

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

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Received December 24, 2002; accepted March 12, 2003

ABSTRACT

The regulation of cardiac and vascular function by the α_{1B} - and α_{1D} -adrenoceptors (ARs) has been assessed in two lines of transgenic mice, one over-expressing a constitutively active α_{1B} -AR mutation (α_{1B} -AR_{C128F}) and the other an α_{1D} -AR knockout line. The advantage of using mice expressing a constitutively active α_{1B} -AR is that the receptor is tonically active, thus avoiding the use of nonselective agonists that can activate all subtypes. In hearts from animals expressing α_{1B} -AR_{C128F}, the activities of the mitogen-activated protein kinases, extracellular signal-regulated kinase, and c-Jun N-terminal kinase were significantly elevated compared with nontransgenic control animals. Mice over-expressing the α_{1B} -AR_{C128F} had echocardiographic evidence of contractile dysfunction and increases in chamber dimensions. In isolated-perfused hearts or left ventricular slices from α_{1B} -AR_{C128F}-expressing animals, the

ability of isoproterenol to increase contractile force or increase cAMP levels was significantly decreased. In contrast to the prominent effects on the heart, constitutive activation of the α_{1B} -AR had little effect on the ability of phenylephrine to induce vascular smooth muscle contraction in the isolated aorta. The ability of phenylephrine to stimulate coronary vasoconstriction was diminished in α_{1D} -AR knockout mice. In α_{1D} -AR knockout animals, no negative effects on cardiac contractile function were noted. These results show that the α_1 -ARs regulate distinctly different physiologic processes. The α_{1B} -AR appears to be involved in the regulation of cardiac growth and contractile function, whereas the α_{1D} -AR is coupled to smooth muscle contraction and the regulation of systemic arterial blood pressure.

G-protein-coupled receptors 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 G-protein-coupled receptor family is the α_1 -adrenergic receptor (α_1 -AR). Three subtypes, the α_{1A} -, α_{1B} -, and α_{1D} -ARs, have been isolated, cloned, and characterized. These receptors are intimately involved in the regulation of peripheral vascular resistance, cardiac function, and vascular and myo-

cardial cell growth (for recent reviews on all aspects of the α_1 -ARs see García-Sáinz et al., 1999; Varma and Deng, 2000; Piascik and Perez, 2001).

Data from heterologous expression systems have shown that all three α_1 -ARs can couple to a variety of G-proteins and second-messenger systems. The α_1 -ARs signal through both pertussis toxin-sensitive G-proteins (Perez et al., 1993) and G-proteins of the G_q family (Wu et al., 1992). Studies in both transiently and stably transfected cells have demonstrated that all α_1 -ARs activate phospholipases C and A₂ (Schwinn et al., 1991; Perez et al., 1993). In addition to mobilizing intracellular calcium (which would occur subsequent to activation of phospholipase C), the α_1 -ARs have also been shown to activate calcium influx via voltage-dependent and -independent calcium channels (Sayet et al., 1993; Lazou

This work was supported by National Institutes of Health Grant HL38120 (M.T.P.), a Predoctoral Fellowship from the Pharmaceutical Research and Manufacturers of America Foundation (D.F.M.), and a Predoctoral Fellowship from the American Heart Association (D.C.).

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Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.102.048553.

ABBREVIATIONS: AR, adrenergic receptor; E-64, *trans*-epoxysuccinyl-leucylamido-[4-guanido]butane; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; LV, left ventricular; PSS, physiologic salt solution; BMY-7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione.

et al., 1994; Minneman and Esbenshade, 1994).

Although these studies have increased our understanding of α_1 -AR regulatory biology, certain caveats must be established. Data from heterologous expression systems indicate the potential properties and regulatory activities of a given receptor. However, these data do not necessarily confirm that these regulatory events have a correlation in mammalian tissues that natively express these receptors. High-density expression of non-native receptors into cells could promote promiscuous coupling to pathways that may not normally be involved in *in vivo* receptor function.

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

Materials and Methods

Animal Use and Care

All animal protocols were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee. Tissues from two transgenic mouse lines were used in all aspects of this work. In one line, mice over-expressed a constitutively active mutation of the α_{1B} -AR, α_{1B} -AR_{C123F}. The over-expression of the constitutively active α_{1B} -AR was driven by the endogenous promoter, and the initial characterization of this mouse line has been described (Zuscik et al., 2000, 2001). The other mouse line was a recently described α_{1D} -AR knockout (Tanoue et al., 2002).

Assessment of MAP Kinase Activity

Tissue Preparation. Transgenic mouse hearts were removed, quick-frozen, and stored in liquid nitrogen. The frozen tissue was homogenized (Dremel, Racine, WI) and incubated on ice for 1 h in 400 μ l of the lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, 20 mM β -glycerophosphate, 0.5% Nonidet P-40, 100 μ M Na₃VO₄, 5 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1.5 nM aprotinin, 10 nM E-64, 10 nM leupeptin, pH 7.4). After the 1-h incubation, the lysate was centrifuged for 15 min at 15,000g at 4°C. The total protein content in the supernatant was determined by the Lowry assay (Lowry et al., 1951).

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, San Diego, CA) 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 β -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. After incubation in renaturation buffer, gels were preincubated in 25 ml of cold kinase buffer (20 mM HEPES, 20 mM MgCl₂, 2 mM dithiothreitol, 5 mM β -glycerophosphate, 0.1 mM Na₃VO₄) for 30 min. Phosphorylation of MBP was performed *in situ* by incubating the gels in kinase buffer containing 20 μ M ATP and 150 to 160 μ Ci of [γ -³²P]ATP for 90 to 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 PhosphorImager (Amersham Biosciences, Inc., Piscataway, NJ), 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 glutathione S-transferase-cJun(1-135). Anisomycin is a known activator of the stress-activated MAPKs; therefore, C6 Anisomycin extracts (Cell Signaling Technology Inc., Beverly, MA) were used as a positive control.

Immunoblotting. Equal amounts of protein samples were resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). 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 Biotechnology, Inc., Santa Cruz, CA) with horseradish peroxidase-conjugated anti-goat IgG at 1:10,000 (Jackson Immunoresearch Laboratories, 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 Biotechnology) with horseradish peroxidase-conjugated donkey anti-rabbit IgG at 1:2,000 (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, U.K.). Following exposure of the membranes to ECL Plus reagent (Amersham Biosciences UK, Ltd.), the chemiluminescent signal was detected with a PhosphorImager (Amersham Biosciences UK, Ltd.). 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 i.p. 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-gauge plastic cannula primed with ice-cold modified Krebs-Hensleit buffer (118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11 mM dextrose, 1.5 mM CaCl₂, and 1 mM pyruvate). The aorta was sutured into position and the cannula placed on a perfusion apparatus (Radnoti Glass Technology Inc., Monrovia, CA). Retrograde (Langendorff) perfusion was immediately performed with oxygenated (95% O₂ and 5% CO₂) modified Krebs-Hensleit buffer at 37.5°C. The hearts were allowed to beat spontaneously. The perfusion pressure was monitored with a pressure transducer (COBE Cardiovascular, 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 minute (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 to 10 mm Hg. The balloon catheter line was connected to a second pressure transducer, and an amplifier module was designated to measure the developed pressure, which was linked to a differentiator. The parameters measured were heart rate, left ven-

tricular (LV) systolic and end-diastolic pressure, and the rise and fall in LV developed pressure as a function of time ($+dP/dt$ and $-dP/dt$, respectively).

Drug-Induced Increases in Inotropy. In both lines of transgenic animals and their respective controls, hearts were perfused at a constant pressure of 75 mm Hg to assess the effects of α_1 -AR modulation on β -AR-induced positive inotropy. After a 25-min equilibration period, an isoproterenol dose-response curve was generated by infusing a stock solution of 100 nM at increasing rates (0.037–2.9 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 to 85 mm Hg was reached, experiments were performed at a constant flow. The protocol was conducted in the presence of 100 nM propranolol to limit the effect of β -AR stimulation on coronary perfusion pressure. After a 25-min equilibration, a stock solution of 1 mM phenylephrine was infused via an infusion pump to attain a final concentration of 100 μ M. 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 5 to 6 months of age (12 with the α_{1B} -AR_{C128F} and 11 controls). 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 a 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 Corporation, Mountain View, CA) 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) were 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), the 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)^{0.5}. 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

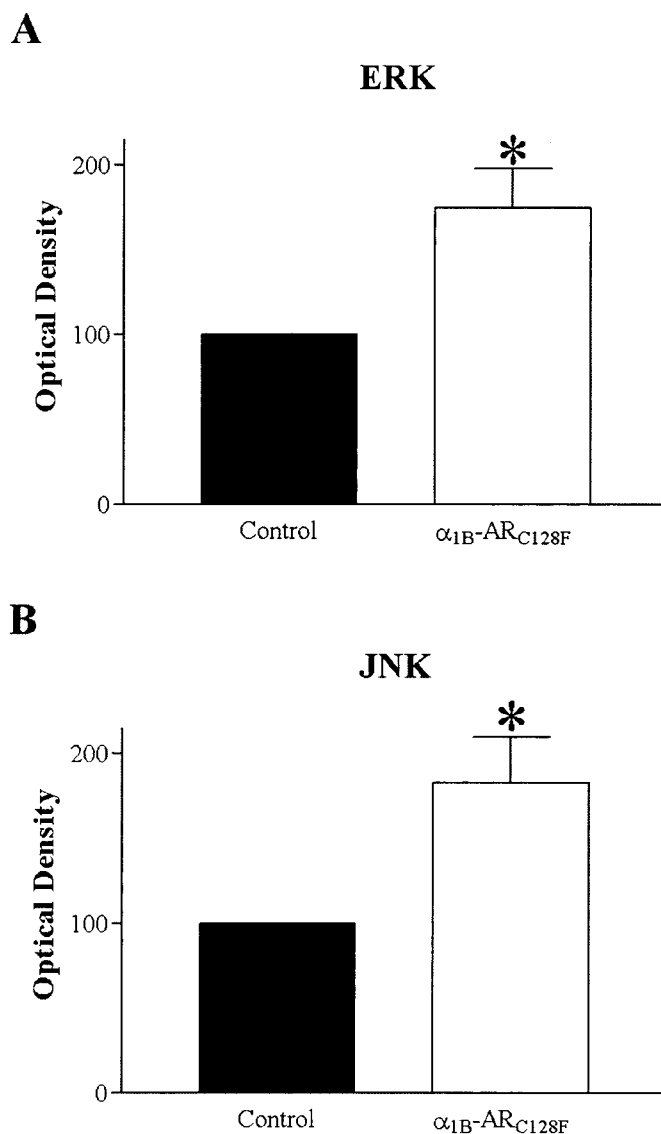


Fig. 1. MAPK activity in transgenic mouse hearts. ERK activity (A) and JNK activity (B) were measured by in-gel kinase assays, where each bar represents the mean and the S.E. of seven independent determinations. The asterisk (*) indicates significantly different values from nontransgenic control values.

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 1.0g 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 0.03 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 after a 20-min incubation with 30 nM BMY-7378, a selective α_{1D} -AR antagonist. The equilibrium dissociation constant for BMY-7378 was calculated as described by Besse and Furchgott (1976).

TABLE 1

Echocardiographic assessment of the murine left ventricular dimensions and function in mice over-expressing the α_{1B} -AR_{C128F}. The values are the mean \pm S.E.

| | n | Body Weight g | Left Ventricular Internal Dimension | | Posterior Wall Thickness | | Left Ventricular Mass g |
|------------------------------------|----|------------------|-------------------------------------|----------------------|--------------------------|---------------------|----------------------------|
| | | | Diastole mm/g | Systole mm/g | Diastole mm/g | Systole mm/g | |
| Control | 10 | 30.03 \pm 1.43 | 0.1364 \pm 0.0046 | 0.0770 \pm 0.0055 | 0.0197 \pm 0.0022 | 0.0358 \pm 0.0041 | 0.0782 \pm 0.0074 |
| α_{1B} -AR _{C128F} | 12 | 27.99 \pm 1.16 | 0.1523 \pm 0.0065* | 0.1038 \pm 0.0066* | 0.0171 \pm 0.0009 | 0.0313 \pm 0.0032 | 0.0683 \pm 0.0051 |

* Statistical difference from the nontransgenic value ($P < 0.05$).

Cyclic AMP Assay in the Mouse Myocardium

Tissue Preparation and Treatment. Mouse hearts were quickly removed and cleaned in nonsupplemented Dulbecco's modified Eagle's medium. The ventricles were sliced and placed in a fresh nonsupplemented Dulbecco's modified Eagle's medium with 100 μ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich, 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 μ l of lysis solution (0.1 M HCl) for 1 h on ice. The lysate was centrifuged for 5 s at 11,750g. 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 μ g/ml with 0.1 M HCl, the lysate was assayed for cAMP levels (nonacetylated) using a commercial enzyme immunoassay cAMP assay kit (BIOMOL Research Laboratories, 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 calorimetric enzymatic reaction with substrate) added = 11.02 \pm 0.35 optical density; percentage of nonspecific binding = 0.0008 \pm 0.0003%; percentage of maximum binding/total activity = 2.92 \pm 0.07%. From cAMP standards, the curves for calculating cAMP concentrations of the unknowns had a 20% intercept = 35.00 \pm 5.85 pmol/ml, 50% intercept = 7.65 \pm 0.59 pmol/ml, and 80% intercept = 1.60 \pm 0.28 pmol/ml. The line obtained had a slope of -32.85 \pm 1.54 with a correlation coefficient of 0.942 \pm 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 followed by Student-Newman-Keuls analysis. A value of $P < 0.05$ was considered statistically significant.

Results

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

Activation of Mouse Myocardial MAPKs. The hearts from α_{1B} -AR_{C128F} mice exhibited significantly elevated levels of ERK and JNK activity when compared with the nontransgenic controls (see Fig. 1, A and B). These results support the idea that the over-expressed α_{1B} -AR_{C128F} is functional and can couple to signaling pathways in the absence of agonists (however, see *Discussion*). ERK activity was not altered in hearts from α_{1D} -AR knockout mice (data not shown).

Echocardiographic Analysis. Activation of MAPKs has been proposed to link the α_1 -ARs to growth responses. Echocardiography was performed as a noninvasive method of

assessing the effect(s) of constitutive activation of the α_{1B} -AR on left ventricular (LV) dimensions and cardiac function (Table 1). The LV dimensions were normalized to the body weight. The transgenic animals showed significantly increased LV internal dimensions during either diastole or systole (Table 1) as well as an increase in chamber diameters. Chamber diameters 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 1). The LV dimensional analysis reveals that there is a significant reduction in the percentage fractional shortening in mice over-expressing the α_{1B} -AR_{C128F} when compared with the nontransgenic controls. The fractional shortening value, an index of contractile function, indicates poor cardiac performance in the transgenic line. The ejection time, heart rate, and mean velocity for circumferential fiber shortening corrected for heart rate were reduced in the animals with the α_{1B} -AR_{C128F} mutation. However these reductions were not statistically significant. Neither the stroke volume nor the cardiac output was found to be statistically different between groups. Therefore, persistent, unregulated activation of the α_{1B} -AR results in a decrease in contractile function and chamber dilation.

Responses in the Isolated-Perfused Heart. To more completely assess the effect of constitutive activation of the α_{1B} -AR on contractile responses, experiments were performed in the isolated-perfused heart. Resting heart rates were 348 \pm 18 and 384 \pm 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 (data not shown). Isoproterenol infusion produced similar increases in heart rate in both groups (Fig. 2A). The ability of isoproterenol (30 and 100 nM) to increase contractile force was significantly decreased in hearts from mice over-expressing the α_{1B} -AR_{C128F} mutation (LVDP and +dP/dt in Fig. 2, B and C). The -dP/dt curves were not significantly different (Fig. 2D).

cAMP Production. The blunted isoproterenol-induced response prompted additional experiments to determine whether there were changes in the β_1 -AR signaling pathway that resulted from α_{1B} -AR overactivity. We therefore assessed the ability of isoproterenol to increase cAMP levels in ventricular slices from control and transgenic animals. The positive control, sodium forskolin, produced similar increases in cAMP in both groups (Fig. 3). In control ventricular slices, isoproterenol (1 and 10 μ M) produced an increase in cAMP levels that was antagonized by 0.1 μ M propranolol. In ventricular segments from α_{1B} -AR_{C128F} mice, the cAMP response to either 1 or 10 μ M isoproterenol was reduced. This

| Left Ventricular Mass/Body Weight (10^{-3}) | Ejection Time | Mean Velocity for Circumferential Fiber Shortening Corrected for Heart Rate | Heart Rate | Stroke Volume | Cardiac Output | % Fractional Shortening |
|---|------------------|---|------------------|---------------------|------------------|-------------------------|
| | <i>ms</i> | $1/\sqrt{s}$ | <i>beats/min</i> | mm^3 | mm^3/min | |
| 2.659 ± 0.302 | 57.23 ± 3.83 | 0.6935 ± 0.1195 | 454.1 ± 22.7 | 0.0273 ± 0.0018 | 12.31 ± 0.83 | 43.10 ± 4.11 |
| 2.441 ± 0.149 | 51.76 ± 2.00 | 0.5550 ± 0.0984 | 484.6 ± 20.9 | 0.0274 ± 0.0023 | 13.21 ± 1.22 | $32.05 \pm 2.44^*$ |

difference was statistically significant at a concentration of 10 μ M.

Contractile Responses in the Mouse Aorta. In the aortae from nontransgenic control mice, phenylephrine produced concentration-dependent increases in developed tension (Fig. 4A). The dose-response curve was shifted to the right by a 30 nM concentration of the α_{1D} -AR selective antagonist BMY-7378. From these data we calculated the equilibrium dissociation constant for BMY-7378 to be 0.294 ± 0.149 nM. This value is in good agreement with that obtained from experiments with cloned α_{1D} -AR as well as the receptor expressed on rat blood vessels (2 nM; Piascik et al., 1995), indicating that, like the rat aorta, the phenylephrine contractile response in the mouse aorta is mediated by the α_{1D} -AR. Over-expression of a constitutively active α_{1B} -AR did not enhance the response of the mouse aortae to phenylephrine (see Fig. 4B). BMY-7378 was also a potent antagonist in the aorta from α_{1B} -AR_{C128F}-expressing mice with an estimated equilibrium dissociation constant of 0.385 ± 0.401 nM (see Table 2), indicating that the α_{1D} -AR still mediates contraction in this blood vessel. These data show that despite over-expression of a constitutively active and signaling competent form of the α_{1B} -AR, the response of the aorta is unaffected and remains under the control of the α_{1D} -AR. Consistent with this lack of effect on vascular smooth muscle contraction, we did not observe any effect on the ability of phenylephrine to induce coronary vasoconstriction in hearts from mice expressing the constitutively active α_{1B} -AR (data not shown).

Experiments in α_{1D} -AR Knockout Mice

Responses in the Isolated-Perfused Heart. The effects of α_{1D} -AR knockout on β -AR-induced responses were assessed in the isolated-perfused heart preparation. The ability of isoproterenol to induce positive chronotropy or inotropy was not significantly different between the control and the mice lacking the α_{1D} -AR (Fig. 5, A and B). (+) or (-) dP/dt curves were also not different in hearts from α_{1D} -AR-deficient mice (Fig. 5, C and D). Echocardiographic analysis also showed no differences in cardiac dimensions or cardiac function in α_{1D} -AR knockout mice (data not shown).

Effects on Coronary Perfusion Pressure. In contrast to having little effect on cardiac contractile responses, knockout of the α_{1D} -AR has prominent effects on coronary vascular responses. The basal coronary flow rate required to maintain the coronary perfusion pressure was found to be significantly greater in α_{1D} -AR knockout animals when compared with nontransgenic controls (Fig. 6). In hearts from control mice, 100 μ M phenylephrine infusion caused a significant increase in coronary perfusion pressure (Fig. 7). Phenylephrine-induced increases in perfusion pressure were significantly re-

duced in hearts from α_{1D} -AR knockout mice. Prominent effects on vascular function were also noted by Tanoue et al., (2002) in α_{1D} -AR knockout animals. These workers noted that the response of the aorta to phenylephrine was significantly impaired in knockout animals (Tanoue et al., 2002).

Discussion

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

These hypotheses were tested using two newly developed lines of transgenic mice. Although 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 nonspecific and occur as a result of interference in the expression of vital signaling molecules unrelated to the α_1 -ARs whose expression was altered.

To examine the regulatory activity of the α_{1B} -AR, we chose a transgenic line of mice over-expressing a constitutively active mutant of this α_{1B} -AR. An α_{1B} -AR knockout line of mice is also available (Cavalli et al., 1997). Studying these knockout animals would essentially be a loss of function protocol. However, by studying constitutively active receptors, we are able to use the gain of function as a readout of receptor activity. The use of constitutively active receptors offers another advantage in studying receptor systems like the α_{1B} -AR for which there are no selective agonists. Without such selective ligands, wild-type receptor activation can only be achieved by administering nonselective agonists such as phenylephrine that would activate all α_1 -AR subtypes. Because constitutively active receptors engage signaling pathways in the absence of agonists, we can observe the results of α_{1B} -AR activation without the need to administer agonist compounds.

In previous work, we showed that in the absence of agonist, the α_{1B} -AR_{C128F} can couple to inositol phosphate formation (Zuscik et al., 2001). In this work we show that there is an increase in the activity of MAPKs (see Fig. 1) in α_{1B} -AR_{C128F} animals. This would imply that this receptor is indeed constitutively coupled to signaling pathways. Coupling of the

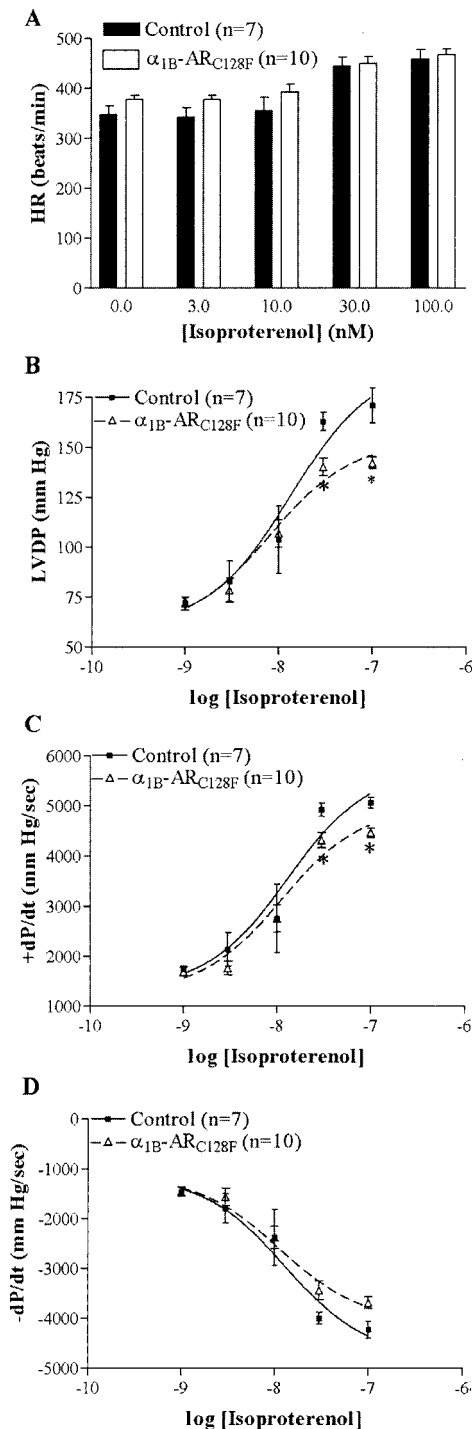


Fig. 2. Functional responses of mouse hearts to 3, 10, 30, and 100 nM isoproterenol. A, heart rate (HR); B, left ventricular developed pressure (LVDP); C, positive change in the developed pressure as a function of time (+dP/dt); and D, negative change in the developed pressure as a function of time (-dP/dt). Each bar or point on the curve represents the mean and the S.E. of 7 and 10 independent experiments for the nontransgenic control and α_{1B} -AR_{C128F} hearts, respectively. The asterisk (*) indicates statistical differences from the nontransgenic control value at the respective isoproterenol concentration.

α_{1B} -AR to MAPKs, would be in agreement with a great deal of data from nontransgenic sources (see reviews of García-Sáinz et al., 1999; Varma and Deng, 2000; Piascik and Perez, 2001). However, considering the uncertainties of experi-

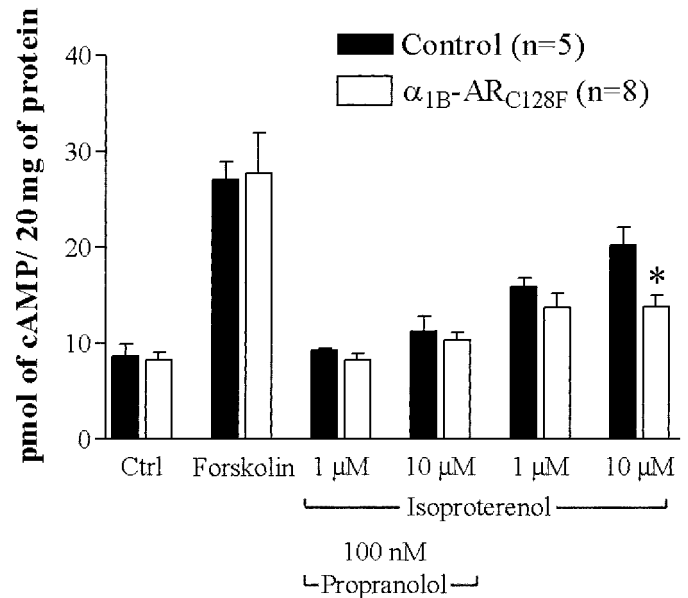


Fig. 3. The ability of isoproterenol to increase cAMP levels in ventricular slices from nontransgenic control and α_{1B} -AR_{C128F} animals. cAMP levels are presented as picomoles of cAMP/20 mg of protein. Data are the mean and the S.E. of five and eight heart samples from experiments performed in duplicate from the nontransgenic control and the α_{1B} -AR_{C128F} hearts, respectively. The asterisk (*) indicates significantly different cAMP levels from nontransgenic control cAMP levels.

ments 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 in cardiac function.

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

Further evidence that over-expression of the α_{1B} -AR_{C128F} interferes with myocardial contractility was obtained in the isolated-perfused heart where we observed that the ability of isoproterenol to increase contractile force was significantly reduced in hearts from transgenic animals (see Fig. 2, B and C). We also noted an impaired ability of isoproterenol to promote increases in cAMP levels (see Fig. 3) in homogenates from transgenic hearts. This indicates the possibility that tonic unregulated activation of the α_{1B} -AR impairs β_1 -AR signaling and could be the underlying reason for the decrease in contractile function.

Activation of members of the α_1 -AR subtype family has been associated with increases in myocardial contraction (see Varma and Deng, 2000, and references therein). The present work and that of others (Akhter et al., 1997; Lemire et al., 2001) shows that the α_{1B} -AR is not the subtype coupled to this positive inotropic effect. In other work with the α_{1B} -AR_{C128F} over-expressing mice, we have shown that it is the α_{1A} -AR that mediates the positive inotropic actions of phenylephrine (S. A. Ross, D. Chalothorn, J. Yun, P. J. Gonzalez-Cabrera, D. F. McCune, B. Rorabaugh, M. T. Piascik, and D. M. Perez, submitted for publication). We have further shown that constitutive activation of the α_{1B} -AR decreases the ability of the α_{1A} -AR to activate myocardial contraction (S. A.

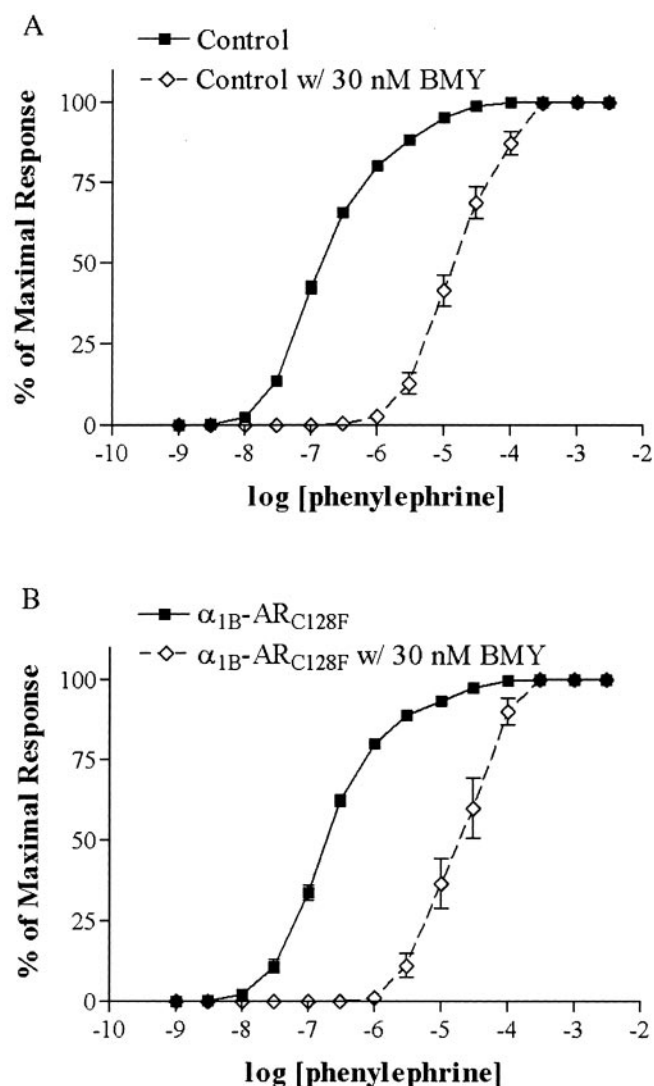


Fig. 4. Log-dose response curves of the phenylephrine-induced contraction in mouse thoracic aortae in the absence and the presence of 30 nM BMY-7378. A) Nontransgenic control, where the curves in the absence and the presence of BMY-7378 are composed of the average and the S.E. of 52 and 23 independent experiments, respectively and B) α_{1B} -AR_{C128F}, where the curves in the absence and the presence of BMY-7378 are composed of the average and the S.E. of 39 and 10 independent experiments, respectively.

Ross, D. Chalothorn, J. Yun, P. J. Gonzalez-Cabrera, D. F. McCune, B. Rorabaugh, M. T. Piascik, and D. M. Perez, submitted for publication) as well as decreasing α_{1A} -AR mRNA levels. Taking into consideration the caveats raised above regarding the use of transgenic models, our data can also be used to argue that tonic unregulated activation of the α_{1B} -AR diminishes cardiac contractile activity by decreasing

the positive inotropic signaling emanating not only from the β_1 -AR but the α_{1A} -AR as well.

In addition to contractile dysfunction, echocardiographic analysis also revealed increases in the left ventricular internal dimensions of the α_{1B} -AR_{C128F} heart. This is evidence of an increase in chamber size. This phenotype of contractile dysfunction and increased chamber dimensions has also been seen in a distinctly different mouse model that uses cardiac targeting to over-express the wild-type α_{1B} -AR (Grupp et al., 1998; Lemire et al., 2001). In contrast to these results, other reports with a cardiac-targeted constitutively active α_{1B} -AR (Milano et al., 1994) or our systemic over-expression model provide evidence of contractile dysfunction and cardiac hypertrophy. It is not clear why studies in the same mouse models reveal differences in cardiac phenotype. What is clear is that tonic unregulated activation of the α_{1B} -AR has significant and negative effects on cardiac function that can progress into hypertrophy or dilated cardiomyopathy. Factors that determine how biosignals emanating from the α_{1B} -AR lead to these pathophysiologies are being investigated.

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

The α_{1D} -AR is an enigmatic and the least well studied member of the α_1 -AR subtype family. In previous work, it has been shown that this receptor is expressed mainly in intracellular compartments (McCune et al., 2000; Chalothorn et al., 2002). We do not yet know the reason for this atypical localization pattern or if the regulatory activities of the α_{1D} -AR are accomplished by these intracellular receptors. Recently, it has been shown that the α_{1D} -AR is constitutively active (García-Sáinz and Torres-Padilla, 1999; Gisbert et al., 2000; McCune et al., 2000). D'Ocon's group has shown that

TABLE 2

Characteristics of the phenylephrine response in control and α_{1B} -AR_{C128F} over-expressing aortae. Calculated pA₂ values and their 95% confidence intervals (CI) are listed along with the dissociation constant (K_i).

| | Phenylephrine | | Characterization of BMY-7378 Antagonism | | | | |
|------------------------------------|---------------|----------------------|---|-----------------|--------|----------------|--------|
| | n | log EC ₅₀ | S.E. | pA ₂ | 95% CI | K _i | 95% CI |
| Control | 52 | -6.869 | 0.0662 | 9.531 | 0.219 | 0.294 | 0.149 |
| α_{1B} -AR _{C128F} | 39 | -6.795 | 0.1036 | 9.414 | 0.452 | 0.385 | 0.401 |

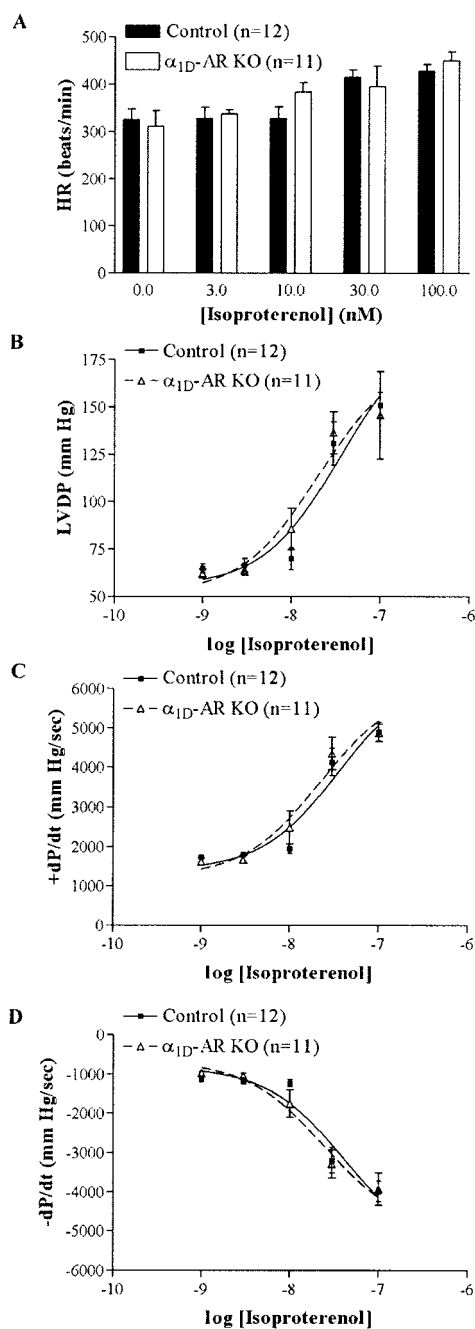


Fig. 5. Functional responses of α_{1D} -AR knockout (KO) mouse hearts to 3, 10, 30, and 100 nM isoproterenol. A, heart rate (HR); B, left ventricular developed pressure (LVDP); C, positive change in the developed pressure as a function of time (+dP/dt); and D, negative change in the developed pressure as a function of time (-dP/dt). Each bar or point on the curve represents the mean and the S.E. of 12 and 11 independent experiments for the control and the α_{1D} -AR KO hearts, respectively.

the constitutively active α_{1D} -ARs are capable of mediating vascular smooth muscle contraction. This constitutive activation could account for the intracellular expression. Other studies have demonstrated that the α_{1D} -AR is expressed throughout the cardiovascular system (Hrometz et al., 1999; Rudner et al., 1999). This includes being expressed on vascular beds such as the renal artery, where the α_{1D} -AR has not been shown to have a function (see Piascik and Perez, 2001). We do not yet understand why members of the α_1 -AR family are expressed on tissues in the cardiovascular system and do

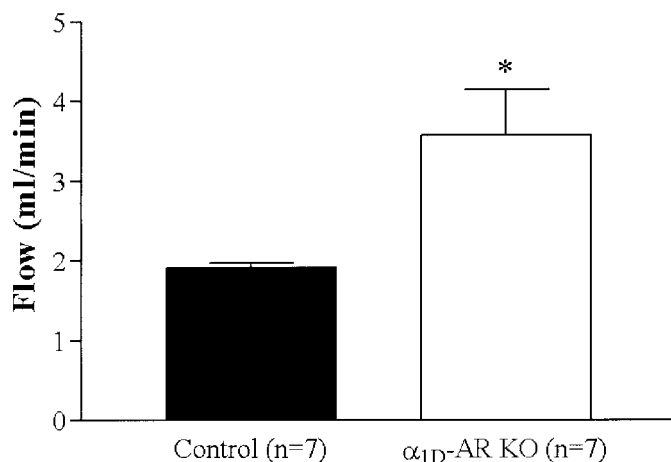


Fig. 6. Basal coronary flow rate required to maintain a constant perfusion pressure. Each bar represents the average and the S.E. of seven independent experiments. The asterisk (*) indicates statistical significance from the control group.

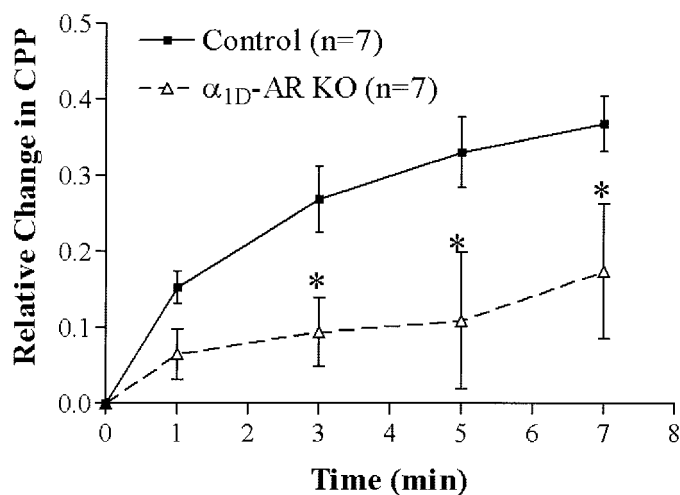


Fig. 7. Effect of 100 μ M phenylephrine on relative changes in the coronary perfusion pressure (CPP) of hearts lacking the α_{1D} -AR. The initial CPPs were 83.3 ± 2.3 and 81.9 ± 2.2 mm Hg for the control and the α_{1D} -AR KO hearts, respectively. The recordings were performed over a 7-min period. Each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

not participate in regulatory events. However, in keeping with this conundrum, we observed little effect of α_{1D} -AR gene deletion on dimensions or contractility as assessed echocardiographically or in the isolated-perfused heart (see also Tanoue et al., 2002).

We hypothesize that the major regulatory activity of the α_{1D} -AR is the regulation of vascular smooth muscle contraction in specific blood vessels (Piascik and Perez, 2001). Evidence supporting this postulate also comes from work with the α_{1D} -AR knockout line of mice (Tanoue et al., 2002). Tanoue et al. (2002) showed that knockout of the α_{1D} -AR significantly decreased systemic arterial blood pressure as well as the pressor responses to norepinephrine and responses in the isolated aorta. In the present work we show that knockout of the α_{1D} -AR significantly impaired the ability of phenylephrine to promote increases in coronary perfusion pressure. Therefore, in the same mouse line, where we can

demonstrate prominent effects on vascular function, we do not see measurable effects on the examined cardiac parameters. This adds support to our hypothesis that the α_{1D} -AR serves predominantly in vascular function.

Acknowledgments

We thank Joseph Tinney (University of Kentucky) for his assistance in the preparation of the animals for the echocardiographic study, and Robert Papay (Cleveland Clinic Foundation) for his help in establishing the α_{1B} -AR_{C128F} transgenic line at the University of Kentucky.

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