2003

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

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ABSTRACT OF DISSERTATION

Dan Chalothorn

The Graduate School
University of Kentucky
2003
CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS
OF THE \( \alpha_1 \)-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 $\alpha_1$-ADRENOCEPTOR SUBTYPES

The $\alpha_1$-adrenoceptors ($\alpha_1$-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: $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-ARs.

This dissertation research investigated the trafficking properties of the $\alpha_1$-ARs at the cellular level as well as physiological relevance of the $\alpha_1$-ARs at the tissue level. In vitro studies using transiently transfected $\alpha_1$-AR/GFP subtypes revealed distinct basal localization patterns and different agonist-mediated activation and desensitization properties. The $\alpha_{1A}$- and the $\alpha_{1B}$-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 $\beta$ arrestin-1, a protein often associated with receptor internalization. In contrast, the $\alpha_{1D}$-AR/GFP did not require $\beta$ arrestin-1 for maintaining the basal receptor orientation pattern.

Although these data increase our knowledge of trafficking properties of the $\alpha_1$-AR subtypes, it is of equal importance to determine the role(s) that each subtype contributes
to cardiovascular function. The lack of subtype-selective $\alpha_1$-AR pharmacological agents prompted the use of genetically manipulated mouse models with a systemic over-expression of a constitutively active $\alpha_{1B}$-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 $\alpha_{1B}$-AR in attenuating the inotropic responses. However, experiments using isolated thoracic aortae from transgenic animals suggested that the $\alpha_{1B}$-AR does not participate in vascular smooth muscle contractile responses. Additional studies investigated the role of $\alpha_{1D}$-AR in cardiovascular function by using animals systemically lacking the $\alpha_{1D}$-AR subtype. Experimental data suggested an $\alpha_{1D}$-AR participation in vascular smooth muscle function since the deficiency of the $\alpha_{1D}$-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 $\alpha_1$-ARs in cellular localization, signal regulation, and trafficking. Additionally, the data provide an investigation into the physiologic significance of both the $\alpha_{1B}$- and the $\alpha_{1D}$-ARs in cardiovascular tissue.

Key Words: $\alpha_1$-Adrenoceptors ($\alpha_1$-ARs), Receptor Localization and Trafficking, Transgenic Animals, Vascular Smooth Muscle, Heart
CELLULAR TRAFFICKING PROPERTIES AND PHYSIOLOGICAL FUNCTIONS
OF THE $\alpha_1$-ADRENOCEPTOR SUBTYPES

By

Dan Chalothorn

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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 19\textsuperscript{th} and early 20\textsuperscript{th} 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 $\alpha$-ARs are responsible for mediating different physiological responses such as myocardial contraction and neurotransmitter release.

In conjunction with the emerging evidence of multiple $\alpha$-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 $\alpha$-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 $\alpha$-ARs as $\alpha_2$ while the post-junctional $\alpha$-ARs as $\alpha_1$. Unlike the previous pharmacological classification that distinguished the $\alpha$-from the $\beta$-ARs, anatomical location was the basis of the early $\alpha$-AR subdivision scheme. Following the discovery of a population of pre-synaptic $\alpha_2$-ARs and the emerging data implicating $\alpha_2$-AR participation in more than inhibition of synaptic transmissions, the criteria for $\alpha$-AR subclassification would evolve to better define the groups of $\alpha$-ARs. In 1977, Berthelsen and Pettinger suggested that physiological functions should serve as the basis for $\alpha$-AR subclassification (Berthelsen and Pettinger, 1977). With this newer classification scheme, the $\alpha_1$-ARs defined the receptors associated with vascular smooth muscle contraction while the $\alpha_2$-ARs included receptors associated with functional inhibition. Redefining the $\alpha$-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 $\alpha_1$-AR is prazosin sensitive and $\alpha_2$-AR is prazosin insensitive. This pharmacological classification also defined the $\alpha_1$-ARs as the group responsive to the agonists as phenylephrine, methoxamine, or cirazoline and the antagonists as prazosin or WB 4101; the $\alpha_2$-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 $\alpha$-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 $\alpha_1$-Adrenoceptors

Following the pharmacological classification of the $\alpha$-ARs into $\alpha_1$- and $\alpha_2$-ARs, several studies using various vascular smooth muscle preparations generated results inconsistent with the notion of a single post-junctional $\alpha_1$-AR. Emerging data favored the notion of $\alpha_1$-AR heterogeneity. In 1977, Ruffolo reported that oxymetazoline could desensitize the $\alpha_1$-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 $\alpha_1$-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 α1-ARs in the rat anococcygeal muscle
using the α1-AR selective imidazoline agonist, SGD 101/75. They showed that
phenoxybenzamine preferentially blocks what would be termed the α1S-AR-mediated
SGD 101/75 response but not the other α1-AR-mediated norepinephrine response.

From data such as those discussed above, McGrath proposed two types of post-
junctional α1-ARs: α1A- and α1B-ARs. Under this classification scheme, the α1A-AR
defined the group mediating the effects of both phenylethylamines at low concentrations
and non-phenylethylamines while the α1B-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 α1-
ARs came from a study observing differences in the extracellular Ca2+-sensitivity to the
two agonists: clonidine and methoxamine (Holck et al., 1983). More evidence of α1-AR
heterogeneity emerged with the finding that Ca2+-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 α1-ARs with a high affinity and another population with a low affinity for
prazosin (Medgett and Langer, 1984).

Although physiological studies indicated α1-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 $[^3H]$-prazosin, suggestive of the existence of two types of $\alpha_1$-ARs (Drew, 1985; Flavahan and Vanhoutte, 1986; Babich et al., 1987; Piascik et al., 1988). In binding studies using $[^3H]$-prazosin with a series of agonists and antagonists, Morrow and Creese (1986) pharmacologically identified the high affinity binding $\alpha_1$-AR as the $\alpha_{1A}$-AR and the $\alpha_{1B}$-AR as the low affinity receptor. In support of this notion, several studies indicated that the $\alpha_{1A}$-AR possesses higher binding affinities than the $\alpha_{1B}$-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 $\alpha_1$-ARs indicated that both dibenamine and benextramine could eliminate the $[^{125}\text{I}]$BE $\alpha_1$-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 $[^{125}\text{I}]$BE $\alpha_1$-AR binding population by approximately 35 to 40%, which suggested that one $\alpha_1$-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 $\alpha_{1A}$-AR population without affecting the $\alpha_{1B}$-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 $\alpha_1$-Adrenoceptor Subtypes

Although the pharmacological characterization of the $\alpha_1$-ARs identified two subtypes, the molecular cloning of the $\alpha_1$-ARs found three receptor subtypes. From the hamster smooth muscle cell line, Cotecchia et al. (1988) isolated and cloned the first $\alpha_1$-AR. Experiments with the cloned $\alpha_1$-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 $\alpha_{1B}$-AR pharmacology because of the ability to bind the antagonist 2-{$\beta$-(4-hydroxy-3-[$^{125}$I]iodophenyl)ethylaminomethyl]-tetralone ($[^{125}$I]HEAT) with high affinity and the $\alpha_{1A}$-AR selective antagonists phentolamine and WB 4101 with low affinity. The cloned receptor possessed low affinity for many $\alpha_1$-AR ligands and demonstrated sensitivity to CEC inactivation. Because of the similarities with the pharmacological profile of the $\alpha_{1B}$-AR defined by Morrow and Creese (1986), Cotecchia et al. (1988) identified the cloned hamster $\alpha_1$-AR as the $\alpha_{1b}$-AR.

Schwinn et al. (1990) isolated and cloned a different $\alpha_1$-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 $\alpha_{1B}$-AR. The pharmacological studies revealed that this cloned $\alpha_1$-AR receptor possesses binding affinities higher than the $\alpha_{1B}$-AR but lower than the defined $\alpha_{1A}$-AR. Schwinn et al. (1990) found no correlations between the properties of this cloned receptor and the previous $\alpha_1$-AR pharmacological binding profiles; in addition, the investigators could not locate the mRNA transcripts for the cloned receptor in tissues known to express the $\alpha_{1A}$-
AR. Further experiments revealed partial receptor sensitivity to CEC inactivation, which the \( \alpha_{1A} \)-AR is resistant. Because experiments with the cloned receptor revealed distinct properties from both the pharmacologically defined \( \alpha_{1A} \)- and \( \alpha_{1B} \)-ARs, these investigators concluded the discovery of a new receptor subtype; thus, they termed the novel cloned receptor as the \( \alpha_{1c} \)-AR. However, other studies using more sensitive ribonuclease protection assays and Northern blots revealed the presence of the mRNA for the \( \alpha_{1c} \)-AR in tissues expressing the \( \alpha_{1A} \)-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 \( \alpha_{1c} \)-AR to the \( \alpha_{1A} \)-AR; consequently, researchers agreed that the \( \alpha_{1c} \)-AR was the \( \alpha_{1a} \)-AR (Hieble et al., 1995).

Similar to the confusion involved in identifying the \( \alpha_{1a} \)-AR, the identification of the third cloned \( \alpha_{1} \)-AR subtype also entailed controversy. Lomasney et al. (1991) isolated and cloned an \( \alpha_{1} \)-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 \( \alpha_{1B} \)-AR membrane spanning domains. In addition, they noted that the cloned receptor lacked a consensus site in the 3\(^{rd}\) cytosolic loop for protein kinase C (PKC) phosphorylation. The pharmacological studies on the receptor revealed a similar profile to the \( \alpha_{1A} \)-AR because of the higher binding affinities for \( \alpha_{1} \)-AR ligands. Additional experiments demonstrated receptor insensitivity to CEC inactivation like the \( \alpha_{1A} \)-AR, thus the investigators termed the cloned \( \alpha_{1} \)-AR as the \( \alpha_{1a} \)-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 \( \alpha_{1A} \)-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 $\alpha_{1A}$-AR yet higher than the $\alpha_{1B}$-AR. In addition, these investigators provided further evidence that the cloned receptor from the rat cerebral cortex could not be the $\alpha_{1A}$-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 $\alpha_{1d}$-AR. Table 1 lists the accepted nomenclature and the pharmacological profiles associated with the $\alpha_1$-ARs.

**Structure of the $\alpha_1$-Adrenoceptors**

From the cloned $\alpha_{1a}$-, $\alpha_{1b}$-, and $\alpha_{1d}$-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 $\alpha_1$-Adrenoceptors.

<table>
<thead>
<tr>
<th></th>
<th>Previous Name(s)</th>
<th>CEC Sensitivity (% inactivated)</th>
<th>Agonist</th>
<th>Antagonist(s)</th>
<th>Agonists</th>
<th>Antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_{1A}$-AR</td>
<td>$\alpha_{1C}$-AR</td>
<td>Low (30)</td>
<td>A 61603</td>
<td>SNAP 6991, 5-MU, Niguldipine, Tamulosin, WB 4104, SZL-49(irrev)</td>
<td>Phenylephrine, Methoxamine, Epinephrine, Norepinephrine, Oxymetazoline, Citrazoline, Imidazolines</td>
</tr>
<tr>
<td>$\alpha_{1B}$-AR</td>
<td>$\alpha_{1B}$-AR</td>
<td>High (90)</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>Prazosin, Doxazosin, Phentolamine, Phenoxybenzamine, Dibenamine, Benextramine</td>
</tr>
<tr>
<td>$\alpha_{1D}$-AR</td>
<td>$\alpha_{1A}$-AR &amp; $\alpha_{1A/D}$-AR</td>
<td>Moderate to High (70)</td>
<td>___</td>
<td>BMY 7378</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>
glycosylation potentially affects the number of receptors on the cellular surface while receptor palmitoylation 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 α1-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 β2-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 α1-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 α1B-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 β2-AR uses both Ser$^{204}$ and Ser$^{207}$; however, the α1A-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 α1- and the β2-ARs. Similar to the Asp$^{113}$ of β2-AR described by Strader et al. (1988), the α1B-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 \(\alpha_{1A}\)-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 α1B-AR, Kjelsberg et al. (1992) documented an increase in signaling associated with the mutation of the Asp293 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 Lys331 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\textsuperscript{125} results in different degrees of constitutive activity depending on the intrinsic properties of the amino acid replacing the Cys\textsuperscript{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\textsuperscript{204} with Val in the \(\alpha_{1B}\)-AR would alter the 3-dimensional arrangement of the transmembrane domains; consequently, both the Ser\textsuperscript{207} and the Ser\textsuperscript{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 \(\alpha_{1B}\)-AR exist, but these receptors have mutations unrelated to the formation of the salt bridge. Cotecchia et al. (1990) generated several constitutively active \(\alpha_{1B}\)-ARs with a combination of mutations on the 3\textsuperscript{rd} 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 \(\alpha_{1}\)-ARs are capable of participating in ionic transport of
molecules across the plasma membrane by modulating Na\textsuperscript{+}/H\textsuperscript{+}-pumps, Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange, Ca\textsuperscript{2+}-, and K\textsuperscript{+}-channels (reviewed in Terzic et al., 1993; Graham et al., 1996).

In cells that endogenously express the \(\alpha_1\)-ARs, the postulated function of these receptors is to modulate the increases of intracellular Ca\textsuperscript{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 \(\alpha_1\)-AR family uses the 3\textsuperscript{rd} 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 (\(\alpha\), \(\beta\), and \(\gamma\)) in which a guanosine diphosphate (GDP) molecule binds the \(\alpha\) 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 \(\alpha\) subunit from the \(\beta\gamma\) complex. The dissociated complexes activate specific downstream effectors until the \(\alpha\) 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 \(\alpha_1\)-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 \(\alpha_{1B}\)-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\textsuperscript{2+}-dependent
transglutaminase activity antagonizable by GTP for cross-linking proteins (Nakaoka et al., 1994).

**Phospholipase Pathways and the Generation of 2\textsuperscript{nd} Messengers**

The $\alpha_1$-AR-activated signal transduction scheme is complex because the $\alpha_1$-ARs can couple to different G-proteins that activate three distinct phospholipase pathways: phospholipase D (PLD), phospholipase A\textsubscript{2} (PLA\textsubscript{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 $\alpha_1$-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 2\textsuperscript{nd} messenger 1,2-diacylglycerol (DAG), which is an activator of protein kinase C (PKC) (see Figure 3).

The ability of the $\alpha_1$-AR to couple to the PTx-sensitive G-proteins permits the receptor to activate the PLA\textsubscript{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 $\alpha_1$-AR activation is the PLC pathway. Three isoforms make up the phospholipase C group: PLC-\(\beta\), PLC-\(\gamma\), and
Figure 3. Diagram of the phospholipase pathways coupled to $\alpha_1$-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 α1-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 (PIP₂). The activation of the PLC-β enzyme results in the cleavage of PIP₂ to two relevant 2nd messengers, DAG and inositol-1,4,5-trisphosphate (IP₃) (reviewed by Terzic et al., 1993; Woodcock 1995; Graham et al., 1996) (see Figure 3). As mentioned previously, DAG activates PKC while IP₃ is responsible for evoking changes in intracellular Ca^{2+} concentrations. IP₃ increases the intracellular Ca^{2+} concentration upon binding to the IP₃ 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 α₁-AR-G_{q}\text{-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 $\alpha_1$-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 2$^{\text{nd}}$ and the 3$^{\text{rd}}$ 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 α1B-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 α1B-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).

**α1-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 α1A- and the α1B-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 α1A-AR localization mainly in the intracellular space in the perinuclear region whereas a diffuse α1B-AR localization was observed predominantly on the plasma membrane. Additionally, they showed that CEC, a highly hydrophilic alkylation antagonist, inactivates approximately the same proportion of α1A- as α1B-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 α1B-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 $\alpha_{1B}$-AR predominantly localizes to the cellular surface whereas the $\alpha_{1D}$-AR localizes to the intracellular region.

**Distribution of the $\alpha_1$-Adrenoceptors**

Many studies reported a wide distribution of the $\alpha_1$-AR mRNA transcripts in several tissues (Ping and Faber, 1993; Piascik *et al.*, 1994; Rokosh *et al.*, 1994; Scofield *et al.*, 1995). More notably $\alpha_1$-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 $\alpha_{1A}$-AR making up approximately 90% of the total $\alpha_1$-AR mRNA (Piascik *et al.*, 1995). Due to the paucity of highly selective ligands that can distinguish between the $\alpha_{1B}$- and the $\alpha_{1D}$-ARs in radioligand-binding experiments, determining the protein expression level for each subtype is difficult. However, many studies generated estimates for the $\alpha_{1A}$-AR subtype population in the rat aorta, caudal artery, mesenteric artery, heart, and brain using $\alpha_{1A}$-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 $\alpha_1$-AR population is the $\alpha_{1A}$-AR, indicating that there is no correlation between mRNA transcript levels and protein expression. In the human heart, the $\alpha_1$-ARs account for up to 15% of the cardiac adrenoceptors (Delhaye *et al.*, 1983).

In addition to the vasculature, the $\alpha_1$-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 α₁-AR subtypes exist (Price et al., 1994; Rokosh et al., 1994; Scofield et al., 1995). Although the transcripts for the α₁-ARs exist in the rat heart, other studies examining the translated products confirmed that the α₁A- and the α₁B-AR make up the α₁-AR population in the heart (Deng et al., 1996a; Deng and Varma, 1997). These studies revealed that the α₁D-AR is nearly undetectable in neonatal rat hearts and virtually non-existent in the adult rat hearts. These findings suggest that the α₁D-AR may play a minor role in mediating physiological responses in the heart.

Functions of the α₁-Adrenoceptors in the Vascular Smooth Muscle

In the vasculature, the α₁-ARs mediate two responses: vasoconstriction and vascular growth. From a physiological standpoint, the α₁-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 α₁-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 $\alpha_1$-AR subtypes, there is a difficulty associated with determining which $\alpha_1$-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 $\alpha_{1D}$-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 $\alpha_{1A}$- and the $\alpha_{1B}$-ARs in contractile responses since later studies revealed that CEC is capable of inactivating the $\alpha_{1A}$- and the $\alpha_{1D}$-ARs to a low and moderate to high degree, respectively (Johnson and Minneman, 1987; Han et al., 1987). Although lacking a selective $\alpha_{1D}$-AR antagonist at the time, several studies that sought to define the vasoconstrictive responses for the $\alpha_{1A}$- and the $\alpha_{1B}$-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 $\alpha_{1A}$-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 $\alpha_{1B}$-AR participation in contraction of the rat thoracic aorta. However, with the discovery of the $\alpha_{1D}$-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 $\alpha_{1D}$-AR is the main mediator of contraction in the rat femoral, iliac, and superior mesenteric arteries. Presently, little evidence exists that conclusively links the $\alpha_{1B}$-AR to vessel constriction, and data indicating that the $\alpha_{1B}$-AR participates in the mesenteric resistance artery resulted from exclusion of the other two $\alpha_1$-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 $\alpha_1$-Adrenoceptors in the Myocardium**

The $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-AR-mediated inotropic effects are the subject of several reviews (see Fedida *et al.*, 1993; Terzic *et al.*, 1993; Li *et al.*, 1997).

The $\alpha_1$-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 papillary muscle (Otani et al., 1988), mouse cardiac
trabeculae (McCloskey et al., 2002), and isolated mouse hearts (Turnbull et al., 2003).
This $\alpha_1$-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 $\alpha_1$-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, $\alpha_1$-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 $\alpha_1$-AR-
mediated inotropic response and extent of alkalinization by $\alpha_1$-AR agonists (Terzic et al.,

To support the finding that the $\alpha_1$-ARs use a different mechanism than the $\beta_1$-AR
in mediating positive inotropy, studies reported that the presence of either a
phosphodiesterase or an adenylyl cyclase inhibitor does not affect the $\alpha_1$-AR-stimulated
response (Endoh and Motomura, 1979; Endoh and Yamashita, 1980; Christiansen et al.,
1987). Thus the $\alpha_1$-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 \textit{et al}., 1980). Skomedal \textit{et al}., (1988) found that the $\alpha_1$-ARs contribute approximately 25% of the norepinephrine-mediated inotropic response. At the cellular level, several studies documented an $\alpha_1$-AR-regulated positive inotropic effect in rat cardiomyocytes (Capogrossi \textit{et al}., 1991; Fedida and Bouchard, 1992; O’Rourke \textit{et al}., 1992; Terzic \textit{et al}., 1992; Gambassi \textit{et al}., 1998).

As mentioned earlier, expression of the $\alpha_{1D}$-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 \textit{et al}., 1997). Other studies have implicated the $\alpha_{1A}$- and the $\alpha_{1B}$-AR in the regulation of positive inotropy (Williamson \textit{et al}., 1994; Deng \textit{et al}., 1996b). Michel \textit{et al}., (1990, 1994) and Yu and Han (1994) reported that the $\alpha_{1B}$-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 $\alpha_{1A}$-AR participation (Rokosh and Sulakhe, 1991; Gambassi \textit{et al}., 1991).

As discussed above, $\beta$-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 $\alpha_1$-AR inotropic responses in the myocardium. However, there are suggestions that during heart failure the $\alpha_1$-ARs play a greater role in inotropic responses to endogenous catecholamines (for review, see Terzic \textit{et al}., 1993; Li \textit{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
β1-AR-Gs-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 β1-AR-mediated response ensues. Consequently, this adaptive reflex becomes a maladaptive process because of the down-regulation of the β1-AR population, which may cause ventricular dysfunction. Many studies suggested that activation of the α1-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 α1-AR densities or α1-AR-mediated PI turnover. Therefore, the question of whether the α1-ARs can sustain myocardial function in the long-run still exists.

α1-Adrenoceptor Signaling in Growth-Related Responses

The α1-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 α1-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 α1-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-ARs promotes DNA
synthesis in quiescent rat smooth muscle cells. Additionally, stimulation of $\alpha_1$-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 $\alpha_1$-ARs
results in an increase of protein synthesis (Chen et al., 1995; Xin et al., 1997). These
studies demonstrated that the activation of the $\alpha_{1B}$- and $\alpha_{1D}$-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 $\alpha_{1B}$-
AR activation, the $\alpha_{1A}$-AR results in attenuation of the $\alpha_{1B}$-AR-mediated growth
response.

The $\alpha_1$-ARs are also capable of mediating events associated with cellular growth
and proliferation in neonatal rat myocardial cells (Simpson, 1983, 1985; Meidell et al.,
revealed two requirements: the up-regulation of the proto-oncogenes and the increased
Figure 5. A depiction of the α₁-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 α1A-AR subtype to cellular hypertrophy. In a study using transgenic mice harboring a constitutively active α1B-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 Gαq (a protein activated as part of the α1-AR signaling cascade) induces cardiac hypertrophy along with cardiac dysfunction. These Gαq 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 α1B-AR antagonizes the hypertrophic actions of the α1A-AR in the rat myocardium (Deng et al., 1998). Despite these conflicting observations, many studies favor the notion that the α1-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 $\alpha_1$-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 $\alpha_1$-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: $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-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 α1-AR/GFP subtype in real-time. In addition, experiments assessed the participation of the receptor trafficking protein, β-arrestin 1, in mediating α1-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 α1-AR subtypes participate in different regulatory activities in the cardiovascular system. Animals with the constitutively active α1B-AR under the control of the endogenous promoter were used to study the role of the α1B-AR participation in aortic smooth muscle contraction. Other experiments used echocardiographic techniques to determine the effect(s) of α1B-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 α1B-AR. In a different series of experiment using mice deficient in the α1D-AR, the study focused on the role of the α1D-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 $\alpha_1$-AR subtypes.

2) Examine the hypothesis that the $\alpha_1$-AR subtypes modulate different physiological responses in the cardiovascular system.
Chapter Three

Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the $\alpha_1$-Adrenoceptor Subtypes

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Introduction

The α1-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 α1-ARs also play a major role in mediating growth responses in cardiac and vascular smooth muscle cells (for reviews on all aspects of the α1-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 α1A-, α1B-, and α1D-ARs, have been cloned and characterized. These subtypes utilize a variety of second messengers and G-proteins to modulate cellular processes. Alterations in normal α1-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.,
Oakley et al. (2000) demonstrated recently that GPCRs have different affinities for the different arrestins. Class A GPCRs, which include the β2-AR, α1B-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 α1-AR subtypes, the desensitization, down-regulation, and internalization characteristics of the α1B-AR have been most extensively examined. For example, agonist-mediated phosphorylation and internalization of the α1B-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 α1A- and α1D-ARs. Vázquez-Prado et al. (2000) showed that the α1A-AR could undergo agonist-mediated phosphorylation, albeit not to the same extent as the α1B-AR. Yang and co-workers (1999) used fibroblasts stably transfected with each of the α1-ARs to show that the increase in inositol phosphates mediated by the α1A- and α1B-ARs could be desensitized, whereas the increase mediated by the α1D-AR was refractory to agonist-mediated desensitization. In contrast to this, García-Sáinz et al. (2001) showed that the α1D-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 α1-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 $\alpha_1$-ARs.
Experimental Procedures

Materials

$\alpha_1$-AR/green fluorescent protein ($\alpha_1$-AR/GFP) vectors were constructed by ligating the coding region of the human $\alpha_1A$-, $\alpha_1B$-, and $\alpha_1D$-AR into the EcoRI-KpnI 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 $\beta$-arrestin 1 and a dominant negative $\beta$-arrestin 1 (319-418) in pcDNA3 has previously been reported (Krupnick et al., 1997b). Rabbit polyclonal antibodies targeted against $\beta$-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 $\alpha_{1A}$-, $\alpha_{1B}$-, or $\alpha_{1D}$-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 α1-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 α1A-, α1B-, or α1D-
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 $\alpha_1$-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 α1-AR/GFP fusion proteins and the living cells were visualized 72 h later. Transfection with a cDNA encoding the α1B-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 α1D-AR/GFP fluorescence was detected intracellularly in a perinuclear orientation (Figure 6). Exhibiting localization properties of each of these subtypes, α1A-AR/GFP fluorescence was observed both on the cell surface and in a perinuclear orientation (Figure 6).

Functional Responses Mediated by the α1-AR/GFP Fusion Proteins

To demonstrate that the expressed α1-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 α1A- or the α1B-AR/GFP constructs (Figures 7, a, b, and d). This indicates that these GFP modified α1-ARs are functional when expressed in HEK-293 cells. Phenylephrine treatment of cells transfected with the α1D-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 α1D-AR is poorly coupled to second
Figure 6. Cellular localization of $\alpha_1$-AR/GFP constructs in transiently transfected HEK-293 cells. Transient transfection of $\alpha_1$-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) α1A-AR/GFP, b) α1B-AR/GFP, and c) α1D-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. Garcia-Cazarin 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 α1B-AR/GFP resulted in a rapid translocation of the receptor from the cell surface to intracellular compartments (Figure 8). The α1-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 α1B-AR internalization occurred in a very rapid fashion.

Receptor activation with phenylephrine also promoted the internalization of the cell surface population of α1A-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 α1A-AR internalization occurred at a slower rate than that seen for the α1B-AR. Treatment of HEK cells expressing the α1D-AR/GFP fusion protein with phenylephrine did not cause a translocation of the cell surface population of α1D-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 $\alpha_{1A}$- or $\alpha_{1B}$-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.

<table>
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<tr>
<th>Time after agonist addition</th>
<th>$\alpha_{1A}$-AR/GFP</th>
<th>$\alpha_{1B}$-AR/GFP</th>
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<td>15 min</td>
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[Images of images captured at different time points after agonist addition]
Figure 8. (continued)

<table>
<thead>
<tr>
<th>Time after agonist addition</th>
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<th>$\alpha_{1B}$-AR/GFP</th>
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Figure 9. Comparison of the effect of 100 µM phenylephrine on changes in intracellular fluorescence intensity in cells transfected with the a) α1A- or b) α1B-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).
Figure 10. Effects of 100 µM phenylephrine on the cellular localization of the α₁D-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 α₁-AR/GFP localization and the ability of the α₁-ARs to stimulate ERK1/2 phosphorylation was assessed. Prolonged incubation with phenylephrine (followed by extensive washout) resulted in an internalization of α₁A- and α₁B-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 α₁A- or α₁B-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 α₁D-AR. Long-term exposure to phenylephrine significantly reduced the level of phospho-ERK1/2 seen following rechallenge with agonist in α₁D-AR expressing cells (Figure 7d).

**Effect of Arrestins on Agonist-Activated Receptor Internalization**

HEK cells were co-transfected with α₁-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 α₁A- or α₁B-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 α₁B-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 α₁B-AR cellular localization (Figure 12). However, the dominant-negative arrestin markedly decreased the ability of phenylephrine to promote α₁B-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 α1A- and the α1B-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 α1A- and α1B-AR/GFP, respectively. The images are representative of four (α1A-AR/GFP) and seven (α1B-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 α1B-AR, β-arrestin 1 (319-418) decreased the magnitude of the phenylephrine-induced α1A-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 α1D-AR internalization (Figure 13). Similarly, β-arrestin 1 (319-418) had no effect on the cellular localization of the α1D-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 α1D-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 α₁-AR/GFP fusion proteins in transiently transfected HEK-293 cells. Previous studies with the α₁B-AR/GFP construct demonstrated that it is fully functional and internalizes in the same manner as a non-GFP tagged α₁B-AR construct (Awaji et al., 1998). In a similar fashion, previous studies showed that both the α₁A- and α₁B-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 α₁A- and α₁B-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 α₁-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 α₁D-AR expressing cells, this increase was not statistically significant. This could indicate that the small population of cell surface α₁D-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 α₁D-AR was the most poorly coupled of the α₁-AR subtypes. In previous work with stably transfected fibroblasts, we showed that the α₁D-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

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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 α₁D-AR. Nonetheless, we must also accept the possibility that the α₁D-AR/GFP construct may not be functionally active (however, see the additional discussion below).

Laser scanning confocal microscopy revealed that the α₁B-AR was expressed predominantly on the cell surface (Figure 6). Although there is some cell surface expression of the α₁D-AR, the majority of this receptor is expressed in intracellular compartments. The α₁A-AR has localization characteristics of both the α₁B- and α₁D-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 α₁-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 α₁-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 α₁-AR accurately portrays the distribution pattern in vascular smooth muscle cells that normally express all three α₁-ARs. Indeed, an elegant series of studies using prazosin labeled with BODIPY-FL to image the α₁-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 α₁-AR population is expressed intracellularly.

Using real-time imaging of living cells, we observed differences in the agonist-
mediated internalization properties of the $\alpha_1$-ARs (Figures 8, 9, and 10). In agreement with previous work (Fonseca et al., 1995; Wang et al., 1997, 2000), we observed that the $\alpha_{1B}$-AR undergoes rapid agonist-mediated internalization. However, there has been little investigation of the effect of agonist activation on the translocation of the other $\alpha_1$-AR subtypes. We noted that the $\alpha_{1A}$-AR also undergoes agonist-mediated internalization. Interestingly, this internalization occurs at a slower rate than for the $\alpha_{1B}$-AR (Figures 9, compare a and b). We were unable to detect any agonist-mediated internalization of the $\alpha_{1D}$-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 $\alpha_{1D}$-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 $\alpha_1$-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 $\alpha_{1A}$- and $\alpha_{1B}$-ARs to promote ERK1/2 phosphorylation (Figure 7). Interestingly, after a 15 h exposure to phenylephrine in $\alpha_{1D}$-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 exposure to agonist and supports the notion that the α1D-AR/GFP construct is functional.

Our results are consistent with other studies that have examined the phosphorylation and desensitization of the α1-ARs. Yang et al. (1999) noted that both the α1A- and α1B-ARs undergo agonist-mediated desensitization. However, these authors noted that the α1B-AR was desensitized by lower concentrations of agonist. Similarly, Vázquez-Prado et al. (2000) found that the α1B-AR underwent more extensive agonist-activated phosphorylation than did the α1A-AR. The more rapid rate of α1B-AR internalization noted here is also consistent with the observation that this receptor is more extensively phosphorylated and readily desensitized than the α1A-AR. Yang et al. (1999) also observed that functional responses mediated by the α1D-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 α1D-AR could be phosphorylated and desensitized. Therefore, a definitive answer regarding the desensitization characteristics of the α1D-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 α1-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 α1A- or α1B-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 $\alpha_{1D}$-AR may be due to continuous internalization of the receptor due to its constitutively active nature.

The three $\alpha_1$-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 $\alpha_{1B}$-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 $\alpha_{1D}$-AR is an atypical GPCR. Although the $\alpha_{1A}$-AR is expressed intracellularly, it appears to have signaling properties expected of a GPCR.
Chapter Four

Differential Cardiovascular Regulatory Activities of the $\alpha_{1B}$- and $\alpha_{1D}$-Adrenoceptor Subtypes

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* 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 \( \alpha_1 \)-adrenergic receptor (\( \alpha_1 \)-AR). Three subtypes, the \( \alpha_{1A} \)-, \( \alpha_{1B} \)-, and \( \alpha_{1D} \)-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 \( \alpha_1 \)-ARs see García-Sáinz et al., 1999; Varma and Deng, 2000; Pi ascik and Perez, 2001).

Data from heterologous expression systems have shown that all three \( \alpha_1 \)-ARs can couple to a variety of G-proteins and second messenger systems. The \( \alpha_1 \)-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 \( \alpha_1 \)-ARs activate phospholipases C and A2 (Perez et al., 1993; Schwinn et al., 1991). In addition to mobilizing intracellular calcium (which would occur subsequent to activation of phospholipase C), the \( \alpha_1 \)-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 \( \alpha_1 \)-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 $\alpha_1$-ARs has been slowed by the availability of selective agonists and antagonists for these receptors. This is especially true for the $\alpha_{1B}$-AR. In this report we have taken advantage of a unique line of transgenic mice systemically over-expressing a constitutively active $\alpha_{1B}$-AR (see Zuscik et al., 2000, 2001) (see Appendix C), to examine the cardiovascular regulatory activities of the $\alpha_{1B}$-AR. A constitutively active receptor is tonically active, thus eliminating the need for agonists that non-selectively activate all $\alpha_1$-ARs. We have also examined regulatory activities in an $\alpha_{1D}$-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 $\alpha_{1B}$- and $\alpha_{1D}$-ARs perform distinctly different regulatory activities. We postulate that the $\alpha_{1B}$-AR is involved in the regulation of cardiac function and that the $\alpha_{1D}$-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 $\alpha_{1B}$-AR, $\alpha_{1B}$-AR$_{C128F}$. The over-expression of the constitutively active $\alpha_{1B}$-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 $\alpha_{1D}$-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 -glycerophosphate, 0.5% NP-40, 100 µM Na$_3$VO$_4$, 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 acrylamide 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 MgCl₂, 2 mM DTT, 5 mM γ-glycerophosphate, 0.1 mM Na₃VO₄) 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 [γ³²P]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 [³²P] 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$_2$PO$_4$, 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%
CO₂) 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 α₁B-AR modulation on β₁-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 α1B-ARc128f 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]·100; LV mass = 1.055[(LVIDd + 2·PWTd)3 – LVIDd3]; and mVcfc = [(LVIDd-LVIDs)/LVIDd]/(ET·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·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% O₂, 5% CO₂; 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 α₁D-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% CO₂ 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 colorimetric 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 α1B-AR\textsubscript{C128F}

Activation of Mouse Myocardial MAPKs

The hearts from α\textsubscript{1B}-AR\textsubscript{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\textsubscript{1B}-AR\textsubscript{C128F} is functional and can couple to signaling pathways in the absence of agonists. ERK activity was not altered in hearts from α\textsubscript{1D}-AR knockout mice (data not shown).

Echocardiographic Analysis

Activation of MAPKs has been proposed to link the α\textsubscript{1}-ARs to growth responses. Echocardiography was performed as a non-invasive method of assessing the effect(s) of constitutive activation of the α\textsubscript{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\textsubscript{1B}-AR\textsubscript{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)

![ERK Activity Chart]

b)

![JNK Activity Chart]
Table 2. Echocardiographic assessment of the murine left ventricular a) dimensions and b) function in mice over-expressing the $\alpha_{1B}$-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)  

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Body Weight (g)</th>
<th>Left Ventricular Internal Dimension (mm/g)</th>
<th>Posterior Wall Thickness (mm/g)</th>
<th>Left Ventricular Mass (g)</th>
<th>Left Ventricular Mass/ Body Weight ($10^{-3}$)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diastole  Systole</td>
<td>Diastole  Systole</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>10</td>
<td>30.03 ± 1.43</td>
<td>0.1364 ± 0.0046  0.0770 ± 0.0055</td>
<td>0.0197 ± 0.0022  0.0358 ± 0.0041</td>
<td>0.0782 ± 0.0074</td>
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<tr>
<td>$\alpha_{1B}$-AR$_{C128F}$</td>
<td>12</td>
<td>27.99 ± 1.16</td>
<td>0.1523* ± 0.0065  0.1038* ± 0.0066</td>
<td>0.0171 ± 0.0009  0.0313 ± 0.0032</td>
<td>0.0683 ± 0.0051</td>
<td>2.441 ± 0.149</td>
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b)  

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<tr>
<th></th>
<th>n</th>
<th>Ejection Time (msec)</th>
<th>Mean velocity for circumferential fiber shortening (1/√sec)</th>
<th>Heart Rate (beats/min)</th>
<th>Stroke Volume (mm³)</th>
<th>Cardiac Output (mm³/min)</th>
<th>Fractional Shortening (%)</th>
</tr>
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<td>Control</td>
<td>10</td>
<td>57.23 ± 3.83</td>
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<td>454.1 ± 22.7</td>
<td>0.0273 ± 0.0018</td>
<td>12.31 ± 0.83</td>
<td>43.10 ± 4.11</td>
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<tr>
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<td>484.6 ± 20.9</td>
<td>0.0274 ± 0.0023</td>
<td>13.21 ± 1.22</td>
<td>32.05* ± 2.44</td>
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reduced in the animals with the α₁B-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 α₁B-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 α₁B-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 α₁B-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 β₁-AR signaling pathway that resulted from α₁B-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 α1B-AR<sub>C128F</sub> hearts, respectively. The asterisk (*) indicates statistical differences from the non-transgenic control value at the respective isoproterenol concentration. Data from Chalothorn et al., 2003.
c)\[+\frac{dP}{dt}\text{ (mm Hg/sec)}\]
\[
\begin{align*}
\log [\text{Isoproterenol}] & \quad \text{Control (n=7)} \\
& \quad \alpha_{1B-AR_{C128F}} (n=10)
\end{align*}
\]
d)\[-\frac{dP}{dt}\text{ (mm Hg/sec)}\]
\[
\begin{align*}
\log [\text{Isoproterenol}] & \quad \text{Control (n=7)} \\
& \quad \alpha_{1B-AR_{C128F}} (n=10)
\end{align*}
\]
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 α1B-AR<sub>C128F</sub> 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<sub>1D</sub>-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<sub>1D</sub>-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<sub>1D</sub>-AR. Over-expression of a constitutively active α<sub>1B</sub>-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<sub>1B</sub>-AR<sub>C128F</sub> expressing mice with an estimated equilibrium dissociation constant of 0.385 ± 0.401 nM (Table 3), indicating that the α<sub>1D</sub>-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<sub>1B</sub>-AR, the response of the aorta is unaffected and remains under the control of the α<sub>1D</sub>-AR (experiments with aorta from α<sub>1D</sub>-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 α1B-AR<sub>C128F</sub> 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 α1B-AR<sub>C128F</sub> 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\textsubscript{1B}-AR\textsubscript{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.
Table 3. Characteristics of the phenylephrine response in control and α1B-ARC128F over-expressing aortae. Calculated pA₂ values and their 95% confidence intervals (C.I.) are listed along with the dissociation constant (Kᵢ). Data taken from Chalothorn et al., 2003.

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<th>Phenylephrine</th>
<th>Characterization of BMY 7378 Antagonism</th>
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<tbody>
<tr>
<td></td>
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<td>Log EC₅₀</td>
<td>S.E.</td>
</tr>
<tr>
<td>Control</td>
<td>52</td>
<td>-6.869</td>
<td>0.0662</td>
</tr>
<tr>
<td>α₁B-ARC₁₂₈F</td>
<td>39</td>
<td>-6.795</td>
<td>0.1036</td>
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with this lack of effect on the ability of phenylephrine to induce coronary vasoconstriction in hearts from mice expressing the constitutively active $\alpha_{1B}$-AR.

Experiments in the $\alpha_{1D}$-AR Knockout Mice

Responses in the Isolated-Perfused Heart

The effects of $\alpha_{1D}$-AR deficiency on $\beta$-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 $\alpha_{1D}$-AR (Figures 18, a and b). (+) or (-) dP/dt curves were also not different in hearts from $\alpha_{1D}$-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 $\alpha_{1D}$-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 $\alpha_{1D}$-AR knockout animals when compared to non-transgenic controls (Figure 19). In hearts from control mice, 100 $\mu$M phenylephrine infusion caused a significant increase in coronary perfusion pressure (Figure 20). Phenylephrine-induced increases in perfusion pressure were significantly reduced in hearts from $\alpha_{1D}$-AR knockout mice.
Figure 18. Functional responses of α₁D-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 α₁D-AR KO hearts, respectively. Data from Chalothorn et al., 2003.
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 α₁D-AR. The initial CPPs were 83.3 ± 2.3 and 81.9 ± 2.2 mm Hg for the control and the α₁D-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 α₁D-AR KO and the control group at the respective time point. Data from Chalothorn et al., 2003.
Discussion

While it is clear that the $\alpha_1$-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 $\alpha_1$-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 $\alpha_{1B}$-AR plays a role in the modulation of cardiac function while the $\alpha_{1D}$-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 $\alpha_1$-ARs whose expression were altered.

To examine the regulatory activity of the $\alpha_{1B}$-AR we chose a transgenic line of mice over-expressing a constitutively active mutant of this $\alpha_{1B}$-AR. An $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_1$-AR subtypes. Because constitutively active receptors engage signaling pathways in the absence of agonists, we can observe the results of $\alpha_{1B}$-AR activation without the need to administer $\alpha_1$-AR agonist compounds.

In previous work we showed that in the absence of agonist, the $\alpha_{1B}$-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 $\alpha_{1B}$-AR$_{C128F}$ animals. This would imply that this receptor is indeed constitutively coupled to signaling pathways. Coupling of the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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).
possibility that tonic unregulated activation of the $\alpha_{1B}$-AR impairs $\beta_1$-AR signaling and could be the underlying reason for the decrease in contractile function.

Activation of members of the $\alpha_1$-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 $\alpha_{1B}$-AR is not the subtype coupled to this positive inotropic effect. In other work with the $\alpha_{1B}$-AR$_{C128F}$ over-expressing mice, we have shown that it is the $\alpha_{1A}$-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 $\alpha_{1B}$-AR decreases the ability of the $\alpha_{1A}$-AR to activate myocardial contraction (Ross et al., 2003 in revision) as well as decreasing $\alpha_{1A}$-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 $\alpha_{1B}$-AR diminishes cardiac contractile activity by decreasing the positive inotropic signaling emanating from both the $\beta_1$- and the $\alpha_{1A}$-ARs.

In addition to contractile dysfunction, echocardiographic analysis also revealed increases in the left ventricular internal dimensions of the $\alpha_{1B}$-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 $\alpha_{1B}$-AR (Grupp et al., 1998; Lemire et al., 2001). In contrast to these results, other reports with a cardiac-targeted constitutively active $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR has minimal activity as a regulator of vascular function. Previously, we showed that over-expression of the $\alpha_{1B}$-AR$_{C128F}$ does not increase resting systemic arterial blood pressure (Zuscik et al., 2001). Knockout of the $\alpha_{1B}$-AR also had no effect on resting blood pressure (Cavalli et al., 1997). Herein we show that over-expression of the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_1$-AR subtype family and that the $\alpha_{1B}$-AR is coupled to regulatory events in the heart without participating in the contraction of vascular smooth muscle.

The $\alpha_{1D}$-AR is an enigmatic and the least well-studied member of the $\alpha_1$-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 α1D-AR are accomplished by these intracellular receptors. Recently, it has been shown that the α1D-AR is constitutively active (Garcia-Sáinz and Torres-Padilla, 1999; Gisbert et al., 2000; McCune et al., 2000). D’Ocon’s group has shown that the constitutively active α1D-ARs are capable of mediating vascular smooth muscle contraction. Other studies have demonstrated that the α1D-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 α1D-AR has not been shown to have a function. We do not yet understand why members of the α1-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 α1D-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 α1D-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 α1D-AR knockout line of mice (Tanoue et al., 2002). Tanoue et al. (2002) showed that knockout of the α1D-AR significantly decreased systemic arterial blood pressure as well as the pressor responses to norepinephrine. In this work we show that knockout of the α1D-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 α1D-AR serves
predominately in vascular function.
Chapter 5

Conclusions

This dissertation work investigated the $\alpha_1$-ARs at the cellular and at the \textit{in vitro} and the \textit{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 \textit{et al.} (2002) will encompass the differences observed among the $\alpha_1$-AR subtypes with respect to basal localization, functional responses, and agonist-mediated internalization, desensitization, and trafficking properties. The discussion of the results in Chalothorn \textit{et al.} (2003) will focus on the roles of specific $\alpha_1$-AR subtypes in physiological responses such as myocardial regulation and function, vascular smooth muscle contraction, and potential roles in cardiac pathophysiology development.

\textbf{CHALOTHORN ET AL. (2002)}

Basal localization of $\alpha_1$-AR/GFPs

Our use of the $\alpha_1$-AR/GFPs in living HEK-293 cells revealed distinct differences in the basal localization patterns of the $\alpha_1$-ARs that were consistent within the subtypes regardless of cell shape or size. We observed a predominant surface orientation for the $\alpha_{1B}$-AR/GFP and an intracellular distribution for the $\alpha_{1D}$-AR/GFP (Chalothorn \textit{et al.}, 2002). Because of the receptor distribution, the $\alpha_{1B}$-AR subtype has characteristics of a typical GPCR whereas the $\alpha_{1D}$-AR has properties of an atypical GPCR. The $\alpha_{1A}$-AR falls between the two classifications because the $\alpha_{1A}$-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 $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFPs are consistent with studies using antibodies targeted against these receptors in fixed cells (Fonseca et al., 1995) or studies using $\alpha_{1A}$- and $\alpha_{1B}$-AR/GFP constructs in different cell lines (Hirasawa et al., 1997; Awaji et al., 1998).

Functional responses of the $\alpha_{1A}$- and the $\alpha_{1B}$-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 $\alpha_{1B}$-AR/GFP construct expression in mouse $\alpha$-T3 cells yields a functional receptor capable of activating phosphatidylinositol/Ca$^{2+}$ signaling (Awaji et al., 1998). In this work, we demonstrated that the $\alpha_{1A}$- and $\alpha_{1B}$-AR/GFP subtypes are coupled functionally to ERK1/2 activation with the $\alpha_{1A}$-AR/GFP showing a greater efficiency of coupling than the $\alpha_{1B}$-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 lysophatidic acid in rat-1 cells (Cook and McCormick, 1996). Thus, we assessed for ERK activation after a 2 hour incubation.

Properties of the $\alpha_{1D}$-AR/GFP

The small population of $\alpha_{1D}$-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 $\alpha_{1A}$- and the $\alpha_{1B}$-ARs than the $\alpha_{1D}$-AR (Zhong and Minneman, 1999). The reason(s) for this atypical behavior of the $\alpha_{1D}$-AR/GFP is(are) not clear, but our observations agree with the work of Theroux et al. (1996) that showed the $\alpha_{1D}$-AR to be inefficiently coupled to both inositol phosphate formation and intracellular $\text{Ca}^{2+}$ release in HEK-293 cells. Another reason for our observations may be related to the predominant intracellular localization of the $\alpha_{1D}$-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 transfected with the $\alpha_{1D}$-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 $\alpha_{1D}$-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 $\alpha_{1D}$-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 $\alpha_{1D}$-AR/GFP did not undergo agonist-mediated internalization (Chalothorn et al., 2002). However, we cannot exclude the possibility that some degree of $\alpha_{1D}$-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 $\alpha_{1D}$-AR/GFP exhibited a minor degree of agonist-mediated desensitization of ERK1/2 activation (Chalothorn et al., 2002). This is further evidence that the $\alpha_{1D}$-AR/GFP is a functionally active protein. The Lomasney et al. (1991) work documented that the $\alpha_{1D}$-AR lacks a consensus sequence for PKC phosphorylation on the 3\textsuperscript{rd} cytosolic loop, and this sequence may affect the ability of the receptor to be desensitized. Desensitization of the $\alpha_{1D}$-AR is controversial because a study by Yang et al. (1999) revealed that in the stably transfected rat-1 fibroblast, the $\alpha_{1D}$-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 $\alpha_{1D}$-AR stably transfected into the rat-1 fibroblast demonstrated that both norepinephrine and phorbol ester could desensitize the functional intracellular Ca\textsuperscript{2+} release. The notion that a constitutively active receptor can be desensitized is supported by a study looking at a constitutively active $\beta_{2}$-AR caused by a point mutation on the 3\textsuperscript{rd} intracellular loop (Pei et al., 1994). The investigators demonstrated that in the absence of an agonist, the constitutively active receptor was phosphorylated by $\beta$-ARK 1 in a manner similar to the agonist-mediated wild-type receptor phosphorylation. Therefore, desensitization of the constitutively activity of the $\alpha_{1D}$-AR/GFP may further explain the poor response to agonist incubation.
Agonist-mediated internalization of the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFP

Differences in the agonist-mediated internalization responses were noted between the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFP subtypes. The $\alpha_{1B}$-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 $\alpha_{1B}$-AR agonist-mediated redistribution (Fonseca et al., 1995; Wang et al., 1997, 2000). The internalization of the $\alpha_{1A}$-AR has not been studied. We showed that the $\alpha_{1A}$-AR/GFP internalizes slower and less extensively than the $\alpha_{1B}$-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 $\alpha_{1B}$- than the $\alpha_{1A}$-AR. This greater degree of $\alpha_{1B}$-AR receptor phosphorylation is consistent with our finding that the $\alpha_{1B}$-AR/GFP internalizes quicker since internalization proteins are more likely to bind to phosphorylated receptors.

Agonist-mediated desensitization of the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFP

Investigation of the agonist-mediated desensitization of ERK1/2 activation revealed differences between the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFP subtypes. Both the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFPs demonstrated characteristics typical of desensitization, in which the $\alpha_{1B}$-AR/GFP was more readily desensitized (Chalothorn et al., 2002). Our observations agree with a study documenting that both the $\alpha_{1A}$- and the $\alpha_{1B}$-ARs undergo functional desensitization of agonist-stimulated inositol phosphate formation in rat-1 fibroblasts.
(Yang et al., 1999). Additionally, our observation that the $\alpha_{1B}$-AR/GFP is desensitized more readily than the $\alpha_{1A}$-AR/GFP is in good agreement with the Vázquez-Prado et al. (2000) study that found a greater degree of PKC phosphorylation for the $\alpha_{1B}$-AR than the $\alpha_{1A}$-AR.

**$\beta$-arrestin 1 participation in $\alpha_1$-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 $\alpha_{1}$-AR-mediated internalization, we performed studies to determine if these proteins are important for internalization. In experiments using the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFPs co-transfected with either $\beta$-arrestin 1 or 2, we found no differences in the ability of either $\beta$-arrestins to influence the $\alpha_{1A}$- or the $\alpha_{1B}$-AR/GFP basal localization and internalization properties (Chalothorn et al., 2002). This suggested that either $\beta$-arrestins do not participate in $\alpha_{1}$-AR internalization or there are already adequate levels of endogenous $\beta$-arrestins in HEK-293 cells. Since over-expression of the $\beta$-arrestins did not conclusively link the $\beta$-arrestins to $\alpha_{1}$-AR/GFP internalization, additional experiments examined the relationship between arrestin/clathrin interaction and $\alpha_{1}$-AR/GFP internalization. To this end, we used a dominant-negative $\beta$-arrestin 1 [\(\beta\)-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 $\beta_2$-AR internalization (Krupnick et al., 1997; Orsini and Benovic, 1998). Expression of $\beta$-arrestin 1 (319-418) did not affect the basal localization patterns for any of the $\alpha_{1}$-AR/GFPs, but it did antagonize the $\alpha_{1A}$- and the $\alpha_{1B}$-AR/GFPs agonist-mediated internalization responses (Chalothorn et al.,
2002). The β-arrestin 1 (319-418) did not produce a detectable change in the α1D-AR/GFP localization or agonist-mediated internalization response. Our findings suggest that arrestin/clathrin interaction is irrelevant for maintaining basal localization patterns for the α1D-AR/GFPs but relevant for α1A- and α1B-AR/GFP agonist-mediated internalization. Although the use of β-arrestin 1 (319-418) confirms the role of arrestin/clathrin interaction in α1A- and α1B-AR/GFP internalization, the specific role that either β-arrestin 1 or 2 plays in α1-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.

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Work from Chalothorn et al. (2002) shows differences in the α1-AR subtype localization, activation, internalization, desensitization, and trafficking properties. Because of these differences, we propose that the α1-AR subtypes subserve different biological responses by coupling to distinct regulatory processes. We believe that the α1B-AR is involved in the modulation of cardiac function whereas the α1D-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 α1-AR subtypes at the physiologic level. We used transgenic animals expressing a constitutively active form of the α1B-AR (α1B-ARC128F) 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 $\alpha_{1B}$-AR without use of agonists that would activate all $\alpha_1$-ARs and investigation of the $\alpha_{1B}$-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 $\alpha_1$-AR expression.

MAPK activity in $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR$_{C128F}$ hearts

The echocardiographic study revealed distinct differences of the left ventricular internal dimensions in animals expressing the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR provided evidence of cardiac hypertrophy. Similarly, the Milano *et al.* (1994) study using cardiac-targeted over-expression of a constitutively active $\alpha_{1B}$-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 $\alpha_{1B}$-AR leads to a cardiac pathophysiology such as cardiac hypertrophy or dilated cardiomyopathy.
**In vivo and in vitro assessment of cardiac function in α\textsubscript{1B}-AR\textsubscript{C128F} hearts**

In the control and the α\textsubscript{1B}-AR\textsubscript{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\textsubscript{1B}-AR\textsubscript{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\textsubscript{1B}-AR\textsubscript{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\textsubscript{1B}-AR\textsubscript{C128F} over-expressing hearts. Additionally, we did observe decreased contractility (altered end-systolic pressure-volume relationship) for β\textsubscript{1}-AR-mediated inotropic responses in isolated-perfused heart experiments in α\textsubscript{1B}-AR\textsubscript{C128F} over-expressing hearts than control hearts. The log-dose response curves for maximal
increases in left ventricular developed pressure as a function of \( \beta_1 \)-AR stimulation were 171.0 ± 8.9 and 142.3 ± 3.0 mm Hg for control and \( \alpha_{1B-AR_{C128F}} \) over-expressing hearts, respectively. In addition, the log EC\(_{50} \) calculated were -7.87 ± 0.20 and -8.09 ± 0.15 for control and \( \alpha_{1B-AR_{C128F}} \) 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 \( \alpha_{1B-AR} \) (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 \( \alpha_{1B-AR_{C128F}} \) ventricular slices**

Since the \( \beta_1 \)-AR inotropic response was impaired in hearts over-expressing the \( \alpha_{1B-AR_{C128F}} \), we examined the \( \beta_1 \)-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 \( \alpha_{1B-AR_{C128F}} \), indicating that the phosphodiesterase activity was not enhanced in ventricle slices as a result of \( \alpha_{1B-AR_{C128F}} \) over-expression. We found attenuated cAMP generation to isoproterenol treatment of hearts with the over-expression of the \( \alpha_{1B-AR_{C128F}} \), which suggests that the tonic unregulated activation of the \( \alpha_{1B-AR} \) impairs the \( \beta_1 \)-AR signaling (Chalothorn et al., 2003). Because Perez et al. (1997) showed that the \( \alpha_{1B-AR_{C128F}} \) is only able to couple to the PLC-\( \beta \) pathway, the
likely mechanism for the reduced isoproterenol-mediated cAMP generation is heterologous desensitization of the β1-AR population by PKC phosphorylation. In support of this, Akhter et al. (1997) and Lemire et al. (2001) indicated that the enhanced α1B-AR activity increases the PKC phosphorylation of the GRKs leading to heterologous desensitization of the β1-AR without reducing the β1-AR population. In addition, Akhter et al. (1997) observed lower cAMP levels in isoproterenol-treated transgenic heart homogenates, which was attributed to α1B-AR coupling with Gs protein to antagonize the β1-AR-Gs pathway.

Inotropic response and α1B-AR activation

Collaborative work with the Perez laboratory has shown that over-expression of the α1B-AR_C128F reduces the mRNA levels of the α1A-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 α1B-AR results in α1A-AR down-regulation. The mechanism the α1B-AR uses to influence α1A-AR mRNA levels is under investigation. Our work and that of others suggest that the increased activity of the α1B-AR negatively modulates α1-AR inotropic signaling in the mouse heart. The down-regulation of the α1A-AR attenuates the α1-AR positive inotropic response, which suggests that the α1A-AR is coupled to positive inotropy (Ross et al., in revision). Our data are consistent with studies showing that the α1A-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 α1B-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 $\alpha_{1B}$-AR$_{C128F}$ over-expressing mice, it is reasonable to believe that $\alpha_{1B}$-AR is a cardiac specific receptor that has the potential to negatively modulate the positive inotropic effects of the $\alpha_{1A}$- and the $\beta_1$-ARs.

**Contractility assessment in $\alpha_{1B}$-AR$_{C128F}$ thoracic aortae**

Since our laboratory hypothesizes that the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR$_{C128F}$ (0.441 ± 0.021g vs. 0.405 ± 0.035g, respectively). This agrees with our earlier finding that the over-expression of the $\alpha_{1B}$-AR$_{C128F}$ does not increase the basal mean arterial pressure (Zuscik et al., 2001). The knockout of the $\alpha_{1B}$-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 $\alpha_{1B}$-AR, and this study demonstrated that the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1D}$-AR KO hearts**

To further test our hypothesis of specificity in the regulatory activities of the $\alpha_1$-ARs, we used 6 to 7 months old mice lacking the expression of the $\alpha_{1D}$-AR ($\alpha_{1D}$-AR KO). Echocardiographic data from these $\alpha_{1D}$-AR KO animals did not indicate anomalies in cardiac dimensions or functions (Chalothorn et al., 2003). The finding that $\alpha_{1D}$-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 $\alpha_{1D}$-AR has little or no effect on cardiac dimensions and/or function. In the isolated-perfused heart experiments, we found no evidence that the $\alpha_{1D}$-AR plays a role in regulating cardiac function since no differences were noted in the ability of the $\alpha_{1D}$-AR KO hearts to respond to isoproterenol when compared to the non-transgenic control responses (Chalothorn et al., 2003).

**Role of $\alpha_{1B}$- and $\alpha_{1D}$-AR in vascular smooth muscle contraction**

In contrast to the minor effect on cardiac function, the $\alpha_{1D}$-AR plays a prominent role in regulating vascular smooth muscle contraction. Studies with the $\alpha_{1B}$-AR$_{C_{128F}}$ aortae and the $\alpha_{1D}$-AR antagonist, BMY 7378, indicate that the primary mediator of the $\alpha_1$-AR-induced contraction is the $\alpha_{1D}$-AR (Chalothorn et al., 2003). This finding further supports the idea that $\alpha_1$-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 $\alpha_{1D}$-AR is the subtype responsible for contraction of the mouse thoracic aorta. Many other studies support the conclusion that the $\alpha_{1D}$-AR is a regulatory receptor involved in vascular smooth muscle contraction (see background).

Additional evidence that the $\alpha_{1D}$-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 $\alpha_{1D}$-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 $\alpha_{1D}$-AR, which indicated that the $\alpha_{1D}$-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 $\alpha_1$-AR agonists. Our study and the Tanoue et al. (2002) study demonstrate that the $\alpha_{1D}$-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 $\alpha_1$-AR subtypes for cellular and physiological responses. Using the $\alpha_1$-AR/GFP constructs, we demonstrate that the $\alpha_{1B}$-AR subtype most approximates the prototypic GPCR with respect to cellular localization, agonist-mediated activation, internalization, desensitization, and trafficking properties. Although the $\alpha_{1A}$-AR subtype displays both
surface and intracellular localization, this subtype possesses properties expected of a GPCR. However, the \( \alpha_{1D} \)-AR subtype seems to be an atypical GPCR. The work done in transgenic animals reveals that the \( \alpha_{1B} \)-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 \( \alpha_{1D} \)-AR subtype is not involved in maintaining cardiac regulation or function, yet the \( \alpha_{1D} \)-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 \( \alpha_{1} \)-AR subtypes. At the cellular level, we noted that there are indeed differences in the \( \alpha_{1} \)-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 \( \alpha_{1D} \)-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 \( \alpha_{1D} \)-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 \( \alpha_{1D} \)-AR/GFP and the chimeric versions may provide support to the notion that the \( \alpha_{1D} \)-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 \( \alpha_1 \)-AR subtypes in a recombinant system mirror the receptors in the endogenous tissue. So additional experiments with a GFP-tagged \( \alpha_1 \)-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 \( \alpha_1 \)-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 $\alpha_1$-ARs at a certain time after the animal has reached adulthood. These studies may offer a better method of assessing the function of the $\alpha_1$-ARs in vivo.

In addressing the reason why there is a decreased $\beta_1$-AR positive inotropic response in the $\alpha_{1B}$-AR$_{C128F}$ expressing mice, binding studies investigating the $\beta_1$-AR density in the heart may be of use. In addition, determining what effects the constitutive activation of the $\alpha_{1B}$-AR has on $\beta_1$-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 $\alpha_{1D}$-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 $\alpha_{1D}$-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 α1-AR/GFP subtype (data generated by Mary L. García-Cazarín). See Methods for the description of α1-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) $\alpha_{1A}$- or b) $\alpha_{1B}$-AR/GFP in the absence (retracing of Figure 9) and the presence of either $\beta$-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)
Appendix C

Hypotension, Autonomic Failure, and Cardiac Hypertrophy in Transgenic Mice Overexpressing the α1B-Adrenergic Receptor

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α1-Adrenergic receptors (α1A, α1D, and α1D) regulate systemic arterial blood pressure and blood flow. Whereas vasoconstrictory action of the α1A and α1D subtypes is thought to be mainly responsible for this activity, the role of the α1B-adrenergic receptor (α1B-AR) in this process is controversial. We have generated transgenic mice that overexpress either wild type or constitutively active α1B-ARs. Transgenic expression was under the control of the isogonic promoter, thus assuring appropriate developmental and tissue-specific expression. Cardiovascular phenotypes displayed by transgenic mice included myocardial hypertrophy and hypotension. Inducible 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 α1B-ARs 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 systemic neural activity. Reduced catecholamine levels together with basal hypotension, bradyarrhythmias and reproducibility 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 α1A-ARs that is important in systemic regulation of blood pressure.

The adrenergic receptor family, which includes 3 α1, 3 α2, and 3 β-receptor subtypes, is a group of G-protein-coupled receptors that mediate the effects of the sympathetic nervous system. Extensive effort has been spent in classifying the three known α1-adrenergic receptor (α1-AR) subtypes (α1A, α1D, and α1D) via molecular cloning techniques (1–4) and pharmacological analysis (5). The most well characterized cardiovascular regulatory actions associated with α1-AR 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 myocardiun (11, 12). In other α1-AR-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 Gq/ phospholipase C pathway (6), which leads to the subsequent activation of downstream signaling molecules including protein kinase C and inositol 1,4,5-triphosphate.

The progress toward elucidating the distinct regulatory role of each α1-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 α1A system where there are no selective agonists or antagonists available. We have alleviated this constraint by examining the unique attributes of the α1A-AR in a transgenic mouse model that exhibits constitutive α1A-AR activity targeted only to tissues that normally express the receptor. The appropriate distribution of receptor overactivity was achieved by using the mouse isogonic α1A-AR promoter (15) to drive the overexpression of a transgene containing cDNAs of either the wild type (W) hamster α1A-AR (3) or the constitutively active mutant forms of the receptor. Two such mutants were employed, a C128F single mutant (S) and a C128F/A354V/A358E triple mutant (T), both of which spontaneously couple to Gq (16, 17). The systemic expression of constitutively active α1A-ARs in three 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 this present study, we extend this examination of phenotype to the cardiovascular system. Discrete overexpression of

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Autonomic Failure of α2a-Adrenoceptors in Cardiovascular Regulation

Constitutive α2aAR activity in the cardiovascular system makes these mice well suited to address questions regarding α2aAR regulation of cardiovascular homeostasis. Our findings not only confirm the involvement of the α2aAR in cardiac hypertrophy but suggest that this subtype is not involved with blood pressure-related vasconstriction. Rather, the hypothesis seems to be that autonomic failure and not the result of a direct action of the α2b subtype in the peripheral vasculature. Understanding the α2aAR control over these processes and the manifestation of disease will further define the therapeutic potential that would come from the development of α2aAR-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 α2aAR overactivity has been described elsewhere (18). Tissue-specific α2aAR overactivity using the mouse α2aAR gene promoter (15) to drive the overexpression of a transgene containing a cDNA coding for the wild-type (W) or activated (R) α2aAR (3) or the constitutively active single-mutant (S166F) α2aAR (16; or triple mutant (T26;G1809;V162) α2aAR (17). The Cleveland Clinic Foundation Transgenic Core Facility injected ~200 copies of each transgene into the pronuclei of one-cell B6CBAF1/Cg mouse embryos, which were surgically implanted into pseudopregnant female mice. Male single, 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 described 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/kg ketamine HCl and 0.1 mg/kg thiamylal. 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 (12 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, \( Q = \pi \times \text{PAP} \times \text{VTI} \times \text{HR} \), where PAP was the diameter of the pulmonary artery, VTI was the diastolic velocity time integral in the pulmonary artery, and HR was the echocardiographically determined heart rate.

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 (20). The mice were anesthetized with an initial intraperitoneal injection of ketamine (0.05 mg/kg) followed 5 min later by an injection of thiobutabarbital (0.1 mg/kg). Supplemental doses of thiobutabarbital were delivered only when necessary to maintain stable anesthesia. A femoral catheter was inserted and connected to a low compliance COX Beckman pressure transducer (Cobe Cardiovascular, Arvada, CO). Blood pressure readings were collected on a Medex 70 Polygraph (Grafs Instruments Division, West Warren, RI). The recording began immediately after anesthesia and continued for 7-8 h.

RESULTS AND DISCUSSION

General Characterization of Mice Possessing α2aAR Overactivity—We have previously described the genotypic and initial phenotypic analysis of systemic α2aAR mice (18), confirming transgene integration. The tissue-specific overexpression of wild-type and mutant α2aARs was confirmed via saturation binding analysis of various tissues from P1 and P2 generation of heterozygous mice. Of the seven transmitting founders, 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 α2aAR 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/+ 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 α2aAR (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 20–50% 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 α2aAR Overactivity—α2aARs have been shown to evoke a hypertrophic response in cultured cardiac myocytes (23, 24) with the regulation of this process predominated by the α2b subtype (25, 26). Because
myocardial-targeted overexpression of constitutively active α1bARs 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. Inductive of a hypertrophic phenotype, W2, S1, T1, and T2 mice showed an increased heart-to-body weight (heart:body) ratio compared with age-matched (4–6 months) NT control mice (Fig. 2). Body weight was not significantly different among the mice 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 1).

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 (25) also shows hypertrophy in the absence of ANF up-regulation, suggesting that the progress of cardiac hypertrophy is not always strictly associated with the up-regulation of ANF (25) and other fetal genes. Another more likely reason for the lack of ANF up-regulation is the low level of α1bAR overexpression present in our model. For example, the Gag overexpression mouse model of cardiac hypertrophy (30) displayed no change in ANF expression with a 2-fold increase in the Gag protein, a circumstance similar to the 2-fold overexpression of cardiac α1bARs in our mice. However, a 4-fold increase in Gag 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 1). This probably is attributed to the decreased heart rate and increased isovolumetric relaxation time displayed by transgenic animals (Table 1). The decreased heart rate, which was confirmed via a tail cuff measurement in conscious mice (Table 1), may be the result of a direct effect on Purkinje fiber automaticity, which is thought to be controlled by the α1bAR (31) and is consistent with the overexpression of the receptor. A similar decrease in heart rate was also found in the heart-targeted Gag overexpressing mouse (30). Overall, because α1bARs are coupled to Goq1, the decrease in heart rate may be directly related to signaling events downstream of α1bAR 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 α1bAR has been shown to cause increased disc glycosaminoglycan content and ANP mRNA without inducing the morphological hallmarks of hypertrophy (12). One conclusion that can be drawn from this earlier study is that only constitutively active α1bARs 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 α1bARs in our mice caused a cardiac hypertrophy that was less robust but similar to that seen in the heart-targeted constitutively active α1bAR mouse. Unlike the heart-targeted model, our model may be exhibiting a phenotype that more genuinely represents the end point impact of α1bAR action in the heart because our use of the isogenic α1bAR promoter facilitates transgene overexpression in all α1bAR-expressing cardiac cell types, not just cardiac myocytes. Overall, because several experimentally distinct approaches to genetically induce α1bAR overactivity have independently led to the manifestation of a somewhat similar cardiac phenotype, the emergence of that phenotype must be α1bAR-dependent and not simply the spurious outcome of transgenic manipulation. Based on this assumption, we assert that in addition to the α1bAR, the α1aAR plays an important regulatory role in the progression of the hypertrophic program in cardiac tissue.

Hypertension in Mice Possessing α1bAR Overactivity—The α1bARs are widely expressed in the peripheral arteries (4, 5) and possess the capacity to regulate vasconstriction (32–36), thus implicating them in the control of blood pressure. Regarding the α1bAR, however, the bulk of the literature suggests that this subtype does not play an important role in the direct regulation of the peripheral vascular tone (6, 9). Rather, the predominant role of α1bARs 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 α1bAR knockout mice showed a decreased pressor response to phenylephrine infusion (8), which indicates the participation of the receptor in the regulation of peripheral vasconstriction. Based on these findings from the knockout model, if α1bARs participate in vasconstriction, our constitutively active α1bAR 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
Autonomic Failure of α1β-Adrenoceptors in Cardiovascular Regulation

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Age-matched NT control mice. Whereas T2 mice showed modest hypotension while still under the influence of the anesthetic agents (Fig. 3A and B), fully conscious and restrained S1 and T2 mice showed a more significant decrease in basal pressure compared with the NT control (Fig. 3A and C). The mean arterial pressure in conscious W2 mice was lower than the NT animals (Fig. 3C); 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. 4A). Overall, our observation of basal hypotension in constitutively active α1β-AR mice contradicts the idea that activation of the α1β-AR can induce vasoconstriction and is the first report to indicate that an α1β-AR 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 α1AR-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. 4B), arguing that the α1-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 α1β-ARs has been identified in peripheral arteries via the use of an α1β-specific antibody (56), these results suggest that vascular α1β-ARs are not directly involved with the regulation of vasoconstriction.

To confirm that the α1β-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. 4B), contractile response curves were generated using cut segments of the mesenteric artery prepared from several lines of mice. The vasoconstrictory activity of phenylephrine was tested in artery segments from α1β-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 those groups were not significantly different from each other (Fig. 5), demonstrating that the α1β-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. 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 α1AR agonist phenylephrine in isolated segments of mouse mesenteric artery (first order branches, external diameter 200–220 μm) taken from non-knockout (NK, closed circles), α1A 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, n = 5 for KO, NT, and W2 each). log EC50 (log[M]) values were −8.41 for NK mice, −5.54 for KO mice, −6.84 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 be significantly different from each other based on a one-way analysis of variance.

Because of the apparent lack of direct α1A AR control over vasoconstriction, the question remains how does systemic α1A 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 norepinephrine, could lead to a lower blood pressure because of a reduced activation of all vascular α1-adrenergic targets. This hypothesis was tested by assessing sympathetic function via 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 catecholamines compared with age-matched NT control mice (Fig. 6A). A similar reduction in total catecholamines was seen after a 1-h period of stable anesthesia (Fig. 6B), 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. 6C). 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 α1AR mice display a Parkinson-like syndrome termed multiple system atrophy with associated neurodegeneration in the substantia nigra, olives pontis, thalami, and locus coeruleus (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 (45).

Besides hypotension, our transgenic mice displayed reproducible problems, weight loss (at 12 months of age), bradyarrhythmia, 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 α1AR-induced neurodegeneration.

Overall, our analysis of α1AR control of blood pressure from a systemic perspective has led us to conclude that α1AR 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 contradictory of a vasocostrictive role for α1ARs. Our findings support an emerging hypothesis, which predicts that α1ARs do not play a major role in contractile regulation in vascular smooth muscle (9) but rather are predominantly coupled to various metabolic and cellular processes at vascular sites where the receptor is expressed (6, 37–38). 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 transgenic injections, which produced founder mice. Blood catecholamine and cortisol levels were determined by the Laboratory Medicine Section of the Cleveland Clinic Foundation.

REFERENCES

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 α₁D-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)

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Appendix F

The Role of the $\alpha_{1B}$-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 *

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Running Head: Role of the $\alpha_{1B}$-AR in myocardium

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ABSTRACT

$\alpha_1$-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 $\alpha_1$-AR subtypes ($\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$) to cardiac function is not clearly defined. In this study, we used a systemically expressing mouse model that over expresses the $\alpha_{1B}$-AR to define the role of this subtype in cardiac function. Using an isolated heart model, we find that a 50% increase of the $\alpha_{1B}$-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 $\alpha_{1A}$-AR antagonist, 5-methylurapidil, which correlated with decreases in $\alpha_{1A}$-AR density, suggesting that the $\alpha_{1B}$-AR had caused a compensatory downregulation of the $\alpha_{1A}$-AR. These results suggest that the $\alpha_{1B}$-AR does not have a major role in the positive inotropic response in the mouse myocardium but may negatively modulate the response of the $\alpha_{1A}$-AR.

Keywords: Adrenergic receptor, heart, myocyte, inotropy
Introduction

$\alpha_1$-Adrenergic receptors (ARs) mediate the effects of the sympathetic nervous system by binding the catecholamines, epinephrine and norepinephrine. $\alpha_1$-AR subtypes ($\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$) are part of the larger and related family of adrenergic receptors which include the $\beta$-ARs ($\beta_1$, $\beta_2$, $\beta_3$) and the $\alpha_2$-ARs ($\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$). 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). $\alpha_1$-AR modulation of cardiac function may become more important in diseased heart where $\beta$-AR responsiveness is often impaired with concomitant upregulation of the $\alpha_1$-AR response (for review, see 2). All three $\alpha_1$-AR subtypes are expressed in the heart of a variety of species (mouse, rat, rabbit, dog, human). Predominant subtypes are the $\alpha_{1A}$- and $\alpha_{1B}$-AR with minor expression (if any) of the $\alpha_{1D}$-AR subtype (23, 26). $\alpha_1$ AR mediated positive inotropic effects have been well-documented in a number of animal models both in vivo and in vitro although this response is considered minor in comparison to $\beta$-AR stimulation (25% versus 75% with norepinephrine stimulation in rat)(11). However, the role of $\alpha_1$-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 $\alpha_{1B}$- and $\alpha_{1A}$-AR
expression, we have developed a transgenic mouse model whereby use of the isogenic mouse $\alpha_{1B}$-AR promoter has allowed us to over express a constitutively active mutant (CAM) form of the hamster $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR leads to decreased ventricular function (10) while over-expression of the $\alpha_{1A}$-AR in the targeted heart was found to increase inotropy dramatically but without evidence of any hypertrophy (14). We wanted to explore the $\alpha_{1B}$-AR subtype in the heart and determine its role in cardiac function in our systemic mouse model.

Since we have found that our $\alpha_{1B}$-AR transgenic mice display neurodegeneration (33) and a corresponding autonomic dysfunction (34), we decided to look at the effects of $\alpha_{1B}$-AR over-expression in the myocardium ex vivo to try to determine what contribution the $\alpha_{1B}$-AR makes to the inotropic effects of the $\alpha_1$-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 $\alpha_{1A}$-AR levels in the transgenic animals due to compensatory effects. Therefore, although the $\alpha_{1B}$-AR may not play a direct role in inotropy in the mouse myocardium, its effects may be related to a negative regulation of the $\alpha_{1A}$-AR subtype, the major determinant of cardiac inotropy.
Materials and Methods

Mice. The generation and genotyping of transgenic mice possessing systemic $\alpha_{1B}$-AR over-activity has been described elsewhere (33). Briefly, tissue-specific distribution of systemic $\alpha_{1B}$-AR over-activity was achieved by utilizing the murine $\alpha_{1B}$-AR gene promoter (32) to drive over-expression of a transgene containing a cDNA coding for a constitutively active mutant of the $\alpha_{1B}$-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 B6/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$_2$PO$_4$, 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 \(\alpha_1\)-AR antagonist 2-\(\beta\)-(4-hydroxyl-3-[\(^{125}\)I]iodophenyl)ethylaminomethyl]-tetralone ([\(^{125}\)I]HEAT) as the radioligand and phentolamine (100 uM) to determine the total \(\alpha_1\)-AR density. The density of the \(\alpha_{1B}\)-AR population was determined by repeating the saturation experiment with 1 nM of 5-methylurapidil to calculate non-specific binding. Total \(\alpha_{1A}\)-AR density was determined by subtracting the \(\alpha_{1B}\)-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 KH2PO4, 1.2 mM MgSO4, 10 mM Hepes, 11 mM Glucose, 0.2 mM pyruvate, pH 7.2, gassed with 100% O2 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 Ca2+-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 [Ca2+] was adjusted to 20 µM. Perfusion of the heart with the Ca2+/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 Ca2+. 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 Ca2+. 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 α₁-AR selective agonist, in the presence of propranolol (1 uM) and rauwolscine (0.1 uM) to block any effects due to β- or α₂-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 μM). 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.

**α₁-AR subtype mediation of inotropy.** To determine the α₁-AR subtype responsible for the observed phenylephrine effects, we used 5-methylyurapidil at a dose (1 nM), which is about 100-fold selective against the α₁A-AR subtype. Figure 2 shows the phenylephrine-response in both control and transgenic hearts in the presence of 5-methylyurapidil (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 α₁A-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 α₁-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 \( \alpha_1 \)-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 \( \alpha_1 \)-AR subtypes. This experiment would determine the total \( \alpha_1 \)-AR population. The experiment is then repeated with a selective concentration of the \( \alpha_{1A} \)-selective blocker, 5-methylurapidil, used at the same concentration as in the functional studies (1 nM). The specific binding would then determine the \( \alpha_{1B} \)-AR population. Since it has been previously shown that the \( \alpha_{1D} \)-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 \( \alpha_{1B} \)-AR densities would determine the amount of \( \alpha_{1A} \)-AR present. Normal and transgenic hearts had total \( \alpha_1 \)-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 \( \pm \) 11 for normal and 140 \( \pm \) 16 for the transgenic). However, the \( \alpha_{1B} \)-AR density increased in the transgenic 72.5 \( \pm \) 13 versus 40.3 \( \pm \) 3.5 fmoles/mg, consistent with the over-expression of this receptor in the heart. This leaves the \( \alpha_{1A} \)-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 \( \alpha_{1B}/\alpha_{1A} \) 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) $\alpha_{1B}$-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 $\alpha_{1A}$-AR antagonist 5-methylurapidil and supported by decreases in $\alpha_{1A}$-AR density by saturation binding. These results suggest that the over-expression of the $\alpha_{1B}$-AR leads to a down-regulation of the $\alpha_{1A}$-AR, ultimately resulting in the observed functional and decreased changes in response to phenylephrine.

The inotropic response to $\alpha_1$-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 $\alpha_1$-AR agonists have been found in whole heart and muscle strips from rat, rabbit, guinea pig, hamster and dog. However, $\alpha_1$-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 $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_1$-ARs. While we report $\alpha_1$-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 $\alpha_{1A}$- and $\alpha_{1B}$-AR subtypes contribute to the positive inotropic effect of $\alpha_1$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR (33). Overexpression is mild, with no significant changes in $\alpha_1$-AR density but resulted in a 50% increase in the $\alpha_{1B}$-AR population (Fig. 5). This is distinctly different from the heart-targeted transgenics of the $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_1$-ARs (7), it is possible that heart fibroblasts as well as the endothelium and smooth muscle cells may contain $\alpha_{1B}$-ARs and be involved in paracrine functions to release factors that contribute to the inotropy. We have recently shown that $\alpha_1$-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 $\alpha_{1A}$-AR binding sites were decreased, the mechanism of action is likely through compensatory effects on the $\alpha_{1A}$-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 $\alpha_{1B}$-AR did not affect basal function. This confirms the finding of previous studies (1) where even 100-fold heart-targeted over-expression of wildtype $\alpha_{1B}$-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 $\alpha_1$-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 papillary 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 papillary
that the negative inotropic effect is mediated by the $\alpha_{1A}$-AR subtype and the
positive inotropic responses are mediated by both the $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_{1}$-AR subtypes (15). This is
contrasted with the $\alpha_{1A}$-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 $\alpha_{1D}$-AR was
responsible for the negative inotropy. Moreover, since trabeculae from these
animals did not show any phenylephrine response, the data suggests that the
$\alpha_{1D}$-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 $\alpha_{1D}$-AR densities.

We did performed similar isolated tissue experiments (data not shown)
and found that in the isolated mouse right ventricle a sustained negative inotropv
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 $\alpha_{1B}$-AR over-expression of Akhter et al. (1) and
Grupp et al. (10) also show positive $\alpha_{1}$-AR mediated inotropy, but isolated mouse
myocytes have also been described to have negative intropy (25). The
discrepancy 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 possibility 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 α₁-AR subtypes, which could be differentially distributed in various heart regions. These studies await use of green fluorescent-tagged receptor transgenics since high avidity antibodies are not yet available. Nevertheless, repeatable and opposite results in α₁-AR mediated inotropy is observed depending upon the system used. Another likely and disturbing possibility is that the α₁-AR subtype distribution may change upon the removal of the organ. This has precedence in guinea pig liver studies in which the α₁-AR subtype switched (α₁A- to α₁D-AR) from the removal to the isolation of the heptacytes (8). The only consistent explanation is that whole or intact heart studies produce an α₁-AR mediated positive inotropy in the mouse (1, 10, 14, 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 α₁B-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 α₁B-AR model. One possibility that could explain this phenomenon was that over-expression of the α₁B-AR may have altered the expression levels of the other α₁-AR subtypes. Indeed, there is some precedence in the literature that this is possible. Prolonged incubation with norepinephrine which causes hypertrophy in normal rat myocytes
also causes the $\alpha_{1B}$- and $\alpha_{1D}$-AR to decrease while the $\alpha_{1A}$-AR increases (24). Deng et al., (6) have shown that there is crosstalk between $\alpha_{1A}$- and $\alpha_{1B}$-AR’s in neonatal rat myocardium, whereby knocking out the $\alpha_{1B}$-AR with CEC caused a potentiation of the $\alpha_{1A}$-AR response. In our studies, the $\alpha_{1A}$-AR antagonist 5-methylurapidil eliminated the functional differences between the transgenic and normal hearts for the effects on cardiac contractility, suggesting that the $\alpha_{1A}$-AR was responsible. The results correlated to the loss of $\alpha_{1A}$-AR binding sites. These results suggest that $\alpha_{1A}$-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 $\alpha_{1A}$-AR results (14) which suggests that the $\alpha_{1A}$-AR is a potent mediator of positive inotropy in the mouse myocardium.

The $\alpha_{1}$-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 $\alpha_{1A}$-AR. Other work has also implicated the $\alpha_{1B}$-AR as being involved in positive inotropic effects. Our data do not support this. Indeed, we demonstrate that over-expression of a constitutively active $\alpha_{1B}$-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 $\alpha_{1B}$-AR to negative inotropic pathways. Rather, the $\alpha_{1B}$-AR appears to interfere with the activity of receptors, such as the $\alpha_{1A}$-AR that mediates positive inotropic responses. Thus, via mechanisms that remain to be elucidated, there is molecular cross talk between the $\alpha_{1B}$-AR and receptors coupled to positive inotropic responses that result in a decrease in contractile function. Thus, we propose that the role of the $\alpha_{1B}$-AR is to negatively modulate contractile activation through indirect mechanisms and that tonic unregulated activation of the $\alpha_{1B}$-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 $\alpha_{1B}$-AR in the myocardium is unlikely to be only a modifier of the $\alpha_{1A}$-AR. Distinct differences are present when constitutively active receptors of the $\alpha_{1A}$- and $\alpha_{1B}$-AR respectively, were transfected into the cardiac murine myocyte cell line (HL-1). In this study, they demonstrate that the $\alpha_{1A}$-AR subtype preferentially couples to cardiac-specific atrial natriuretic factor gene expression, while the $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-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 $\alpha_{1B}$-AR does not lead to any basal functional changes in the isolated heart. However with $\alpha_{1}$-AR stimulation, we see a depressed response from the transgenic hearts compared to controls. We attribute this difference to a decrease in $\alpha_{1A}$-AR expression levels in the transgenic hearts. Therefore, we suggest that the $\alpha_{1B}$-AR does not modulate cardiac contraction directly but may be a negative modifier of positive inotropy.
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Footnotes:
\(^1\)While our work was in revision, the work of Turnbull et al., 2002 was published ahead of print, describing similar findings.
REFERENCES


Table 1: Baseline Physiological Parameters.

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<th>Parameters</th>
<th>Normal (n=6)</th>
<th>Transgenic (n=8)</th>
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<tbody>
<tr>
<td>Age (weeks)</td>
<td>37 ± 1</td>
<td>39 ± 1</td>
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<tr>
<td>Body Weight (g)</td>
<td>39 ± 2</td>
<td>28.3 ± 2 *</td>
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<td>Dry Ht. Wt. (g)</td>
<td>0.19 ± 0.01</td>
<td>0.15 ± 0.02</td>
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<td>Ht. Wt./Body Wt. (mg/g)</td>
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<td>5.6 ± 0.3</td>
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<td>CF/Ht. Wt. (ml/g)</td>
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<td>11.20 ± 0.47</td>
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</table>

Values are Mean ± SEM; Ht, heart; WT, weight; CF, coronary flow;
* = P < 0.05 versus normal.
Table 2: Baseline Cardiac Parameters.

<table>
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<tr>
<th>Parameters</th>
<th>Normal (n=6)</th>
<th>Transgenic (n=8)</th>
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<tr>
<td>HR (bpm)</td>
<td>345 ± 11</td>
<td>354 ± 12</td>
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<td>DP (mmHg)</td>
<td>93 ± 7</td>
<td>89 ± 5</td>
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<td>CF (ml.min⁻¹)</td>
<td>2.08 ± 0.19</td>
<td>1.98 ± 0.25</td>
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</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (n=6)</th>
<th>Transgenic (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>23 ± 2%</td>
<td>10 ± 3%*</td>
</tr>
<tr>
<td>DP (mmHg)</td>
<td>45 ± 10%</td>
<td>14 ± 5%*</td>
</tr>
<tr>
<td>+dP/dT (mmHg.ms⁻¹)</td>
<td>66 ± 14%</td>
<td>22 ± 7%*</td>
</tr>
<tr>
<td>- dP/dT (mmHg.ms⁻¹)</td>
<td>74 ± 18%</td>
<td>19 ± 8%*</td>
</tr>
<tr>
<td>CF (ml.min⁻¹)</td>
<td>45 ± 12%</td>
<td>59 ± 32%</td>
</tr>
</tbody>
</table>

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 20 min 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. α₁-AR subtype density in normal and transgenic hearts.** Membranes were prepared as described from individual mouse hearts. Total α₁-AR density was determined by saturation binding experiments using the nonselective α₁-AR-antagonist ¹²⁵I-HEAT as the radioligand and phenolamine (100 uM) to determine non-specific binding. α₁B-AR density was determined by performing the identical saturation study with the α₁A-AR selective blocker, 5-methylurapidil (1 nM). The α₁A-AR population was determined by subtracting the α₁B-AR density from the total α₁-AR pool. Bₘₐₓ was determined using the non-linear regression analysis of GraphPad Prism. Bars represent the mean Bₘₐₓ ± SEM (n=6-8). *, P < 0.05 versus normal.
Figure 1A

Phenylephrine $10^{-5}$M

$LVP$ (mmHg)

$CPP$ (mmHg)

dP/dT (mmHg/S)

BPM

CF (ml/min)

10 mins
Figure 1B
Figure 2
Figure 3

[Graph showing the effect of log phenylephrine (M) on % change in myocyte length, comparing normal and transgenic conditions.]
Figure 4

% change in myocyte length

Log Isoproterenol (M)

- Normal
- Transgenic
Figure 5
Appendix G
Contributions to papers


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


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


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* indicates equal contributions

Abstracts


Signature _____________________________