Constitutively Active Mutants of the α_{1a}- and the α_{1b}-Adrenergic Receptor Subtypes Reveal Coupling to Different Signaling Pathways and Physiological Responses in Rat Cardiac Myocytes*

(Received for publication, July 18, 1999, and in revised form, October 29, 1999)

Charlene McWhinney‡§, Dean Wenham‡, Sujata Kanwal, Vivian Kalman, Carl Hansen, and Janet D. Robishaw¶

From the Henry Hood Research Program, Pennsylvania State College of Medicine, Danville, Pennsylvania 17822-2614 and §Oklahoma State University, School of Medicine, Department of Physiology and Pharmacology, Tulsa, Oklahoma 74107

Activation of α_{1}-adrenergic receptors influences both the contractile activity and the growth potential of cardiac myocytes. However, the signaling pathways linking activation of specific α_{1}-adrenergic receptor (AR) subtypes to these physiological responses remain controversial. In the present study, a molecular approach was used to identify conclusively the signaling pathways activated in response to the individual α_{1a}- and α_{1b}-AR subtypes in cardiac myocytes. For this purpose, a mutant α_{1a}-AR subtype (α_{1a}-S290/292R-AR) was constructed based on analogy to the previously described constitutively active mutant α_{1b}-AR subtype (α_{1b}-S288–284-AR). The mutant α_{1a}-S290/292R-AR subtype displayed constitutively active behavior based on four criteria. To introduce the constitutively active α_{1a}-AR subtypes into cardiac myocytes, recombinant Sindbis viruses encoding either the α_{1a}-S290/293-AR or α_{1b}-S288–284-AR subtypes were used to infect the whole cell population with >90% efficiency, thereby allowing the biochemical activities of the various signaling pathways to be measured. When expressed at comparable levels, the α_{1a}-S290/293-AR subtype exhibited a significantly elevated basal level as well as agonist-stimulated level of inositol phosphate accumulation, coincident with activation of atrial natriuretic factor-luciferase gene expression. By contrast, the α_{1a}-S288–284-AR subtype displayed a markedly increased serum response element-luciferase gene expression but no activation of atrial natriuretic factor-luciferase gene expression. Taken together, this study provides the first molecular evidence for coupling of the α_{1a}-AR and the α_{1b}-AR subtypes to different signaling pathways in cardiac myocytes.

Activation of α_{1}-adrenergic receptors (AR)\(^1\) influences both the contractile activity and the growth potential of cardiac myocytes. However, despite intense investigation, the signaling pathways linking activation of specific α_{1}-AR subtypes to these particular physiological responses remain controversial (1). The situation is complicated by the diversity of α_{1}-AR subtypes. Three distinct α_{1}-AR subtypes have been identified by molecular cloning (2–4). Recently, the relationships between the cloned and native α_{1}-AR subtypes have been established by comparison of their affinity constants for a wide variety of α_{1}-AR subtype-selective antagonists (5, 6). From this comparison, it has been suggested that the cloned α_{1a}-AR represents the native α_{1a1}-AR subtype; the cloned α_{1a2}-AR\(^2\) corresponds to the native α_{1a2}-AR subtype; and the cloned α_{1b}-AR is considered to represent a novel α_{1b2}-AR subtype. With the recognition that multiple α_{1}-AR subtypes exist, the roles of the individual α_{1}-AR subtypes in mediating specific physiological effects need to be investigated further.

The assignment of particular physiological responses and signaling pathways first requires the elucidation of the specific α_{1}-AR subtypes present in cardiac myocytes. In a previous study, we showed that all three α_{1}-AR subtypes are expressed at the mRNA level, but only the α_{1a}- and α_{1b}-AR subtypes are detectable at the protein level in neonatal rat cardiac myocytes (7). Based on a wide range of subtype-selective receptor antagonists, this study suggested that the α_{1a}-AR subtype appeared to be largely responsible for the stimulation of the phosphatidylinositol hydrolysis pathway in response to agonist treatment of these cells, whereas activation of the mitogen-activated protein kinase (MAPK) pathway appeared to occur through a subtype whose pharmacological profile most closely resembled that of the α_{1b}-AR subtype. Although intriguing, the limited selectivities of the currently available α_{1}-AR antagonists do not allow the definitive assignments of functional responses and signaling pathways to be made. Thus, for a given α_{1}-AR antagonist, the binding constants for inhibition of signaling responses show a range of differences among published reports that is almost as great as the range of putative differences among the cloned α_{1}-AR subtypes (8). Therefore, to circumvent

\(^*\) This research was supported by National Institutes of Health Grants HL 49278 (to J. D. R.) and DK 45417 (to C. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^\dagger\) Both authors contributed equally to this work and should be considered as joint first authors.

\(^\ddagger\) To whom correspondence should be addressed: Henry Hood Research Program, Pennsylvania State College of Medicine, Weis Center for Research, 100 North Academy Ave., Danville, PA 17822. Tel.: 570-271-6684; Fax: 570-271-6701.

\(^1\) The abbreviations used are: AR, adrenergic receptors; AEBSF, 4-((2-aminoethyl)benzenesulfonylfluoride); BMY 7378, 8-(2-(4-(2-methoxy-phenyl)-1-piperazinyl)-ethyl)-8-azaspiro (4,5)decane-7,9-dione dihydrochloride; ERK, extracellular signal-regulated kinase; SRE, serum response element; ANF, atrial natriuretic factor; [\(^{125}\)I]HEAT, (2,8-4-hydroxy-3-[[4-iodophenylamino]methyl](tetralone); G protein, guanine nucleotide-binding regulatory protein; JNK, c-Jun NH\(_2\)-terminal kinase; cAMP, cyclic AMP; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; WB-4101, (2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride; kb, kilobase pair; PI, phosphatidylinositol; MEC(K), MAPK/ERK kinase (kinase).

\(^2\) Nomenclature used for the α_{1}-ARs is that recommended by the International Union of Pharmacology (Hieble, J. P., Bylund, D. B., Clarke, D. E., Elkenburg, D. C., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., and Ruffolo, R. R. (1995) Pharmacol. Rev. 47, 267–270) where native α_{1}-AR subtypes are denoted by uppercase subscripts (e.g. α\(_{1a}\)) and cloned α_{1}-AR subtypes are denoted by lowercase subscripts (e.g. α\(_{1b}\)). Also, in accordance with these recommendations, the recombinant α_{1a}-AR subtype is renamed α\(_{1a1}\)-AR subtype, which is believed to represent the native α\(_{1a1}\)-AR subtype.
the limitations of a pharmacologic approach, we sought to develop a molecular approach that could be used to identify conclusively which signaling pathways, and ultimately which functional responses, are activated in response to the individual α1A- and α1B-AR subtypes in cardiac myocytes. To this end, we constructed a constitutively active mutant of the α1B-AR subtype by analogy to a previously described constitutively active mutant of the α1A-AR subtype (9). Such constitutively active receptors have the advantage that their signaling properties can be examined in the absence of agonist. Following introduction of the individual constitutively active α1-AR subtypes into the normal cellular context of cardiac myocytes in which the wild type receptor subtypes are expressed, we determined which signaling pathways are activated in response to each mutant receptor subtype in the absence of agonist.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: BMY 7378, prazosin, 5-methylurapidil, oxymetazoline, and WB-4101 (Research Biochemicals International); phenylephrine, propranolol, and phentolamine (Sigma); [3H]prazosin, [125I]HEAT (NEN Life Science Products); myo-[3H]lisinol (Amersham Pharmacia Biotech); AG1-X8 resin (Bio-Rad); and pREP4 and pREP8 expression vectors and Sindbis Virus Expression Kit (Invitrogen).

Construction and Subcloning of the Mutant α1-AR Subtypes in COS-m6 Cells—The α1A- and α1B-AR cDNA was constructed by site-directed mutagenesis of the bovine α1-AR cDNA, which was generously provided by Dr. Jon Lomasney, Northwestern University Medical School. The oligonucleotides 5'-CCGCCACATAAAGGCCTGAAAGCCCTG-3' and 5'-CTTGTAGCCCTTGTATCCGGC-3' were used to generate the appropriate mutations at Lys290 → His and Ala293 → Leu. The mutant α1A- and α1B-AR cDNA was verified by DNA sequencing.

For expression studies in COS-m6 cells, the cDNA encoding the mutant α1A- or α1B-AR subtype was subcloned into the BamHI site of the empty expression vector. The cDNA fragment encoding the mutant α1A- or α1B-AR subtype in the pBC121 expression vector was kindly provided by Dr. Susanna Cotechici, Lausanne, Switzerland. The α1B-AR should be phenotypically identical to the α1A-AR, since only substitutions at Lys290 → His and Ala293 → Leu were shown to be responsible for constitutive activity (9). The cDNAs encoding the wild type α1-AR and α1A- and α1B-AR subtypes in the pREP4 and pREP8 expression vectors, respectively, were generously provided by Dr. Kenneth Minnerman (Emory University Medical School).

For expression studies in cardiac myocytes, the cDNA fragments encoding the mutant α1A- or α1B-AR subtype were subcloned into the psiRep5 vector (Invitrogen). The 1.5-kb cDNA fragment encoding the mutant α1A- or α1B-AR was released from the pBC121 vector by digestion with BamHI. After filling in the sticky ends with Klenow polymerase, this cDNA fragment was ligated into the StuI site of the psiRep5 vector. Similarly, the 2-kb cDNA fragment encoding the mutant α1A- or α1B-AR was released from the pBC121 vector by digestion with EcoRI and PstI. The 2-kb cDNA fragment was isolated and further digested with ApaLI to generate a 1.9-kb cDNA fragment. After filling in the sticky ends with dNTPs and Klenow polymerase, this cDNA fragment was ligated into the StuI site of the psiRep5 vector.

Differential Coupling of Active α1-AR Subtypes in Rat Cardiac Myocytes—Cardiac myocytes were prepared from hearts of 1–2 day-old Harlan Sprague-Dawley rats. Briefly, the ventricles were removed, digested with a mixture of trypsin, chymotrypsin, and elastase in a Celtirist apparatus at 37 °C, and subjected to Percoll step gradients to obtain an enriched fraction of greater than 94% myocytes, as described previously (11). Myocytes were suspended in modified Eagle’s medium (MEM) containing 5% newborn calf serum, 100 μM 5-bromo-2′-deoxyuridine, 50 units/ml penicillin, and 50 μg/ml streptomycin and plated at a density of 5 × 10^6/18-mm well, 1 × 10^6/35-mm well, and 2 × 10^6/60-mm dish. Following overnight incubation, the serum-containing medium was removed and replaced with a defined serum-free medium, as detailed previously (11). Myocytes were maintained in the defined serum-free medium for 24 h before being used for viral infection.

By standard gene transfer methods, cardiac myocytes are not easily transfected. Therefore, it was necessary to develop a viral infection procedure in order to be able to measure activation of signaling pathways in response to the introduction of a specific mutant receptor subtype in the whole cell population. In another study, we demonstrated the utility of a recombinant Sindbis virus to infect greater than 90% of the cardiac myocytes, as measured by positive β-galactosidase staining. Therefore, in the present study, recombinant Sindbis viruses encoding the mutant α1A- or α1B-AR subtypes were used to infect cardiac myocytes. For construction of recombinant Sindbis viruses, the pSinRep vector containing the cDNA for either the mutant α1A- or α1B-AR was linearized with XhoI. The cDNA fragment containing the cDNA for the LacZ was linearized with XhoI. The capped RNA transcripts were generated by in vitro transcription of the pSinRep cDNAs as well as the DH-BB helper virus DNA, as described by the manufacturer (Invitrogen). The recombinant Sindbis viruses were harvested from the medium of baby hamster kidney cells that had been electroporated 28 h earlier with the capped RNA transcripts.

For measurement of receptor expression, neonatal cardiac myocytes growing on 60-mm dishes were infected with recombinant Sindbis virus encoding either LacZ, mutant α1A- or α1B-AR subtypes in COS-m6 cells—Expression of the wild type and mutant receptors in COS-m6 cells was carried out by the DEAE-dextran transfection procedure (10). On day 0, COS-m6 cells were plated at a density of 2 × 10^5 cells/100-mm dish in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. On day 1, cells were transfected with varying concentrations of plasmid DNA and DEAE-dextran. The cells were harvested 48 h later for measurement of total or cell surface receptor binding. For total receptor binding, the cells were lysed with a Polytron homogenizer in ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, and 5 mM EDTA, and the cell lysates were centrifuged at 10,000 × g for 20 min at 4 °C. The resulting membrane pellets were resuspended in buffer containing 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA at a protein concentration of 1–3 mg/ml. Receptor expression was monitored by competitive binding of the α1-AR antagonist, [3H]prazosin, from these membranes. Briefly, 0.03 mg of membrane protein was incubated in a total volume of 90 μl of assay buffer consisting of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% bovine serum albumin, and [3H]prazosin for 45 min at room temperature. In the competition binding experiments, membranes were incubated with 1 nM of the [3H]prazosin in the presence or absence of varying concentrations of competing ligands. In the saturation binding experiments, membranes were incubated with 0.2–35 nM [3H]prazosin in the presence or absence of 10 μM phentolamine to determine nonspecific binding. The incubation was terminated by the addition of 1 ml of ice-cold assay buffer, followed by rapid filtration over Whatman GF/C glass fiber filters. After washing the tubes and filters three times with 1 ml of ice-cold assay buffer, membrane-bound [3H]prazosin retained on the filters was counted by liquid scintillation spectrometry.

For measurement of receptor binding, the cells were washed 8 times with Dulbecco’s phosphate-buffered saline to remove antagonists that were present in the growth media (see “Results” for details) and then gently released from the dishes by incubation in 1 ml of Dulbecco’s phosphate-buffered saline containing 0.05% trypsin and 0.5 mM EDTA for 2 min at 37 °C. The cell suspensions were treated with 5 ml of Dulbecco’s modified Eagle’s medium containing 10% serum to inactivate the trypsin and centrifuged at 1,500 × g for 20 min at 4 °C. The cell pellets were resuspended in assay buffer to give approximately 2.5 × 10^8 cells/ml. The binding of the α1A- or α1B-AR antagonist, [3H]prazosin, to the resuspended cells was performed as described above in a final volume of 500 μl containing approximately 1 × 10^6 cells. An aliquot of cells was counted by trypan blue staining to ensure the intactness of the cells.

3 C. A. Hansen, V. Kaliman, J. Asundi, W. Reinheimer, T. Smink, and J. D. Robishaw, manuscript in preparation.
assayed for protein concentration, and used for measurement of receptor expression. Briefly, 0.01 mg of membranes were incubated with a saturating concentration of \(^{[32P]P}\)HEAT (4 nM) in a total volume of 90 μl of incubation buffer consisting of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1% bovine serum albumin for 45 min at 25 °C (7). The reactions were terminated by addition of 1 ml of ice-cold trichloroacetic acid filtered through Whatman GF/F filters. The tubes and filters were counted three times with ice-cold incubation buffer, and the filters were counted in a Beckman gamma counter. Nonspecific binding was determined by the inclusion of 1 μM prazosin.

Measurement of Phosphatidylinositol Hydrolysis—In the studies employing COS-m6 cells, cells growing on 18-mm wells were transfected with 5 μg of plasmid using the calcium-phosphate precipitation method and then labeled for 24 h with medium containing myo-[\(^{3}H\)]inositol (2 μCi/ml). After 24 h labeling, the medium was replaced with medium containing 20 mM LiCl followed by addition of agonist or vehicle for another 45 min. The cells were stopped by the addition of ice-cold trichloroacetic acid (final concentration of 6%). The precipitated proteins were removed by centrifugation, solubilized in 0.25 M NaOH, 0.2% SDS, and protein concentrations were determined. The supernatants were extracted three times with 3 volumes of water-saturated diethyl ether and incubated at 55 °C for 60 min, and the neutralized aqueous extracts were applied to 1-ml columns of Dowex AG-1-X8, formate form. Total inositol phosphates were eluted using a standard procedure (13) and counted by liquid scintillation spectrometry.

For the neonatal myocardium studies, cells growing on 18-mm wells were infected with the various recombinant Sindbis viruses. Twenty-four hours later, the cells were labeled with media supplemented with myo-[\(^{3}H\)]inositol (2 μCi/ml). At 48 h post-Sindbis virus infection, the cells were treated with 10 mM LiCl in the presence or absence of 100 μM phenylephrine and 1 μM propranolol for 30 min at 37 °C. The cells were stopped by two rapid 1-ml washes of ice-cold phosphate-buffered saline followed by the addition of 1 ml of 6% ice-cold trichloroacetic acid. After scraping, the cells were centrifuged at 14,000 × g for 10 min to remove the precipitated proteins. The supernatants were extracted three times with diethyl ether, and the extracts were applied to 0.5-ml columns of Dowex AG-1-X8, formate form. Inositol phosphates were eluted by a standard procedure (13) and counted by liquid scintillation spectrometry.

Measurement of MAPK Activation—Neonatal rat cardiac myocytes growing on 35-mm wells were treated in the presence or absence of 100 μM phenylephrine and 1 μM propranolol for 30 min at 37 °C. Reactions were stopped by two washes with ice-cold phosphate-buffered saline followed by addition of 75 μl of MAPK extraction buffer, consisting of 20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM vanadate, 10 μg/ml leupeptin, 10 μM PMSF, 1 μM AEBSF, 25 μg/ml leupeptin, and 5 μg/ml pepstatin A. After scraping, the cell extracts were incubated on ice for 15 min and centrifuged at 14,000 × g for 10 min at 4 °C. The supernatant fractions were assayed for protein concentration using the Coomassie Plus Protein Assay (Pierce). Equal amounts of protein were electrophoresed on 11% SDS-polyacrylamide gels, and the proteins were transferred to Immobilon-P membrane (Millipore). MAPK activation was determined by immunoblotting with a polyclonal antisera that recognizes only the doubly phosphorylated forms of ERK1 and ERK2 (Anti-active MAPK, Promega, 1:10,000), which is the form of MAPK that possesses myelin basic protein phosphorylating activity. Western blots stripped and reprobed with a monoclonal pan-ERK1/ERK2 antibody (Promega) showed equivalent amounts of ERK1/ERK2 protein across the lanes. The amounts of active and total ERK1 and ERK2 were visualized by incubating the immunoblot with a goat anti-rabbit horseradish peroxidase conjugate (1:5000) followed by enhanced chemiluminescence detection using the SuperSignal Chemiluminescent Substrate Kit (Pierce).

Luciferase Gene Expression—Cultured cardiac myocytes were transfected with 1 μg of plasmid DNA using either the SRE-luciferase reporter plasmid (Stratagene, Inc.) or the ANF promoter-luciferase reporter plasmid, which was the generous gift of Dr. J. H. Brown. An aliquot of 1 μl of luciferase reporter plasmids (SRE or ANF) was mixed with 7 μl of the “Plus” component of LipofectAmine Plus reagent (Life Technologies, Inc.) and 100 μl of Opti-MEM medium (Life Technologies, Inc.) and incubated for 15 min at room temperature. In a separate tube, 7 μl of the lipid component of LipofectAmine Plus reagent was added to 100 μl of Opti-MEM medium and incubated at room temperature for 15 min. The contents of the two tubes were then mixed together and incubated an additional 30 min at room temperature. The cardiac myocyte tissue culture plates (60 mm) were washed with Opti-MEM medium and then 1 ml of fresh Opti-MEM medium without antibiotics was added to each cell plate. The contents of the DNA/LipofectAmine Plus mixture was then added to each dish of myocytes. After 5 h, the transfection media were removed and the myocytes maintained in Opti-MEM medium without antibiotics.

For luciferase gene expression assays, the cardiac myocytes were lysed in 0.5 ml of 1× lysis buffer (Promega Corp.). The cell lysates were centrifuged at 15,000 × g for 1 min at 4 °C, and the supernatants were transferred to a new tube. An aliquot of 50 μl of the supernatant was assayed in duplicate for SRE- and/or ANF-luciferase gene expression using a Luciferase Assay System with Reporter Lysis Buffer (Promega Corp.). Relative light units were measured utilizing a Lumat LB9507 EG&G Berthold luminometer with myocyte cell lysates. Following luciferase assays, β-galactosidase enzyme assays were performed in duplicate samples. The luciferase relative light units were corrected for the β-galactosidase enzyme assay numbers to correct for the transfection efficiency in the myocytes. The cardiac cell lysate from the luciferase assays was assayed spectrophotometrically for the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega).

Data Analysis—Values represent the mean ± S.E. Saturation binding and competition binding curves were analyzed using the nonlinear least squares regression analysis program GraphPad Prism (GraphPad Software). Estimates of ligand binding affinity (K_a) and receptor density (B_max) were obtained from saturation isotherms by fitting the data to a rectangular hyperbola. IC_50 values from competition binding experiments were converted to K_a values using the Cheng and Prusoff equation (14). IC_50 = IC_50/(1 + [A]/K_a) (14), where [A] is the concentration of ligand used and K_a was determined from the saturation binding studies.

RESULTS

Construction and Characterization of the Constitutively Active α_1a-S290/293-AR—To address whether constitutively active mutants of the α_1a-AR and the α_1b-AR subtypes could be used to probe their subtype-specific coupling to signaling pathways, a constitutively active mutant of the α_1a-AR needed to be constructed. A constitutively active mutant α_1a-AR subtype has already been constructed and characterized by Dr. Coteccchia (9). Accordingly, the α_1a-AR subtype was mutated at Lys^290 and Ala^395 (α_1a-S290/293-AR) to mirror the substitutions previously found to result in maximal constitutive activation of the mutant α_1a-AR subtype (9). Properties that have been shown to be characteristic of constitutive activation include the following: 1) an increased affinity of a receptor for agonist but not for antagonist; 2) an elevated basal level of signaling; and 3) an increased agonist-stimulated level of signaling. To determine whether the mutant α_1a-S290/293-AR subtype exhibited any or all of these properties, the mutant α_1a-S290/293-AR subtype was expressed and then characterized in COS-m6 cells. Since COS-m6 cells do not express wild type α_1-ARs, these cells provide a convenient background in which to study the properties of the mutant α_1a-S290/293-AR subtype.

Increased Affinity for Agonist—COS-m6 cells transfected with either the wild type α_1a-AR or the mutant α_1a-S290/293-AR were characterized in terms of their affinity for various agonists and antagonists by monitoring the displacement of [H]prazosin through agonist or antagonist competition binding assays. Phenylephrine and norepinephrine, both full α_1-AR agonists (15), displaced [H]prazosin from membranes expressing the α_1a-S290/293-AR mutant with approximately 40-fold higher affinities than those expressing the wild type receptor (Fig. 1; Table I). The partial imidazoline agonist, oxymetazoline (15), also demonstrated a higher affinity for the α_1a-S290/293-AR (Fig. 1), although the difference in affinity, in this case, was only 6-fold (Table I). The antagonist, 5-methylurapidil, demonstrated similar affinity for both the wild type and mutant receptors (Fig. 1; Table I). The agonist with antagonist competition curves fitted best to a single-site model, where Hill coefficients were between 0.7 and 1.0. These data demonstrate that the mutant α_1a-S290/293-AR showed an increase in binding affinity for agonist but not for antagonist compared with its wild type counterpart. These data provide the first confirmation that the mutant α_1a-S290/293-AR possesses properties reminis-
FIG. 1. Competition binding relationships of agonists and antagonists to the wild type α₁a-AR and α₁a-S²⁹⁰/²⁹³ mutant receptors expressed in COS-m6 cells. The ability of α₁-AR agonists and antagonists to displace specific [³H]prazosin binding from membranes prepared from COS-m6 cells transiently expressing homogeneous populations of either wild type α₁a-AR or α₁a-S²⁹⁰/²⁹³ mutant receptor was performed and analyzed as described under “Experimental Procedures.” Each value represents the mean ± S.E. of three to four individual experiments performed in duplicate. The corresponding Kᵰ values are reported in Table I. Mean receptor expression levels were 1400 (α₁a-AR) and 300 (α₁a-S²⁹⁰/²⁹³) fmol/mg membrane protein obtained using 0.2 and 2.6 μg/ml of cDNA/transfection, respectively.

TABLE I

| Competitor          | Wild type α₁a-AR | α₁a-S²⁹⁰/²⁹³ Mutant |
|---------------------|------------------|------------------|
| Norepinephrine      | 8099 ± 1328      | 187 ± 25° (43)   |
| Phenylephrine       | 17936 ± 1895     | 453 ± 134° (40)  |
| Oxymetazoline       | 111 ± 12         | 17 ± 1° (6)      |
| 5-Methylurapidil    | 2 ± 0.2          | 3 ± 0.3 (0.7)    |

*P < 0.01. **P < 0.001 compared with wild type α₁a-AR.

Previously, it was shown that an increased activity observed between the wild type and mutant α₁a-ARs may be related to the intrinsic activity of the agonist (i.e. a larger difference is observed for the full agonist, phenylephrine, compared with the partial agonist, oxymetazoline), and this observation is in agreement with that previously described for a constitutively active mutant of the β₂-AR (16).

Enhanced Basal and Agonist-stimulated Phosphatidylinositol Hydrolysis—Previously, it was shown that all α₁-AR subtypes couple to the phosphatidylinositol hydrolysis pathway when overexpressed at high, “non-physiological” levels (e.g. >1500 fmol of receptor/mg of protein) (17–19). In the present report, we decided to study the signaling properties of both the constitutively active and wild type α₁-ARs when they were expressed at more physiological levels in order to get a better idea of how these receptors operate in vivo. By altering the amount of plasmid DNA used in the transfections, COS-m6 cells expressing various levels of receptor were obtained. In particular, cells expressing receptor in the 300 fmol/mg protein range were studied, since this density seems comparable with many intact cellular systems that normally express α₁-ARs. Examples of values for various tissues are as follows: 256 fmol/mg protein in rat cardiac myocytes (20); 158 fmol/mg protein in rat cerebral cortex (21); and 233, 313, and 690 fmol/mg protein in rat hippocampus, vas deferens, and liver, respectively (22).

The second confirmation that the mutant α₁a-S²⁹⁰/²⁹³-AR exhibits properties characteristic of other constitutively active receptors was a reproducible and marked increase in the basal level of phosphatidylinositol hydrolysis (i.e. in the absence of agonist). As shown in Fig. 2, COS-m6 cells expressing the mutant α₁a-S²⁹⁰/²⁹³-AR displayed a high basal level of inositol phosphate accumulation at the lowest receptor density examined of 199 fmol/mg protein. By contrast, cells expressing the wild type α₁a-AR showed no detectable basal level of inositol phosphate accumulation over a wide range of receptor densities from 308 to 2098 fmol/mg protein. These data demonstrate that the mutant α₁a-S²⁹⁰/²⁹³-AR had an increased ability to interact with G protein to stimulate inositol phosphate accumulation in the absence of agonist, which is indicative of constitutive activity. Moreover, since the mutant α₁a-S²⁹⁰/²⁹³-AR was expressed at a lower level than the wild type α₁a-AR, these data likely underestimate the greater constitutive activity of the mutant α₁a-S²⁹⁰/²⁹³-AR.

Also, as shown in Fig. 2, COS-m6 cells expressing the mutant α₁a-S²⁹⁰/²⁹³-AR displayed a higher agonist-stimulated level of inositol phosphate accumulation compared with cells expressing the wild type α₁a-AR. In fact, the wild type α₁a-AR had to be expressed at a 10-fold higher level than the mutant α₁a-S²⁹⁰/²⁹³-AR before their maximal responses were comparable. These data are consistent with previous studies of wild type and mutant β₂-ARs, which showed that greatly elevated expression of the wild type receptor was able to increase agonist-stimulated cAMP accumulation to a level comparable to that achieved with the constitutively active mutant receptor (19).

Increased Potency of Agonist-stimulated Phosphatidylinositol Hydrolysis—Previously, it was shown that an increased...
agonist potency is yet another feature of a constitutively active receptor (9, 16). Fig. 3 compares the dose-response curves for the wild type α1a-AR and mutant α1a-S290/293-AR to agonist. As can be seen, phenylephrine exhibits a 13-fold greater potency for stimulating phosphatidylinositol hydrolysis via the mutant α1a-S290/293-AR (EC50 = 15 ± 8 nM, n = 3) compared with the wild type α1a-AR (EC50 = 192 ± 64 nM, n = 3, p < 0.01). These results demonstrate that the mutant α1a-S290/293-AR also fulfilled this criterion of a constitutively active receptor.

Increased Receptor Expression in the Presence of Antagonist—We observed that for any given plasmid DNA concentration used for transfection, the level of expression of the mutant α1a-S290/293-AR was considerably lower than that of the wild type receptor. A similar finding was recently reported for a constitutively active mutant of the β2-AR (23). Moreover, inclusion of antagonist in the post-transfection period was shown to increase the level of expression of the constitutively active mutant of the β2-AR. To determine whether this property was shared by the constitutively active mutant of the α1a-S290/293-AR, inclusion of a variety of antagonists in the post-transfection period was studied. These antagonists were removed just prior to collection of the cells by exhaustive washing (8 times) with Dulbecco’s phosphate-buffered saline. As shown in Fig. 4, the α1a-AR-selective antagonists, 5-methylurapidil and WB-4101, in the post-transfection period led to a significant increase in the number of mutant α1a-S290/293-ARs on the cell surface. The α1b-AR selective antagonist, BMY 7378, resulted in a similar increase. Interestingly, the non-selective α1-AR antagonist, prazosin, was unique in that it did not elevate the density of mutant α1a-S290/293-AR. These data suggest that the ability of antagonists to enhance receptor expression is a property shared by constitutively active mutants of the β2-AR and α1a-S290/293-AR. The mechanism by which antagonists result in higher receptor expression is not yet clear but may relate to stabilization of receptor conformation (23). In this regard, the finding that not all antagonists display this property is interesting in that it might support the existence of multiple receptor conformations of the mutant α1a-S290/293-AR.

Comparison of the Signaling Properties of the Constitutively Active α1a-S290/293 and α1b-S288/294-AR Subtypes in COS-1a-S290/293-AR was considerably lower than that of the wild type α1a-AR (9, 16). Fig. 3 compares the dose-response curves for the wild type α1a-AR and mutant α1a-S290/293-AR to agonist. As can be seen, phenylephrine exhibits a 13-fold greater potency for stimulating phosphatidylinositol hydrolysis via the mutant α1a-S290/293-AR (EC50 = 15 ± 8 nM, n = 3) compared with the wild type α1a-AR (EC50 = 192 ± 64 nM, n = 3, p < 0.01). These results demonstrate that the mutant α1a-S290/293-AR also fulfilled this criterion of a constitutively active receptor.

Increased Receptor Expression in the Presence of Antagonist—We observed that for any given plasmid DNA concentration used for transfection, the level of expression of the mutant α1a-S290/293-AR was considerably lower than that of the wild type receptor. A similar finding was recently reported for a constitutively active mutant of the β2-AR (23). Moreover, inclusion of antagonist in the post-transfection period was shown to increase the level of expression of the constitutively active mutant of the β2-AR. To determine whether this property was shared by the constitutively active mutant of the α1a-S290/293-AR, inclusion of a variety of antagonists in the post-transfection period was studied. These antagonists were removed just prior to collection of the cells by exhaustive washing (8 times) with Dulbecco’s phosphate-buffered saline. As shown in Fig. 4, the α1a-AR-selective antagonists, 5-methylurapidil and WB-4101, in the post-transfection period led to a significant increase in the number of mutant α1a-S290/293-ARs on the cell surface. The α1b-AR selective antagonist, BMY 7378, resulted in a similar increase. Interestingly, the non-selective α1-AR antagonist, prazosin, was unique in that it did not elevate the density of mutant α1a-S290/293-AR. These data suggest that the ability of antagonists to enhance receptor expression is a property shared by constitutively active mutants of the β2-AR and α1a-S290/293-AR. The mechanism by which antagonists result in higher receptor expression is not yet clear but may relate to stabilization of receptor conformation (23). In this regard, the finding that not all antagonists display this property is interesting in that it might support the existence of multiple receptor conformations of the mutant α1a-S290/293-AR.

Comparison of the Signaling Properties of the Constitutively Active α1a-S290/293 and α1b-S288/294-AR Subtypes in COS-1a-S290/293-AR was considerably lower than that of the wild type α1a-AR (9, 16). Fig. 3 compares the dose-response curves for the wild type α1a-AR and mutant α1a-S290/293-AR to agonist. As can be seen, phenylephrine exhibits a 13-fold greater potency for stimulating phosphatidylinositol hydrolysis via the mutant α1a-S290/293-AR (EC50 = 15 ± 8 nM, n = 3) compared with the wild type α1a-AR (EC50 = 192 ± 64 nM, n = 3, p < 0.01). These results demonstrate that the mutant α1a-S290/293-AR also fulfilled this criterion of a constitutively active receptor.

Increased Receptor Expression in the Presence of Antagonist—We observed that for any given plasmid DNA concentration used for transfection, the level of expression of the mutant α1a-S290/293-AR was considerably lower than that of the wild type receptor. A similar finding was recently reported for a constitutively active mutant of the β2-AR (23). Moreover, inclusion of antagonist in the post-transfection period was shown to increase the level of expression of the constitutively active mutant of the β2-AR. To determine whether this property was shared by the constitutively active mutant of the α1a-S290/293-AR, inclusion of a variety of antagonists in the post-transfection period was studied. These antagonists were removed just prior to collection of the cells by exhaustive washing (8 times) with Dulbecco’s phosphate-buffered saline. As shown in Fig. 4, the α1a-AR-selective antagonists, 5-methylurapidil and WB-4101, in the post-transfection period led to a significant increase in the number of mutant α1a-S290/293-ARs on the cell surface. The α1b-AR selective antagonist, BMY 7378, resulted in a similar increase. Interestingly, the non-selective α1-AR antagonist, prazosin, was unique in that it did not elevate the density of mutant α1a-S290/293-AR. These data suggest that the ability of antagonists to enhance receptor expression is a property shared by constitutively active mutants of the β2-AR and α1a-S290/293-AR. The mechanism by which antagonists result in higher receptor expression is not yet clear but may relate to stabilization of receptor conformation (23). In this regard, the finding that not all antagonists display this property is interesting in that it might support the existence of multiple receptor conformations of the mutant α1a-S290/293-AR.

Comparison of the Signaling Properties of the Constitutively Active α1a-S290/293 and α1b-S288/294-AR Subtypes in COS-1a-S290/293-AR was considerably lower than that of the wild type α1a-AR (9, 16). Fig. 3 compares the dose-response curves for the wild type α1a-AR and mutant α1a-S290/293-AR to agonist. As can be seen, phenylephrine exhibits a 13-fold greater potency for stimulating phosphatidylinositol hydrolysis via the mutant α1a-S290/293-AR (EC50 = 15 ± 8 nM, n = 3) compared with the wild type α1a-AR (EC50 = 192 ± 64 nM, n = 3, p < 0.01). These results demonstrate that the mutant α1a-S290/293-AR also fulfilled this criterion of a constitutively active receptor.

Increased Receptor Expression in the Presence of Antagonist—We observed that for any given plasmid DNA concentration used for transfection, the level of expression of the mutant α1a-S290/293-AR was considerably lower than that of the wild type receptor. A similar finding was recently reported for a constitutively active mutant of the β2-AR (23). Moreover, inclusion of antagonist in the post-transfection period was shown to increase the level of expression of the constitutively active mutant of the β2-AR. To determine whether this property was shared by the constitutively active mutant of the α1a-S290/293-AR, inclusion of a variety of antagonists in the post-transfection period was studied. These antagonists were removed just prior to collection of the cells by exhaustive washing (8 times) with Dulbecco’s phosphate-buffered saline. As shown in Fig. 4, the α1a-AR-selective antagonists, 5-methylurapidil and WB-4101, in the post-transfection period led to a significant increase in the number of mutant α1a-S290/293-ARs on the cell surface. The α1b-AR selective antagonist, BMY 7378, resulted in a similar increase. Interestingly, the non-selective α1-AR antagonist, prazosin, was unique in that it did not elevate the density of mutant α1a-S290/293-AR. These data suggest that the ability of antagonists to enhance receptor expression is a property shared by constitutively active mutants of the β2-AR and α1a-S290/293-AR. The mechanism by which antagonists result in higher receptor expression is not yet clear but may relate to stabilization of receptor conformation (23). In this regard, the finding that not all antagonists display this property is interesting in that it might support the existence of multiple receptor conformations of the mutant α1a-S290/293-AR.

Comparison of the Signaling Properties of the Constitutively Active α1a-S290/293 and α1b-S288/294-AR Subtypes in COS-
Comparison of the Signaling Properties of the Constitutively Active \( \alpha_1 \)-AR Subtypes in Rat Cardiac Myocytes—In a previous study, we showed that both the \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes are expressed in neonatal rat cardiac myocytes (7). Activation of both the phospholipase C and MAPK signaling pathways has been demonstrated upon addition of \( \alpha_1 \) agonists to cardiac myocytes (1, 7, 27, 30). Moreover, several studies have demonstrated that the combined activation of these receptor subtypes is associated with stimulation of several signaling pathways, including stimulation of phosphatidylinositol hydrolysis (19) and activation of ERK (24), JNK (25), and p38 kinase (26). However, the definitive assignment of these signaling pathways to the activation of the individual \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes has been hampered by the lack of pharmacological tools with the requisite subtype specificity. With the development of constitutively activated \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes, we now have the molecular tools to examine their respective signaling properties in the appropriate cellular context in which the wild type \( \alpha_1 \)-AR receptors are normally expressed, but in the absence of agonist to stimulate the various wild type \( \alpha_1 \)-AR subtypes.

Whereas standard gene transfer methods were effective for introducing luciferase reporter plasmids into cardiac myocytes, they were ineffective for expression of the constitutively active \( \alpha_1 \)-AR subtypes in myocytes. However, we recently demonstrated the first successful use of recombinant Sindbis viruses to express G protein \( \beta \) subunits in cardiac myocytes. This viral infection procedure was found to provide a rapid and efficient method to introduce genes into cardiac myocytes, with greater than 90% of cardiac myocytes being successfully targeted. Thus, activation of signaling pathways in response to the introduction of the individual constitutively activated receptor subtypes can be measured on the whole cell population. Therefore, to utilize this method, we constructed recombinant Sindbis viruses encoding the constitutively activated \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes. To control for any nonspecific effects of Sindbis virus infection, a recombinant Sindbis virus containing the bacterial lacZ gene or pSinRep5 vector was utilized. The expression of the constitutively activated \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes was quantitated by radioligand binding. As shown in Fig. 6, uninfected cardiac myocytes and myocytes infected with the LacZ Sindbis virus expressed similar numbers of \( \alpha_1 \)-ARs. Under the basal condition, \( \alpha_1 \)-AR subtypes were quantitated by radioligand binding. As shown in Fig. 6, uninfected cardiac myocytes and myocytes infected with the LacZ Sindbis virus expressed similar numbers of \( \alpha_1 \)-ARs. Under the agonist-stimulated condition, phenylephrine-stimulated phosphatidylinositol hydrolysis in cardiac myocytes expressing similar levels of constitutively activated \( \alpha_1 \)-AR and \( \alpha_1 \)-AR subtypes. Under the basal condition (30 min with 10 mM LiCl in the absence of agonist), cells expressing the constitutively activated \( \alpha_1 \)-AR subtype displayed a 2.4-fold increase in inositol phosphate accumulation compared with the LacZ cells, which showed no enhancement of inositol phosphates accumulation during the 30 min of incubation with 10 mM LiCl. On the other hand, cells expressing the constitutively activated \( \alpha_1 \)-AR subtype showed no increase in inositol phosphates accumulation during the 30 min of incubation with 10 mM LiCl, which makes them indistinguishable from the LacZ cells (Fig. 7A). Under the agonist-stimulated condition, phenylephrine produced a 6-fold increase in inositol phosphates accumulation in the LacZ cells. Cells expressing the constitutively active \( \alpha_1 \)-AR subtype showed an additional reduction in inositol phosphates accumulation compared with the LacZ cells.
subtype couples to the phosphatidylinositol hydrolysis signaling pathway in both the absence and presence of agonist in cardiac myocytes, which confirms and extends previous pharmacological data from our own laboratory (7). By contrast, the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype was not able to couple to the phosphatidylinositol hydrolysis signaling pathway in cardiac myocytes.

Comparison of the MAPK Signaling Pathways of the Constitutively Active $\alpha_{1a}$-S$^{290/293}$ and $\alpha_{1b}$-S$^{288-294}$-AR Subtypes in Rat Cardiac Myocytes—Next, we examined the MAPK signaling pathway. Fig. 8 shows a comparison of basal and phenylephrine-induced activation of MAPK activity in cardiac myocytes expressing similar levels of the activated $\alpha_{1a}$-S$^{290-293}$-AR and $\alpha_{1b}$-S$^{288-294}$-AR subtypes. Under the basal condition (in the absence of agonist), cardiac myocytes expressing the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR subtype showed a slight activation (1.5-fold), with the level of active MAPK being similar to that observed in LacZ cells (Fig. 8A). By contrast, cells expressing the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype displayed marked activation, with the level of active MAPK being 5.8-fold above that observed in LacZ cells. Under the agonist-stimulated condition, phenylephrine produced a modest increase in the level of active MAPK in the LacZ cells, which was less than the level of active MAPK observed in the $\alpha_{1b}$-S$^{288-294}$-AR subtype in the absence of agonist. In cells expressing the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR subtype, phenylephrine stimulation increased the level of active MAPK only marginally (1.3-fold) compared with that in the phenylephrine-stimulated LacZ cells. In contrast, cells expressing the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype showed a further 3.3-fold increase in the agonist-stimulated level of active MAPK compared with that in the phenylephrine-stimulated LacZ cells (Fig. 8B). These results clearly demonstrate that the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype effectively couples to the MAPK signaling pathway in both the absence and presence of agonist in cardiac myocytes, whereas the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR subtype shows little or no ability to couple to the MAPK signaling pathway in cardiac myocytes. Not only was the strict and specific coupling of the $\alpha_{1b}$-S$^{288-294}$ AR subtype to the MAPK kinase signaling pathway, and the $\alpha_{1a}$-S$^{290/293}$-AR subtype to the phosphatidylinositol hydrolysis pathway unexpected, this specificity was observed even though the mutant receptor subtypes were being expressed at 10-fold higher levels than the wild type receptor subtypes.

Comparison of Gene Activation by the Signaling Pathways of the Constitutively Active $\alpha_{1a}$-S$^{290/293}$ and $\alpha_{1b}$-S$^{288-294}$-AR Subtypes in Rat Cardiac Myocytes—We next examined the effect of the two signaling pathways on downstream events at the level of gene regulation in cardiac myocytes. In order to assess the impact of the two divergent signaling pathways activated by the constitutively activated $\alpha_{1a}$-AR and $\alpha_{1b}$-AR subtypes, we chose to measure the downstream effect on ANF and c-fos gene transcription. Both of these genes are activated during norepinephrine-induced cardiac hypertrophy in the heart, and as such represent important targets for the $\alpha_{1b}$-AR and $\alpha_{1b}$-AR subtype signaling pathways. The ANF gene is activated by the $\alpha_{1b}$-AR receptor agonist, phenylephrine, through multiple promoter elements (AP-1, SP1, A/T, and SRE, see Refs. 27–31), and is linked to activation of PI hydrolysis (32–36). Moreover, previous reports have suggested that the $\alpha_{1b}$-AR subtype mediates the activation of ANF gene expression (33, 37). The early response genes (c-fos, c-jun, and c-myc) are activated by stimulation of $\alpha_{1b}$-ARs (36, 38–43). The c-fos gene is up-regulated during cardiac hypertrophy of myocytes (44–45). Therefore, we investigated the affect of the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR and $\alpha_{1b}$-S$^{288-294}$-AR subtypes on c-fos gene regulation in cardiac myocytes.

As the c-fos gene is regulated by the (SRE) element in the promoter, we utilized luciferase gene expression reporter constructs coupled to the SRE promoter element. To measure ANF and c-fos gene activation, we measured the activation of the SRE- and the ANF-luciferase gene reporter constructs in cardiac myocytes co-transfected with the activated $\alpha_{1a}$-S$^{290/293}$-AR and $\alpha_{1b}$-S$^{288-294}$-AR subtypes. Fig. 9 shows a comparison of luciferase gene activity in transfected cardiac myocytes expressing similar levels of the activated $\alpha_{1a}$-S$^{290/293}$-AR and $\alpha_{1b}$-S$^{288-294}$-AR subtypes. In the absence of agonist, cells expressing the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR subtype and the ANF-luciferase reporter construct showed a 2.4-fold increase in the level of luciferase activity (Fig. 9). By contrast, cells expressing the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype and the ANF-luciferase reporter construct displayed no significant increase in the level of luciferase activity compared with control. In similar experiments in the absence of agonist, cells expressing the constitutively activated $\alpha_{1b}$-S$^{288-294}$-AR subtype and the SRE-luciferase reporter plasmid displayed a marked 4.7-fold increase in the level of luciferase activity. By contrast, the constitutively activated $\alpha_{1a}$-S$^{290/293}$-AR and the SRE-luciferase construct showed no significant increase in the level of luciferase activity compared with control. Taken together, these data have several important ramifications. First, the $\alpha_{1a}$-S$^{290/293}$-AR subtype preferentially activates ANF-luciferase activity compared with SRE/c-fos-
The effect of phenylephrine on the level of active MAPK in LacZ, analyzed for the presence of active MAPK as described under “Experimental Procedures.” A shows the basal level of active MAPK, whereas B shows the effect of phenylephrine on the level of active MAPK in LacZ, in the \( \alpha_{1a} \)-S\(^{290/293} \)-AR mutant and/or in the \( \alpha_{1b} \)-S\(^{288–294} \)-AR mutant expressing cardiac myocytes. The lower section of each panel shows a representative immunoblot of the detection of active MAPK (ERK1 and ERK2) by the anti-active MAPK polyclonal antisera. Immunoblots stripped and reprobed with pan-ERK1/2 monoclonal antibody for determination of protein loading/lane showed equal amounts of ERK1/2 per lane (data not shown). Bar graphs represent quantification of the amount of active ERK2 (p42 kDa MAPK) by densitometric quantification and are the mean ± S.E. for 5–9 experiments.

**DISCUSSION**

**Constitutively Activated Receptors**—Constitutively activated receptors demonstrate various properties that set them apart from their wild type counterparts, but it is their ability to activate signaling pathways in the absence of agonist that makes them valuable molecular tools. Particularly in the case of the \( \alpha_{1} \)-ARs, where pharmacological tools do not have the requisite specificity to allow the individual subtypes to be studied in isolation, the use of constitutively activated receptor subtypes provides a strategy to study the signaling properties of the individual receptor subtypes. In the present study, we constructed a constitutively active \( \alpha_{1a} \)-S\(^{290/293} \)-AR subtype by making the analogous mutations in the COOH-terminal end of the third cytoplasmic loop that produced the previously described constitutively active \( \alpha_{1b} \)-S\(^{288–294} \)-AR subtype by Dr. Cotecchia (9). We did not characterize the properties of the constitutively active \( \alpha_{1b} \)-S\(^{288–294} \)-AR subtype as it has already been reported (9). Characterization of the functional properties of this mutant \( \alpha_{1b} \)-S\(^{288–294} \)-AR subtype in COS-m6 cells revealed that it possesses the three criteria that define constitutive activation as follows: 1) an increased affinity of the mutant receptor for agonist but not for antagonist; 2) an elevated basal level of signaling; and 3) an increased agonist-stimulated level of signaling; and 3) an increased agonist-stimulated level of signaling (9, 23). Clearly, these data indicate that mutations in the analogous region of the third cytoplasmic loop previously reported to induce constitutive activation of the \( \alpha_{1b} \)-AR subtype also evoke constitutive activation of the \( \alpha_{1a} \)-AR subtype. Furthermore, an additional feature of the mutant \( \alpha_{1a} \)-S\(^{290/293} \)-AR subtype was observed, namely the ability of antagonists to enhance the level of receptor expression. Since this feature has also been observed in the case of the constitutively active \( \beta \)-AR (23), it may represent a fourth property characteristic of constitutively active receptors. With regard to this property, it is interesting that only some antagonists increase the level of receptor expression, suggesting multiple conformational states may exist that can be distinguished by the various antagonists.
Future studies will be necessary to elucidate the mechanism by which various antagonists can increase receptor density.

The Mutant \( \alpha_{1A} \text{S}^{290/293} \text{-AR and} \) \( \alpha_{1B} \text{-S}^{288/294} \text{-AR Subtypes Couple to Different Signaling Pathways and Gene Expression in Cardiac Myocytes—With the development of constitutively activated \( \alpha_{1A} \text{-AR subtype probes, we applied these molecular tools to cardiac myocytes in order to decipher which signaling pathways and physiological responses (i.e. gene transcription) were activated by each of the endogenous} \alpha_{1A} \text{-AR subtypes.} \) Rat cardiac myocytes express comparable levels of the endogenous \( \alpha_{1A} \text{-AR and} \alpha_{1B} \text{-AR subtypes (138.7 and 147.3 fmol/mg protein, respectively, see Ref. 7). Therefore, they must also possess the appropriate complement of heterotrimeric G proteins, effector molecules, and other ancillary proteins that constitute physiologically relevant} \alpha_{1A} \text{-AR stimulus-response pathways. Thus, following the introduction of either the constitutively activated} \alpha_{1A} \text{-S}^{290/293} \text{-AR or} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtypes in these cells, the recombinant activated receptor subtype should compete with its native receptor subtype to couple to its appropriate signaling pathway(s) in a physiologically relevant manner. Since the constitutively activated receptor subtypes do not require the presence of agonist, the signaling properties of the individual} \alpha_{1A} \text{-S}^{290/293} \text{-AR and} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtypes should be evident as an enhanced basal level of stimulation of the signaling pathways (i.e. in the absence of agonist).}

Analysis of the effects of expressing either the constitutively activated \( \alpha_{1A} \text{-S}^{290/293} \text{-AR or} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype} \) not only confirms our recent pharmacological results (7) but provides definitive molecular evidence that the \( \alpha_{1A} \text{-S}^{290/293} \text{-AR subtype is preferentially coupled to the phosphatidylinositol (PI) hydrolysis signaling pathway and ANF-luciferase gene expression. The ANF gene is activated by the} \alpha_{1A} \text{-AR receptor agonist, phenylephrine, through multiple promoter elements (AP-1, SP1, A/T, and SRE, see Refs. 27–31), is re-expressed during cardiac hypertrophy, and is linked to activation of PI hydrolysis (32–36). Our finding that the} \alpha_{1A} \text{-S}^{290/293} \text{-AR subtype mediates coupled to ANF gene expression is consistent with previous reports, which suggested that the} \alpha_{1A} \text{-AR subtype mediated the activation of ANF gene expression (33, 37). It has been reported that phenylephrine induces the ANF gene expression by activation of the Ras-MEK-JNK signaling pathway (46).}

A novel finding of this study is that the \( \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype is responsible for activation of SRE/c-fos-luciferase gene expression and MAPK signaling pathway. The SRE from the c-fos promoter has been shown to be the point of integration of MAPK signaling pathways (47–51). The Ras-Raf-MEK-ERK signaling pathway activates transcription factors Elk-1 and Sap-1a, which bind to the SRE of the c-fos promoter (47–51, 52–61). Interestingly, the SRE from the ANF promoter is different than that from the c-fos promoter in that it does not contain the sequences necessary for the binding of Elk-1 transcription factor (62). Our finding that the \( \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype is responsible for SRE/c-fos gene activation in cardiac myocytes is in contrast with the previous report of Deng et al. (40). They demonstrated, using pharmacological methods, that the} \alpha_{1B} \text{-S}^{290/293} \text{-AR subtype was responsible for c-fos gene activation in cardiac myocytes (40). However, our results are in agreement with the previously reported pharmacological studies in vascular smooth muscle cells, where the} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype was responsible for c-fos gene activation (39).}

The discrepancy in findings may be due to the limitations of the pharmacological approach, where the \( \alpha_{1A} \text{-AR subtype agonist and antagonists are not sufficiently selective.}

These data, as well as previously published pharmacological data (7), indicate that the \( \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype couples to the MAPK/SRE/c-fos signaling pathway in rat cardiac myocytes. In Fig. 8, we observed the} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype preferentially activates of the MAPK signaling pathway in cardiac myocytes. Finally, these data are consistent with another study in NIH-3T3 cells that reported differential coupling of the} \alpha_{1A} \text{- and} \alpha_{1B} \text{-AR subtypes to the MAPK signaling pathways (63).]

Previously, differential coupling to the PI signaling pathway has been shown to exist between the wild type \( \alpha_{1A} \text{- and} \alpha_{1B} \text{-AR subtypes when heterologously expressed in COS cells (19). This differential coupling to the PI signaling pathway was also observed in the present study (Fig. 2) when heterologously expressed in COS cells. Moreover, both the wild type and constitutively active mutant \( \alpha_{1A} \text{- and} \alpha_{1B} \text{-AR subtypes showed this differential coupling indicating the difference is a property of the receptor subtypes themselves and not a characteristic of the constitutive activating mutation. Finally, this is the first conclusive demonstration of differential coupling of the} \alpha_{1A} \text{- and} \alpha_{1B} \text{-AR subtypes in a primary cell type (i.e. cardiac myocytes) where the two receptor subtypes are normally expressed. These data indicate that the two receptor subtypes must possess differences in their abilities to interact with downstream components of these pathways. The basis for these differences may involve the selective interaction of each} \alpha_{1A} \text{-AR subtype with a different heterotrimeric G protein to produce distinct bifurcating signals in the form of Gα and Gβγ subunits. Since the phenylephrine-mediated stimulation of the phosphatidylinositol hydrolysis pathway in cardiac myocytes is insensitive to pertussis toxin (64), it is likely that the} \alpha_{1A} \text{-AR subtype couples though a member of the Gq11 protein family to regulate phospholipase C-β. The predominant phospholipase C-β isoform in rat neonatal cardiac myocytes is phospholipase C-β3 (65), which can be regulated by either the α or the β subunits of the Gq11 protein family in vitro (66). Whether the α or the β subunits of the Gq11 protein family are responsible for in vivo regulation will be the subject of future investigations.}

The underlying mechanism for activation of the MAPK pathway in rat cardiac myocytes has been controversial. The present study sheds new insights on this mechanism. Since agonist stimulation of the MAPK pathway is insensitive to pertussis toxin in cardiac myocytes (67), this suggests that the \( \alpha_{1B} \text{-AR subtype associates with a member of the Gq11 or G12/13 protein family rather than the Gα protein family. However, other than the activation of phospholipase C-β, the downstream components regulated by the} \alpha \text{ or} \beta \text{ subunits of the Gq11 or G12/13 have yet to be conclusively identified. Studies by Thorburn and colleagues (68) suggest that agonist-induced activation of the MAPK pathway is mediated by Raf-1 kinase in cardiac myocytes. Since Raf-1 kinase can be activated by protein kinase C (24, 69–71), which, in turn, can be activated by products of the PI hydrolysis pathway (72), this could provide a mechanism for activation of the MAPK pathway. However, this mechanism is difficult to reconcile with the results of the present study showing the} \alpha_{1B} \text{-S}^{288/294} \text{-AR subtype potently stimulates SRE-luciferase gene expression without any activation of the PI hydrolysis pathway or ANF gene expression. This argument is further supported by the fact that protein kinase C has been shown to activate ANF gene expression in myocytes (29–30). Additionally, the recent finding that transgenic hearts overexpressing the Gq11 protein subunit exhibited marked stimulation of the PI hydrolysis pathway but no activation of the MAPK pathway also argues against this mechanism (73). Alternatively, the} \beta \text{ subunits rather than the} \alpha \text{ subunits of Gq11 or G12/13 could be involved in stimulation of the MAPK pathway, since it has been shown that the} \beta \text{ subunits released from Gq11 can activate a Ras-dependent pathway leading to stimulation of Raf-1 kinase (74–76). Whether the} \beta \text{ subunits released from Gq11 or G12/13 can similarly activate a Ras-dependent pathway in cardiac myocytes.}
myocytes will be the subject of future investigations. The Mutant α1-S288S AR and α1a-S288A S290A AR Subtypes May Mediate Different Physiological Responses in the Heart—The demonstration that the α1a-AR and α1b-AR subtypes couple to different signaling pathways may explain the wide variety of contractile and cell growth processes that are altered upon addition of α1 agonists to cardiac myocytes. Activation of both the phospholipase C and MAPK signaling pathways has been demonstrated upon addition of α1 agonists to cardiac myocytes (1, 7, 27, 30). Interestingly, transgenic hearts overexpressing the wild type Gα1 subunit showed stimulation of the phosphatidylinositol hydrolysis pathway but no activation of the MAPK pathway (73). Stimulation of the phosphatidylinositol hydrolysis pathway was associated with severe contractile defects as well as an increased cell size with enhanced expression of ANF, β-myosin heavy chain, and α-skeletal actin. In addition, the expression of the constitutively activated Gα1 subunit in the heart caused cardiac hypertrophy, which was followed by apoptosis of myocytes through an increase in p38 and JNK activities (76).

The results of the present study would predict that myocytes overexpressing the constitutively activated α1a-S290A-AR subtype through its activation of the PI hydrolysis pathway would produce similar physiological effects. This is the topic of ongoing investigations. By contrast, myocytes overexpressing the constitutively activated α1b-S288–294-AR subtype through its activation of a different effector signaling pathway should produce a different repertoire of physiological effects. Previous studies on this point are controversial. On the one hand, Milano and colleagues (77) reported that transgenic hearts overexpressing the constitutively activated α1b-S288–294-AR subtype showed a modest degree of cell hypertrophy, although stimulation of the phospholipase C and MAPK signaling pathways was not examined. Investigators (78) have demonstrated that the overexpression of α1b-adrenergic receptors in the heart induced left ventricular dysfunction but not activation of β-myosin heavy chain and/or myosin light chain-2v gene expression. On the other hand, Akhtar and colleagues (12) showed that transgenic hearts overexpressing the wild type α1b-AR subtype did not develop hypertrophy despite an activation of ANF gene expression. Although the MAPK signaling pathway was not examined, these investigators reported an elevated diacylglycerol content that was attributed to stimulation of the PI hydrolysis pathway. However, in view of the results of the present study, additional analysis of these transgenic hearts will be needed to determine whether the elevated diacylglycerol content was in fact due to stimulation of a phopholipase C pathway or perhaps due to activation of a phospholipase D pathway. Finally, the results of the present study showing the constitutively activated α1a-S290T/293-AR and α1b-S288–294-AR subtypes couple to different signaling pathways raise the possibility that activation of both receptor subtypes may be necessary to reproduce all the features of the α1-AR-induced hypertrophic phenotype. Future experiments employing constitutively activated α1a-S290T/293-AR and α1b-S288–294-AR subtypes should provide a definitive assessment of the roles of these receptor subtypes in mediating α1-AR-induced cardiac hypertrophy.

REFERENCES
1. Post, G. R., and Brown, J. H. (1996) FASEB J. 10, 741–749
2. Schwinn, D. A., Lomasney, J. W., Lorenz, W., Saklat, P. J., Fremeau, R. T., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J., and Cotechie, S. (1996) J. Biol. Chem. 271, 8183–8189
3. Cotechie, S., Schwinn, D. A., Randall, R. L., Lefkowitz, R. J., and Kohlka, B. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7159–7163
4. Perez, D. M., DeYoung, M. B., and Graham, R. M. (1996) Mol. Pharmacol. 49, 843–851
5. Haymond, M. W., et al. (1996) Circ. Res. 78, 77–80
6. Michel, M. C., and Insel, P. A. (1994) Nature 371, 667–670
7. Michel, M. C., and Insel, P. A. (1994) Mol. Pharmacol. 45, 530–537
8. Michel, M. C., and Insel, P. A. (1994) Mol. Pharmacol. 46, 7159–7163
9. Cotecchia, S., Schwinn, D. A., Randall, R. L., Lefkowitz, R. J., and Kohlka, B. K. (1996) Mol. Pharmacol. 49, 843–851
10. Perez, D. M., DeYoung, M. B., and Graham, R. M. (1996) Mol. Pharmacol. 49, 784–795
11. Schwinn, D. A., Page, S. O., Mullen, J. P., Lorenz, W., Liggett, S. B., Yamamoto, K., Lapetina, E. G., Caron, M. G., Lefkowitz, R. J., and Cotechie, S. (1999) Mol. Pharmacol. 46, 618–624
12. Stewart, A. F. R., et al. (1996) Circ. Res. 79, 796–802
Differential Coupling of Active $\alpha_1$-AR Subtypes

55. Horban, A., Kolbeck-Ruhmkorff, C., and Zimmer, H.-G. (1997) J. Mol. Cell. Cardiol. 29, 2903–2914
56. Karns, L. R., Kariya, K., and Simpson, P. C. (1995) J. Biol. Chem. 270, 410–417
57. Kariya, K., Karns, L. R., and Simpson, P. C. (1994) J. Biol. Chem. 269, 3775–3782
58. Long, C. S., Or Dahl, C. P., and Simpson, P. C. (1989) J. Clin. Invest. 83, 1076–1082
59. Moalic, J. M., Bauters, C., Himbert, D., Bercovici, J., Mouas, C., Guicheney, P., Baudoin-Legros, M., Rappaport, L., Mezger, V., and Swynghedauw, B. (1989) J. Hypertens. 7, 195–201
60. Parker, T. G., and Schneider, M. D. (1991) Annu. Rev. Physiol. 53, 179–200
61. Schunkert, H., Jahn, L., Izumo, S., Apstein, C., and Lorell, B. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11480–11484
62. Thuerauf, D. J., Arnold, N. D., Zechner, D., Hanford, D. S., DeMartin, K. M., McDonough, P. M., Prywes, R., and Glembotski, C. C. (1998) J. Biol. Chem. 273, 20636–20643
63. Hu, Z. W., Shi, X. Y., Lin, R. Z., and Hoffman, B. B. (1999) Mol. Endocrinol. 13, 3–4
64. Schwinn, D. A., Johnston, G. I., Page, S. O., Mosley, M. J., Wilson, K. H., Worman, N. P., Campbell, S., Fidock, M. D., Furness, L. M., Parry-Smith, D. J., Peter, B., and Bailey, D. S. (1995) J. Pharm. Exp. Ther. 272, 134–142
65. Hansen, C. A., Schroerling, A. G., and Robishaw, J. D. (1995) J. Mol. Cell. Cardiol. 27, 471–484
66. Smrcka, A. V., and Sternweis, P. C. (1993) J. Biol. Chem. 268, 9667–9674
67. Bogojevitich, M. A., Clerk, A., and Sugden, P. H. (1995) Biochem. J. 309, 437–443
68. Thorburn, J., McMahon, M., and Thorburn, A. (1994) J. Biol. Chem. 269, 30580–30586
69. Downward, J., Graves, J. D., Warne P. H., Rayter, S., and Cantrell, D. A. (1990) Nature 346, 719–723
70. Nakafuku, M., Satoh, T., and Kaziro, Y. (1992) J. Biol. Chem. 267, 19448–19454
71. Kolch, W., Heledecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marne, D., and Rapp, U. (1995) J. Biol. Chem. 270, 410–417
72. Harrington, E. O., and Ware, J. A. (1995) Trends Cardiovasc. Med. 5, 153–159
73. D'Angelo, D. S., Sakata, Y., Lorenz, J. N., Boivin, G. P., Walsh, R. A., Liggett, S. B., and Dorn, G. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8121–8126
74. Koch, W. J., Hawes, B. E., Allen, L. F., and Leffkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12706–12710
75. Van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Portiri, E., Sakaue, M., Luttrell, L. M., and Leffkowitz, R. J. (1995) Nature 376, 781–784
76. Adams, J. W., Sakata, Y., Davis, M. G., Sah, V. P., Wang, Y., Liggett, S. B., Chien, K. R., Brown, J. H., and Dorn, G. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10140–10145
77. Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F., and Leffkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10109–10113
78. Grupp, I. L., Lorenz, J. N., Walsh, R. A., Boivin, G. P., Rindt, H. (1998) Am. J. Physiol. 275, H1338–H1350