The \( \alpha_{1A} \)-Adrenergic Receptor Subtype Mediates Biochemical, Molecular, and Morphologic Features of Cultured Myocardial Cell Hypertrophy*

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\( \alpha_{1A} \)-Adrenergic agonists induce a hypertrophic phenotype in cultured neonatal rat ventricular myocytes. Quantifiable markers of this phenotype include stimulation of phosphoinositide hydrolysis, transcriptional induction of atrial natriuretic factor (ANF) gene expression, and an increase in myocardial cell size. The aim of the present work was to determine which \( \alpha_{1A} \)-adrenergic receptor subtype mediates the acquisition of these parameters of myocardial hypertrophy. Phosphoinositide hydrolysis is inhibited by low concentrations of 5-methylurapidil (log \( K_i = -8.7 \)) and (+)-niguldipine (log \( K_i = -10.6 \)). The \( \alpha_{1A} \)-adrenergic receptor-induced increase in transcriptional activation of an ANF luciferase reporter gene is inhibited over the same range of concentrations of 5-methylurapidil (log \( K_i = -8.2 \)) and (+)-niguldipine (log \( K_i = -11.2 \)) that inhibit phosphoinositide hydrolysis. In addition, the increase in cell size that accompanies \( \alpha_{1A} \)-adrenergic receptor stimulation of cultured ventricular myocytes is blocked by similar concentrations of 5-methylurapidil (log \( K_i = -8.0 \)) and (+)-niguldipine (log \( K_i = -10.6 \)). In contrast, treatment with the \( \alpha_{1B} \) selective antagonist chlorehthylclonidine at a concentration of 10 \( \mu \)M had no effect on the adrenergically mediated induction of ANF luciferase reporter gene expression or the adrennergically induced increase in myocardial cell size. These findings demonstrate that pharmacologically identifiable \( \alpha_{1A} \)-adrenergic receptors mediate not only the early effects of \( \alpha_{1A} \)-adrenergic stimulation such as phosphoinositide hydrolysis, but that they activate the signaling pathways that control transcriptional induction of the ANF luciferase reporter gene and an increase in myocardial cell size. Studies using \( \alpha_{1A} \)-adrenergic receptor cDNAs to delineate and alter the direct interaction of this receptor subtype with proximal signaling molecules, e.g., GTP binding proteins, should provide a powerful means of assessing their role in the induction of the molecular and morphologic parameters of myocardial cell hypertrophy.

Activation of cardiac \( \alpha_{1A} \)-adrenergic receptors leads to rapid changes in contractility, electrophysiological properties, and metabolic responses of the myocardium (1). \( \alpha_{1A} \)-Adrenergic agonists stimulate cardiac phosphoinositide metabolism (2), activate glycogenolysis (3), induce positive inotropic effects which are related either to an increase in free cytosolic Ca\(^{2+} \) levels (4) or to an increase in the responsiveness of myofilaments to Ca\(^{2+} \) (5), and elicit a cascade of other acute effects on cardiac intermediary metabolism (6).

In addition to these relatively acute effects, recent studies have suggested that \( \alpha \)-adrenergic stimuli also have long term effects on cardiac structure and function. Chronic exposure of cultured neonatal rat ventricular muscle cells to \( \alpha \)-adrenergic agonists leads to the acquisition of several genetic and morphologic features of hypertrophy. These include induction of a distinct immediate early gene program, appearance of markers of an embryonic program of gene expression, accumulation and assembly of constitutively expressed contractile proteins into sarcomeric units, and an increase in cell size without concomitant proliferation (7, 8). Activation of a subset of constitutively expressed contractile proteins and markers of an embryonic program of gene expression is largely due to selective transcriptional activation (9). The precise signaling pathways which mediate these chronic effects of \( \alpha \)-adrenergic stimulation on cardiac ventricular muscle cells are unknown. Recent studies have implicated protein kinase C (10–12), the Ras protooncogene (13), and the GTP binding protein, \( G_{\alpha} \) (14), in the signaling pathways which activate genetic markers of the hypertrophic response. A detailed analysis of the \( \alpha \)-adrenergic signaling pathways which directly or indirectly interact with these proximal signals to activate distinct features of myocardial cell hypertrophy would be facilitated by the identification of the precise \( \alpha_{1A} \)-adrenergic receptor(s) that mediate this chronic effect.

Recent studies have revealed that \( \alpha_{1A} \)-adrenergic receptors comprise a family of closely related receptor subtypes (15, 16), which regulate cellular responses through diverse signaling pathways (17). Both the \( \alpha_{1A} \) and \( \alpha_{1B} \)-adrenergic receptor subtypes are expressed in rat heart as assessed by radioiodinated binding (18, 19). Northern blotting with specific \( \alpha_{1A} \)-adrenergic receptor subtype cDNA probes demonstrates that two distinct \( \alpha_{1A} \)-adrenergic receptors are expressed in rat heart (20). The functional presence of other members of the \( \alpha_{1A} \)-adrenergic
receptor family has not been detected in rat heart. The multitude of cardiac responses to α1-adrenergic stimulation such as its effect on inotropy, phosphoinositide hydrolysis, and gene expression raises the question as to whether distinct receptor subtypes mediate these individual responses. It is possible that activation of multiple ventricular muscle α1-adrenergic receptor subtypes is required to elicit integrated cellular responses that occur in response to α-adrenergic receptor agonists. Alternatively, a single α1-adrenergic receptor subtype might mediate the acquisition of many of the well-characterized features of the hypertrophic response. In previous studies, identification of α1-adrenergic receptor subtype-dependent signaling pathways has focused on the relatively acute effects of α1-adrenergic stimulation on phosphoinositide metabolism or Ca2+ mobilization, using tissue slices, cultured cell lines, or surrogate cell lines which stably express cloned α1-receptor subtype cDNAs (21-23). Since α1-adrenergic stimulation of ventricular muscle cells leads to transcriptional activation of well-defined genetic markers of the hypertrophic response, as well as structural/morphologic changes which can be distinguished at a single cell level, the myocyte model system may ultimately allow the implementation of genetic approaches to identify signaling pathways that lead to long-term phenotypic alterations which are specific for distinct α1-adrenergic receptor subtypes.

The current study provides direct evidence that a pharmacologically identified α1A-adrenergic receptor subtype activates biochemical, genetic, and morphologic features of adrenergically mediated ventricular cell hypertrophy, thereby suggesting that the entire repertoire of the hypertrophic phenotype can be activated through an α1A-adrenergic receptor-dependent pathway. Identification of the α1A-adrenergic receptor as the adrenergic receptor responsible for hypertrophy in cultured cardiac ventricular muscle cells, coupled with the availability of well-defined genetic markers of this important adaptive cardiac muscle response, will allow genetic approaches to characterize the potentially complex interaction of the α1A-adrenergic receptor with other proximal signaling molecules such as the GTP binding proteins, Gs, and Rap, in cardiac muscle cells.

MATERIALS AND METHODS

Cell Culture—Neonatal rat ventricular cells were cultured as previously described with minor modifications (24, 25). Briefly, hearts from 1- to 2-day-old Sprague-Dawley rat pups were recovered, atria were removed, and the ventricles were pooled and dissected. Myocytes were dispersed by digestion with collagenase II (Worthington) and pancreatin (GIBCO). Myocardial cell suspensions were centrifuged through Percoll step gradients to obtain cell preparations with >95% myocytes, as assessed by immunofluorescence with MLC-2 antisera (24, 25). Myocytes were plated at a density of 3.0 × 10^6 cells/cm^2, in either 100- or 150-mm tissue culture dishes precoated with gelatin, in 4:1 Dulbecco's modified Eagle's medium/medium 199 (GIBCO), supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (ampicillin at 25 μg/ml and gentamicin at 3 μg/ml). Following a 24-h incubation in serum-containing medium, cells were washed and incubated in serum-free medium in the presence or absence of the indicated α1-adrenergic agonists and antagonists. 0.1% bovine serum albumin was added to prevent nonspecific adsorption of antigens to plasticware. Transfected cells were treated as described below.

Transfection of Myocardial Cells—Cells were plated in 100-mm gelatinized tissue culture dishes at a density of 3.0 × 10^6 cells/cm^2 and allowed to attach for 18-20 h after plating. The cells were transfected with 6 μg of pON249 and 20 μg of pANF683BL utilizing a modified calcium phosphate precipitation method (36). After washing, the cells were maintained in 4:1 Dulbecco's modified Eagle's medium 199 in the presence or absence of the indicated adrenergic agonist and antagonist until harvested 38-48 h later.

Luciferase and β-Galactosidase Assays—Transfected cells were washed twice with PBS' without Ca2+ or Mg2+ and then harvested in 1.0 ml of extraction buffer (100 mM Tricine, 10 mM MgSO4, 2 mM EDTA, pH 7.8, 1 mM dithiothreitol). Cells from each dish were collected by centrifugation, resuspended in 200 μl of extraction buffer, lysed by five cycles of freezing in dry ice, and thawing at 37 °C, and cell debris was removed by centrifugation. The cleared extracts were combined with 275 μl of 73 μM luciferin (Analytical Luminescence Laboratory) and 2 mM ATP (Sigma) in extraction buffer. Luciferase activity (27) was measured in triplicate in a Monolight 2011 luminometer (Analytical Luminescence Laboratory). β-Galactosidase activity was assayed on 50-μl samples as described (26).

 luciferase and β-galactosidase activities were normalized to their corresponding β-galactosidase activities.

**Immunofluorescence Techniques**—Indirect immunofluorescence protocols were performed by a minor modification of a previously described procedure (29). Briefly, the myocardial cells were grown on Lab-Tek plastic chamber slides precoated with 4 μg of laminin (Sigma) per cm^2. 48 h after incubation in the indicated medium with or without α1-agonists and antagonists, the cells were rinsed with PBS without Ca2+ or Mg2+ and fixed for 15 min at room temperature with 3% paraformaldehyde in 10 mM sodium phosphate, 150 mM NaCl, 1 mM MgCl2, pH 7.4. The cells were then incubated in 50 mM Tris and washed four times with PBS. Alternatively, the slides were incubated for 10 min with 1% bovine serum albumin to block nonspecific sites, incubated with TrpE/MLC-2 antisera (24) for 60 min at 37 °C, rinsed and washed four times with PBS. Subsequently, the cells were rinsed with Ca2+/Mg2+-free PBS, washed four times with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, followed by three additional washings with PBS. Chamber slides were incubated with 1% bovine serum albumin for 10 min to block nonspecific sites, incubated with TrpE/MLC-2 antisera (24) for 60 min at 37 °C, and then rinsed and washed four times with PBS. Subsequently, the slides were incubated for 10 min in a 1:2 dilution of normal goat serum (TAGO, Inc.) in PBS and then incubated for 60 min at 37 °C with fluorescein-conjugated affinity-purified goat anti-rabbit IgG (Cappel Laboratories). The slides were subsequently rinsed and then washed four times with PBS, mounted on glass coverslips with 15% (w/v) Airvol 205 polyvinyl alcohol (Air Products and Chemicals, Inc.), 33% glycerol, and 0.1% sodium azide in PBS, pH 7.4, and viewed with fluorescence microscopy. Cellular areas were determined by planimetry of individual cell areas in high power fields.

**Fluoride/Calcium Imaging—**To identify cis sequences which mediate α1-adrenergic-induced changes in the ANF promoter, the ANF promoter expression vector was created. The ANF683BL chimeric reporter construct was as described (9) by ligating an EcoRI (−638)/StuI (+62) fragment of the rat ANF gene (a generous gift of Peter L. Davies) (30) into pHBluescript (Stratagene). An EcoRI/NcoI fragment was then blunt-end-ligated into the HindIII site of the pSVOAL5' (27). pON249, a β-galactosidase expression vector under the control of the human cytomegalovirus promoter (31), was used to control for transfection efficiency.

**Radioligand Binding—**Neonatal rat hearts were excised, macroscopically freed from connective tissue, placed into ice-cold 5 mM KHCO3 and homogenized with a Teflon homogenizer for 10 s at full speed and then twice for 20 s at 2/3 speed. The homogenate was washed twice at 40,000 × g for 30 min at 4 °C, and the final pellet was resuspended in binding buffer (50 mM Tris, 0.5 mM EDTA, pH 7.5) and rehomogenized for 10 s at full speed. [3H]Prazosin binding was carried out for 45 min at 25 °C in 1 ml of binding buffer. The incubation was terminated by rapid vacuum filtration over Whatman GF/C filters which were washed twice with 10 ml of binding buffer. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine. Nonspecific binding was typically 10-15% of total binding. Additional washes with 0.1% bovine serum albumin content were determined by the method of Bradford (32) using bovine serum albumin as standard.

**Drug Affinities at the Cardiac α1-Adrenergic Receptor and Receptor Subtype Distribution**—We evaluated drug affinities at the cardiac α1-adrenergic receptor and receptor subtype distribution in competition binding experiments using [3H]prazosin and pooled hearts of 18-25 neonatal rats (−300-500 μg of membrane protein per assay tube). The radioligand competition curves were analyzed by iterative nonlinear regression analysis to fit the experimental data to a mono- or biphasic sigmoid curve using the In Plot program (Graph PAD Software, San Diego, CA). The biphasic fit was accepted if it yielded statistically significant improvement in an F-test. From these curves, the apparent affinity (IC50) of the high and low affinity sites, the percentage of total binding at 0.1 nM [3H]prazosin, and the Hill coefficient were determined from competition curves. IC50 and Hill coefficient values from the competition curves into Kd and n values, Kd values for [3H]prazosin were determined in saturation binding experiments using similar methods. The Hill coefficient values were used to fit the competition data into a Hill equation. These values were used to assess whether the high affinity site was of a monovalent or divalent nature. This analysis was performed on duplicate experiments and the results were expressed as mean ± S.E. when n > 4. The abbreviations used are: PBS, phosphate-buffered saline; ANF, atrial natriuretic factor; GTPγS, guanosine 5'-O-(thio)triphosphate; G-proteins, guanine nucleotide binding proteins.
conditions using six concentrations of ligand ranging from 0.1 to 1.6 nm. Affinity \((K_d)\) and number \((B_{max})\) of \(\alpha_1\)-adrenergic receptors were determined from these saturation binding experiments with fitting of the untransformed data to a rectangular hyperbolic function (Fig. 1A).

**Phosphoinositide Hydrolysis**—Myocardial cells were plated at a density of \(3 \times 10^4\) cells/cm\(^2\) in 35-mm plates. Following a 24-h incubation in serum-containing medium, cells were washed and maintained in serum-free medium containing 5–30 nCi/ml \([3H]\)inositol for 24 h. The cells were then rinsed in fresh serum-free medium and preincubated for 15 min with 2 mM DL-propranolol and 5-methylurapidil or \((+)-niguldipine. 100 \mu M\) Phenylephrine and 10 mM LiCl were then added for 30 min, the medium was aspirated, the cells were harvested by scraping into 10% trichloroacetic acid and sonicated, and the cell homogenates were centrifuged for 10 min at 4 °C. Supernatants were neutralized by ether extraction and \([3H]\)inositol phosphates were fractionated by anion exchange column chromatography as described (33).

Chemicals—\([3H]Prazosin (specific activity 70–80 Ci/mmol) was obtained from Du Pont-New England Nuclear and phenylephrine and oxymetazoline from Sigma. The following drugs were gifts of the respective companies: \((+)-niguldipine and 5-methylurapidil (BYK Pharma, Konstanz, FRG), phentolamine (Ciba Geigy, Basel, Switzerland).

**Data Analysis**—Results of multiple experiments are shown as mean ± S.E. \(IC_{50}\) data from competition binding or inhibition experiments were converted to \(K_i\) values using the Cheng and Prusoff (34) equation: 

\[
K_i = \frac{IC_{50}}{1 + [A]/K_d},
\]

where \([A]\) is the concentration of ligand or agonist used and \(K_d\) is its affinity as determined in our binding studies. The \(EC_{50}\) values determined for phenylephrine-stimulated phosphoinositide hydrolysis and activation of ANF luciferase expression were similar to the \(K_d\) determined for phenylephrine in the binding studies. \(IC_{50}\) was calculated using iterative nonlinear regression analysis using the InPlot program (GraphPAD Software, San Diego, CA).

**RESULTS**

**Radioligand Binding Studies**—To determine the relative composition of \(\alpha_1\)-adrenergic subtypes in ventricular cells, radioligand binding studies were performed with a membrane fraction isolated from neonatal rat hearts. Saturation studies with \([3H]Prazosin detected 99 ± 10 fmol \((K_d=72 ± 12 \text{ pm}, n = 3)\) of \(\alpha_1\)-adrenoceptors/mg of protein in neonatal rat heart membranes (Fig. 1A). In competition studies, the nonselective \(\alpha_1\)-adrenergic agonist, phenylephrine, competed for \([3H]Prazosin binding with a steep monophasic curve. However, the \(\alpha_1A\)-selective antagonists \((+)-niguldipine, 5-methylurapidil, and oxymetazoline competed for \([3H]Prazosin binding in cardiac membranes from neonatal rats with shallow biphasic curves (Fig. 1B). A relative distribution of approximately 32% \(\alpha_{1A}\)- and 68% \(\alpha_{1B}\)-adrenergic receptors was calculated from the ratio of high/low affinity sites for the subtype-selective compounds.

**Phosphoinositide Hydrolysis**—To determine the consequences of activation of specific \(\alpha_1\)-adrenergic receptor subtypes, we examined inhibition of phenylephrine-stimulated phosphoinositide hydrolysis by \(\alpha_1\)-adrenergic receptor antagonists. \((+)-niguldipine inhibited phenylephrine-stimulated inositol phosphate formation with high affinity \((\log K_i = -10.6)\) (Fig. 2). Decreasing the extracellular free \(Ca^{2+}\) concentration by addition of EGTA to \(-100 \text{ nm}\) partially inhibited the response to phenylephrine; however, the \(K_i\) for \((+)-niguldipine was unchanged, suggesting that the \(Ca^{2+}\) channel blocking properties of niguldipine do not contribute to its potency in blocking phosphoinositide hydrolysis (data not shown). 5-Methylurapidil inhibited phosphoinositide hydrolysis with a log \(K_i\) of \(-8.7\). The \(K_i\) values for \((+)-niguldipine and 5-methylurapidil-mediated inhibition of phosphoinositide hydrolysis are similar to those anticipated for the high affinity \(\alpha_{1A}\)-adrenergic receptor. Phenylephrine also increased inositol

![Fig. 1. \([3H]Prazosin binding to neonatal cardiac membranes. A\), data from a representative experiment in which each point is the mean of duplicate determinations. Similar data were obtained in two other experiments. Inset, Scatchard transformation of the data. B, each point is the mean ± S.E. of values from three experiments. Quantitative evaluation of the data is in Table I.](image)

![Fig. 2. Effects of 5-methylurapidil and (+)-niguldipine on phosphoinositide hydrolysis. Cultured neonatal rat ventricular cells were incubated overnight with \([3H]\)inositol and then for 15 min in medium containing 2 nM propranolol, and either the \(\alpha_{1B}\)-antagonist \((+)-niguldipine (\text{----})\) or 5-methylurapidil (\text{---}) at the indicated concentration. Phenylephrine (100 \mu M) and LiCl were then added for 30 min, cells were harvested, and \([3H]\)inositol phosphates were fractionated by anion exchange column chromatography (see "Materials and Methods"). The \(K_i\) for \((+)-niguldipine is 2.4 × 10^{-11} \text{ log } K_i = -10.6\) and that for 5-methylurapidil is 1.9 × 10^{-9} \text{ log } K_i = -8.7. These values were calculated from the \(IC_{50}\) of 8.0 × 10^{-10} for \((+)-niguldipine and 6.2 × 10^{-4} for 5-methylurapidil using the Cheng Prusoff equation (see "Materials and Methods"). Data are the means ± S.E. of counts per min from three separate experiments with \((+)-niguldipine and two with 5-methylurapidil (each experiment performed in triplicate).](image)

**Table I**

| Drug              | \(\log K_{1\text{max}}\) | \(\log K_{1\text{low}}\) | % high |
|-------------------|--------------------------|--------------------------|--------|
| Phenylephrine     | -5.5 ± 0.1               | -6.8 ± 0.3               | 27 ± 6 |
| 5-Methylurapidil  | -8.8 ± 0.3               | -8.8 ± 0.3               | 5 ± 1  |
| (+)-Niguldipine   | -5.9 ± 0.4               | -7.6 ± 0.4               | 33 ± 3 |
| Oxymetazoline     | -8.5 ± 0.2               | -6.7 ± 0.0               | 37 ± 5 |

**Data are the means ± S.E. of values from three experiments in which 18–25 hearts were pooled. The competition curve for phenylephrine was monophasic.**
bis- and trisphosphate formation. Similar $K_i$ values were determined for inhibition of inositol tris-, bis-, or monophosphate (data not shown). These data suggest that the increase in inositol monophosphate induced by phenylephrine results primarily from $\alpha_{1A}$-adrenergic receptor-mediated polyphosphoinositide hydrolysis.

Activation of an ANF Luciferase Fusion Vector via an $\alpha_{1A}$-Receptor Subtype—Neonatal rat ventricular cells were transfected as described under "Materials and Methods" with the pANF638L luciferase reporter gene containing ANF 5′-flanking sequences from -638 to +62 ligated upstream of the promoterless pSVOAL5′ luciferase reporter gene (9). The 638 base pairs of ANF 5′-flanking sequence is sufficient to confer the maximum level of $\alpha_{1A}$-adrenergic inducibility to the luciferase reporter gene in transient assays. In all experiments, transfection efficiency was monitored by co-transfection of the cytomegalovirus $\beta$-galactosidase vector, and luciferase activity was normalized to $\beta$-galactosidase activity. Following transfection, the cells were maintained in the absence or the presence of the 100 $\mu$M phenylephrine plus 2 $\mu$M DL-propranolol and either 10 $\mu$M chlorothelyclonidine or various concentrations of the $\alpha_{1A}$-antagonists 5-methylurapidil or (+)-niguldipine. 5-Methylurapidil and (+)-niguldipine decreased luciferase expression in these transfected cells in a concentration-dependent manner with a log $K_i$ of -8.2 and -11.2, respectively (Fig. 3). Values are similar to the high affinity binding site observed in the radioligand binding studies in Table I and in previous studies of high affinity $\alpha_{1A}$-adrenergic receptors in other cell types (18, 35, 36). Similar concentrations of 5-methylurapidil and (+)-niguldipine were required for inhibition of phosphoinositide hydrolysis (Table II). Treatment of cells with 10 $\mu$M chlorothelyclonidine had no effect on phenylephrine-stimulated activation of the ANF luciferase reporter gene (data not shown). $\alpha_1$-Adrenergic stimulation does not induce luciferase expression in cells transfected with the constitutively expressed Rous sarcoma virus luciferase fusion gene or the promoterless pSVOAL5′, demonstrating the specificity of adrenergic stimulation on the transcriptional activation of the ANF luciferase fusion gene (9).

Effects on Cell Size and Assembly of MLC-2 into Organized Contractile Units—Since previous studies have demonstrated that $\alpha_1$-adrenergic stimulation results in cultured myocardial cell hypertrophy, it was of interest to examine the effects of $\alpha_{1A}$-adrenergic antagonists on the acquisition of defined morphologic features of the hypertrophic phenotype. Organization of an individual contractile protein (MLC-2) into sarcomeric units is a hallmark of the hypertrophic phenotype in cultured neonatal rat ventricular cells (24, 29). Immunocytofluorescent analysis with antibodies against a TrpE/MLC-2 fusion protein demonstrated that myocytes maintained in serum-free media without phenylephrine had a random distribution of MLC-2 with few detectable striations. Treatment with phenylephrine (48 h) increased the cell size and the assembly of MLC-2 into organized contractile units, and a greater proportion of the cells had linear striations. The presence of the $\alpha_{1A}$-antagonist 5-methylurapidil or (+)-niguldipine caused a concentration-dependent block in the phenylephrine-stimulated increase in cell size, organization of contractile units, and the proportion of cells demonstrating linear striations (Figs. 4 and 5). Quantitative analysis of the inhibition of phenylephrine-induced increases in cell size by 5-methylurapidil or (+)-niguldipine demonstrated that both drugs had high potency for the inhibition of this response (log $K_i$ = -8.0 and -10.6 for 5-methylurapidil and (+)-niguldipine, respectively). Chlorothelyclonidine (10 $\mu$M) had no significant effect on the phenylephrine-stimulated increase in cell size (data not shown). These findings correspond with the effect of the $\alpha_{1A}$-antagonists on other markers of cultured ventricular cell growth (Table II) and suggest that the $\alpha_{1A}$-adrenergic receptor activates the morphologic features of the hypertrophic phenotype in $\alpha_1$-adrenergically stimulated cultured ventricular myocytes.

### Table II

|                        | 5-Methylurapidil | (+)-Niguldipine |
|------------------------|------------------|-----------------|
| **Log $K_i$ (M)**      | -8.0             | -8.0            |
| **Inhibition of induction** | -10.6            | -10.6           |
| **Inhibition of increase in cell size** | -8.2             | -11.2           |
| **A1-affinity binding site** | -7.6 ± 0.3       | -7.6 ± 0.4      |
| **A2-affinity binding site** | -8.8 ± 0.3       | -9.9 ± 0.4      |

**DISCUSSION**

Neonatal rat myocardium contains high and low affinity binding sites for the $\alpha_{1A}$-adrenergic receptor-selective drugs oxymetazoline, 5-methylurapidil, and (+)-niguldipine. The drug affinities at the high and low affinity sites are in agreement with those previously described for $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptors (18, 35, 36). Thus, $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptors coexist in rat neonatal ventricular myocardium in an approximately 30:70% ratio. This ratio is similar to that found previously in whole adult rat myocardium using the same ligand (18, 19), but the percent of $\alpha_{1A}$-adrenergic receptors is somewhat lower than that reported in neonatal rat myocardium using $[^{35}S]$IBE 2254 as the ligand (37). The effect of stimulation of these receptor subtypes in myocardial cells is not well characterized. Therefore, the effect of $\alpha_{1A}$-adrenergic receptor subtype antagonists on phosphoinositide hydrolysis, transcriptional activation of an ANF luciferase reporter gene, and cell size, three quantifiable markers of $\alpha_1$-adrenergic-
mediated cultured ventricular cell hypertrophy, were analyzed. Our data demonstrate that the $\alpha_{1A}$-selective antagonists inhibited all three responses with high affinity (Table II).

Previous classifications for $\alpha_1$-adrenergic receptors have coupled the $\alpha_{1B}$-adrenergic receptor to the formation of inositol phosphates and release of intracellular Ca$^{2+}$; whereas, the $\alpha_{1A}$-adrenergic receptor has been coupled to Ca$^{2+}$ influx (17, 38). For example, formation of 1,4,5-inositol trisphosphate which has been implicated in the release of Ca$^{2+}$ from intracellular stores was detected with stimulation of $\alpha_{1B}$-adrenergic receptor in hepatocytes, but not with $\alpha_{1A}$-adrenergic receptors in renal cells (39). More recent data, however, suggest that the response to $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptor stimulation may be dependent on the cellular context. For example, it has been shown that $\alpha_{1A}$-adrenergic receptor stimulation can elicit inositol monophosphate formation in renal cells (21, 39, 40) and in primary cultures from rat brain (41). In renal cells, the $\alpha_{1A}$-adrenergic receptor-stimulated inositol monophosphate formation was inhibited by removal of extracellular Ca$^{2+}$ and was not associated with increases in inositol 1,4,5-trisphosphate (39) suggesting that the $\alpha_{1A}$ effect on inositol monophosphate formation was secondary to increases in intracellular Ca$^{2+}$ and involved a different phosphoinositide hydrolysis pathway from that described for $\alpha_{1B}$-adrenergic receptors. There is also recent data which demonstrate that in cultured ventricular myocytes, adrenergically induced polyphosphoinositide hydrolysis is inhibited by the $\alpha_{1A}$-antagonist WB 4101 and not the irreversible $\alpha_{1B}$-antagonist chlorcyclizidine (37). Our data similarly show that the $\alpha$-adrenergic-mediated production of inositol trisphosphate in cardiac myocytes is inhibited by 5-methylurapidil and (+)-niguldipine at concentrations that block the $\alpha_{1A}$-adrenergic receptor, but not the $\alpha_{1B}$-adrenergic receptor. In other studies, we have demonstrated that an antibody to the $\alpha$-subunit

2. K. U. Knowlton, V. LaMorte, J. H. Brown, J. Thorborn, J. Feramisco, and K. R. Chien, in preparation.
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Fig. 5. Effects of α₁-antagonists on relative cell area. Cultured neonatal rat ventricular cells were maintained in serum-free medium containing 0.1% bovine serum albumin for 48 h with 100 μM phentolamine, 2 μM propranolol, and 5-methylurapidil (○—○) or (+)-niguldipine (▲—▲) at the indicated concentrations. Control cells were maintained for 48 h in serum-free medium without adrenergic agonists or antagonists. Cells were subsequently stained with TrpE/MLC-2 antibody as described under “Experimental Procedures.” Profiles were assessed by plasmometry of individual cells in high power fields. Data are means ± S.E. of values from 50–60 cells. All myocytes within the photographic field were measured.

of G_{q/11} blocks phenylephrine- and GTPγS-stimulated inositol trisphosphate formation in membranes from neonatal ventricular myocytes. Taken together, these data suggest that a pharmacologically identifiable α₁A-adrenergic receptor subtype couples to the G_{q/11} protein and stimulates phosphoinositide hydrolysis through activation of a polyphosphoinositide-specific phospholipase C in ventricular myocytes.

α₁-Adrenergic agonists initiate a program of hypertrophy in neonatal rat ventricular muscle cells that has molecular and morphologic characteristics similar to ventricular hypertrophy in the intact animal (7, 8). The constellation of biochemical, genetic, and morphologic effects of α-adrenergic stimulation on cardiac ventricular muscle cells, and the previously mentioned diversity of α₁-adrenergic receptor subtypes, suggests the possibility that these effects may be the result of the simultaneous activation of multiple α₁-adrenergic receptor subtypes, and that the phenotypic effects represent an integrated response to the activation of a variety of signaling pathways. However, the results of the present study suggest that not only are proximal signaling mechanisms in adrenergic receptor-mediated cultured ventricular cell hypertrophy under the control of the α₁A-adrenergic receptor, but that the α₁A-adrenergic receptor also functions like a growth factor receptor in neonatal cardiac myocytes, stimulating long-term genetic and morphologic features of cultured myocardial cell hypertrophy.

A variety of cell types and conditions have been used to evaluate the response to α-adrenergic stimulation that occurs within minutes of ligand binding (17, 21, 22, 38–41). However, little is known regarding the pathways which mediate the long-term effects of α₁-adrenergic stimulation on transcriptional activation that contribute to myocardial cell growth. The α₁A-, α₂A-, and β-adrenergic receptor subtypes are each coupled to distinct G-proteins which act to transduce G-protein-specific responses, such as activation or inhibition of adenylylcyclase, or activation of phospholipase C. The amino-terminal portion of the third intracellular domain of the adrenergic receptor plays a major role in determining the G-protein specificity of a given adrenergic receptor. β-Adrenergic receptors in which the third intracytoplasmic domain is replaced by the third intracytoplasmic domain from the α₁A-adrenergic receptor can activate phosphoinositide hydrolysis in COS7 cells (22, 42). In addition, a single amino acid substitution in the third intracellular domain of the α₁A-adrenergic receptor renders the receptor constitutively active as assessed by phosphoinositide hydrolysis (43). Transfection of mutated α₁A-adrenergic receptor expression vectors into Rat-1 and NIH 3T3 fibroblasts induces proliferation even in the absence of catecholamines (44). These previously reported effects of mutations in the α₁B-adrenergic receptor in noncardiac cells and the findings of the present manuscript demonstrating the effects of stimulation of the α₁A-adrenergic receptor in cardiac myocytes suggest that the adrenergically mediated model of myocardial cell hypertrophy can be utilized to precisely characterize the effects of mutations in an α₁A-adrenergic receptor.

There is evidence by expression cloning for the presence of at least three distinct α₁-adrenergic receptor subtypes (45, 46), but the gene corresponding to the pharmacologically defined rat cardiac α₁A-adrenergic receptor has not been isolated (46). Microinjection and transfection of mutant α₁A-adrenergic receptor expression vectors into cultured ventricular myocytes will allow quantitative assessment of the effects of these mutations on ANF gene expression and myocardial cell size.

Recent studies have documented that activation of another G-protein-coupled receptor, endothelin-1, will also activate the hypoxic response of cultured ventricular muscle cells, in which adrenergic agonists and antagonists both quantitatively and qualitatively affect the effects of α₁A-adrenergic receptor stimulation. The endothelin-1 receptor is linked to phosphoinositide hydrolysis and the up-regulation of both the ANF and MLC-2 genes. Furthermore, similar cis regulatory elements within the ANF and MLC-2 promoters appear to be required for inducible expression by either endothelin or α-adrenergic agonists (29, 47). The addition of α-adrenergic agonists and endothelin-1 does not have an additive effect on cultured ventricular muscle cells. Thus, it appears that these two distinct stimuli converge on similar signaling pathways for the activation of genetic markers of the hypertrophic response. Identification of the specific receptors which activate these quantitative and qualitative features of neonatal ventricular cell hypertrophy will now allow genetic manipulation of each receptor macromolecule to precisely characterize its potential interaction with other proximal signaling molecules involved in the hypertrophic response.

The current studies, therefore, demonstrate that the pharmacologically identified α₁A-adrenergic receptor activates biochemical, genetic, and morphologic markers of ventricular muscle cell hypertrophy. The precise role of this G-protein-coupled receptor as a myocardial growth factor receptor, and its interaction with other proximal signaling molecules such as the G-proteins, G_{q/11} and Ras, can now be assessed using co-transfection and microinjection in cultured ventricular myocardial cells, and in transgenic mice (48). These studies are currently in progress and should shed further light on the α₁A-adrenergic receptor-dependent signaling pathways which lead to the activation of ventricular muscle cell hypertrophy.

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