,5-trisphosphate levels were significantly higher in kidneys from W2+/−, S1+/−, and T2+/− mice than in age-matched NT mice (Fig. 1). Similar constitutive stimulation of inositol 1,4,5-trisphosphate metabolism has been previously shown in the liver (18). The rank order increase in inositol 1,4,5-trisphosphate pool size seen among the various lines (T2 > S1 > W2) corresponds with the strength of constitutive signaling that was found for these receptors *in vitro* (16, 17).

It should be noted that when bred to homozygosity, mice overexpressing constitutively active mutant forms of the α_{1B}AR (S1 and T2) displayed reproductive problems. This was not seen in the W2 line, suggesting that reproductive failure was unlikely the result of breeding artifacts. Therefore, all phenotypic analyses were performed on heterozygotes. All transgenic lines also displayed a 20–30% reduction in body weight, but this was only apparent in older mice that were more than 12 months of age (18).

Cardiac Hypertrophy in Mice Possessing α_{1B}AR Overactivity—α_1ARs have been shown to evoke a hypertrophic response in cultured cardiac myocytes (23, 24) with the regulation of this process predominated by the α_{1A} subtype (25, 26). Because

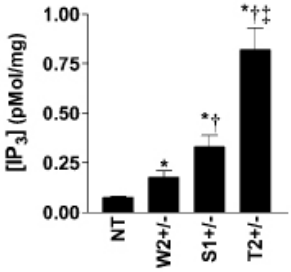


FIG. 1. **IP_3 levels were determined in kidneys from 6-month-old NT, W2+/−, S1+/−, and T2+/− mice as described under "Experimental Procedures" ($n = 3$).** The *asterisk* indicates the significance from the NT group. The *dagger* indicates significant increases compared with the W2+/− group. The *double dagger* indicates significant increases compared with the S1+/− group. Significance was determined using a two-tailed Student's *t* test ($p < 0.05$).

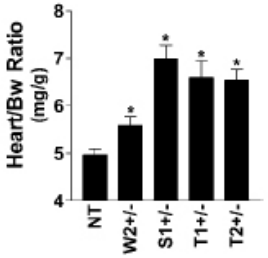


FIG. 2. **Heart to body weight (*Heart/Bw*) ratios were calculated in NT, W2+/−, S1+/−, T1+/−, and T2+/− mice at 4–6 months of age ($n > 12$ for each *line*).** The *asterisk* indicate the significance from the NT group based on a two-tailed Student's *t* test ($p < 0.05$).

myocardial-targeted overexpression of constitutively active α_{1B}ARs has also been shown to cause cardiac hypertrophy in mice (11), we performed morphologic and echocardiographic analyses in the context of our systemic transgenic model. Indicative of a hypertrophic phenotype, W2, S1, T1, and T2 mice showed an increased heart to body weight (*heart/Bw*) ratio compared with age-matched (4–6 months) NT control mice (Fig. 2). Body weight was not significantly different among the lines at 4–6 months of age. It should be noted that other organs including the liver, kidneys, lungs, and brain did not exhibit a change in mass relative to body weight (data not shown). Increases in heart mass ranged between 12 and 41% with S1 mice showing the largest increase. These findings were confirmed echocardiographically in W2, S1, and T2 mice, which showed an increased thickness of the posterior wall and interventricular septum compared with age-matched NT control mice (Table I).

Molecular confirmation of cardiac hypertrophy was attempted by measuring *ANF* message levels via Northern blot analysis of poly(A) mRNA purified from 8-month-old NT and T2 mouse hearts. *ANF*, a gene often associated with cardiac hypertrophy (27), was not up-regulated in T2 mice relative to the NT controls (data not shown), suggesting that the morphologic and echocardiographic findings are indicative of an early stage hypertrophy. Besides our model, the hypertrophic cardiomyopathy mouse (28) also shows hypertrophy in the absence of *ANF* up-regulation, suggesting that the progression of cardiac hypertrophy is not always strictly associated with the up-regulation of *ANF* (29) and/or other fetal genes. Another more likely reason for the lack of *ANF* up-regulation is the low level of α_{1B}AR overexpression present in our model. For example, the Gαq overexpression mouse model of cardiac hypertrophy (30) displayed no change in *ANF* expression with a 2-fold increase in the Gαq protein, a circumstance similar to the 2-fold overexpression of cardiac α_{1B}ARs in our mice. However, a 4-fold increase in Gαq was sufficient to invoke *ANF* transcription. These findings collectively indicate that a threshold of expression may be necessary to evoke changes in fetal gene transcription.

Despite an increased ventricular diameter in both diastole and systole, the cardiac output in the transgenic lines was lower than that seen in NT mice (Table I). This probably is attributed to the decreased heart rate and increased isovolumetric relaxation time displayed by transgenic animals (Table I). The decreased heart rate, which was confirmed via a tail cuff measurement in conscious mice (Table I), may be the result of a direct effect on Purkinje fiber automaticity, which is thought to be controlled by the α_{1B}AR (31) and is consistent with the overexpression of the receptor. A similar decrease in heart rate was also found in the heart-targeted Gαq-overexpressing mouse (30). Overall, because α_{1B}ARs are coupled to Gαq, the decrease in heart rate may be directly related to signaling events downstream of α_{1B}AR activation, or it may be part of the autonomic dysfunction, which we describe later.

Interestingly, a robust myocardial overexpression (>40-fold) of the wild type α_{1B}AR has been shown to cause increased diacylglycerol content and *ANF* mRNA without inducing the morphological hallmarks of hypertrophy (12). One conclusion that can be drawn from this earlier study is that only constitutively active α_{1B}ARs can induce hypertrophy. This raises the possibility that constitutively active receptors may signal through different pathways than wild type receptors. However, in arguing against this possibility, modest developmental and tissue-specific overexpression (2-fold in the heart) of wild type α_{1B}ARs in our mice caused a cardiac hypertrophy that was less robust but similar to that seen in the heart-targeted constitutively active α_{1B}AR mouse. Unlike the heart-targeted model, our model may be exhibiting a phenotype that more genuinely represents the end point impact of α_{1B}AR action in the heart because our use of the isogenic α_{1B}AR promoter facilitates transgene overexpression in all α_{1B}AR-expressing cardiac cell types, not just cardiac myocytes. Overall, because several experimentally distinct approaches to genetically induce α_{1B}AR overactivity have independently led to the manifestation of a somewhat similar cardiac phenotype, the emergence of that phenotype must be α_{1B}AR-dependent and not simply the spurious outcome of transgenic manipulation. Based on this assumption, we assert that in addition to the α_{1A}AR, the α_{1B}AR plays an important regulatory role in the progression of the hypertrophic program in cardiac tissue.

Hypotension in Mice Possessing α_{1B}AR Overactivity—The α_1ARs are widely expressed in the peripheral arteries (4, 5) and possess the capacity to regulate vasoconstriction (32–36), thus implicating them in the control of blood pressure. Regarding the α_{1B}AR, however, the bulk of the literature suggests that this subtype does not play a significant role in the direct regulation of the peripheral vascular tone (5, 9). Rather, the predominant role of α_{1B}ARs expressed in the vasculature has been proposed to include the regulation of growth and metabolic activity (6, 37–39). Contrary to these studies, however, the α_{1B}AR knockout mouse showed a decreased pressor response to phenylephrine infusion (8), which indicates the participation of the receptor in the regulation of peripheral vasoconstriction. Based on these findings from the knockout model, if α_{1B}ARs participate in vasoconstriction, our constitutively active α_{1B}AR mice should display hypertension because of the constitutive activation of this process. However, our mice displayed the opposite phenotype, a significantly reduced systemic arterial blood pressure.

4–6-month-old S1 and T2 mice were hypotensive relative to

TABLE I
Echocardiographic analyses

12-month-old mice were anesthetized with 0.05 mg/gBw ketaset and 0.1 mg/gBw inactin, and the chest area was shaved and swabbed with ultrasound gel. Several cardiac parameters were echocardiographically determined including interventricular septal thickness (*IVS*), posterior wall thickness (*PW*), left ventricular internal dimension in diastole (*LVIDd*) and in systole (*LVIDs*), isovolumetric relaxation time (*IVRT*), and heart rate (*HR (echo)*). For comparison, HR was also determined in conscious mice via a tail cuff. (*HR (cuff)*), IVS, PW, LVIDd, and LVIDs were normalized to body weight and percent fractional shortening (*%FS*) was calculated as 100 × [(LVIDd − LVIDs)/LVIDd]. Cardiac output (*CO*) was calculated as described under "Experimental Procedures." The *asterisks* denote significance from NT ($n \geq 5$) based on a two-tailed Student's t test ($p < 0.05$).

	Wall thickness		Left ventricle dimensions			Heart function			
	IVS	PW	LVIDd	LVIDs	%FS	IVRT	HR (echo)	HR (cuff)	CO
	mm/gBw	*mm/gBw*	*mm/gBw*	*mm/gBw*		*ms*	*beats/min*	*beats/min*	*ml/min*
NT	0.031 ± 0.002	0.027 ± 0.001	0.074 ± 0.002	0.028 ± 0.002	62.2 ± 1.2	15.70 ± 1.2	573 ± 53	681 ± 67	24.0 ± 3.3
W2+/−	0.036 ± 0.002	0.034 ± 0.001*	0.113 ± 0.011*	0.057 ± 0.010*	49.6 ± 6.2	25.50 ± 1.7*	344 ± 42*	607 ± 46*	10.4 ± 1.2*
S1+/−	0.040 ± 0.002*	0.034 ± 0.001*	0.102 ± 0.003*	0.044 ± 0.006*	57.9 ± 5.0	29.17 ± 3.1*	337 ± 16*	590 ± 63*	18.3 ± 1.1
T2+/−	0.041 ± 0.002*	0.039 ± 0.003*	0.100 ± 0.008*	0.049 ± 0.008*	51.2 ± 4.6	27.06 ± 3.2*	382 ± 41*	588 ± 55*	15.2 ± 1.8*

age-matched NT control mice. Whereas T2 mice showed modest hypotension while still under the influence of the anesthetic agents (Fig. 3, *A* and *B*), fully conscious and unrestrained S1 and T2 mice showed a more significant decrease in basal pressure compared with the NT control (Fig. 3, *A* and *C*). The mean arterial pressure in conscious W2 mice was lower than that in NT animals (Fig. 3*C*); however, this was not statistically significant. Confirming these measurements made in conscious animals, the basal mean femoral artery pressure was also significantly lower in 4-month-old anesthetized S1 mice than in age-matched NT control mice (Fig. 4*A*). Overall, our observation of basal hypotension in constitutively active α_{1B}AR mice contradicts the idea that activation of the α_{1B}AR can induce vasoconstriction and is the first report to indicate that an α_1AR can affect resting arterial blood pressure. It should be noted that although all transgenic lines demonstrated a hypertrophic phenotype in the heart, only the two constitutively active lines (S1 and T2) demonstrated hypotension. This was probably attributed to the intermediate level of constitutive signaling (see Fig. 1) and variability in the data collected from W2 mice. For example, some parameters of hypertrophy were not significant for the W2 line, and blood pressure was reduced but was highly variable and not significant.

To extend these findings, we compared the potency of the α_1AR-selective agonist phenylephrine to evoke a pressor response in NT and transgenic mice. Phenylephrine produced a dose-dependent increase in systemic arterial blood pressure in the NT and all transgenic groups. The pressor response in the transgenic group was no greater than that seen in NT animals. Indeed the pressor dose response curve in transgenic animals was shifted to the right of that seen in NT mice (Fig. 4*B*), arguing that the α_{1B}AR does not transduce the phenylephrine pressor response. This rightward shift seen in the transgenic lines was probably because of the decrease in basal blood pressure. It should be noted that the dose-response curves could not be completed to saturation because of the lethal effect of high doses of phenylephrine. Because the expression of α_{1B}ARs has been identified in peripheral arteries via the use of an α_{1B}-specific antibody (36), these results suggest that vascular α_{1B}ARs are not directly involved with the regulation of vasoconstriction.

To confirm that the α_{1B}AR is not directly involved in blood pressure regulation either via vasoconstriction or somehow via a negative influence on the pressor response (*i.e.* vasodilation, Fig. 4*B*), contractile-response curves were generated using *ex vivo* segments of the mesenteric artery prepared from several lines of mice. The vasoconstrictory action of phenylephrine was tested in artery segments from α_{1B}AR knockout mice (8) from our W2 line of mice and from the appropriate non-knockout and non-transgenic control groups. The phenylephrine dose response curves generated for each of these groups were not significantly different from each other (Fig. 5), demonstrating that the α_{1B}AR does not participate in blood pressure-related vasoconstriction and confirming that the hypotension seen in our transgenic animals is not the result of an arterial event.

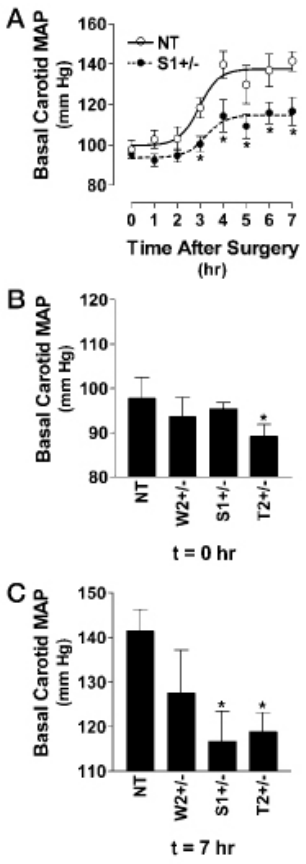


FIG. 3. **Mean carotid pressure (*Basal Carotid MAP*) was determined under basal conditions in conscious NT, W2, S1, and T2 mice via an in-dwelling catheter as described under "Experimental Procedures."** *A*, time course of carotid MAP in NT (*open circles*) and S1 (*closed circles*) recovering from anesthesia ($n > 8$ for each point). *B*, a summary of carotid MAP in NT, W2, S1, and T2 mice immediately after surgery while still under anesthesia ($n > 8$ for each *line*). *C*, a summary of carotid MAP in fully conscious NT, W2, S1, and T2 mice 7 h after surgery ($n > 8$ for each *line*). The *asterisks* in each part of the figure indicate the significance from the NT group based on a two-tailed Student's t test ($p < 0.05$).

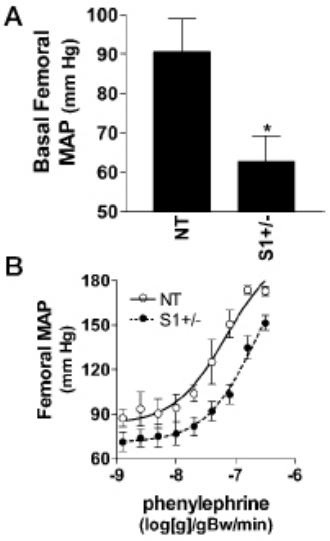


FIG. 4. **Mean femoral artery pressure (*Basal Femoral MAP*) was determined in NT and S1 mice via an in-dwelling catheter under basal conditions and following intravenous presentation of phenylephrine as described under "Experimental Procedures."** *A*, basal femoral MAP in NT and S1 mice under anesthesia ($n > 6$ for each *line*). The *asterisk* indicates the significance from the NT group based on a two-tailed Student's t test ($p < 0.05$). *B*, phenylephrine dose effect on femoral MAP in NT (*open circles*) and S1 (*closed circles*) mice under anesthesia ($n > 6$ for each *line*). Dose response data was analyzed using the non-linear regression functions of the non-iterative curve fitting program GraphPad Prism.

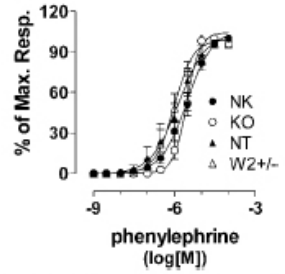


FIG. 5. **Concentration-response curves for the α_1AR agonist phenylephrine in isolated segments of mouse mesenteric artery (first order branches, external diameter 200–220 μm) taken from non-knockout (*NK, closed circles*), α_{1B}AR knockout (*KO, open circles*), non-transgenic (*NT, closed triangles*), and W2 transgenic mice (*W2, open triangles*).** Data points represent the mean for each group ($n = 11$ for NK and $n = 5$ for KO, NT, and W2 each). log(EC_{50}) (*log[M]*) values were −5.53 for NK mice, −5.54 for KO mice, −5.84 for NT mice, and −6.0 for W2 mice. Dose response data were analyzed using the non-linear regression functions of the non-iterative curve fitting program GraphPad Prism. Groups were determined to not be significantly different from each other based on a one-way analysis of variance.

Because of the apparent lack of direct α_{1B}AR control over vasoconstriction, the question remains how does systemic α_{1B}AR overactivity lead to a reduction in blood pressure? It is well established that peripheral vascular tone is partially regulated by sympathetic nervous system activity (40). Lower sympathetic activity, as measured by a reduction of plasma catecholamines, could lead to a lower blood pressure because of a reduced activation of all vascular α_1-adrenergic targets. This hypothesis was tested by assessing sympathetic function via

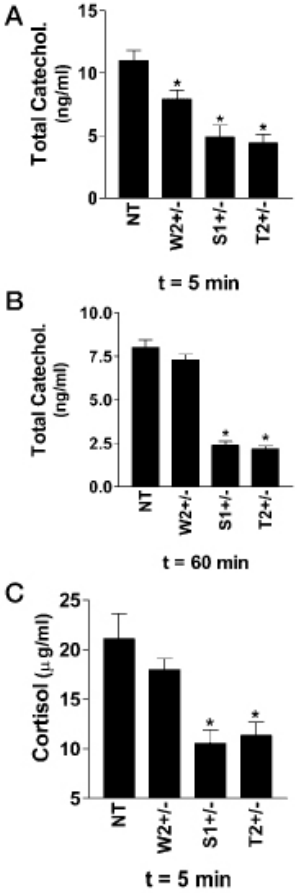


FIG. 6. Total plasma epinephrine and norepinephrine levels in NT, W2, S1, and T2 mice were determined as described under "Experimental Procedures" either 5 min after application of anesthesia (*A*) or after 1 h of stable anesthesia (*B*) ($n = 5$ for each *line*). (*C*) total plasma cortisol levels in NT, W2, S1, and T2 mice were also determined 5 min after application of anesthesia as described under "Experimental Procedures" ($n = 3$ for each *line*). The *asterisks* in each part of the figure indicate the significance from the NT group based on a two-tailed Student's t test ($p < 0.05$).

the measurement of total blood levels of norepinephrine, epinephrine, and cortisol in the transgenic lines. Indicating reduced sympathetic output in transgenic animals, 6-month-old S1 and T2 mice showed a roughly 50% reduction of total blood catecholamine compared with age-matched NT control mice (Fig. 6*A*). A similar reduction in total catecholamines was seen after a 1-h period of stable anesthesia (Fig. 6*B*), suggesting that the reduction was not a result of indirect effects of anesthesia or of altered reactivity/stress induced by handling. It should be noted that catecholamine levels seen in our mice (ng/ml) are in the same range as those reported in other transgenic mouse models (41). As expected, because cortisol is released from the adrenal medulla in response to sympathetic stimulation, we also found a corresponding reduction in plasma cortisol (50%) in S1 and T2 mice relative to the NT control (Fig. 6*C*). Because the reduced plasma catecholamine and cortisol levels were correlated to blood pressure effects, it is possible that these two events may be linked. These data suggest that the hypotension seen in our transgenic mice may be, at least in part, because of a reduction in the sympathetic nerve activity. It is also possible

that the decrease in heart rate and cardiac output may also contribute to the hypotension seen in our transgenic mice. This possibility does not seem probable given the fact that W2 mice, which displayed a reduced heart rate and cardiac output, were not hypotensive.

Our transgenic α_{1B}AR mice display a Parkinsonian-like syndrome termed multiple system atrophy with associated neurodegeneration in the substantia nigra, olive pontine, thalamus, and locus coerulus (18). Symptomatically, the presence of multiple system atrophy often involves autonomic failure because of these extensive neurodegenerative lesions in the brain. Therefore, a probable reason for the hypotension seen in our mice is a lowered sympathetic output caused by autonomic dysfunction. Although some patients with autonomic failure have hypertension (42), autonomic neuropathy is a common cause of orthostatic hypotension (43) and is also responsible for the hypotension commonly seen in Parkinson's disease and multiple system atrophy patients (44). Accordingly, these patients also exhibit low plasma levels of norepinephrine (44). Besides hypotension, our transgenic mice displayed reproductive problems, weight loss (at 12 months of age), bradycardia, depressed heart function, and low cortisol and catecholamine levels that are all associated with autonomic dysfunction. Autonomic failure produces distinct abnormalities depending upon the location of the lesions (44). Therefore, our model is probably the outcome of autonomic dysfunction that is caused by α_{1B}AR-induced neurodegeneration.

Overall, our analysis of α_{1B}AR control of blood pressure from a systemic perspective has led us to conclude that α_{1B}AR overactivity does not cause an elevation in pressure but rather induces a net hypotension. The mechanism driving this hypotension is probably rooted in an autonomic failure because many of the symptoms displayed by our mice are consistent with this diagnosis. The data presented in this study are also counterindicative of a vasoconstrictive role for α_{1B}ARs. Our findings support an emerging hypothesis, which predicts that α_{1B}ARs do not play a major role in contractile regulation in vascular smooth muscle (9) but rather are predominately coupled to various metabolic and cellular processes at vascular sites where the receptor is expressed (6, 37–39). This realization has important implications in the pharmacotherapeutic approach to the manipulation of blood pressure.

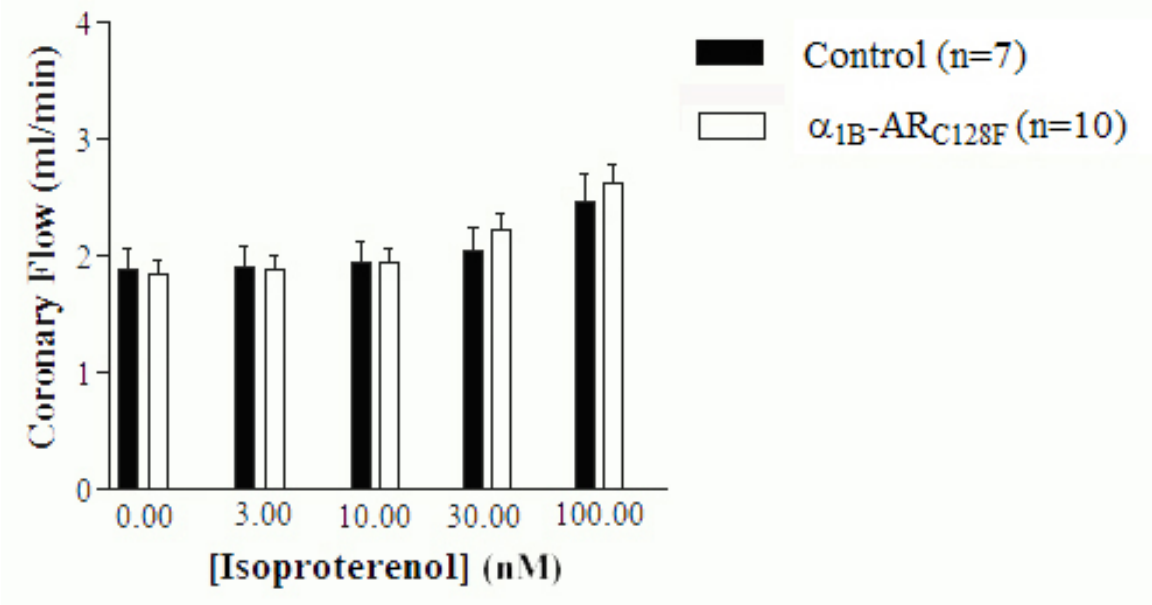
Acknowledgments—We thank the Cleveland Clinic Foundation Transgenic Core Service for performing the transgene injections, which produced founder mice. Blood catecholamine and cortisol levels were determined by the Laboratory Medicine Section of the Cleveland Clinic Foundation.

REFERENCES

1. Lomasney, J. W., Cotecchia, S., Lorenz, W., Leung, W. Y., Schwinn, D. A., Yang-Feng, T. L., Braunstein, M., Lefkowitz, R. J., and Caron, M. G. (1991) *J. Biol. Chem.* **266,** 6365–6369
2. Perez, D. M., Piascik, M. T., and Graham, R. M. (1991) *Mol. Pharmacol.* **40,** 876–883
3. Cotecchia, S., Schwinn, D. A., Randall, R. R., Lefkowitz, R. J., Caron, M. G., and Kobilka, B. K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7159–7163
4. Perez, D. M., Piascik, M. T., Malik, N., Gaivin, R. J., and Graham, R. M. (1994) *Mol. Pharmacol.* **46,** 823–831
5. Guarino, R. D., Perez, D. M., and Piascik, M. T. (1996) *Cell. Signalling* **8,** 323–333
6. Chen, L., Xin, X., Eckhart, A. D., Yang, N., and Faber, J. E. (1995) *J. Biol. Chem.* **270,** 30980–30988
7. Leech, C. J., and Faber, J. E. (1996) *Am. J. Physiol.* **270,** H710–H722
8. Cavalli, A., Lattion, A.-L., Hummler, E., Nenniger, M., Pedrazzini, T., Aubert, J.-F., Michel, M. C., Yang, M., Lembo, G., Vecchione, C., Mostardini, M., Schmidt, A., Beermann, F., and Cotecchia, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11589–11594
9. Hrometz, S. L., Edelmann, S. E., McCune, D. F., Olges, J. R., Hadley, R. W., Perez, D. M., and Piascik, M. T. (1999) *J. Pharmacol. Exp. Ther.* **290,** 452–463
10. Anyukhovsky, E. P., Rybin, V. O., Nikashin, A. V., Budanova, O. P., and Rosen, M. P. (1992) *Circ. Res.* **71,** 526–534
11. Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F., and Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10109–10113
12. Akhter, S. A., Milano, C. A., Shotwell, K. F., Cho, M.-C., Rockman, H. A., Lefkowitz, R. J., and Koch, W. J. (1997) *J. Biol. Chem.* **272,** 21253–21259
13. Kunos, G., and Ishac, E. J. N. (1987) *Biochem. Pharmacol.* **36,** 1185–1191
14. Kopp, U. G., and Dibona, G. F. (1996) in *The Kidney* (Brenner, B. M., ed) pp. 789–814, W. B. Saunders Co., Philadelphia, PA
15. Zuscik, M. J., Piascik, M. T., and Perez, D. M. (1999) *Mol. Pharmacol.* **56,** 1288–1297
16. Perez, D. M., Hwa, J., Gaivin, R. J., Mathur, M., Brown, F., and Graham, R. M. (1996) *Mol. Pharmacol.* **49,** 112–122
17. Hwa, J., Gaivin, R. J., Porter, J. E., and Perez, D. M. (1997) *Biochemistry* **36,** 633–639
18. Zuscik, M. J., Sands, S., Ross, S. A., Waugh, D. J. J., Gaivin, R. J., Morilak, D. A., and Perez, D. M. (2000) *Nat. Med.* **6,** 1388–1394
19. Tanaka, N., Dalton, N., Mao, L., Rockman, H. A., Peterson, K. L., Gottshall, K. R., Hunter, J. J., Chien, K. R., and Ross, J., Jr. (1996) *Circulation* **94,** 1109–1117
20. Link, R. E., Desai, K., Hein, L., Stevens, M. E., Chruscinski, A., Bernstein, D., Barsh, G. S., and Kobilka, B. K. (1996) *Science* **273,** 803–805
21. Lorenz, J. N., and Robbins, J. (1997) *Am. J. Physiol.* **272,** H1137–H1146
22. Mulvany, M. J., and Halpern, W. (1977) *Circ. Res.* **41,** 19–26
23. Kariya, K., Karns, L. R., and Simpson, P. C. (1991) *J. Biol. Chem.* **266,** 10023–10026
24. Knowlton, K. U., Baracchini, E., Chien, K. R., Harris, A. N., Henderson, S. A., Evans, S. M., and Glembotski, C. C. (1991) *J. Biol. Chem.* **266,** 7759–7768
25. Knowlton, K. U., Michel, M. C., Itani, M., Shubeita, H. E., Ishihara, K., Brown, J. H., and Chien, K. R. (1993) *J. Biol. Chem.* **268,** 15374–15380
26. Autelitano, D. J., and Woodcock, E. A. (1998) *J. Mol. Cell. Cardiol.* **30,** 1515–1523
27. Chien, K. R., Knowlton, K. U., Zhu, H., and Chien, S. (1991) *FASEB. J.* **5,** 3037–3046
28. Vikstrom, K. L., Factor, S. M., and Leinwand, L. A. (1996) *Mol. Med.* **2,** 556–567
29. Vikstrom, K. L., Bohlmeyer, T., Factor, S. M., and Leinwand, L. A. (1998) *Circ. Res.* **82,** 773–778
30. D'Angelo, D. D., Sakata, Y., Lorenz, J. N., Boivin, G. P., Walsh, R. A., Liggett, S. B., and Dorn, G. W., II. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8121–8126
31. Balzo, U. D., Rosen, M. R., Malfatto, G., Kaplan, L. M., and Steinberg, S. F. (1990) *Circ. Res.* **67,** 1535–1551
32. Elhawary, A. M., Pettinger, W. A., and Wolff, D. W. (1992) *J. Pharmacol. Exp. Ther.* **260,** 709–713
33. Piascik, M. T., Guarino, R. D., Smith, M. S., Soltis, E. E., Saussy, D. L., Jr., and Perez, D. M. (1995) *J. Pharmacol. Exp. Ther.* **275,** 1583–1589
34. Bylund, D. B., Regan, J. W., Faber, J. E., Hieble, J. P., Triggle, C. R., and Ruffolo, R. R., Jr. (1995) *Can. J. Physiol. Pharmacol.* **73,** 533–543
35. Zhou, L., and Vargas, H. M. (1996) *Eur. J. Pharmacol.* **305,** 173–176
36. Piascik, M. T., Hrometz, S. L., Edelmann, S. E., Guarino, R. D., Hadley, R. W., and Brown, R. D. (1997) *J. Pharmacol. Exp. Ther.* **283,** 854–868
37. Nakaki, T., Nakayama, M., Yamamoto, S., and Kato, R. (1990 *Mol. Pharmacol.* **37,** 30–36
38. Siwik, D. A., and Brown, R. D. (1996) *J. Cardiovasc. Pharmacol.* **27,** 508–518
39. Sasaguri, T., Teruya, H., Ishida, A., Abumiya, T., and Ogata, J. (2000) *Biochem. Biophys. Res. Commun.* **268,** 25–30
40. Burt, A. M. (1993) *Textbook of Neuroanatomy*, pp. 367–399 W. B. Saunders Co., Philadelphia, PA
41. Makaritsis, K. P., Johns, C., Gavras, I., Altman, J. D., Handy, D. E., Bresnahan, M. R., and Gavras, H. (1999) *Hypertension* **34,** 403–407
42. Shannon, J. R., Jordan, J., Diedrich, A., Pohar, B., Black, B. K., Robertson, D., and Biaggioni, I. (2000) *Circulation* **101,** 2710–2715
43. Robertson, D., and Robertson, R. M. (1994) *Arch. Intern. Med.* **154,** 1620–1624
44. Montastruc, J. L., Senard, J.-M., Rascol, O., and Rascol, A. (1996) in *Advances in Neurology* (Battistin, L., Searlato, T., Caraceni, T., and Ruggieri, S., eds) pp. 377–381, Lippincott Williams & Wilkins, Philadelphia, PA

Appendix D

Measured effects of coronary flow to increasing concentrations of isoproterenol using the isolated-perfused heart preparation. Refer to *Methods* for measuring coronary flow. All bars represent the mean and the standard error of the mean for a minimum of 7 independent experiments.



Appendix E

Echocardiographic assessment of the murine left ventricular **a)** dimensions and **b)** function in α_{1D}-AR KO mice. The values are the mean ± S.E., and * indicates statistical difference from the non-transgenic value ($P<0.05$). Data generated with the help of Dr. Kimimasa Tobita.

a)

	n	Body Weight (g)	Left Ventricular Internal Dimension (mm/g)		Posterior Wall Thickness (mm/g)		Left Ventricular Mass (g)	Left Ventricular Mass/ Body Weight (10^{-3})
			Diastole	Systole	Diastole	Systole		
Control	3	41.20 ± 2.33	0.1186 ± 0.0060	0.0855 ± 0.0067	0.0147 ± 0.0026	0.0212 ± 0.0019	0.0595 ± 0.0078	1.464 ± 0.271
α_{1D}-AR KO	3	33.23* ± 1.04	0.1252 ± 0.0057	0.0792 ± 0.0008	0.0211 ± 0.0025	0.0332* ± 0.0039	0.0553 ± 0.0088	1.665 ± 0.266

b)

	n	Ejection Time (msec)	Mean velocity for circumferential fiber shortening (1/√sec)	Heart Rate (beats/min)	Stroke Volume (mm^3)	Cardiac Output (mm^3/min)	Fractional Shortening (%)
Control	3	66.00 ± 6.96	0.3438 ± 0.0594	382.7 ± 46.7	0.0379 ± 0.0028	14.52 ± 2.19	28.06 ± 2.01
α_{1D}-AR KO	3	52.67 ± 1.63	0.6210* ± 0.0542	477.3* ± 6.5	0.0290 ± 0.0067	13.86 ± 3.22	36.58* ± 2.62

Appendix F

The Role of the α_{1B}-Adrenergic Receptor in the Inotropic Response of the Mouse Myocardium

Sean A. Ross, Dan Chalothorn[†], June Yun, Pedro J. Gonzalez-Cabrera, Dan F. McCune, Boyd Rorabaugh, Michael T. Piascik[†] and Dianne M. Perez *

The Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, Ohio 44195 and [†] The Department of Pharmacology and the Vascular Biology Research Group, The University of Kentucky College of Medicine, Lexington, Kentucky 40536.

Running Head: *Role of the α_{1B}-AR in myocardium*

Correspondence: Dianne M. Perez, Department of Molecular Cardiology NB50, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. Phone: 216-444-2058. Fax: 216-444-9263. E-mail: perezd@ccf.org.

ABSTRACT

α_1-Adrenergic receptors (ARs) are known mediators of a positive inotropy in the heart which may play even more important roles in heart disease. Due to a lack of sufficiently selective ligands, the contribution of each of the three α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D}) to cardiac function is not clearly defined. In this study, we used a systemically expressing mouse model that over expresses the α_{1B}-AR to define the role of this subtype in cardiac function. Using an isolated heart model, we find that a 50% increase of the α_{1B}-AR in the heart does not change basal cardiac parameters compared to age-matched normals (heart rate, + or - dp/dt, and coronary flow). However, the inotropic response to phenylephrine is blunted. The same results were obtained in isolated adult myocytes. The difference in inotropy could be blocked by the selective α_{1A}-AR antagonist, 5-methylurapidil, which correlated with decreases in α_{1A}-AR density, suggesting that the α_{1B}-AR had caused a compensatory downregulation of the α_{1A}-AR. These results suggest that the α_{1B}-AR does not have a major role in the positive inotropic response in the mouse myocardium but may negatively modulate the response of the α_{1A}-AR.

Keywords: Adrenergic receptor, heart, myocyte, inotropy

Introduction

α_1-Adrenergic receptors (ARs) mediate the effects of the sympathetic nervous system by binding the catecholamines, epinpehrine and norepinephrine. α_1-AR subtypes (α_{1A}, α_{1B}, α_{1D}) are part of the larger and related family of adrenergic receptors which include the β-ARs (β_1, β_2, β_3) and the α_2-ARs (α_{2A}, α_{2B},α_{2C}). Adrenergic receptors are also members of the much larger family of G-protein coupled Receptors (GPCRs) of which over 80% of hormones use to transduce their signals.

Alpha-1 adrenergic receptors (AR) play many roles in the myocardium ranging from positive inotropic and chronotropic effects , cardiac preconditioning , arrhythmogenesis and cardiac hypertrophy (12, 23). α_1-AR modulation of cardiac function may become more important in diseased heart where β-AR responsiveness is often impaired with concomitant upregulation of the α_1-AR response (for review, see 2). All three α_1-AR subtypes are expressed in the heart of a variety of species (mouse, rat, rabbit, dog, human). Predominant subtypes are the α_{1A}- and α_{1B} AR with minor expression (if any) of the α_{1D}-AR subtype (23, 26). α_1 AR mediated positive inotropic effects have been well-documented in a number of animal models both *in vivo* and i*n vitro* although this response is considered minor in comparison to β-AR stimulation (25% versus 75% with norepinephrine stimulation in rat)(11). However, the role of α_1-ARs in mediating cardiac contractility has been controversial due to the substantial variations (i.e. both negative and positive inotropy) between different species and the preparation used.

Elucidating the roles of the individual subtypes pharmacologically has proved difficult with the lack of subtype selective ligands and only with the advent of genetic manipulation have we begun to make significant progress. Recent advances in transgenic and knock-out technologies have allowed investigators to dissect out some of the contributions of the various subtypes to physiological responses in the vasculature and cardiac tissues (23). While some labs have focussed on heart-targeted transgenic models of α_{1B}- and α_{1A}-AR over-

expression, we have developed a transgenic mouse model whereby use of the isogenic mouse α_{1B}- AR promoter has allowed us to over express a constitutively active mutant (CAM) form of the hamster α_{1B}-AR only to tissues that normally express the receptor (33). Advantages of using this model is that over-expression is targeted to natural cells/tissues that express the subtype, over-expression is not dramatic, and multiple systems (i.e. heart, vasculature, CNS) are affected and can be studied in the same animal. Of the previous transgenic and knockout models developed, there is only one report that explores the role of the α_{1B}-AR subtype in cardiac function *per se* with most reports focusing on either tissue contractility, hypertrophy or blood pressure regulation. Heart-targeted over-expression of the wild-type α_{1B}-AR leads to decreased ventricular function (10) while over-expression of the α_{1A}-AR in the targeted heart was found to increase inotropy dramatically but without evidence of any hypertophy (14). We wanted to explore the α_{1B}-AR subtype in the heart and determine its role in cardiac function in our systemic mouse model.

Since we have found that our α_{1B}-AR transgenic mice display neurodegeneration (33) and a corresponding autonomic dysfunction (34), we decided to look at the effects of α_{1B}-AR over-expression in the myocardium *ex vivo* to try to determine what contribution the α_{1B} -AR makes to the inotropic effects of the α_1-AR pool of receptors. We find that there is no change in basal cardiac parameters (developed pressure, heart rate, coronary flow) in the transgenic animals compared to controls, however there is a significant decrease in the response to phenylephrine, suggesting a negative inotropy. We propose that this difference may be attributed to a decrease in α_{1A}-AR levels in the transgenic animals due to compensatory effects. Therefore, although the α_{1B}-AR may not play a direct role in inotropy in the mouse myocardium, its effects may be related to a negative regulation of the α_{1A}-AR subtype, the major determinant of cardiac inotropy.

Materials and Methods

Mice. The generation and genotyping of transgenic mice possessing systemic α_{1B}-AR over-activity has been described elsewhere (33). Briefly, tissue-specific distribution of systemic α_{1B}-AR over-activity was achieved by utilizing the murine α_{1B}-AR gene promoter (32) to drive over-expression of a transgene containing a cDNA coding for a constitutively active mutant of the α_{1B}-AR, called T for triple mutant (12). The Cleveland Clinic Foundation Transgenic Core Facility injected approximately 200 copies of each transgene into the pronuclei of one-cell B_6/CBA mouse embryos, which were surgically implanted into pseudo-pregnant female mice. Founder mice were identified and subsequent generations were genotyped by southern analysis of genomic DNA extracted from tail biopsies. Mice are used at approximately 9-10 months of age with equal numbers of male and female mice.

Measurement of cardiac function in intact heart preparations. After intravenous injection of heparin sodium, i.p. (500 U/kg) and intraperitoneal anesthetization with pentobarbital sodium (150 mg/kg), the heart, with all major vessels and lungs attached, was excised. The aorta was then cannulated with a flared PE10 catheter and was positioned above the coronary ostia. A water-filled latex balloon was inserted into the lumen of the left ventricle via the left atrium. The distal end of the balloon-attached catheter was connected to a pressure transducer for measurement of intraventricular pressure and $\pm dP/dt$. The balloon was inflated to a constantly-held diastolic pressure of 3-10 mm Hg. The retrograde perfusion via the aorta was carried out by a perfusion pump maintaining a column of Krebs-Henseleit solution (KHS) composed of (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.5 EDTA, 25 $NaHCO_3$, 5 pyruvic acid, and 11 glucose; pH 7.4 (following gassing with 95% O_2-5% CO_2 at 37°C) to provide a constant coronary perfusion pressure of 75 mmHg. We confirmed the coronary perfusion pressure by using a pressure transducer connected via a side port to the aorta perfusion cannula. Coronary flow was measured via an in-line Transonic flow probe (Transonics 1N) connected to a

Transonics flow meter (T106). Drugs were added by infusion pump through an injection port directly above the aortic cannula. Data were continually recorded and displayed on a Powerlab data acquisition system. Cardiac parameters were measured off-line at the end of the experiment. The preparation was allowed to stabilize for 30 min prior to the start of the experiment. Propranolol (1 uM), rauwolscine (0.1 uM) and 5-methylurapidil (1 nM) were administered 20 min before the administration of phenylephrine. Phenylephrine (10 uM) was infused for a period of 10 min.

Membrane preparation and binding experiments. Individual hearts were placed in ice cold buffer A (0.2 to 0.3 mg/ml final) composed of 10 mM Hepes (pH 7.4), 250 mM sucrose, 5 mM EGTA, 12.5 mM $MgCl_2$ and a cocktail of protease inhibitors. After a 30 sec disruption with a polytron, material was transferred to a dounce homogenizer, diluted 1:7 in buffer A, and homogenized 10 times each with a loose and tight pestle. Homogenates were spun for 5 min at 300 x g to remove fat and for 5 min at 1250 x g to remove nuclei and then incubated for 15 min at 4°C in an equal volume of 0.5 M KCl. The KCl wash breaks down myosin, resulting in a purer membrane preparation. Homogenates were spun for 15 min at 35,000 x g to pellet membranes. Pellets were resuspended in ice cold buffer B composed of 20 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM EGTA 12.5 mM $MgCl_2$ and a cocktail of protease inhibitors. This spin/resuspension was repeated twice. After resuspension in buffer B containing 10% glycerol, the final pellet was homogenized again, analyzed for protein concentration by Bradford, and frozen at –70°C (< 5 mg/ml final). Saturation binding was performed using the α_1-AR antagonist 2-[β-(4-hydroxyl-3-[^{125}I]iodophenyl)ethylaminomethyl]-tetralone ([^{125}I]HEAT) as the radioligand and phentolamine (100 uM) to determine the total α_1-AR density. The density of the α_{1B}-AR population was determined by repeating the saturation experiment with 1 nM of 5-methylurapidil to calculate non-specific binding. Total α_{1A}-AR density was determined by subtracting the α_{1B}-AR density from the total. Bmax

(maximum receptor density) and Kd (affinity) values were obtained using the non-linear regression function of GraphPad Prism.

Isolation of mouse ventricular myocytes. Murine ventricular myocytes were enzymatically dissociated from mouse (25-40 g) hearts using a slightly modified protocol (Wolska and Solaro 1996). In brief, mice were heparinized (200 U IP) and anesthetized with sodium pentobarbital (100 mg/kg IP). Mouse hearts were rapidly excised and perfused through the aorta with a calcium-free modified Hepes buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM Hepes, 11 mM Glucose, 0.2 mM pyruvate, pH 7.2, gassed with 100% O_2 at 37°C). A constant pressure of 40 mm Hg, which was monitored with a pressure transducer/digital polygraph (Windo Graf 900; Gould Instruments, Valley View, OH), was maintained by varying the flow into the coronaries with a peristaltic pump (Masterflex Digital Console Drive; Cole-Palmer, Vernon Hills, IL). Following 4 min of retrograde perfusion with a Ca^{2+}-free modified Hepes buffer, a HEPES buffer containing Liberase Blendzyme (14 mg/ml; Roche Diagonistic, Indianapolis, IN) was further perfused for 8 min after which the [Ca^{2+}] was adjusted to 20 μM. Perfusion of the heart with the Ca^{2+}/collagenase Hepes buffer was halted at approximately 15 min later when a dramatic fall in coronary perfusion pressure (when 40 mm Hg could not be maintained with increased coronary flow) was detected. The ventricles were minced and gently titurated, then filtered through a 250 μm mesh using a collagenase-free Hepes buffer with 200 μM Ca^{2+}. The filtrate was placed in a 37°C water bath for 5 min, and then the supernatant was discarded and the wash was repeated. The final pellet was resuspended in a collagenase free Hepes buffer containing 1 mM Ca^{2+}. Myocytes were used within 4 hours of isolation.

Myocyte contractility measurements. Myocytes were incubated on a laminin-coated glass coverslip in a recording chamber (RC-24, Warner Instrument, Hamden, CT) and mounted onto the stage of an inverted microscope (IX-70, Olympus America, Melville, NY). Rod-shaped, calcium tolerant myocytes

were bathed in Hepes buffer (1 mM Ca^{2+}) at a rate of 1 ml/min (Masterflex L/S; Cole Parmer, Vernon Hills, IL) from an inline heater (37°C, TS 28; Warner Instrument, Hamden, CT), and field stimulated (0.5 Hz, SD9 stimulator; Grass Instruments, Quincy, MA). Myocytes were imaged with a charge-coupled device camera, and the changes in cell length were quantified by an edge-motion detection with a video dimension analyzer (Coyote Bay, Manchester, NH). The myocyte twitch amplitude was defined as a percentage of the diastolic length. The baseline measurements were quantitated (Inspector 3.0; Matrox Electronic Systems, Ltd., Canada) after a 5 min equilibrium period. The effect of increasing concentrations of phenylephrine (1 nM to 10 μM) and isoproterenol (1 nM to 10 μM) on twitch amplitude were analyzed in two cell types (Normal (n=24 myocytes from 3 hearts), and transgenic (n=25 from 4 hearts). The effect on twitch amplitude was digitally recorded after 2 min of drug perfusion, and later quantitated with Matrox Inspector 3.0.

Statistics. Significant differences are obtained using unpaired student's t-test.

Results

Baseline Parameters. To determine if the transgenic mice displayed changes in baseline values, we first determined physiological parameters (Table 1). The age matched groups (≈ 9 months) have a significant difference in body weight with the transgenic mice having a reduced body weight (28%) compared to normals. Heart weight, when normalized to body weight, is slightly greater in the transgenic group, however this is not significant. There were also no differences in the coronary flow between the two when corrected for heart weight differences.

We next determined changes in baselines cardiac parameters (Table 2). There was no significant difference in baseline cardiac parameters between the normal and transgenic hearts. Heart rate (≈ 350 bpm) and developed pressure (≈ 90mm Hg) parameters are similar to previously reported values obtained using this isolated heart preparation (27).

Cardiac Parameters in response to phenylephrine. Figure 1A shows a typical response of hearts from control animals to phenylphrine (10 uM), an α_1-AR selective agonist, in the presence of propranolol (1 uM) and rauwolscine (0.1 uM) to block any effects due to β- or α_2-ARs. There is a triphasic response with an initial increase in LVP, dP/dT, HR and CF followed by a rapid decrease then a sustained increase. The rapid decrease falls below baseline pressure of about 10-20 mm Hg but is transient, only last about 2-3 sec, and is not different in normal versus transgenic (Figure 1B). The sustained increased in pressure is taken as the point of comparison for inotropic responses.

Comparisons of the phenylephrine response between normal and transgenic are shown in Table 3. There are significant differences between the transgenic and normal hearts in all cardiac parameters except coronary flow with addition of phenylephrine (10 uM). Although all parameters increase due to phenylephrine stimulation in the transgenic, the values are below those normally seen in the control mice. Heart rate increases 23% in the normal hearts whereas there is only a 10% increase in the transgenic hearts. Indices of contractility such as developed pressure and +dP/dT show greater increases in the normal hearts (45% and 66%, respectively) compared to transgenic hearts (14% and

22%, respectively). Myocardial relaxation as assessed by –dP/dT followed the same trend with a 74% increase in the normal hearts compared with 19% in the hearts from transgenic animals. Coronary flow tended to increase in the transgenic heart (likely due to the decreased pressure) but this was not significant.

α_1-AR subtype mediation of inotropy. To determine the α_1-AR subtype responsible for the observed phenylephrine effects, we used 5-methlyurapidil at a dose (1 nM), which is about 100-fold selective against the α_{1A}-AR subtype. Figure 2 shows the phenylephrine-response in both control and transgenic hearts in the presence of 5-methylurapidil (1 nM). There are no significant differences between the transgenic and normal hearts in any of the cardiac parameters with this treatment, suggesting that the α_{1A}-AR selective antagonist equalized the two systems.

Phenylephrine response in isolated myocytes. To determine if a decrease in the drug-induced inotropic response was also apparent in isolated myocytes, we measured changes in myocyte cell-length as an index of contractility (Fig. 3). Increasing amounts of phenylephrine resulted in a concentration-dependent increase in myocyte contractility (≈65%) in the normal myocytes whereas there was no increase and even a significant decrease in myocyte contractility in the transgenic myocytes. This suggests that the transgenic myocyte has no positive but a negative inotropy. To assess possible changes in the β-AR response, changes in myocyte length with increasing concentrations of isoproterenol can be seen in Figure 4. As isoproterenol concentration increases , there is an increase in myocyte contractility (≈200%) in the normal myocytes with similar results in the transgenics.

To determine if there were changes in the α_1-AR subtype protein population, as suggested by the data, we first attempted to perform competition ligand binding studies to determine the percentage of high and low affinity sites.

There are no high avidity antibodies available to determine protein expression. However, since the total α_1-AR population in the mouse heart is very low (86 fmoles/mg membrane protein, Figure 5), this was not feasible to simultaneously discriminate accurately two receptor populations. We then decided to perform differential saturation binding studies using phentolamine at a concentration of 100 uM which would block all the α_1-AR subtypes. This experiment would determine the total α_1-AR population. The experiment is then repeated with a selective concentration of the α_{1A}-selective blocker, 5-methylurapidil , used at the same concentration as in the functional studies (1 nM). The specific binding would then determine the α_{1B}-AR population. Since it has been previously shown that the α_{1D}-AR is not present in the heart or is present at very low numbers, as determined by binding (26), the difference between the total and α_{1B}-AR densities would determine the amount of α_{1A}-AR present. Normal and transgenic hearts had total α_1-AR Bmax values of 86 $\pm$ 11 and 99 $\pm$ 9 fmoles/mg membrane protein, respectively (Figure 5). This difference between normal and the transgenic heart is not significant. The affinities of the radioligand for the membrane preparations were also not significantly different between groups (in pM; 150 ± 11 for normal and 140 ± 16 for the transgenic). However, the α_{1B}-AR density increased in the transgenic 72.5 ± 13 versus 40.3 ± 3.5 fmoles/mg, consistent with the over-expression of this receptor in the heart. This leaves the α_{1A}-AR population in the transgenic at 26 fmoles/mg compared to 46 fmoles in normal hearts. This represents about a 70/30 ratio of the α_{1B}/α_{1A} populations in the transgenics while the normal mice has a 50/50 ratio.

Discussion

Our results indicate that the systemic over-expression of a constitutive active mutant (CAM) α_{1B}-AR in the murine myocardium leads to a functional change in the inotropic, chronotropic and lusitropic response to phenylephrine compared to normal hearts. These differences could be eliminated by the α_{1A}-AR antagonist 5-methylurapidil and supported by decreases in α_{1A}-AR density by saturation binding. These results suggest that the over-expression of the α_{1B}-AR leads to a down-regulation of the α_{1A}-AR, ultimately resulting in the observed functional and decreased changes in response to phenylephrine.

The inotropic response to α_1-AR stimulation has been studied in a variety of animal species and preparations. However, the responses have considerable variation (for review, see 13). Positive inotropy to α_1-AR agonists have been found in whole heart and muscle strips from rat, rabbit, guinea pig, hamster and dog. However, α_1-AR mediated negative intropy has recently been described in mouse muscle strips and isolated myocytes (15, 18, 20, 25). The predominant subtypes in the myocardium are the α_{1A}- and α_{1B}-AR subtypes with densities varying according to species (19), cardiac region (29) and developmental stage (5). The mouse heart has been documented to contain the lowest levels of α_1-ARs. While we report α_1-AR density levels of approximately 80-90 fmoles/mg, previous reports are in the 5-10 fmole/mg range (14, 26). This difference may be due to our use of radioactive iodine which has a higher specific activity and greater sensitivity than tritium and our purer membrane preparation which removes most of the contaminating myosin with the KCl wash.

There have been a number of pharmacological studies looking at the effects of the various subtypes on myocardial function (reviewed in 11). Using antagonists (WB-4101, 5-methylurapidil and CEC, chloroethylclonidine) to isolate the contributions of the subtypes, these studies postulate that both the α_{1A}- and α_{1B}-AR subtypes contribute to the positive inotropic effect of α_1-AR stimulation. One possible drawback of these pharmacological studies is the lack of subtype selectivity of these antagonists for these receptors. We decided to use a genetic

approach in combination with a pharmacological one to determine the contribution of the α_{1B}-AR subtype to myocardial function. We took a unique approach in creating transgenic mice using the isogenic mouse promoter to drive systemic over-expression of the α_{1B}-AR (33). Overexpresssion is mild, with no signficant changes in α_1-AR density but resulted in a 50% increase in the α_{1B}-AR population (Fig. 5). This is distinctly different from the heart-targeted transgenics of the α_{1A}- and α_{1B}-AR (10, 14, 17) which display dramatic over-expression of the receptors (100-fold) and are only expressed in the myocyte. Since vascular fibroblasts have recently been shown to contain α_1-ARs (7), it is possible that heart fibroblasts as well as the endothelium and smooth muscle cells may contain α_{1B}-ARs and be involved in paracrine functions to release factors that contribute to the inotropy. We have recently shown that α_1-ARs subtypes may be involved in the paracrine functions of IL-6 (9), which are associated with cardiac function. In this regard, we cannot rule out other contributing factors to the negative inotropy, but since the responses were equalized by 5-methylurapidil and α_{1A}-AR binding sites were decreased, the mechanism of action is likely through compensatory effects on the α_{1A}-AR.

Baseline cardiac parameters (DP, +/-dP/dt, CF) from transgenic animals were comparable to those in normal hearts suggesting that the systemic over-expression of the α_{1B}-AR did not affect basal function. This confirms the finding of previous studies (1) where even 100-fold heart-targeted over-expression of wildtype α_{1B}-AR did not result in any change in basal parameters (LV systolic pressure, +/-dP/dt). However, using the same mouse model, the studies of Grupp et al. (10) did produce a lower basal LV function.

Stimulation of α_1-AR's with phenylephrine resulted in an increase in all cardiac parameters (DP, HR, dP/dt and CF) in both transgenic and normal hearts. In both groups, phenylephrine elicited a triphasic response (brief positive inotrophy followed by brief negative inotrophy and then sustained positive inotrophy). This is the first study to show this type of triphasic response in the whole murine heart[1]. However, this triphasic response is consistent with previous

findings found in isolated rat papilliary muscle (21), mouse cardiac trabeculae (15) and now the isolated mouse heart (27). The mechanism of the triphasic response has not been clearly established. It has been proposed in rat papilliary that the negative inotropic effect is mediated by the α_{1A}-AR subtype and the positive inotropic responses are mediated by both the α_{1A}- and α_{1B}-AR subtypes (28). In the mouse trabeculae, both subtypes are thought to mediate the negative phase, but there is no positive inotropy but only a partial recovery from the negative inotropy, again mediated by both α_1-AR subtypes (15). This is contrasted with the α_{1A}-AR transgenic, which showed dramatic increases in inotropy (14). Interestingly Nishimaru et al. (20) showed that in isolated mouse right ventricle, phenylephrine stimulation results in a sustained negative inotrophy mediated by enhanced Ca^{2+} efflux through the Na^{+}/Ca^{2+} exchanger. The most recent work of Turnbull et al.(27) utilized the $\alpha_{1A/B}$ double knockout mice. Convincingly, they show that the $\alpha_{1A/B}$ double knockout did not have any phenylephrine-induced positive inotropy but only a sustained negative inotropy, which could be reversed with BMY7378, suggesting that the α_{1D}-AR was responsible for the negative inotropy. Moreover, since trabeculae from these animals did not show any phenylephrine response, the data suggests that the α_{1D}-AR was located in the coronary vasculature and the observed decreased coronary flow caused the negative inotropy. This interpretation would also be consistent with our results which show similar intensities of the transient negative inotropy between normal and transgenic mice (Fig. 1B) which would have maintained equal α_{1D}-AR densities.

We did performed similar isolated tissue experiments (data not shown) and found that in the isolated mouse right ventricle a sustained negative inotropic response is observed with phenylephrine stimulation. However, in the isolated myocyte, phenylephrine does induce a positive inotropic effect (Fig. 3). The whole heart mouse model of α_{1B}-AR over-expression of Akhter et al. (1) and Grupp et al. (10) also show positive α_1-AR mediated inotropy, but isolated mouse myocytes have also been described to have negative intropy (25). The

discrepency between the two systems (isolated or intact heart and myocyte vs isolated ventricular tissue) cannot be attributed to different perfusate solutions or temperatures since they were consistent between our two preparations. One possiblity is that the frequency of stimulation may be responsible for the difference. It has been shown that frequency of pacing can affect the response of tissues to phenylephrine (3), and tissue/myocyte preparations are stimulated at significantly lower frequencies (0.33 Hz – 1Hz) than the spontaneously beating heart. However, this does not account for our own differences between the isolated heart and tissue-bath studies. Another possibility is the localization of the α_1-AR subtypes , which could be differentially distributed in various heart regions. These studies await use of green flourescent-tagged receptor transgenics since high avidity antibodies are not yet available. Nevertheless, repeatable and opposite results in α_1-AR mediated inotropy is observed depending upon the system used. Another likely and disturbing possibility is that the α_1-AR subtype distribution may change upon the removal of the organ. This has precedence in guinea pig liver studies in which the α_1-AR subtype switched (α_{1A}- to α_{1D}-AR) from the removal to the isolation of the heptacytes (8). The only consistent explanation is that whole or intact heart studies produce an α_1-AR mediated positive inotropy in the mouse (1, 10,1 4, 27) while tissue and/or myocyte preparations may produce the negative inotropy (15, 20, 25), suggesting that whole heart studies may be more physiologically relevant.

In this study, we found that the sustained increase in inotropic response to phenylephrine in the transgenic hearts is significantly less than that found in the normal hearts. This result was unexpected since α_{1B}-ARs have been implied to have a positive inotropic response to phenylephrine in whole heart. However, our work is consistent to Grupp et al., (10) that demonstrated an impaired left ventricular contraction in the heart-targeted wild type α_{1B}-AR model. One possibility that could explain this phenomenon was that over-expression of the α_{1B}-AR may have altered the expression levels of the other α_1-AR subtypes. Indeed, there is some precidence in the literature that this is possible. Prolonged incubation with norepinephrine which causes hypertrophy in normal rat myocytes

also causes the α_{1B}- and α_{1D}-AR to decrease while the α_{1A}-AR increases (24). Deng et al., (6) have shown that there is crosstalk between α_{1A}- and α_{1B}-AR's in neonatal rat myocardium, whereby knocking out the α_{1B}-AR with CEC caused a potentiation of the α_{1A}-AR response. In our studies, the α_{1A}-AR antagonist 5-methylurapidil eliminated the functional differences between the transgenic and normal hearts for the effects on cardiac contractility, suggesting that the α_{1A}-AR was responsible. The results correlated to the loss of α_{1A}-AR binding sites. These results suggest that α_{1A}-AR levels are decreased in the transgenic hearts and that this subtype is responsible for the differences in cardiac responsiveness observed between the transgenic and normal hearts to phenylephrine. This result would be consistent to the heart-targeted transgenic α_{1A}-AR results (14) which suggests that the α_{1A}-AR is a potent mediator of positive inotropy in the mouse myocardium.

The α_1-ARs have prominent effects on cardiac function. In this communication, we show that the major positive inotropic activity for this subtype family resides in the α_{1A}-AR. Other work has also implicated the α_{1B}-AR as being involved in positive inotropic effects. Our data do not support this. Indeed, we demonstrate that over-expression of a constitutively active α_{1B}-AR is associated with a negative effect on myocardial contraction. The mechanism underlying these effects does not appear to be a direct linkage of the α_{1B}-AR to negative inotropic pathways. Rather, the α_{1B}-AR appears to interfere with the activity of receptors, such as the α_{1A}-AR that mediates positive inotropic responses. Thus, via mechanisms that remain to be elucidated, there is molecular cross talk between the α_{1B}-AR and receptors coupled to positive inotropic responses that result in a decrease in contractile function. Thus, we propose that the role of the α_{1B}-AR is to negatively modulate contractile activation through indirect mechanisms and that tonic unregulated activation of the α_{1B}-AR has the potential to lead to contractile dysfunction and pathophysiology. This is supported by data from the cardiac-targeted transgenics as well as from our own systemic over-

expression model which show that these mice develop contractile dysfunction and hypertrophy (34).

The role of the α_{1B}-AR in the myocardium is unlikely to be only a modifier of the α_{1A}-AR. Distinct differences are present when constitutively active receptors of the α_{1A}- and α_{1B}-AR respectively, were transfected into the cardiac murine myocyte cell line (HL-1). In this study, they demonstrate that the α_{1A}-AR subtype preferentially couples to cardiac-specific atrial natriuretic factor gene expression, while the α_{1B}-AR preferentially couples to activation of mitogen-activated protein kinase, Ets-like transcription factor-1 and serum response element signaling pathways (16). This implicates the α_{1B}-AR as being involved in mitogenic signals or remodeling scenarios as is proposed for the vasculature (reviewed in 23). We also published an oligonucleotide microarray study of the changes associated with α_{1B}-AR mediated gene expressions in the hearts of the same transgenic animals used this study (31). We found changes in the gene expressions for growth, Src-related signals, development, cell-cycle, apoptosis as well as inflammation, consistent with the role of this subtype in growth, development and potential for pathology.

In conclusion we have shown that over-expression of the α_{1B}-AR does not lead to any basal functional changes in the isolated heart. However with α_1-AR stimulation, we see a depressed response from the transgenic hearts compared to controls. We attribute this difference to a decrease in α_{1A}-AR expression levels in the transgenic hearts. Therefore, we suggest that the α_{1B}-AR does not modulate cardiac contraction directly but may be a negative modifier of positive inotropy.

Acknowledgements: This work was funded by RO1HL614380-04 (DMP), R01HL 31820-11(MTP), a local American Heart Association fellowship to (SAR, PJG-C, and DC), an NRSA to (DFM) and a T32 HL07914 training grant in Vascular Cell Biology to (BR and JY). (SAR) Present Address: GlaxoSmithKline, Research Triangle Park, NC.

Footnotes:

[1]While our work was in revision, the work of Turnbull et al., 2002 was published ahead of print, describing similar findings.

REFERENCES

1. **Akhter SA, Milano CA, Shotwell KF, Cho MC, Rockman HA, Lefkowitz RJ, and Koch WJ.** Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signaling.*J Biol Chem* 272: 21253-9, 1997.

2. **Brodde OE and Michel MC.** Adrenergic and muscarinic receptors in the human heart. *Pharmacol Rev* 51: 651-690, 1999.

3. **Chu L, and Endoh M.** Positive inotropic effect of alpha 1-adrenoceptor stimulation in dog ventricular myocardium. *J Cardiovasc Pharmacol.* 38:S13-6, 2001.

4. **Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG, and Kobilka BK.** Molecular cloning and expression of the cDNA for the hamster alpha 1-adrenergic receptor. *Proc Natl Acad Sci U S A* 85:7159-63, 1988.

5. **del Balzo U, Rosen MR, Malfatto G, Kaplan LM, and Steinberg SF.** Specific alpha 1-adrenergic receptor subtypes modulate catecholamine-induced increases and decreases in ventricular automaticity. *Circ Res* 67: 1535-51, 1990.

6. **Deng XF, Sculptoreanu A, Mulay S, Peri KG, Li JF, Zheng WH, Chemtob S, and Varma DR.** Crosstalk between alpha-1A and alpha-1B adrenoceptors in neonatal rat myocardium: implications in cardiac hypertrophy. *J Pharmacol Exp Ther* 286: 489-96, 1998.

7. **Faber JE, Yang N, and Xin X.** Expression of alpha-adrenoceptor subtypes by smooth muscle cells and adventitial fibroblasts in rat aorta and in cell culture. *J Pharmacol Exp Ther* 298: 441-52, 2001.

8. **Gonzalez-Espinosa C, Gonzalez-Espinosa D, Romero-Avila MT, Garcia-Sainz JA.** Inverse alpha(1A) and alpha(1D) adrenoceptor mRNA expression during isolation of hepatocytes. *Eur J Pharmacol.* 384(2-3): 231-7,1999.

9. **Gonzalez-Cabrera PJ, Gaivin R, Yun J, Ross SA, Papay RS, McCune DF, Rorabaugh BR, and Perez DM**. Genetic profiling of α_1-adrenergic receptor subtypes by oligonucleotide microarrays: Coupling to IL-6 secretion but differences in STAT 3 phosphorylation and gp-130. (*Mol. Pharmacol.* in press).

10. **Grupp IL, Lorenz JN, Walsh RA, Boivin GP, Rindt H**. Overexpression of alpha1B-adrenergicreceptor induces left ventricular dysfunction in the absence of hypertrophy. *Am J Physiol.* 275: H1338-50, 1998.

11. **Hwa J, DeYoung M, Perez DM, and Graham RM.** Autonomic control of myocardium: Alpha adrenoceptor mechanisms. In: *The Autonomic Nervous System.* G. Burnstock, Ed, Volume VIII: The nervous control of the heart, Volume editors: J Shepherd and SF Vatner. Harvard Academic Press, 1996.

12. **Hwa J, Gaivin R, Porter J and Perez DM.** Synergism of constitutive activity in α_1-adrenergic receptor activation. *Biochemistry* 36: 633-639, 1997.

13. **Li K, He H, Li C, Sirois P and Rouleau JL.** Myocardial a1-adrenoceptor: inotropic effect and physiological and pathologic implications. *Life Sci* 60:1305-1318, 1997.

14. **Lin F, Owens WA, Chen S, Stevens ME, Kesteven S, Arthur JF, Woodcock EA, Feneley MP, and Graham RM.** Targeted alpha(1A)-adrenergic receptor overexpression induces enhanced cardiac contractility but not hypertrophy. *Circ Res* 89: 343-50, 2001.

15. **McCloskey DT, Rokosh DG, O'Connell TD, Keung EC, Simpson PC, and Baker AJ.** Alpha(1)-adrenoceptor subtypes mediate negative inotropy in myocardium. *J. Mol Cell Cardiol.* 34: 1007017,2002.

16. **McWhinney CD, Hansen C and Robishaw JD.** Alpha1-adrenergic signaling in a cardiac murine atrial myocyte (HL-1) cell line. *Mol Cell Biochem* 214: 111-9, 2000.

17. **Milano CA, Dolber PC, Rockman HA, Bond RA, Venable ME, Allen LF, and Lefkowitz RJ.** Myocardial expression of a constitutively active α_{1B}-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad Sci USA.* 91: 10109-10113, 1994.

18. **Montgomery DE, Wolska BM, Pyle WG, Roman BB, Dowell JC, Buttrick PM, Koretsky AP, Del Nido P, and Solaro RJ.** α-Adrenergic response and myofilament activity in mouse hearts lacking PKC phosphorylation sites on cardiac TnI. *Am. J. Physiol Heart Circ Physiol* 282: H2397-2405, 2002.

19. **Mukherjee A, Haghani Z, Brady J, Bush L, McBride W, Buja LM, and Willerson JT.** Differences in myocardial alpha- and beta-adrenergic receptor numbers in different species. *Am J Physiol.* 245: H957-61,1983.

20. **Nishimaru K, Kobayashi M, Matsuda T, Tanaka Y, Tanaka H, and Shigenobu K.**
Alpha-Adrenoceptor stimulation-mediated negative inotropism and enhanced Na(+)/Ca(2+) exchange in mouse ventricle. *Am J Physiol Heart Circ Physiol* 280: H132-41, 2001.

21. **Otani H, Otani H, and Das DK**. Alpha 1-adrenoceptor-mediated phosphoinositide breakdown and inotropic response in rat left ventricular papillary muscles. *Circ Res* 62: 8-17,1988.

22. **Perez DM, Piascik MT, Malik N, Gaivin RJ, and Graham RM**. Cloning, expression and tissue distribution of the rat homolog of the bovine α_{1C}-adrenergic receptor provide evidence for its classification as the α_{1A}-subtype. *Mol Pharmacol* 46: 823-831, 1994.

23. **Piascik MT and Perez DM.** α_1-Adrenergic receptors: New Insights and Directions. *J Pharmacol Exp Ther* 298: 403-410, 2001.

24. **Rokosh DG, Stewart AF, Chang KC, Bailey BA, Karliner JS, Camacho SA, Long CS, and Simpson PC.** Alpha1-adrenergic receptor subtype mRNAs are differentially regulated by alpha1-adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and in vivo. Repression of alpha1B and alpha1D but induction of alpha1C. *J. Biol. Chem.* 271: 5839-43, 1996.

25. **Sakurai K, Norota I, Tanaka H, Kubota I,Tomoike H , Endoh M.** Negative inotropic effects of angiotensin II,endothelin-1 and phenylephrine in indo-1 loaded
adult mouse ventricular myocytes. *Life Sciences* 70: 1173–1184,2002.

26. **Tanoue A, Nasa Y, Koshimizu T, Shinoura H, Oshikawa S, Kawai T, Sunada S, Takeo S, and Tsujimoto G.** The alpha(1D)-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction. *J Clin Invest* 109: 765-75, 2002.

27. **Turnbull L, McCloskey DT, O'Connell TD, Simpson PC, Baker AJ.** {alpha}1-adrenergic receptor (AR) responses in {alpha}1AB-AR knockout mouse hearts suggests presence of {alpha}1D-AR. *Am J Physiol Heart Circ Physiol.* 2002 Dec 5 (ahead of print)

28. **Williamson AP, Seifen E, Lindemann JP, and Kennedy RH**. Effects of WB4101 and chloroethylclonidine on the positive and negative inotropic actions of phenylephrine in rat cardiac muscle. *J Pharmacol Exp Ther* 268:1174-82, 1994.

29. **Wolff DW, Dang HK, Liu MF, Jeffries WB, and Scofield MA**. Distribution of alpha1-adrenergic receptor mRNA species in rat heart. *J Cardiovasc Pharmacol* 32:117-22, 1998.

30. **Wolska BM and Solaro RJ.** Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Am. J. Physiol.* 271: H1250-H1255, 1996.

31. **Yun J, Zuscik MT, Gonzalez-Cabrera P, Ross SA, McCune DF, Piascik MT, and Perez DM.** Gene expression profiling of α_{1b}-adrenergic receptor-induced cardiac hypertrophy by oligonucleotide arrays *Cardiovascular.Research* (in press).

32. **Zuscik MJ, Piascik MT, and Perez DM**. Cloning, cell-type specificity and regulatory function of the murine α_{1b}-adrenergic receptor promoter. *Mol. Pharmacol.* 56: 1288-1297, 1999.

33. **Zuscik MJ, Sand S, Ross SA, Waugh DJJ, Gaivin RJ, Morilak D, and Perez DM.** Overexpression of the α_{1b}-Adrenergic receptor causes apoptotic neurodegeneration: A multiple system atrophy. *Nature Medicine* 6: 1388-1394, 2000.

34. **Zuscik MJ, Chalothorn D, Hellard D, Deighan C, McGee A, Daly C, Waugh DJJ, Ross SA, Gaivin RJ, Moorehead, A., Thomas J, Plow EF, McGrath JC, Piascik MT, and Perez DM.** Hypotension, autonomic failure and cardiac hypertrophy in transgenic mice over-expressing the α_{1b}-adrenergic receptor. *J Biol Chem* 276: 13738-13743, 2001.

Table 1: Baseline Physiological Parameters.

Parameters	Normal (n=6)	Transgenic (n=8)
Age (weeks)	37 ± 1	39 ± 1
Body Weight (g)	39 ± 2	28.3 ± 2 *
Dry Ht. Wt. (g)	0.19 ± 0.01	0.15 ± 0.02
Ht. Wt./Body Wt. (mg/g)	5.0 ± 0.3	5.6 ± 0.3
CF/Ht. Wt. (ml/g)	10.44 ± 0.55	11.20 ± 0.47

Values are Mean ± SEM; Ht, heart; WT, weight; CF, coronary flow;
* = $P < 0.05$ versus normal.

Table 2: Baseline Cardiac Parameters.

Parameters	Normal (n=6)	Transgenic (n=8)
HR (bpm)	345 ± 11	354 ± 12
DP (mmHg)	93 ± 7	89 ± 5
+dP/dT ($mmHg.ms^{-1}$)	2.86 ± 0.37	2.74 ± 0.14
- dP/dT ($mmHg.ms^{-1}$)	1.72 ± 0.16	1.68 ± 0.11
CF ($ml.min^{-1}$)	2.08 ± 0.19	1.98 ± 0.25

Values are Mean ± SEM; HR, heart rate; DP, developed pressure; ± dP/dT, maximal rates of the rise and fall in ventricular pressures; CF, coronary flow.

Table 3: Changes in Cardiac Parameters with the Addition of Phenylephrine (10 uM).

Parameters (n=8)	Normal (n=6)	Transgenic
HR (bpm)	23 ± 2%	10 ± 3%*
DP (mmHg)	45 ± 10%	14 ± 5%*
+dP/dT ($mmHg.ms^{-1}$)	66 ± 14%	22 ± 7%*
- dP/dT ($mmHg.ms^{-1}$)	74 ± 18%	19 ± 8%*
CF ($ml.min^{-1}$)	45 ± 12%	59 ± 32%

Values are mean % change from its own baseline ± SEM; HR, heart rate; DP, developed pressure; ± dP/dT, maximal rates of the rise and fall in ventricular pressures; CF, coronary flow. * , $P < 0.05$ versus normal.

Figure Legends

Figure 1: Recorded changes in cardiac parameters after the addition of phenylephrine (10 uM). **A,** Recorded changes in various cardiac parameters of a normal mouse heart are obtained using the Powerlab data acquisition system. Cardiac parameters are measured off-line at the end of the experiment. The preparation was allowed to stabilize for 30 minutes prior to the start of the experiment. Propranolol (1 uM), and rauwolscine (0.1 uM) were administered 20 min before the administration of phenylephrine. Phenylephrine (10 uM) was infused for a period of 10 min. LVP, left ventricular pressure; CPP, coronary perfusion pressure; dp/dt, rise and fall in ventricular pressure; BPM, beats per min; CF, coronary flow. **B,** changes in LVP observed in normal (top) and transgenic (bottom) after phenylephrine infusion. The transient negative inotropy was similar in both samples.

Figure 2: Changes in cardiac parameters after the addition of phenylephrine
(10 uM) in the presence of 5-Methylurapidil (1 nM). The preparation was allowed to stabilize for 30 min prior to the start of the experiment. Propranolol (1 uM), rauwolscine (0.1 uM) and 5-methylurapidil (1 nM) were administered 20min before the administration of phenylephrine. Phenylephrine (10 uM) was infused for a period of 10 min. Cardiac parameters are measured off-line at the end of the experiment. Light bars, normal mice; dark bars, transgenic mice; DP, developed pressure; + or - dp/dt, the maximum rise and fall in ventricular pressure; HR, heart rate. Results are mean % change ± SEM (n=6 in both groups).

Figure 3: Changes in myocyte cell-length with increasing phenylephrine concentrations. Myocytes are incubated on a laminin-coated glass coverslip in a recording chamber and imaged with a charge-coupled device camera. Changes in cell length were quantified by an edge-motion detection with a video dimension analyzer. The effect of increasing concentrations of phenylephrine (1

nM to 10 μM) on the changes in cell length was analyzed in normal (n=24 myocytes from 3 hearts), and transgenic (n=25 from 4 hearts) mice. Results are mean % change ± SEM; *, P < 0.05 versus normal.

Figure 4: Changes in myocyte cell-length with increasing isoproterenol concentrations. Myocytes are incubated on a laminin-coated glass coverslip in a recording chamber and imaged with a charge-coupled device camera. Changes in cell length were quantified by an edge-motion detection with a video dimension analyzer. The effect of increasing concentrations of isoproterenol (1 nM to 10 μM) on changes in cell length was analyzed in normal (n=24 myocytes from 3 hearts), and transgenic (n=25 from 4 hearts) mice. Results are mean % change ± SEM; *, P < 0.05 versus normal.

Figure 5. α_1-AR subtype density in normal and transgenic hearts. Membranes were prepared as described from individual mouse hearts. Total α_1-AR density was determined by saturation binding experiments using the nonselective α_1-AR-antagonist ^{125}I-HEAT as the radioligand and phentolamine (100 uM) to determine non-specific binding. α_{1B}-AR density was determined by performing the identical saturation study with the α_{1A}-AR selective blocker, 5-methylurapidil (1 nM). The α_{1A}-AR population was determined by subtracting the α_{1B}-AR density from the total α_1-AR pool. B_{max} was determined using the non-linear regression analysis of GraphPad Prism. Bars represent the mean B_{max} ± SEM (n=6-8). *, P < 0.05 versus normal.

Figure 1A

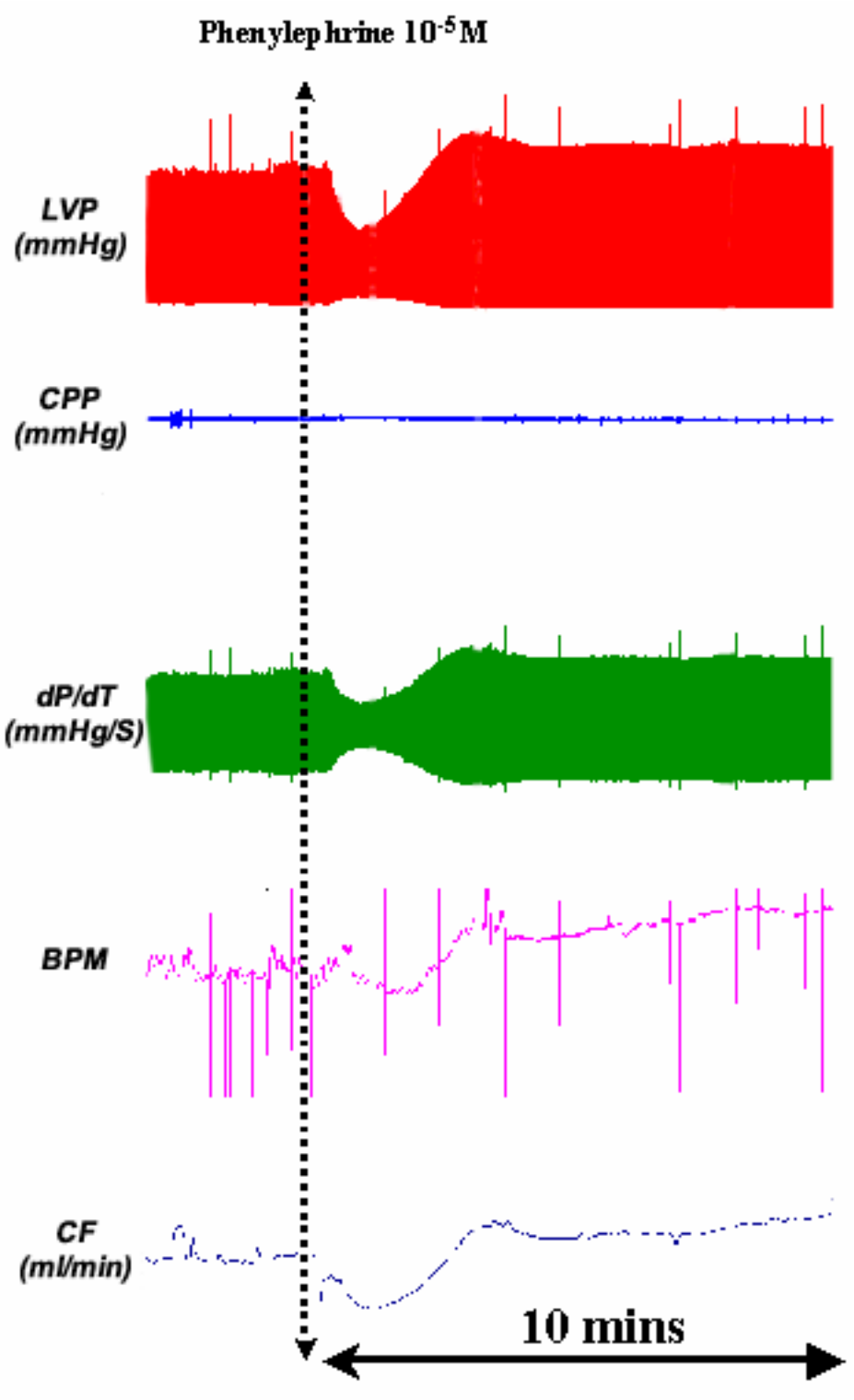

Phenylephrine 10-5M
LVP
(mmHg)
CPP
(mmHg)
dP/dT
(mmHg/S)
BPM
CF
(ml/min)
10 mins

Figure 1B

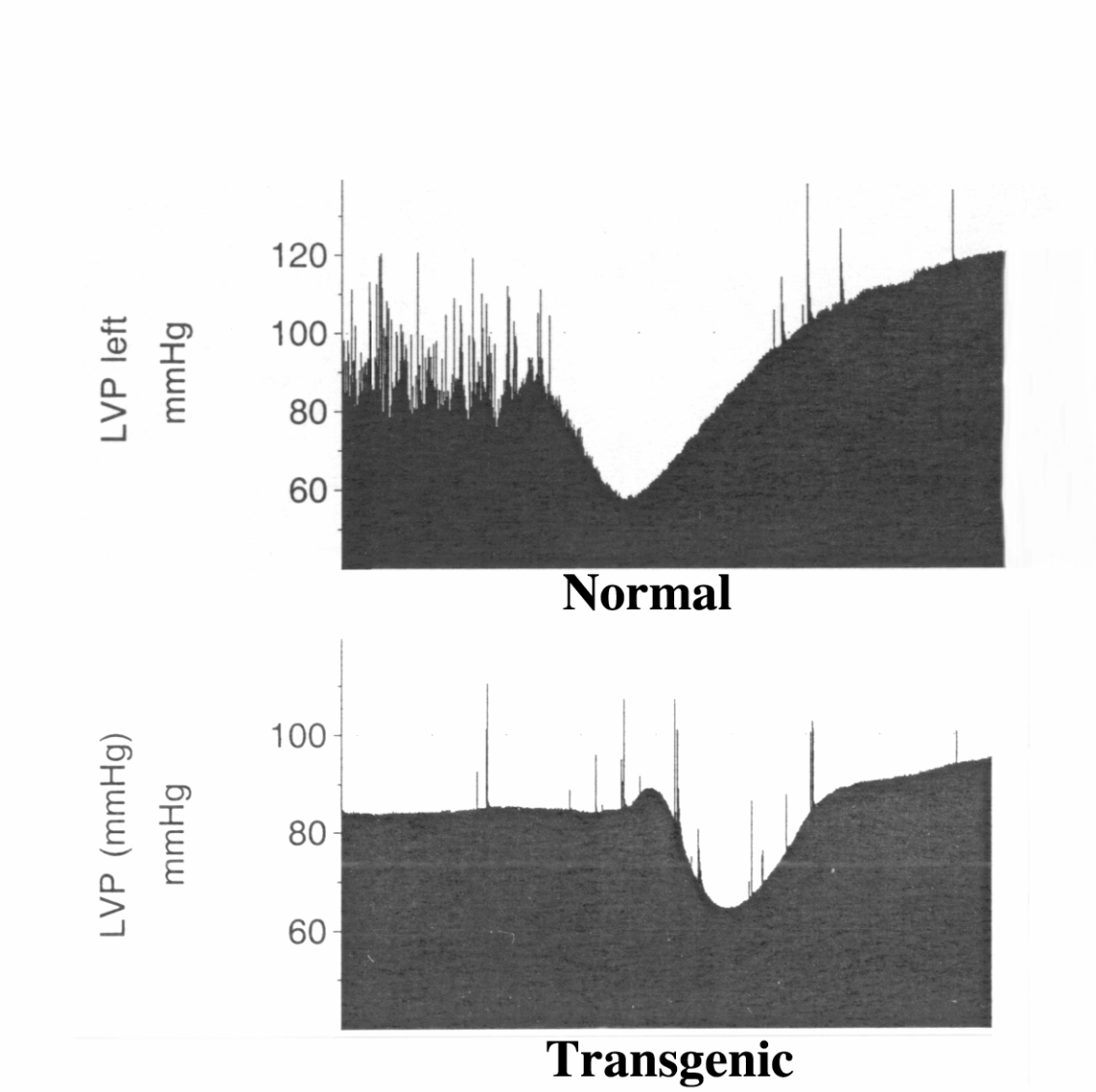

LVP left
mmHg
120
100
80
60
Normal
LVP (mmHg)
mmHg
100
80
60
Transgenic

Figure 2

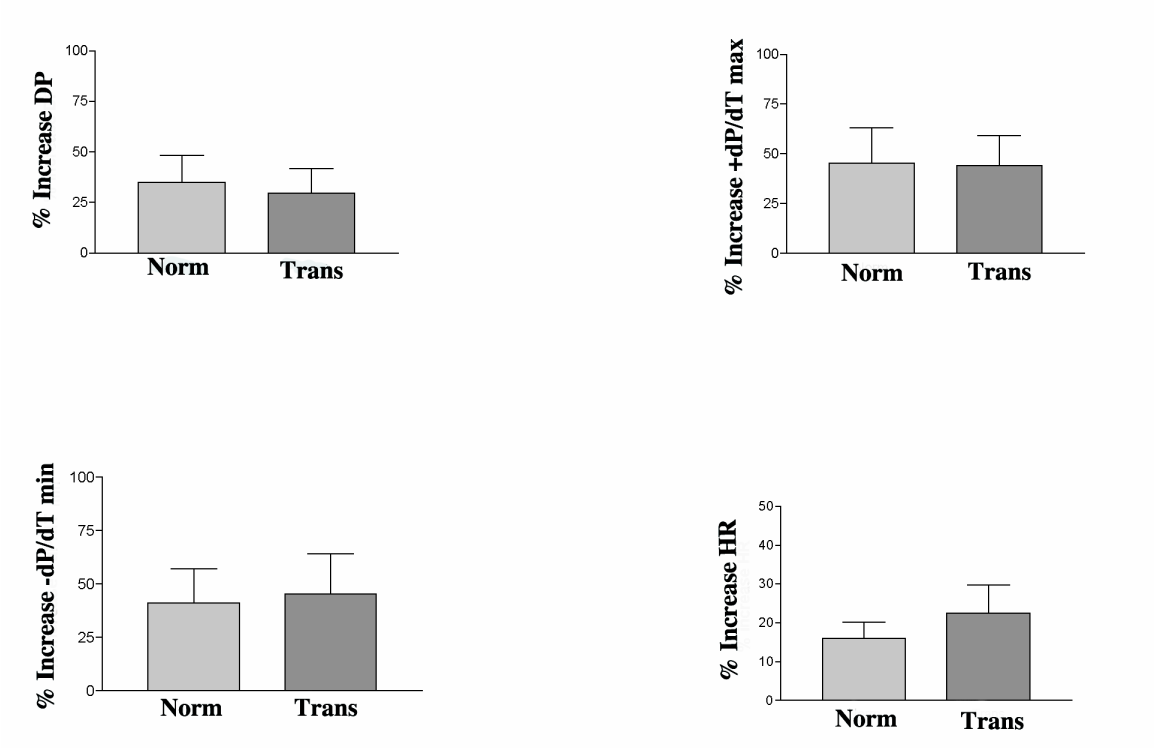

% Increase DP
100
75
50
25
0
Norm
Trans
% Increase +dP/dT max
100
75
50
25
0
Norm
Trans
% Increase -dP/dT min
100
75
50
25
0
Norm
Trans
% Increase HR
50
40
30
20
10
0
Norm
Trans

Figure 3

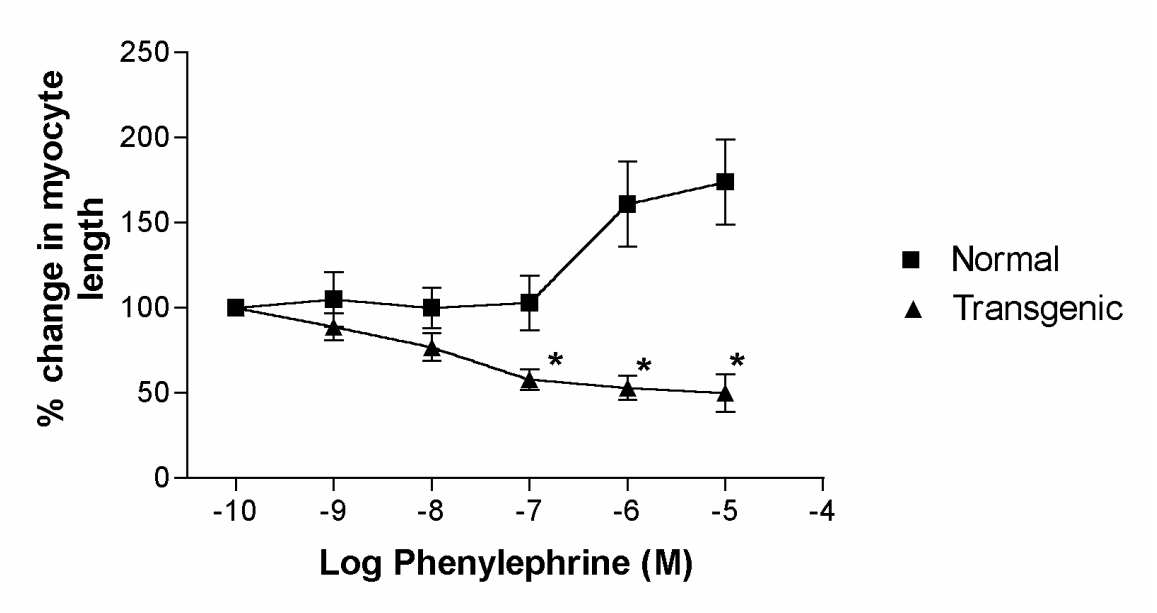

250
200
150
100
50
0
% change in myocyte length
-10
-9
-8
-7
-6
-5
-4
Log Phenylephrine (M)
*
*
*
Normal
Transgenic

Figure 4

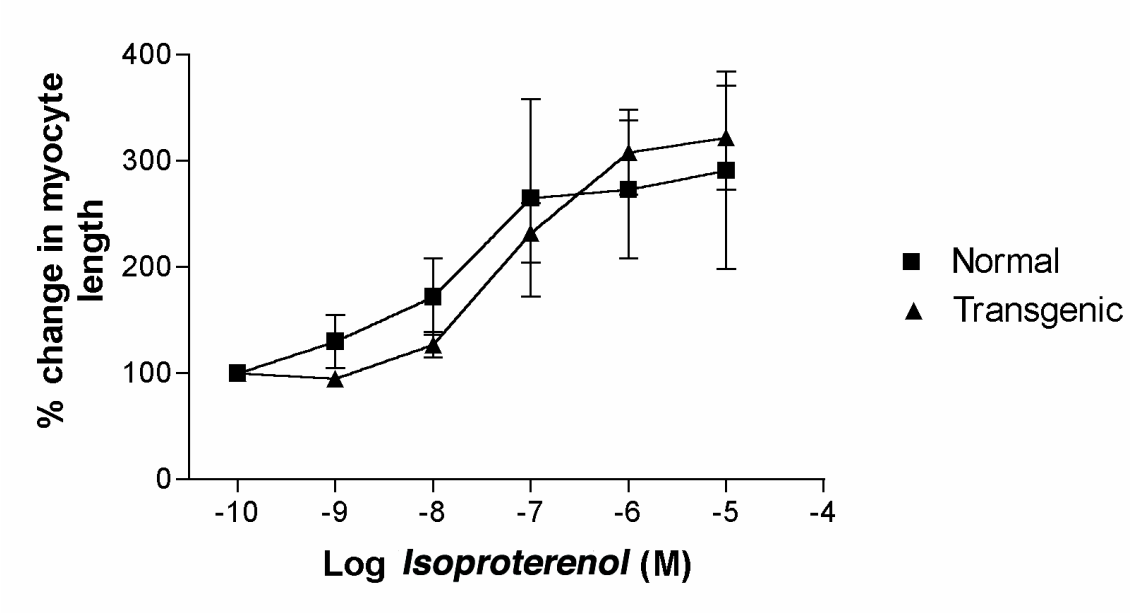
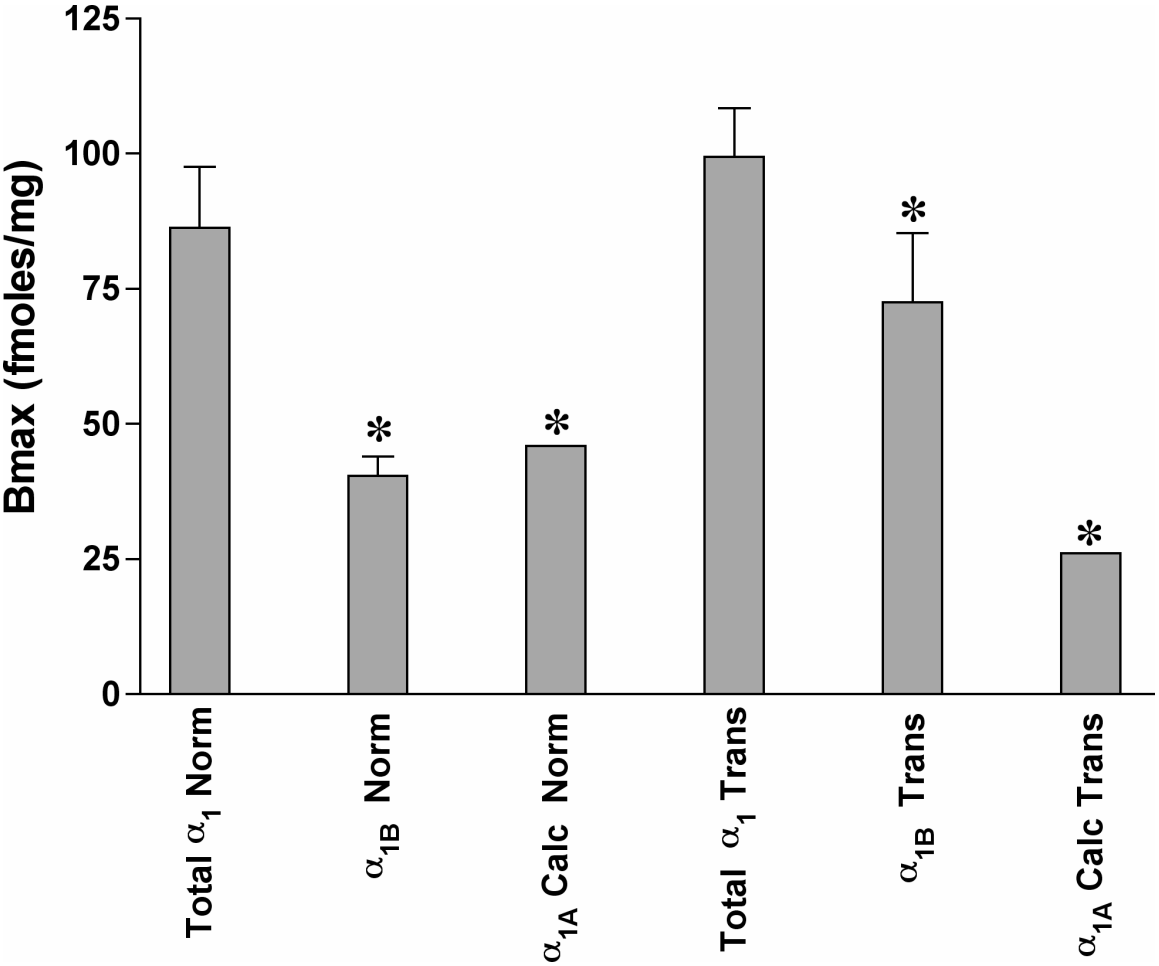

400
300
200
100
0
% change in myocyte length
-10
-9
-8
-7
-6
-5
-4
Log *Isoproterenol* (M)
Normal
Transgenic

Figure 5


Bmax (fmoles/mg)
125
100
75
50
25
0
*
*
*
*
Total α1 Norm
α1B Norm
α1A Calc Norm
Total α1 Trans
α1B Trans
α1A Calc Trans

Appendix G
Contributions to papers

Zuscik, M.J., Chalothorn, D., Hellard, D., Deigham, C., McGee, A., Daly, C.J., Waugh, D.J.J., Ross, S.A., Gaivin, R.J., Morehead, A., Thomas, J.D., Plow, E.F., McGrath, J.C., Piascik, M.T., and Perez, D.M. (2001) Hypotension, Autonomic Failure and Cardiac Hypertrophy in Transgenic Mice Overexpressing the α_{1B}-Adrenergic Receptor. *J. Biol. Chem.* **276**: 13738-13743.

Responsible for generation of the data on the carotid mean arterial pressure (Figure 4).

Ross, S.A., Chalothorn, D., Gonzalez-Cabrera, P.J., Yun, J., Gaivin, R., McCune, D.F., Papay, R., Rorabaugh, B., Piascik, M.T., and Perez, D.M. The Role of the α_{1B}-Adrenergic Receptor in the Inotropic Response of the Mouse Myocardium. In revision at *Am. J. Physiol.*

Responsible for generation of the data related to myocyte contraction (Figures 3 and 4).

References

Aboud, R., Shafii, M., and Docherty, J.R. (1993) Investigation of the subtypes of alpha 1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.* **109**: 80-87.

Adelstein, R.S. and Eisenberg, E. (1980) Regulation and kinetics of the actin-myosin-ATP interaction. *Annu. Rev. Biochem*. **49**: 921-956. Review.

Ahlquist, R.P. (1948) A study of the adrenotropic receptors. *Am. J. Physiol.* **153**: 586-600.

Akhter, S.A., Milano, C.A., Shotwell, K.F., Cho, M.C., Rockman, H.A., Lefkowitz, R.J., and Koch, W.J. (1997) Transgenic mice with overexpression of the alpha1B-adrenergic recptors. In vivo regulation alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signaling. *J. Biol. Chem.* **272**: 21253-21259.

Apkon, M. and Nerbonne, J.M. (1988) Alpha 1-adrenergic agonists selectively suppress voltage-dependent K+ current in rat ventricular myocytes. *Proc. Natl. Acad. Sci. U S A*. **85**: 8756-8760.

Awaji, T., Hirasawa, A., Kataoka, M., Shinoura, H., Nakayama, Y., Sugawara, T., Izumi, S., and Tsujimoto, G. (1998) Real-time optical monitoring of ligand-mediated internalization of alpha1b-adrenoceptor with green fluorescent protein. *Mol. Endocrinol*. **12**: 1099-1111.

Awaji, T., Hirasawa, A., Kataoka, M., Shinoura, H., Yasuhisa, N., Sugawara, T., Izumi, S., and Tsujimoto, G. (1998). Real-time optical monitoring of ligand-mediated internalization of α_{1b}-adrenoceptor with green fluorescent protein. *Mol. Endocrinol.* **12**: 1099-111.

Babich, M., Pedigo, N.W., Butler, B.T., and Piascik, M.T. (1987) Heterogeneity of alpha 1 receptors associated with vascular smooth muscle: evidence from functional and ligand binding studies. *Life Sci*. **41**: 663-673.

Bell, R.M. and Burns, J.D. (1991) Lipid activation of protein kinase C. *J. Biol. Chem*. **266**: 4661-4664. Review.

Benfey, B.G. and Varma, D.R. (1967) Interactions of sympathomimetic drugs, propranolol and phentolamine, on atrial refractory period and contractility. *Br. J. Pharmacol.* **30**: 603-611.

Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc. Natl. Acad. Sci. U S A*. **84**: 8879-8882.

Benovic, J.L., Strasser, R.H., Caron, M.G., and Lefkowitz, R.J. (1986) Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. U S A*. **83**: 2797-2801.

Bernard, C. (1864) Physiological studies on certain American poisons. *La revue des deux mondes*.

Berstein, G., Bilank, J.L., Smrcka, A.V., Higashijima, T., Sternweis, P.C., Exton, J.H., and Ross, E.M. (1992) Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11, and phospholipase C-beta 1. *J. Biol. Chem*. **267**: 8081-8088.

Berthelsen, S. and Pettinger, W.A. (1977) A functional basis for classification of alpha-adrenergic receptors. *Life Sci*. **21**: 595-606.

Besse, J.C. and Furchgott, R.F. (1976) Dissociation constant and relative efficacies of agonists on alpha adrenergic receptors in rabbit aorta. *J. Pharmacol. Expt. Ther.* **197**: 66-78

Bevan, J.A. (1981) A comparison of the contractile responses of the rabbit basilar and pulmonary arteries to sympathomimetic agonists: further evidence for variation in vascular adrenoceptor characteristics. *J. Pharmacol. Exp. Ther.* **216**: 83-89.

Billah, M.M. and Anthes, J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269**: 281-291. Review.

Blaes, N. and Boissel, J.P. (1983) Growth-stimulating effect of catecholamines on rat aortic smooth muscle cells in culture. *J. Cell. Physiol*. **116**: 167-172.

Blank, J.H., Ross, A.H., and Exton, J.H. (1991) Purification and characterization of two G-proteins that activate the beta 1 isozyme of phosphoinositide-specific phospholipase C. Identification as members of the Gq class. *J. Biol. Chem*. **266**: 18206-18216.

Boer, R. Grassegger, A., Schudt, C., and Glossman, H. (1989) (+)-Niguldipine binds with very high affinity to Ca2+ channels and to a subtype of alpha 1-adrenoceptors. *Eur. J. Pharmacol*. **172**: 131-145.

Bogoyevitch, M.A., Marshall, C.J., and Sugden, P.H. (1995) Hypertrophic agonists stimulate the activities of the protein kinases c-Raf and A-Raf in cultured ventricular myocytes. *J. Biol. Chem*. **270:** 26303-26310.

Bohm, M., Schmitz, W., and Scholz, H. (1987) Evidence against a role of a pertussis toxin-sensitive guanine nucleotide-binding protein in the $alpha_1$-adrenoceptor mediated positive inotropic effect in the heart. *Naunyn Schmiedebergs Arch. Pharmacol*. **335**: 476-479.

Braun, A.P., Fedida, D., Clark, R.B., and Giles, W.R. (1990) Intracellular mechanisms for alpha 1-adrenergic regulation of the transient outward current in rabbit atrial myocytes. *J. Physiol*. **431**: 689-712.

Bristow, M.R., Ginsburg, R., Minobe, W., Cubicciotti, R.S., Sageman, W.S., Lurie, K., Billingham, M.E., Harrison, D.C., and Stinson, E.B. (1982) Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N. Engl. J. Med*. **307**: 205-211.

Bristow, M.R., Minobe, W., Rasmussen, R., Hershberger, R.E., and Hoffman, B.B. (1988) Alpha-1 adrenergic receptors in the nonfailing and failing human heart. *J. Pharmacol. Exp. Ther*. **247**: 1039-1045.

Brodde, O.E., Michel, M.C., and Zerkowski, H.R. (1995) Signal transduction mechanisms controlling cardiac contractility and their alterations in chronic heart failure. *Cardiovasc. Res*. **30**: 570-584. Review.

Brown, G.L. and Gillespie, J.S. (1957) The output of sympathetic transmitter from the spleen of the cat. *J. Physiol. (Lond.)*. **138**: 81-102.

Brown, R.D., Prendiville, C., and Cain, C. (1986) Alpha 1-adrenergic and H1-histamine receptor control of intracellular Ca2+ in a muscle cell line: the influence of prior agonist exposure on receptor responsiveness. *Mol. Pharmacol*. **29**: 531-539.

Buckner, S.A., Oheim, K.W., Morse, P.A., Knepper, S.M., and Hancock, A.A. (1996) Alpha 1-adrenoceptor-induced contractility in rat aorta is mediated by the alpha 1D subtype. *Eur. J. Pharmacol*. **297**: 241-248.

Capogrossi, M.C., Kachadorian, W.A., Gambassi, G., Spurgeon, H.A., and Lakatta, E.G. (1991) Ca2+ dependence of alpha-adrenergic effects on the contractile properties and Ca2+ homeostasis of cardiac myocytes. *Circ. Res*. **69**: 540-550.

Carman, C.V. and Benovic, J.L. (1998) G-protein-coupled receptors: turn-ons and turn-offs. *Curr. Opin. Neurobiol*. **8**: 335-344. Review.

Caron, M.G. and Lefkowitz, R.J. (1993) Catecholamine receptors: structure, function, and regulation. *Recent Prog. Horm. Res*. **48**: 277-290. Review.

Cavalli, A., Lattion, A.L., Hummler, E., Nenniger, M., Pedrazzini, T., Aubert, J.F., Michel, M.C., Yang, M., Lembo, G., Vecchione, C., Mostardini, M., Schmidt, A., Beermann, F., and Cotecchia, S. (1997) Decreased blood pressure response in mice deficient of the alpha1b-adrenergic receptor. *Proc. Natl. Acad. Sci. U. S. A*. **94**: 11589-11594.

Chalothorn, D., McCune, D.F., Edelmann, S.E., García-Cazarín, M.L., Tsujimoto, G., and Piascik, M.T. (2002) Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the α_1-Adrenoceptor Subtypes. *Mol. Pharmacol.* **61**: 1008-1016.

Chalothorn, D., McCune, D.F., Edelmann, S.E., Tobita, K., Keller, B.B., Lasley, R.D., Perez, D.M., Tanoue, A., Tsujimoto, G., Post, G.R., and Piascik, M.T. (2003) Differential cardiovascular regulatory activities of the $alpha_{1B}$- and $alpha_{1D}$-adrenoceptor subtypes. *J. Pharmacol. Exp. Ther.*

Chen, C.A. and Manning, D.R. (2001) Regulation of G proteins by covalent modification. *Oncogene*. **20**: 1643-1652. Review.

Chen, L., Xin, X., Eckhart, A.D., Yang, N., and Faber, J.E. (1995) Regulation of vascular smooth muscle growth by alpha 1-adrenoreceptor subtypes in vitro and in situ. *J. Biol. Chem.* **270**: 30980-30988.

Chen, S. Xu, M., Lin, F., Lee, D., Riek, P., and Graham, R.M. (1999) Phe310 in transmembrane VI of the alpha1B-adrenergic receptor is a key switch residue involved in activation and catecholamine ring aromatic bonding. *J. Biol. Chem*. **274**: 16320-16330.

Christiansen, H.B., Horgmo, G.T., Skomedal, T., and Osnes, J.B. (1987) Enhancement of the alpha-adrenergic inotropic component of noradrenaline by simultaneous stimulation of muscarinic acetylcholine receptors in rat myocardium. *Eur. J. Pharmacol*. **142**: 93-102.

Chuang, T.T., LeVine, H., and DeBlasi, A. (1995) Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. *J. Biol. Chem*. **270**: 18660-18665.

Clerk, A., Bogoyevitch, M.A., Anderson, M.B., and Sugden, P.H. (1994) Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. *J. Biol. Chem*. **269**: 32848-32857.

Coates, J. and Weetman, D.F. (1983) Occurrence of alpha 1s-adrenoceptors in the mouse but not in the rabbit isolated anococcygeus preparations. *Br. J. Pharmacol*. **78**: 117-122.

Coates, J., Jahn, U., and Weetman, D.F. (1982) The existence of a new subtype of alpha-adrenoceptor on the rat anococcygeus is revealed by SGD 101/75 and phenoxybenzamine. *Br. J. Pharmacol*. **75**: 549-552.

Cook, S.J. and McCormick, F. (1996) Kinetic and biochemical correlation between sustained p44ERK1 (44 kDa extracellular signal-regulated kinase 1) activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells. *Biochem. J.* **320**: 237-245.

Cotecchia, S., Exum, S., Caron, M.G., and Lefkowitz, R.J. (1990) Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. U.S.A*. **87**: 2896-2900.

Cotecchia, S., Schwinn, D.A., Randall, D.D., Lefkowitz, R.J., Caron, M.G., and Kobilka, B.K. (1988) Molecular cloning and expression of the cDNA for the hamster alpha 1-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 7159-7163.

Cowlen, M.S. and Toews, M.L. (1987) Evidence for alpha 1-adrenergic receptor internalization in DDT1 MF-2 cells following exposure to agonists plus protein kinase C activators. *Mol. Pharmacol*. 34: 340-346.

D' Angelo, D.D., Sakata, Y., Lorenz, J.N., Boivin, G.P., Walsh, R.A., Liggett, S.B., and Dorn, G.W. (1997) Transgenic Gαq overexpression induces cardiac contractile failure in mice. *Proc. Natl. Acad. Sci. USA*. **94**: 8121-8126.

Dale, H. (1906) On some physiological actions of ergot. *J. Physiol. (Lond.).* **34**: 1-35.

Deng, X.F. and Varma, D.R. (1997) Alpha 1D-adrenoceptors do not contribute to inotropic responses of neonatal rat myocardium. *J. Cardiovasc. Pharmacol*. **29**: 57-60.

Deng, X.F., Chemtob, S. Almazan, G., and Varma, D.R. (1996b) Ontogenic differences in the functions of myocardial alpha1 adrenoceptor subtypes in rats. *J. Pharmacol. Exp. Ther*. 276: 1155-1161.

Deng, X.F., Chemtob, S., and Varma, D.R. (1996a) Characterization of alpha 1 D-adrenoceptor subtype in rat myocardium, aorta and other tissues. *Br. J. Pharmacol*. **119**: 269-276.

Deng, X.F., Sculptureanu, A., Mulay, S., Peri, K.G., Li, J.F., Zheng, W.H., Chemtob, S., and Varma, D.R. (1998) Crosstalk between alpha-1A and alpha-1B adrenoceptors in neonatal rat myocardium: implications in cardiac hypertrophy. *J. Pharmacol. Exp. Ther*. **286**: 489-496.

Diviani, D., Lattion, A.L., Larbi, N., Kunapuli, P., Pronin, A., Benovic., J.L., and Cotecchia, S. (1996) Effect of Different G Protein-coupled Receptor Kinases on Phosphorylation and Desensitization of the α_{1B}-Adrenergic Receptor. *J. Biol. Chem.* **271**: 5049-5058.

Docherty, J.R. (1998) Subtypes of functional alpha1- and alpha2-adrenoceptors. *Eur. J. Pharmacol*. **361**: 1-15. Review

Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1991) Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem*. **60**: 653-688. Review.

Drew, G.M. (1985) What do antagonists tell us about alpha-adrenoceptors? *Clin. Sci.* **68**: 15s-19s.

Drew, G.M. and Whiting, S.B. (1979) Evidence for two distinct types of postsynaptic α-AR in vascular smooth muscle *in vivo*. *Br. J. Pharmacol.* **67**: 207-215.

Dubocovich, M.L. and Langer, S.Z. (1974) Negative feed-back regulation of noradrenaline release by nerve stimulation in the perfused cat's spleen: differences in potency of phenoxybenzamine in blocking the pre- and post-synaptic adrenergic receptors. *J. Physiol*. **237**: 505-519.

Dzau, V.J. and Gibbons, G.H. (1993) Vascular remodeling: mechanisms and implications. *J. Cardiovasc. Pharmacol*. **11**: S1-S5.

Ehrlich, P. (1913) Chemotherapeutics: Scientific principles, methods and results. *Lancet.* **2**: 445.

Endoh, M. and Motomura, S. (1979) Differentiation by cholinergic stimulation of positive inotropic actions mediated via alpha- and beta-adrenoceptors in the rabbit heart. *Life Sci*. **25**: 759-768.

Endoh, M. and Yamashita, S. (1980) Adenosine antagonizes the positive inotropic action mediated via beta-, but not alpha-adrenoceptors in the rabbit papillary muscle. *Eur. J. Pharmacol*. **65**: 445-448.

Exton, J.H. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem*. **265**: 1-4. Review.

Fedida, D. and Bouchard, R.A. (1992) Mechanisms for the positive inotropic effect of alpha 1-adrenoceptor stimulation in rat cardiac myocytes. *Circ. Res.* **71**: 673-688.

Fedida, D., Braun, A.P., and Giles, W.R. (1993) Alpha 1-adrenoceptors in myocardium: functional aspects and transmembrane signaling mechanisms. *Physiol. Rev*. **73**: 469-487. Review.

Ferguson, S.S., Barak, L.S., Zhang, J., and Caron, M.G. (1996) G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* **74**: 1095-1110.

Ferguson, S.S., Menard, L., Barak, L.S., Koch, W.J., Colapietro, A.M., and Caron, M.G. (1995) Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1. *J. Biol. Chem*. **270**: 24782-24789.

Flavahan, N.A. and Vanhoutte, P.M. (1986) $Alpha_1$-adrenoceptor subclassification in vascular smooth muscle. *Trends Pharmacol.* **7**: 347-349.

Fliegel, L. and Wang, H. (1997) Regulation of the Na^+/H^+ exchanger in the mammalian myocardium. *J. Mol. Cell. Cardiol.* **29**: 1991-1999.

Fonseca, M.I., Button, D.C., and Brown, R.D. (1995) Agonist regulation of alpha 1B-adrenergic receptor subcellular distribution and function. *J. Biol. Chem*. **270**: 8902-8909.

Forray, C., Bard, J.A., Wetzel., J.M., Chiu, G., Shapiro, E., Tang, R., Lepor, H., Hartig., P.R., Weinshank, R.L., Branchek., T.A., and Gluchowski, C. (1994) The alpha 1-adrenergic receptor that mediates smooth muscle contraction in human prostate has the pharmacological properties of the cloned human alpha 1c subtype. *Mol. Pharmacol*. **45**: 703-708.

Fratelli, M. and DeBlasi, A. (1988) Agonist-induced alpha 1-adrenergic receptor changes. Evidence for receptor sequestration. *FEBS Lett*. **212**: 149-153.

Fronek, K. (1983) Trophic effect of the sympathetic nervous system on vascular smooth muscle. *Ann. Biomed. Eng*. **11**: 607-615.

Gambassi, G., Berenholtz, S., Ziman, B., Lakatta, E.G., and Capogrossi, M.C. (1991) Opposing effects of α_{1A} and α_{1B} receptors on the inotropic response to α_1-adrenergic stimulation in adult rat myocytes. *Circulation*. **84**: II403.

Gambassi, G., Spurgeon, H.A., Lakatta, E.G., Blank, P.S., and Capogrossi, M.C. (1992) Different effects of α- and β-adrenergic stimulation on cytosolic pH and myofilament responsiveness to Ca^{2+} in cardiac myocytes. *Circ. Res*. **71**: 870-882.

Gambassi, G., Spurgeon, H.A., Ziman, B.D., Lakatta, E.G., and Capogrossi, M.C. (1998) Opposing effects of alpha 1-adrenergic receptor subtypes on Ca2+ and pH homeostasis in rat cardiac myocytes. *Am. J. Physiol*. **274**: H1152-H1162.

Gagnon, A.W., Kallal, L., and Benovic, J.L. (1998) Role of clathrin-mediated endocytosis in agonist-induced downregulation of the beta-2-adrenergic receptor. *J. Biol. Chem.* **273**: 6976-6981.

Gao, Y., Ye, L.H., Kishi, H., Okagaki, T., Samizo, K, Nakamura, A., and Kohama, K. (2001) Myosin light chain kinase as a multifunctional regulatory protein of smooth muscle contraction. *IUBMB Life*. **51**: 337-344. Review.

García-Sáinz, J.A. and Torres-Padilla, M.E. (1999) Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblasts stably expressing alpha1d-adrenoceptors. *FEBS Lett*. **443**: 277-281.

García-Sáinz, J.A., Vázquez-Cuevas, F.G., and Romero-Avila, M.T. (2001) Phosphorylation and desensitization of α_{1d}-adrenergic receptors. *Biochem. J.* **353**: 603-610.

García-Sáinz, J.A., Vázquez-Prado, J., and Medina, L.C. (2000) Alpha 1-adrenoceptors: function and phosphorylation. *Eur. J. Pharmacol*. **389**: 1-12. Review.

García-Sáinz, J.A., Vázquez-Prado, J., and Villalobos-Molina, R. (1999) Alpha 1-adrenoceptors: subtypes, signaling, and roles in health and disease. *Arch. Med. Res.* **30**: 449-458. Review.

Gardin, J.M., Siri, F.M., Kitsis, R.N., Edwards, J.G., and Leinwand, L.A. (1995) Echocardiographic Assessment of Left Ventricular Mass and Systolic Function in Mice. *Circ. Res.* **76**: 907-914.

Gether, U. and Kobilka, B.K. (1998) G protein-coupled receptors. II. Mechanism of agonist activation. *J. Biol. Chem*. **273**: 17979-17982. Review.

Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem*. **56**: 615-649. Review.

Gisbert, R., Noguera, M.A., Ivorra, M.D., and D'Ocon, P. (2000) Functional evidence of a constitutively active population of alpha(1D)-adrenoceptors in rat aorta. *J. Pharmacol. Exp. Ther.* **295**: 810-817.

Goodman, O.B., Krupnick, J.G., Gurevich, V.V., Benovic, J.L., and Keen, J.H. (1997) Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J. Biol. Chem*. **272**: 15017-15022.

Goodman, O.B., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H., and Benovic, J.L. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature*. **383**: 447-450.

Goodman, O.B., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H., and Benovic, J.L. (1998) Role of arrestins in G-protein-coupled receptor endocytosis. *Adv. Pharmacol*. **42**: 429-433.

Govier, W.C., Mosal, N.C., Whittington, P., and Broom, A.H. (1966) Myocardial alpha and beta adrenergic receptors as demonstrated by atrial functional refractory-period changes. *J. Pharmacol. Exp. Ther.* **154**: 255-263.

Graham, R.M., Perez, D.M., Hwa, J., and Piascik, M.T. (1996) alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling. *Circ. Res*. **78**: 737-749. Review.

Gross, G., Hanft, G., and Kolassa, N. (1988) Urapidil and some analogues with hypotensive properties show high affinities for 5-hydroxytryptamine (5-HT) binding sites of the 5-HT1A subtype and for alpha 1-adrenoceptor binding sites. *Naunyn. Schmiedebergs Arch. Pharmacol*. **336**: 597-601.

Grupp, I.L., Lorenz, J.N., Walsh, R.A., Boivin, G.P., and Rindt, H. (1998) Overexpression of the α_{1B}-adrenergic receptor induces left ventricular dysfunction in the absence of hypertrophy. *Am. J. Physiol.* **275** (*Heart Circ. Physiol.* **44**): H1338-H1350.

Gurevich, V.V., Dion, S.B., Onorato, J.J., Ptasienski, J., Kim, C.M., Sterne-Marr, R., Hosey, M.M., and Benovic, J.L. (1995) Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, beta 2-adrenergic, and m2 muscarinic cholinergic receptors. J. *Biol. Chem*. **270**: 720-731.

Gutkind, J.S. (1998) Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. *Oncogene*. **17**: 1331-1342. Review.

Han, C., Abel, P.W., and Minneman, K.P. (1987a) Heterogeneity of alpha 1-adrenergic receptors revealed by chlorethylclonidine. *Mol. Pharmacol*. **32**: 505-510.

Han, C., Abel, P.W., and Minneman, K.P. (1987b) Alpha 1-adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca2+ in smooth muscle. *Nature*. **329**: 333-335.

Han, C.D. and Minneman, K.P. (1991) Interaction of subtype-selective antagonists with alpha 1-adrenergic receptor binding sites in rat tissues. *Mol. Pharmacol*. **40**: 531-538.

Hanft, G. and Gross, G. (1989) Subclassification of alpha 1-adrenoceptor recognition sites by urapidil derivatives and other selective antagonists. *Br. J. Pharmacol*. **97**: 691-700.

Harrison, S.N., Autelitano, D.J., Wang, B.H., Milano, C., Du, X.J., and Woodcock, E.A. (1998) Reduced reperfusion-induced Ins(1,4,5)P3 generation and arrhythmias in hearts expressing constitutively active alpha1B-adrenergic receptors. *Circ. Res*. **83**: 1232-1340.

Hausdorff, W.P., Caron, M.G., and Lefkowitz, J.R. (1990) Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J.* **4**: 2881-2889. Review.

Hieble, J.P., Bylund, D.B., Clarke, D.E., Eikenburg, D.C., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., and Ruffolo, R.R. (1995) International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. *Pharmacol. Rev*. **47**: 267-270. Review.

Hirasawa, A., Sugawara, T., Awaji, T., Tsumaya, K., Ito, H., and Tsujimoto, G. (1997) Subtype-specific differences in subcellular localization of alpha1-adrenoceptors:

chlorethylclonidine preferentially alkylates the accessible cell surface alpha1-adrenoceptors irrespective of the subtype. *Mol. Pharmacol*. **52**: 764-770.

Hoffman, B.B. and Hu, Z.W. (2000) Alpha(1)-adrenoceptors (alpha(1)-AR) and vascular smooth muscle cell growth. *Prostate* **9**: 29-33. Review.

Holck, M.I., Jones, C.H.M., and Haeusler, G. (1983) Differential interactions of clonidine and methoxamine with the postsynaptic alpha-adrenoceptor of rabbit main pulmonary artery. *J. Cardiovasc. Pharmacol.* **5**: 240-248.

Horie, K., Obika, K., Folgar, R., and Tsujimoto, G. (1995) Selectivity of the imidazoline alpha-adrenoceptor agonists (oxymetazoline and cirazoline) for human cloned alpha 1-adrenoceptor subtypes. *Br. J. Pharmacol*. **116**: 1611-1618.

Hrometz, S.L., Edelmann, S.E., McCune, D.F., Olges, J.R., Hadley, R.W., Perez, D.M., and Piascik, M.T. (1999) Expression of multiple alpha1-adrenoceptors on vascular smooth muscle: correlation with the regulation of contraction. *J. Pharmacol. Exp. Ther*. **290**: 4524-63.

Hughes, R.J. and Insel, P.A. (1986) Agonist-mediated regulation of alpha 1- and beta 2-adrenergic receptor metabolism in a muscle cell line, BC3H-1. *Mol. Pharmacol*. **29**: 521-530.

Hughes, R.J., Boyle, M.R., Brown, R.D., Taylor, P., and Insel, P.A. (1982) Characterization of coexisting alpha 1- and beta 2-adrenergic receptors on a cloned muscle cell line, BC3H-1. *Mol. Pharmacol*. **22**: 258-266.

Hunter, J.J., Tanaka, N., Rockman, H.A., Ross, J., and Chien, K.R. (1995) Ventricular Expression of a MLC-2v-ras Fusion Gene Induces Cardiac Hypertrophy and Selective Diastolic Dysfunction in Transgenic Mice. *J. Biol. Chem.* **270**: 23173-23178.

Hwa, J. and Perez, D.M. (1996) The unique nature of the serine interactions for alpha 1-adrenergic receptor agonist binding and activation. *J. Biol. Chem*. **271**: 6322-6327.

Hwa, J., Gaivin, R.J., Porter, J.E., and Perez, D.M. (1997) Synergism of constitutive activity in alpha 1-adrenergic receptor activation. *Biochemistry*. **36**: 633-639.

Hwa, J., Graham, R.M., and Perez, D.M. (1995) Identification of critical determinants of alpha 1-adrenergic receptor subtype selective agonist binding. *J. Biol. Chem*. **270**: 23189-23195.

Hwa, J., Graham, R.M., and Perez, D.M. (1996) Chimeras of alpha1-adrenergic receptor subtypes identify critical residues that modulate active state isomerization. *J. Biol. Chem.* **271**: 7956-7964.

Ikeda, U., Tsuruya, T., and Yaginuma, T. (1991) Alpha 1-adrenergic stimulation is coupled to cardiac myocyte hypertrophy. *Am. J. Physiol*. **260**: H953-H956.

Im, M.J., Rieck, R.P., and Graham, R.M. (1990) A novel guanine nucleotide-binding protein coupled to the alpha 1-adrenergic receptor. II. Purification, characterization, and reconstitution. *J. Biol. Chem*. **265**: 18952-18960.

Inayatulla, A., Li, D.Y., Chemtob, S., and Varma, D.R. (1994) Ontogeny of positive inotropic responses to sympathomimetic agents and of myocardial adrenoceptors in rats. *Can. J. Physiol. Pharmacol*. **72**: 361-367.

Inglese, J., Freedman, N.J., Koch, W.J., and Lefkowitz, R.J. (1993) Structure and mechanism of the G-protein-coupled receptor regulation. *J. Biol. Chem.* **268**: 23735-23738.

Insel, P.A., Weiss, B.A., Slivka, S.R., Howard, M.J., Waite, J.J., and Godson, C.A. (1991) Regulation of phospholipase A2 by receptors in MDCK-Dl cells. *Biochem. Soc. Trans*. **19**: 329-333. Review.

Jackson, C.L. and Schwartz, S.M. (1992) Pharmacology of smooth muscle cell replication. *Hypertension*. **20**: 713-736. Review.

Johnson, R.D. and Minneman, K.P. (1987) Differentiation of alpha 1-adrenergic receptors linked to phosphatidylinositol turnover and cyclic AMP accumulation in rat brain. *Mol. Pharmacol*. **31**: 239-246.

Kennedy, M.E. and Limbird, L.E. (1993) Mutations of the alpha 2A-adrenergic receptor that eliminate detectable palmitoylation do not perturb receptor-G-protein coupling. *J. Biol. Chem*. **268**: 8003-8011.

Kenny, B.A., Chalmers, D.H., Philpott, P.C., and Naylor, A.M. (1995) Characterization of an alpha 1D-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. *Br. J. Pharmacol*. **115**: 981-986.

Kjelsberg, M.A., Cotecchia, S., Ostrowski, J., Caron, M.G., and Lefkowitz, R.J. (1992) Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J. Biol. Chem*. **267**: 1430-1433.

Knepper , S.M., Buckner, S.A., Brune, M.E., DeBernardis, J.F., Meyer, M.D., and Hancock, A.A. (1995) A-61603, a potent alpha 1-adrenergic receptor agonist, selective for the alpha 1A receptor subtype.
J. Pharmacol. Exp. Ther. **274**: 97-103.

Knowlton, K.U., Michel, M.C., Itani, M., Shubeita, H.E., Ishihara, K., Brown, J.H., and Chien, K.R. (1993) The alpha 1A-adrenergic receptor subtype mediates biochemical,

molecular, and morphologic features of cultured myocardial cell hypertrophy. *J. Biol. Chem*. **268**: 15374-15380.

Kraft and Anderson (1983) Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. *Nature*. **301**: 621-623.

Krupnick, J.G. and Benovic, J.L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol*. **38**: 289-319. Review.

Krupnick, J.G., Goodman, O.B., Keen, J.H., and Benovic, J.L. (1997a) Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. *J. Biol. Chem*. **272**: 15011-15016.

Krupnick, J.G., Santini, F., Gagnon, A.W., Keen, J.H., and Benovic, J.L. (1997b) Modulation of the arrestin-clathrin interaction in cells. Characterization of beta-arrestin dominant-negative mutants. *J. Biol. Chem*. **272**: 32507-32512.

Kusiak, J.W., Pitha, J., and Piascik, M.T. (1989) Interaction of a chemically reactive prazosin analog with alpha-1 adrenoceptors of rat tissues. *J. Pharmacol. Exp. Ther*. **249**: 70-77.

Lachnit, W.G., Tran, A.M., Clarke, D.E., and Ford, A.P. (1997) Pharmacological characterization of an alpha 1A-adrenoceptor mediating contractile responses to noradrenaline in isolated caudal artery of rat. *Br. J. Pharmacol*. **120**: 819-826.

Langley, J.N. (1878) On the physiology of the salivary secretion. *J. Physiol. (Lond.)*. **1**: 339-369.

Langley, J.N. (1905) On the reaction of cells and of nerve endings to certain poisons, chiefly as regards to the reaction of striated muscle to nicotines and curare. *J. Physiol. (Lond.)* **33**: 163-206.

Lattion, A.L., Diviani, D., and Cotecchia., S. Truncation of the Receptor Carboxyl Terminus Impairs Agonist-dependent Phosphorylation and Desensitization of the α_{1B}-Adrenergic Receptor. *J. Biol. Chem*. **269**: 22887-22893.

Laz, T.M., Forray, C., Smith, K.E., Bard, J.A., Vaysse, P.J., Branchek, T.A., and Weinshank, R.L. (1994) The rat homologue of the bovine alpha 1c-adrenergic receptor shows the pharmacological properties of the classical alpha 1A subtype. *Mol. Pharmacol*. **46**: 414-422.

Lazou, A., Bogoyevitch, M.A., Clerk, A., Fuller, S.J., Marshall, C.J.,and Sugden, P.H. (1994) Regulation of mitogen-activated protein kinase cascade in adult rat heart preparations in vitro. *Circ. Res*. **75**: 932-941.

Leeb-Lundberg, L.M., Cotecchia, S., DeBlasi, A., Caron, M.G., and Lefkowitz, R.J. (1987) Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of alpha 1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT1 MF-2 smooth muscle cells. *J. Biol. Chem*. **262**: 3098-3105.

Lefkowitz, R.J. (1996) G-protein-coupled receptors: new roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* **273**: 18677-18680. Minireview.

Lemire, I., Allen, B.G., Rindt, H., and Hebert, T.E. (1998) Cardiac-specific overexpression of alpha1BAR regulates betaAR activity via molecular crosstalk. *J. Mol. Cell. Cardiol*. **30**: 1827-1839.

Lemire, I., Ducharme, A., Tardif, J.C., Poulin, F., Jones, L.R., Allen, B.G., Hebert, T.E., and Rindt, H. (2001) Cardiac-directed overexpression of wild-type alpha1B-adrenergic receptor induces dilated cardiomyopathy. *Am. J. Physiol. Heart Circ. Physiol*. **281**: H931-H938.

Li, K., He, H., Li, C., Sirois, P., and Rouleau, J.L. (1997) Myocardial alpha1-adrenoceptor: inotropic effect and physiologic and pathologic implications. *Life Sci*. **60**: 1305-1318. Review.

Llahi, S. and Fain, J.N. (1992) Alpha 1-adrenergic receptor-mediated activation of phospholipase D in rat cerebral cortex. *J. Biol. Chem*. **267**: 3679-3685.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science*. 248: 1547-1550.

Lomasney, J.W., Cotecchia, S., Lorenz, W., Leung, W.Y., Schwinn, D.A., Yang-Feng, T.L, Brownstein, M., Lefkowitz, R.J., and Caron, M.G. (1991) Molecular cloning and expression of the cDNA for the alpha 1A-adrenergic receptor. The gene for which is located on human chromosome 5. *J. Biol. Chem*. **266**: 6365-6369.

Long, C.S., Ordahl, C.P., and Simpson, P.C. (1989) Alpha 1-adrenergic receptor stimulation of sarcomeric actin isogene transcription in hypertrophy of cultured rat heart muscle cells. *J. Clin. Invest*. **83**: 1078-1082.

Lurie, K.B., Tsujimoto, G., and Hoffman, B.B. (1985) Desensitization of alpha-1 adrenergic receptor-mediated vascular smooth muscle contraction. *J. Pharmacol. Exp. Ther*. **234**: 147-152.

MacKenzie, J.F., Daly, C.J., Pediani, J.D., and McGrath, J.C. (2000) Quantitative imaging in live human cells reveals intracellular α_1-adrenoceptor ligand-binding sites. *J. Pharmacol. Expt. Ther.* **294**: 434-443.

Majesky, M.W., Daemen, M.J., and Schwartz, S.M. (1990) Alpha 1-adrenergic stimulation of platelet-derived growth factor A-chain gene expression in rat aorta. J. Biol. Chem. **265**: 1082-1088.

McCloskey, D.T., Rokosh, D.G., O'Connell, T.D., Keung, E.C., Simpson, P.C., and Baker, A.J. (2002) Alpha(1)-adrenoceptor subtypes mediate negative inotropy in myocardium. *J. Mol. Cell. Cardiol.* **34**: 1007-1017.

McCune, D.F., Edelmann, S.E., Olges, J.R., Post, G.R., Waldrop, B.A., Waugh, D.J.J., Perez, D.M., and Piascik, M.T. (2000) Regulation of the cellular localization and signaling properties of the alpha(1B)- and alpha(1D)-adrenoceptors by agonists and inverse agonists. *Mol. Pharmacol*. **57**: 659-666.

McGrath, J.C. (1982) Evidence for more than one type of post-junctional alpha-adrenoceptor. *Biochem. Pharmacol*. **31**: 467-484. Review.

Medgett, I.C. and Langer, S.Z. (1984) Heterogeneity of smooth muscle alpha adrenoceptors in rat tail artery in vitro. *J. Pharmacol. Exp. Ther.* **229**: 823-830.

Meidell, R.S., Sen, A., Henderson, S.A., Slahetka, M.F., and Chien, K.R. (1986) Alpha 1-adrenergic stimulation of rat myocardial cells increases protein synthesis. *Am .J. Physiol*. **251**: H1076-H1084.

Mhaouty-Kodja, S., Barak, L.S., Scheer, A., Liliane, A., Diviani, D., Caron, M.G., and Cotecchia, S. (1999) Constitutively active alpha-1b adrenergic receptor mutants display different phosphorylation and internalization features. *Mol. Pharmacol*. **55**: 339-347.

Michel, M.C., Hanft, G., and Gross, G. (1994) Functional studies on alpha 1-adrenoceptor subtypes mediating inotropic effects in rat right ventricle. *Br. J. Pharmacol*. **111**: 539-546.

Michel, M.C., Knowlton, K.U., Gross, G., and Chien, K.R. (1990) α_1-Adrenergic receptor subtypes mediate distinct functions in adult rat heart. *Circulation* **82**: III561.

Milano, C.A., Dolber, P.C., Rockman, H.A., Bond, R.A., Venable, M.E., Allen, L.F., and Lefkowitz, R.J. (1994) Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. Proc. *Natl. Acad. Sci. U S A*. **91**: 10109-10113.

Milligan, G., Shah, B.H., Mullaney, I., and Grassie, M.A. (1995) Biochemical approaches to examine the specificity of interactions between receptors and guanine nuclotide binding proteins. *J. Recept. Signal. Transduct. Res*. **15**: 253-265. Review

Minden, A., and Karin, M. (1997) Regulation and function of the JNK subgroup of MAP kinases. *Biochem. Biophys. Acta*. **1333**: F85-F104. Review.

Minneman, K.P. (1988) Alpha 1-adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca2+. *Pharmacol. Rev*. **40**: 87-119. Review.

Minneman, K.P. and Esbenshade, T.A. (1994) Alpha 1-adrenergic receptor subtypes. *Ann. Rev. Pharmacol. Toxicol.* **34**: 117-133. Review.

Minneman, K.P., Han, C., and Abel, P.W. (1988) Comparison of alpha 1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB 4101. *Mol. Pharmacol*. **33**: 509-514.

Morano, I., Hofmann, F., Zimmer, M., and Ruegg, J.C. (1985) The influence of P-light chain phosphorylation by myosin light chain kinase on the calcium sensitivity of chemically skinned heart fibres. *FEBS Lett*. **189**: 221-224.

Morrow, A.L. and Creese, I. (1986). Characterization of α_1-adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H]WB4104 and [^{3}H]prazosin binding. *Mol. Pharmacol*. **29**: 321-330.

Nakaki, T., Nakayama, M., Yamamoto, S., and Kato, R. (1990) Alpha 1-adrenergic stimulation and beta 2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol*. **37**: 30-36.

Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J., and Graham, R.M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science*. **264**: 1593-1596.

Nichols, A.J. and Ruffolo, R.R. (1991) Functions mediated by α-adrenoceptors. *Prog. Basic Clin. Pharmacol.* **8**: 115-179. Review.

Nishimura, J., Khalil, R.A., Drenth, J.P., and Van Breeman, C. (1990) Evidence for increased myofilament Ca2+ sensitivity in norepinephrine-activated vascular smooth muscle. *Am. J. Physiol*. **259**: H2-H8.

Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*. **258**: 607-614. Review.

Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J*. **9**: 484-496. Review.

Noguera, M.A., Calatayud, S., and D'Ocon, M.P. (1993) Actions of alpha-adrenoceptor blocking agents on two types of intracellular calcium stores mobilized by noradrenaline in rat aorta. *Naunyn Schmiedeberg's Arch. Pharmacol.* **348**: 472-7.

O'Dowd, B.F., Hantowich, M., Caron, M.G., Lefkowitz, R.L., and Bouvier, M. (1989) Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the

carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J. Biol. Chem*. **264**: 7564-7569.

O'Dowd, B.F., Lefkowtiz, R.J., and Caron, M.G. (1989) Structure of the adrenergic and related receptors. *Annu. Rev. Neurosci*. **12**: 67-83. Review.

O'Rourke, B., Reibel, D.K., and Thomas, A.P. (1992) Alpha-adrenergic modification of the Ca2+ transient and contraction in single rat cardiomyocytes. *J. Mol. Cell. Cardiol*. **24**: 809-820.

Oakley, R.H., Laporte, S.A., Holt, J.A., Caron, M.G., and Barak, L.S. (2000) Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem*. **275**: 17201-17210.

Orsini, M.J. and Benovic, J.L. (1998) Characterization of dominant negative arrestins that inhibit beta2-adrenergic receptor internalization by distinct mechanisms. *J. Biol. Chem*. **273**: 34616-34622.

Oshita, M., Kigoshi, S., and Muramatsu, I. (1993) Pharmacological characterization of two distinct alpha 1-adrenoceptor subtypes in rabbit thoracic aorta. *Br. J. Pharmacol*. **108**: 1071-1076.

Otani, H., Otani, H., and Das, D.K. (1988) Alpha 1-adreonceptor-mediated phosphoinositide breakdown and inotropic response in rat ventricular papillary muscles. *Circ. Res*. **62**: 9-17.

Pei, G., Samama, P., Lohse, M., Wang, M., Codina, J., and Lefkowitz, R.J. (1994) A consititutively active mutant β_2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc. Natl. Acad. Sci. U.S.A*. **91**: 2699-2702.

Perez, D.M., DeYoung, M.B., and Graham, R.M. (1993) Coupling of expressed alpha 1B- and alpha 1D-adrenergic receptor to multiple signaling pathways is both G protein and cell type specific. *Mol. Pharmacol*. **44**: 784-795.

Perez, D.M., Hwa, J., Gaivin, R., Mathur, M., Brown, F., and Graham, R.M. (1996) Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein-coupled receptor. *Mol. Pharmacol*. **49**: 112-122.

Perez, D.M., Piascik, M.T., and Graham, R.M. (1991) Solution-phase library screening for the identification of rare clones: isolation of an alpha 1D-adrenergic receptor cDNA. *Mol. Pharmacol*. **40**: 876-883.

Perez, D.M., Piascik, M.T., Malik, N., Gaivin, R., and Graham, R.M. (1994) Cloning, expression, and tissue distribution of the rat homolog of the bovine alpha 1C-adrenergic

receptor provide evidence for its classification as the alpha 1A subtype. *Mol. Pharmacol*. **46**: 823-831.

Piascik, M.T., Butler, B.T., Pruitt, T.A., and Kusiak, J.W. (1990) Agonist interaction with alkylation-sensitive and -resistant alpha-1 adrenoceptor subtypes. *J. Pharmacol. Exp. Ther*. **254**: 982-991.

Piascik, M.T., Guarino, R.D., Smith, M.S., Soltis, E.E., Saussy, D.L., and Perez, D.M. (1995) The specific contribution of the novel alpha-1D adrenoceptor to the contraction of vascular smooth muscle. *J. Pharmacol. Exp. Ther*. **275**: 1583-1539.

Piascik, M.T., Hrometz, S.L., Edelmann, S.E., Guarino, R.D., Hadley, R.W., and Brown, R.D. (1997) Immunocytochemical localization of the alpha-1B adrenergic receptor and the contribution of this and the other subtypes to vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. *J. Pharmacol. Exp. Ther*. **283**: 854-868.

Piascik, M.T., Kusiak, J.W., Pitha, J., Butler, B.T., and Babich, M. (1988) Alkylation of alpha-1 receptors with a chemically reactive analog of prazosin reveals low affinity sites for norepinephrine in rabbit aorta. *J. Pharmacol. Exp. Ther*. **246**: 1001-1011.

Piascik, M.T. and Perez, D.M. (2001) α_1-Adrenergic Receptors: New Insights and Directions. *J. Pharmacol. Expt. Ther.* **298**: 403-410.

Piascik, M.T., Smith, M.S., Solitis, E.E., and Perez, D.M. (1994) Identification of the mRNA for the novel alpha 1D-adrenoceptor and two other alpha 1-adrenoceptors in vascular smooth muscle. *Mol. Pharmacol*. **46**: 30-40.

Piascik, M.T., Sparks, M.S., and Pruitt, T.A. (1991) Interaction of imidazolines with alkylation-sensitive and -resistant alpha-1 adrenoceptor subtypes. *J. Pharmacol. Exp. Ther*. **258**: 158-165.

Piascik, M.T., Sparks, M.S., Pruitt, T.A., and Soltis, E.E. (1991) Evidence for a complex interaction between the subtypes of the alpha 1-adrenoceptor. *Eur. J. Pharmacol*. **199**: 279-289.

Ping, P. and Faber, J.E. (1993) Characterization of alpha-adrenoceptor gene expression in arterial and venous smooth muscle. *Am. J. Physiol.* **265**: H1501-H1509.

Pitcher, J.A., Freedman, N.J., and Lefkowitz, R.J. (1998) G protein-coupled receptor kinases. *Annu. Rev. Biochem*. **67**: 653-692. Review.

Pombo, J.F., Troy, B.L., and Russell, R.O. (1971) Left ventricular volumes and ejection fraction by echocardiography. *Circulation.* **43**: 480-490.

Porter, J.E., Hwa, J., and Perez, D.M. (1996) Activation of the alpha1b-adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint. *J. Biol. Chem*. **271**: 28318-28323.

Premont, R.T., Inglese, J., and Lefkowitz, R.J. (1995) Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J*. **9**: 175-182. Review.

Price, D.T., Chari, R.S., Berkowitz, D.E., Meyers, W.C., and Schwinn, D.A. (1994a) Expression of alpha 1-adrenergic receptor subtype mRNA in rat tissues and human SK-N-MC neuronal cells: implications for alpha 1-adrenergic receptor subtype classification. *Mol. Pharmacol*. **46**: 221-226.

Price, D.T., Lefkowitz, R.J., Caron, M.G., Berkowitz, D., and Schwinn, D.A. (1994) Localization of mRNA for three distinct alpha 1-adrenergic receptor subtypes in human tissues: implications for human alpha-adrenergic physiology. *Mol. Pharmacol*. **45**: 171-175.

Puceat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L.L., and Brown, J.H. (1994) Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J. Biol. Chem*. **269**: 16938-16944

Ramirez, M.T., Sah, V.P., Zhao, X.L., Hunter, J.J., Chien, K.R., and Brown, J.H. (1997) The MEKK-JNK pathway is stimulated by alpha1-adrenergic receptor and ras activation and is associated with in vitro and in vivo cardiac hypertrophy. *J. Biol. Chem*. **272**: 14057-14061.

Robberecht, P., Delhaye, M., Taton, G., De Neef, P., Waelbroeck, M., De Smet, J.M., Leclerc, J.L., Chatelain, P., and Christophe, J. (1983) The Human Heart β-Adrenergic Receptors. *Mol. Pharmacol*. **24**: 169-173.

Rokosh, D.G. and Sulakhe, P.V. (1991) Characteristics of alpha$_1$-adrenoceptors coupled to inotropic response and phosphoinositide metabolism in rat myocardium. *Circulation*. **84**: II389.

Rokosh, D.G., Stewart, A.F., Chang, K.C., Bailey, B.A., Karliner, J.S., Camacho, S.A., Long, C.S., and Simpson, P.C. (1994) Distribution of alpha 1C-adrenergic receptor mRNA in adult rat tissues by RNase protection assay and comparison with alpha 1B and alpha 1D. *Biochem. Biophys. Res. Commun*. **200**: 1177-1184.

Ross, S.A., Chalothorn, D., Yun, J., Gonzalez-Cabrera, P.J., McCune, D.F., Rorabaugh, B., Piascik, M.T., and Perez, D.M. The Role of the α_{1B}-Adrenergic Receptor in the Inotropic Response of the Mouse Myocardium. In revision at *Am. J. Physiol.*

Rudner, X.L., Berkowitz, D.E., Booth, J.V., Funk, B.L., Cozart, K.L., D'Amico, E.B., El-Moalem, H., Page, S.O., Richardson, C.D., Winters, B., Marucci, L., and Schwinn, D.A.

(1999) Subtype specific regulation of human vascular alpha(1)-adrenergic receptors by vessel bed and age. *Circulation.* **100**: 2336-2343.

Ruffolo, R.R., Motley, E.D., and Hieble, J.P. (1991) The effect of pertussis toxin on alpha 1-adrenoceptor-mediated vasoconstriction by the full agonist, cirazoline, and the partial agonist, (-)-dobutamine, in pithed rats. *Fundam. Clin. Pharmacol*. **5**: 11-23.

Ruffolo, R.R., Turowski, B.S., and Patil, P.N. (1977) Lack of cross-desensitization between structurally dissimilar alpha-adrenoceptor agonists. *J. Pharm. Pharmacol*. **29**: 378-380.

Saussy, D.L., Goetz, A.S., Queen, K.L., King, H.K., Lutz, M.W., and Rimele, T.J. (1996) Structure activity relationships of a series of buspirone analogs at alpha-1 adrenoceptors: further evidence that rat aorta alpha-1 adrenoceptors are of the alpha-1D-subtype. *J. Pharmacol. Exp. Ther*. **278**: 136-144.

Savarese, T.M. and Fraser, C.M. (1992) In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem. J.* **283**: 1-19. Review.

Sayet, I., Neuilly, G., Rakotoarisoa, L., Mironneau, C., and Mironneau, J. (1993) Relation between alpha 1-adrenoceptor subtypes and noradrenaline-induced contraction in rat portal vein smooth muscle. *Br. J. Pharmacol.* **110**: 207-212.

Schluter, K.D. and Piper, H.M. (1999) Regulation of growth in the adult cardiomyocytes. *FASEB J*. **13**: S17-22. Review.

Schwinn, D.A., Lomasney, J.W., Lorenz, W., Szklut, P.J., Fremeau, R.T., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J., and Cotecchia, S. (1990) Molecular cloning and expression of the cDNA for a novel alpha 1-adrenergic receptor subtype. *J. Biol. Chem*. **265**: 8183-8189.

Schwinn, D.A., Page, S.O., Middleton, J.P., Lorenz, W., Liggett, S.B., Yamamoto, K., Lapetina, E.G., Caron, M.G., Lefkowitz, R.J., and Cotecchia, S. (1991) The alpha1C-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.* **40**: 619-626.

Schwinn, D.A. and Price, R.R. (1999) Molecular pharmacology of human alpha1-adrenergic receptors: unique features of the alpha 1a-subtype. *Eur. Urol*. **36**: Suppl 1:7-10.

Scofield, M.A., Liu, F., Abel, P.W., and Jeffries, W.B. (1995) Quantification of steady state expression of mRNA for alpha-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. *J. Pharmacol. Exp. Ther*. **275**: 1035-1042.

Shah, A., Cohen, I.S., and Rosen, M.R. (1988) Stimulation of cardiac α-receptors increases Na/K pump current and decreases g_k via a pertussis-sensitive pathway. *Biophys*. **54**: 219-225.

Shukla, S.D. and Halenda, S.P. (1991) Phospholipase D in cell signalling and its relationship to phospholipase C. *Life Sci*. **48**: 851-866. Review.

Simpson, P. (1983) Norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an alpha 1 adrenergic response. *J. Clin. Invest*. **72**: 732-738.

Simpson, P. (1985) Stimulation of hypertrophy of cultured neonatal rat heart cells through an alpha 1-adrenergic receptor and induction of beating through an alpha 1- and beta 1-adrenergic receptor interaction. Evidence for independent regulation of growth and beating. *Circ. Res*. **56**: 884-894.

Simpson, P.C. (1988) Role of proto-oncogenes in myocardial hypertrophy. *Am. J. Cardiol*. **62**: 13G-19G. Review

Siwik, D.A. and Brown, R.D. (1996) Regulation of protein synthesis by alpha 1-adrenergic receptor subtypes in cultured rabbit aortic vascular smooth muscle cells. *J. Cardiovasc. Pharmacol*. **27**: 508-518.

Skomedal, T., Aass, H., and Osnes, J.B. (1980) Competitive blockade of α-adrenergic receptors in rat heart by prazosin. *Acta. Pharmacol. Toxicol.* **47**: 217-222.

Skomedal, T., Aass, H., and Osnes, J.B. (1983) Qualitative differences between the inotropic responses in rat papillary muscles to α-adrenoceptor and β-adrenoceptor stimulation by both noradrenaline and adrenaline. *Acta. Pharmacol. Toxicol*. **52**: 57-67.

Skomedal, T., Schiander, I.G., and Osnes, J.B. (1988) Both alpha and beta adrenoceptor-mediated components contribute to final inotropic response to norepinephrine in rat heart. *J. Pharmacol. Exp. Ther.* **247**: 1204-1210.

Slivka, S.R. and Insel, P.A. (1987) Alpha 1-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E2 formation in Madin-Darby canine kidney cells. Possible parallel activation of phospholipase C and phospholipase A2. *J. Biol. Chem*. **262**: 4200-4207.

Starke, K. (1977) Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.* **77**: 1-124. Review.

Steinberg, S.F., Chow, Y.K., Robinson, R.B., and Bilezikian, J.P. (1987) A pertussis toxin substrate regulates α_1-adrenergic dependent phosphatidylinositol hydrolysis in cultured rat myocytes. *Endocrinology*. **120**: 1889-1895.

Steinberg, S.F., Drugge, E.D., Bilezikian, J.P., and Robinson, R.B. (1985) Acquisition by innervated cardiac myocytes of a pertussis toxin-specific regulatory protein linked to the α_1-receptor. *Science*. **230**: 186-188.

Strader, C.D., Candelore, M.R., Rands, E., Hill, W.S., and Dixon, R.A. (1989) Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J. Biol. Chem*. **264**: 13572-13578.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D., and Dixon, R.A. (1994) Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem*. **63**: 101-132. Review.

Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S., and Dixon, R.A. (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J. Biol. Chem*. **263**: 10267-10271.

Strader, C.D., Sigal, I.S., Register, R.B., Candelore, M.R., Rands, E., and Dixon, A.F. (1987) Identification of residues required for ligand binding to the beta-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A*. **84**: 4384-4388.

Tanoue, A., Nasa, Y., Koshimizu, T., Shinoura, H., Oshikawa, S., Kawai, T., Sunada, S., Takeo, S., and Tsujimoto, G. (2002). The α_{1D}-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction. *J.Clin. Invest.* **109**: 765-775.

Terzic, A., Puceat, M., Clement, O., Scamps, F., and Vassort, G. (1992) Alpha 1-adrenergic effects on intracellular pH and calcium and on myofilaments in single rat cardiac cells. *J. Physiol*. **447**: 275-292.

Terzic, A., Puceat, M., Vassort, G., and Vogel, S.M. (1993) Cardiac α_1-Adrenoceptors: An Overview. *Pharmacol. Rev.* **45**: 147-175 Review.

Testa, R., Destefani, C., Guarneri, L., Poggesi, E., Simonazzi, I., Taddei, C., and Leonardi, A. (1995) The alpha 1d-adrenoceptor subtype is involved in the noradrenaline-induced contractions of rat aorta. *Life Sci*. **57**: PL159-PL163.

Theroux , T.L., Esbenshade, T.A., Peavy, R.D., and Minneman, K.P. (1996) Coupling efficiencies of human alpha 1-adrenergic receptor subtypes: titration of receptor density and responsiveness with inducible and repressible expression vectors. *Mol. Pharmacol*. **50**: 1376-1387.

Tian, N.W., Gupta, S., and Deth, R.C. (1990) Species differences in chlorethylclonidine antagonism at vascular alpha-1 adrenergic receptors. *J. Pharmacol. Exp. Ther*. **253**: 877-883.

Timmermans, P.B. and Van Zwieten, P.A. (1980) Postsynaptic alpha 1- and alpha 2-adrenoceptors in the circulatory system of the pithed rat: selective stimulation of the alpha 2-type by B-HT 933. *Eur. J. Pharmacol*. **63**: 199-202.

Timmermans, P.B., Thoolen, M.J., Mathy, M.J., Wilffert, B., De Jonge, A., and Van Zwieten, P.A. (1983) Sgd 101/75 is distinguished from other selective alpha 1-adrenoceptor agonists by the inhibition of its pressor responses by calcium entry blockade and vasodilatation in pithed rats and cats. *Eur. J. Pharmacol*. **96** :187-192.

Tsujimoto, G., Bristow, M.R., and Hoffman, B.B. (1984) Identification of alpha 1 adrenergic receptors in rabbit aorta with [125I] BE2254. *Life Sci*. **34**: 639-646.

Turnbull, L., McCloskey, D.T., O'Connell, T.D., Simpson, P.C., and Baker, A.J. (2003) α_1-adrenergic receptor (AR) responses in $\alpha_{1A/B}$-AR knockout mouse hearts suggests presence of α_{1D}-AR. *Am. J. Physiol. Heart Circ. Physiol.* **284**: H1104-H1109.

Van Meel, J.C., De Jonge, A., Kalkman, H.O., Wilffert, B., Timmermans, P.B., and Van Zwieten, P.A. (1981) Organic and inorganic calcium antagonists reduce vasoconstriction in vivo mediated by postsynaptic alpha 2-adrenoceptors. *Naunyn. Schmiedebergs Arch. Pharmacol*. **316**: 288-293.

Van Zwieten, P.A. and Timmermans, P.B. (1987) Alpha-adrenoceptor stimulation and calcium movements. *Blood Vessels*. **24**: 271-280. Review.

Vargas, H.M. and Gorman, A.J. (1995) Vascular alpha-1 adrenergic receptor subtypes in the regulation of arterial pressure. *Life Sci*. **57**: 2291-2308. Review.

Varma, D.R. and Deng, X.F. (2000) Cardiovascular alpha1-adrenoceptor subtypes: functions and signaling. *Can. J. Physiol. Pharmacol*. **78**: 267-292. Review

Vázquez-Prado, J., Medina, L.C., Romero-Avila, M.T., González-Espinosa, C., and Garcia-Sáinz, J.A. (2000) Norepinephrine- and phorbol ester-induced phosphorylation of alpha(1a)-adrenergic receptors. Functional aspects. *J. Biol. Chem*. **275**: 6553-6559.

Venema, R.C., Raynor, R.L., Noland, T.A.J., and Kuno, J.F. (1993) Role of protein kinase C in the phosphorylation of cardiac myosin light chain 2. *Biochem. J.* **294**: 401-406.

von Zastrow, M. and Kobilka, B.K. (1992) Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**: 3530-3538.

Wang, B.H., Du, X.J., Autelitano, D.J., Milano, C.A., and Woodcock, E.A. (2000) Adverse effects of constitutively active α_{1B}-adrenergic receptors after pressure overload in mouse hearts. *Am. J. Physiol. Heart Circ. Physiol.* **279**: H1079-H1086.

Wang, J., Wang, L., Zheng, J., Anderson, J.L., and Toews, M.L. (2000) Identification of distinct carboxyl-terminal domains mediating internalization and down-regulation of the hamster alpha(1B)- adrenergic receptor. *Mol. Pharmacol*. **57**: 687-694.

Wang, J., Zheng, J., Anderson, J.L., and Toews, M.L. (1997) A mutation in the hamster alpha1B-adrenergic receptor that differentiates two steps in the pathway of receptor internalization. *Mol Pharmacol*. **52**: 306-313.

Weber, J.D., Raben, D.M., Phillips, P.J., and Baldassare, J.J. (1997) Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem. J.* **326**: 61-68.

Weiss, B.A. and Insel, P.A. (1991) Intracellular Ca2+ and protein kinase C interact to regulate alpha 1-adrenergic- and bradykinin receptor-stimulated phospholipase A2 activation in Madin-Darby canine kidney cells. *J. Biol. Chem*. **266**: 2126-2133.

Wenham, D., Rahmatullah, M., Hansen, C.A., and Robishaw, J.D. (1997) Differential coupling of alpha1-adrenoreceptor subtypes to phospholipase C and mitogen activated protein kinase in neonatal rat cardiac myocytes. *Eur. J. Pharmacol*. **339**: 77-86.

Wenzel, D.G. and Su, J.L. (1966) Interactions between sympathomimetics amines and blocking agents on the rat ventricle strips. *Arch. Int. Pharmacodyn. Ther.* **160**: 379-389.

Wickberg, J.E., Akers, M., Caron, M.G., and Hagen, P.O. (1983) Norepinephrine-induced down regulation of alpha 1 adrenergic receptors in cultured rabbit aorta smooth muscle cells. *Life Sci*. **33**: 1409-1417.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev*. **79**: 143-180. Review.

Wikberg, J.E., Akers, M., Caron, M.G., and Hagen, P.O. (1983) Norepinephrine-induced down regulation of alpha 1 adrenergic receptors in cultured rabbit aorta smooth muscle cells. *Life Sci*. **33**: 1409-1417.

Wilden, U., Hall, S.W., and Kuhn, H. (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA* **83**: 1174-1178.

Willamson, A.P., Seifen, E., Lindermann, J.P., and Kennedy, R.H. (1994) Alpha 1a-adrenergic receptor mediated positive chronotropic effect in right atria isolated from rats. *Can. J. Physiol. Pharmacol*. **72**: 1574-1479.

Winstel, R., Freund, S., Krasel, C., Hoppe, E., and Lohse, M.J. (1996) Protein kinase cross-talk: membrane targeting of the beta-adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. U. S. A*. **93**: 2105-2109.

Woodcock, E.A. (1995) Inositol phosphates in the heart: controversy and consensus. *J. Mol. Med*. **73**: 313-323. Review.

Wu, D., Katz, A., Lee, C.H., and Simon, M.I. (1992a) Activation of phospholipase C by alpha 1-adrenergic receptors is mediated by the alpha subunits of Gq family. *J. Biol. Chem*. **267**: 25798-25802.

Wu, D.Q., Lee, C.H., Rhee, S.G., and Simon, M.I. (1992b) Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J. Biol. Chem*. **267**: 1811-1817.

Xin, X., Yang, N., Eckhart, A.D., and Faber, J.E. (1997) Alpha1D-adrenergic receptors and mitogen-activated protein kinase mediate increased protein synthesis by arterial smooth muscle. *Mol. Pharmacol*. **51**: 764-775.

Yamamoto, Y. and Koike, K. (2001) Characterization of alpha1-adrenoceptor-mediated contraction in the mouse thoracic aorta. *Eur. J. Pharmacol*. **424**: 131-140.

Yang, M., Ruan, J., Voller, M., Schalken, J., and Michel, M.C. (1999) Differential regulation of human alpha1-adrenoceptor subtypes. *Naunyn Schmiedebergs Arch. Pharmacol*. **359**: 439-446.

Yu, G.S. and Han, C. (1994) Role of alpha 1A- and alpha 1B-adrenoceptors in phenylephrine-induced positive inotropic response in isolated rat left atrium. *J. Cardiovasc. Pharmacol*. **24**: 745-752.

Zechner, D., Thuerauf, D.J., Hanford, D.S., McDonough, P.M., and Glembotski, C.C. (1997) A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression. *J. Cell. Biol.* **139**: 115-127.

Zhong, H. and Minneman, K.P. (1999) Differential activation of mitogen-activated protein kinase pathways in PC12 cells by closely related alpha1-adrenergic receptor subtypes. *J. Neurochem*. **72**: 2388-2396.

Zuscik, M.J., Chalothorn, D., Hellard, D., Deighan, C., McGee, A., Daly, C.J., Waugh, D.J.J., Ross, S.A., Gaivin, R.J., Morehead, A.J., Thomas, J.D., Plow, E.F., McGrath, J.C., Piascik, M.T., and Perez, D.M. (2001) Hypotension, autonomic failure, and cardiac hypertrophy in transgenic mice overexpressing the alpha 1B-adrenergic receptor. *J. Biol. Chem*. **276**: 13738-13743.

Zuscik, M.J., Piascik, M.T., and Perez, D.M. (1999) Cloning, cell-type specificity, and regulatory function of the mouse alpha(1B)-adrenergic receptor promoter. *Mol. Pharmacol*. **56**: 1288-1297.

Zuscik, M.J., Sands, S., Ross, S.A., Waugh, D.J.J., Gaivin, R.J., Morilak, D.A., and Perez, D.M. (2000). Overexpression of the alpha 1B-adrenergic receptor causes apoptotic neurodegeneration: multiple system atrophy. *Nat. Med.* **6**: 1388-1394.

Vita

Dan Chalothorn

Date of Birth

September 2nd, 1976

Place of Birth

Stephensville, Texas

Education

08/95-05/98 Vanderbilt University, Nashville, Tennessee
Bachelor of Science in Chemistry
Bachelor of Science in Molecular Biology

09/94-06/95 Georgia Institute of Technology, Atlanta, Georgia

Honors and Awards

American Heat Association Pre-Doctoral Fellowship 07/01-07/03

University of Kentucky Fellowship 05/99-05/00

Gamma Beta Phi Honor Society

Publications

Chalothorn, D.*, McCune, D.F.*, Edelmann, S.E., Tobita, K., Keller, B.B., Lasley, R.D., Perez, D.M., Tanoue, A., Tsujimoto, G., Post, G.R., and Piascik, M.T. Differential Cardiovascular Regulatory Activities of the $Alpha_{1B}$- and $Alpha_{1D}$-Adrenoceptor Subtypes. Accepted at *J. Pharmacol. Exp. Ther.*

Ross, S.A., Chalothorn, D., Gonzalez-Cabrera, P.J., Yun, J., Gaivin, R., McCune, D.F., Papay, R., Rorabaugh, B., Piascik, .T., and Perez, D.M. The Role of the α_{1B}-Adrenergic Receptor in the Inotropic Response of the Mouse Myocardium. In revision at *Am. J. Physiol.*

Chalothorn, D., McCune, D.F., Edelmann, S.E., García-Carzarín, M.L., Tsujimoto, G., and Piascik, M.T. (2002) Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the α_1-Adrenoceptor Subtypes. *Mol. Pharm.* **61**: 1008-1016.

Zuscik, M.J., Chalothorn, D., Hellard, D., Deigham, C., McGee, A., Daly, C.J., Waugh, D.J.J., Ross, S.A., Gaivin, R.J., Morehead, A., Thomas, J.D., Plow, E.F., McGrath, J.C., Piascik, M.T., and Perez, D.M. (2001) Hypotension, Autonomic Failure and Cardiac Hypertrophy in Transgenic Mice Overexpressing the α_{1B}-Adrenergic Receptor. *J. Biol. Chem.* **276**: 13738-13743.

* indicates equal contributions

Abstracts

Chalothorn, D., McCune, D.F., Edelmann, S.E., Tobita, K., Keller, B.B., Lasley, R.D., Perez, D.M., Tanoue, A., Tsujimoto, G., Post, G.R., and Piascik, M.T. Differential Cardiovascular Regulatory Activities of the α_{1B}- and the α_{1D}-Adrenoceptor Subtypes. Federation of American Societies of Experimental Biology, American Society for Pharmacological and Experimental Therapeutics, San Diego, 2003.

Chalothorn, D., McCune, D.F., Edelmann, S.E., Tobita, K., Keller, B.B., Perez, D.M., Post, G.R., and Piascik, M.T. Differential Regulation of Contraction and Activation of Mitogen-Activated Protein Kinases in

Vascular Smooth Muscle by the Alpha 1-Adrenoceptor Subtypes. Federation of American Societies of Experimental Biology, American Society for Pharmacological and Experimental Therapeutics, New Orleans, 2002.

Chalothorn, D., McCune, D.F., Edelmann, S.E., Tsujimoto, G., Benovic, J.L., and Piascik, M.T. Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the α_1-Adrenoceptor Subtypes. Federation of American Societies of Experimental Biology, American Society for Pharmacological and Experimental Therapeutics, Orlando, 2001.

Chalothorn, D., McCune, D.F., Edelmann, S.E., Zuscik, M.J., Perez, D.M., and Piascik, M.T. Effect of the Wild-Type or Constitutively Active Alpha 1B-Adrenoceptor on Blood Pressure Regulation and Vascular Smooth Muscle Contraction. 73rd Scientific Sessions, American Heart Association, New Orleans, 2000.

Signature _____