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

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Activation of \( \alpha \)-adrenergic receptors influences both the contractile activity and the growth potential of cardiac myocytes. However, the signaling pathways linking activation of specific \( \alpha \)-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 \( \alpha_{1a} \)- and \( \alpha_{1b} \)-AR subtypes in cardiac myocytes. For this purpose, a mutant \( \alpha_{1a} \)-AR subtype \((\alpha_{1a}S^{290\text{g}}/293\text{g})\)-AR) was constructed based on analogy to the previously described constitutively active \( \alpha_{1d} \)-AR subtype \((\alpha_{1d}S^{288\text{g}}-294\text{g}) \). The mutant \( \alpha_{1a}S^{290\text{g}}/293\text{g}-AR \) subtype displayed constitutive activity based on four criteria. To introduce the constitutively active \( \alpha \)-AR subtypes into cardiac myocytes, recombinant Sindbis viruses encoding either the \( \alpha_{1a}S^{290\text{g}}/293\text{g} \)-AR or \( \alpha_{1b}S^{288\text{g}}-294\text{g} \)-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 \( \alpha_{1a}S^{290\text{g}}/293\text{g} \)-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 \( \alpha_{1b}S^{288\text{g}}-294\text{g} \)-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 \( \alpha_{1a} \)-AR and the \( \alpha_{1b} \)-AR subtypes to different signaling pathways in cardiac myocytes.

Activation of \( \alpha \)-adrenergic receptors (AR) influences both...
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 \(\alpha_{1A}\) and \(\alpha_{1D}\) subtypes in cardiac myocytes. To this end, we constructed a constitutively active mutant of the \(\alpha_{1D}\) subtype by analogy to a previously described constitutively active mutant of the \(\alpha_{1C}\) 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 \(\alpha_{1A}\) 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-[\(^{3}H\)]inositol (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 \(\alpha_{1A}\) cDNA**—The \(\alpha_{1A}\)-specific cDNA was constructed by site-directed mutagenesis of the bovine \(\alpha_{1A}\)-AR cDNA, which was generously provided by Dr. Jan Lomasney, Northwestern University Medical School. The oligonucleotides 5'-GCCGAAGCATATAACGCCCTAAACCGCTG-3' and 5'-CTTTGTAGCCTTTAGATGCGCCAAG-3' were used to generate the appropriate mutations at \(\text{Lys}^{299}\)→His and Ala\(^{293}\)→Leu. The mutant \(\alpha_{1A}\)-S\(^{299}\)AR cDNA was verified by DNA sequencing.

For expression studies in COS-m6 cells, the cDNA encoding the mutant \(\alpha_{1A}\)-S\(^{299}\)AR subclone was subcloned into the BamHI site of the pSinRep vector. The DH-BB-3′-deoxyuridine, 50 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin and plated at a density of \(5 \times 10^5/18\)-mm well, 1 \(\times 10^5/35\)-mm well, and 2 \(\times 10^5/60\)-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 feasibility of a recombinant Sindbis virus to infect greater than 90% of the cardiac myocytes, as measured by positive \(\beta\)-galactosidase staining.3 Therefore, in the present study, recombinant Sindbis viruses encoding the mutant \(\alpha_{1A}\)-S\(^{299}\)AR or mutant \(\alpha_{1A}\)-S\(^{299}\)AR subtypes were used to infect cardiac myocytes. For construction of recombinant Sindbis viruses, the pSinRep vector containing the cDNA for either the mutant \(\alpha_{1A}\)-S\(^{299}\)AR or mutant \(\alpha_{1A}\)-S\(^{299}\)AR was linearized with XhoI. The cDNA corresponding to the \(\text{LacZ}\) gene was ligated to the XhoI site of the linearized pSinRep vector. The capped RNA transcripts were generated by in vitro transcription of the pSinRep cDNAs as well as the DH-BB helper virus RNA, 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 \(\alpha_{1A}\)-S\(^{299}\)AR- or mutant \(\alpha_{1A}\)-S\(^{299}\)AR. The appropriate dilutions of recombinant Sindbis virus were determined empirically to yield comparable levels of mutant \(\alpha_{1A}\)-S\(^{299}\)AR or mutant \(\alpha_{1A}\)-S\(^{299}\)AR expression. After the initial 1-h infection period, the medium was supplemented with the \(\alpha_{1A}\)-AR antagonist WB-4101, to a final concentration of 1 \(\mu\)M. The inclusion of WB-4101 in the medium was found to increase significantly the number of mutant receptors expressed on the cell surface. At 48 h post-infection, the cell lysates were centrifuged at 50,000 \(\times \) rpm to pellet the membrane fractions. The membrane pellets were resuspended in 100 \(\mu\)l of 50 mM Tris-HCl, pH 7.4, and 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 \([3H]\)prazosin in the presence or absence of varying concentrations of competing ligands. In the saturation binding experiments, membranes were incubated with 0.2–15 nM \([3H]\)prazosin in the presence or absence of 10 \(\mu\)M phentolamine (Sigma) 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.

In the whole cell receptor assay, 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 \(\times \) rpm. The cell pellets were resuspended in assay buffer to give approximately 2.5 \(\times\) \(10^6\) cells/ml. The binding of the \(\alpha_{1A}\)-AR antagonist, \([3H]\)prazosin, to the resuspended cells was performed as described above in a final volume of 500 \(\mu\)l containing approximately 1 \(\times 10^5\) cells. An aliquot of cells was counted by trypsin blue staining to ensure the intactness of the cells.

**Differential Coupling of Active \(\alpha_{1A}\)-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 Celstrir 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 \(\mu\)M 5-bromo-2'-deoxyuridine, 50 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin and plated at a density of \(5 \times 10^5/18\)-mm well, 1 \(\times 10^5/35\)-mm well, and 2 \(\times 10^5/60\)-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.

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asayed for protein concentration, and used for measurement of receptor expression. Briefly, 0.01 mg of membranes were incubated with a saturating concentration of [3H]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 the addition of 1 ml of MAPK extraction buffer, filtration of the mixture over Whatman GF/C filters. The tubes and filters were washed 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 Phosphorylating Activity**—In the studies employing COS-m6 cells, cells growing on 18-mm wells were washed three times with 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% sodium dodecyl sulfate (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 neonatal rat cardiac myocytes, 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 [3H]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 5 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% sodium deoxycholate, 1 mM vanadate, 10 μM DTT, 10 μM EDTA, 1 mM 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 active form of MAPK (anti-phospho-MAPK, New England Biolabs). MAPK activation was determined by probing their subtype-specific coupling to signaling pathways, a constitutively active mutant of the α1-AR needed to be constructed. A constitutively active mutant α1-AR subtype has already been constructed and characterized by Dr. Cotecchia (9). Accordingly, the α1-AR subtype was mutated at Lys290 and Ala295 (α1-S290/295-AR) to mirror the substitutions previously found to result in maximal constitutive activation of the mutant α1-AR subtype (9). Properties that have been shown to be characteristic of constitutive activation include the following: 1) an increased affinity for 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 α1-S290/295-AR subtype exhibited any or all of these properties, the mutant α1-S290/295-AR subtype was expressed and then characterized in COS-m6 cells. Since COS-m6 cells do not express wild type α1-ARs, these cells provide a convenient background in which to study the properties of the mutant α1-S290/295-AR subtype.

**Increased Affinity for Agonist**—COS-m6 cells transfected with either the wild type α1A-AR or the mutant α1-S290/295-AR were characterized in terms of their affinity for various agonists and antagonists by monitoring the displacement of [3H]prazosin through agonist or antagonist competition binding assays. Phenylephrine and norepinephrine, both full agonists and antagonists by monitoring the displacement of [3H]prazosin from membranes expressing the α1A-S290/295 mutant with approximately 40-fold higher affinities than those expressing the wild type receptor (Fig. 1; Table 1). The partial imidazoline agonist, oxymetazoline (15), also demonstrated a higher affinity for the α1-S290/295-AR (Fig. 1), although the difference in affinity, in this case, was only 6-fold (Table 1). The antagonist, 5-methylurapidil, demonstrated similar affinity for both the wild type and mutant receptors (Fig. 1; Table 1). The agonist bound at 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 α1-S290/295-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 α1-S290/295-AR possesses properties reminiscent of constitutive activation.
FIG. 1. Competition binding relationships of agonists and antagonists to the wild type α₅-AR and α₁₅-S²⁹⁰/²⁹₃ mutant receptors expressed in COS-m6 cells. The ability of α₅-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 α₁₅-AR or α₁₅-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 Kᵣ values are reported in Table I. Mean receptor expression levels were 1400 (α₁₅-AR) and 300 (α₁₅-S²⁹⁰/²⁹₃) fmol/mg membrane protein obtained using 0.2 and 2.6 μg/ml of cDNA/transfection, respectively.

Values are Kᵣ (nM) calculated from the data shown in Fig. 1. The ratio between wild type α₁₅-AR and α₁₅-S²⁹⁰/²⁹₃, Kᵣ values are shown in parentheses. Each value represents the mean ± S.E. of at least three individual experiments performed in duplicate.

Table I

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Wild type α₁₅-AR</th>
<th>α₁₅-S²⁹⁰/²⁹₃ Mutant</th>
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<tbody>
<tr>
<td>Norepinephrine</td>
<td>8099 ± 1328</td>
<td>187 ± 25³ (43)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>17936 ± 1895</td>
<td>453 ± 13⁴ (40)</td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>111 ± 12</td>
<td>17 ± 1* (6)</td>
</tr>
<tr>
<td>5-Methylurapidil</td>
<td>2 ± 0.2</td>
<td>3 ± 0.3 (0.7)</td>
</tr>
</tbody>
</table>

*p < 0.01.

† p < 0.001 compared with wild type α₁₅-AR.

Enhanced Basal and Agonist-stimulated Phosphatidylinositol Hydrolysis—Previously, it was shown that all α₁₅-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 α₁₅-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 α₁₅-ARs. Examples of Bmax 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 α₁₅-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 α₁₅-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 α₁₅-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 α₁₅-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 α₁₅-S²⁹⁰/²⁹₃-AR was expressed at a lower level than the wild type α₁₅-AR, these data likely underestimate the greater constitutive activity of the mutant α₁₅-S²⁹⁰/²⁹₃-AR.

Also, as shown in Fig. 2, COS-m6 cells expressing the mutant α₁₅-S²⁹⁰/²⁹₃-AR displayed a higher agonist-stimulated level of inositol phosphate accumulation compared with cells expressing the wild type α₁₅-AR. In fact, the wild type α₁₅-AR had to be expressed at a 10-fold higher level than the mutant α₁₅-S²⁹⁰/²⁹₃-AR before their maximal responses were comparable. These data are consistent with previous studies of wild type and mutant β₁-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
Cells—By showing that the mutant $\alpha_{1a}$S290/293-AR possesses all of the characteristics of a constitutively active receptor, the second aim of this study was to compare the signaling properties of the constitutively active mutant $\alpha_{1a}$S290/293-AR subtype with that of the analogous constitutively active mutant $\alpha_{1b}$S290/293-AR subtype developed by Dr. Coteccia (9), since it is their ability to activate signaling pathways in the absence of agonist that makes them useful tools for linking a specific receptor subtype to a particular signaling pathway. Activation of both the phospholipase C and MAPK signaling pathways has been demonstrated upon addition of $\alpha_1$-AR agonists to cardiac myocytes (1, 7, 27, 30). Fig. 5 shows a comparison of basal and agonist-stimulated phosphatidylinositol hydrolysis in COS-m6 cells expressing either the mutant $\alpha_{1a}$S290/293-AR or the $\alpha_{1b}$S290/293-AR subtype. As can be seen, the mutant $\alpha_{1a}$S290/293-AR subtype displayed an elevated basal activity and a similar increase in agonist-stimulated phosphatidylinositol hydrolysis compared with the mutant $\alpha_{1b}$S298/294-AR subtype. In fact, the mutant $\alpha_{1b}$S298/294-AR subtype displayed an elevated basal activity and a higher agonist-stimulated phosphatidylinositol hydrolysis activity compared with the mutant $\alpha_{1b}$S298/294-AR subtype. At a five times greater receptor density in COS-m6 cells, the mutant $\alpha_{1b}$S298/294-AR subtype had to be expressed to observe similar levels of basal and agonist-stimulated phosphatidylinositol hydrolysis activity as the mutant $\alpha_{1a}$S290/293-AR subtype, suggesting that the mutant $\alpha_{1b}$S298/294-AR subtype had a lower potency in activating the phosphatidylinositol hydrolysis signaling pathway. These data indicate that, even though they are closely related structurally, the mutant $\alpha_{1a}$S290/293-AR and $\alpha_{1b}$S298/294-AR subtypes differ functionally in terms of their relative abilities to stimulate the phosphatidylinositol hydrolysis signaling pathway. Although interesting, meaningful interpretation of these results is difficult since COS-m6 cells do not normally express the $\alpha_{1a}$- and $\alpha_{1b}$-ARs and, thus, are unlikely to possess the physiologically appropriate G proteins and downstream signaling components. To circumvent this problem, we decided to study the signaling properties of the mutant $\alpha_{1a}$S290/293-AR and $\alpha_{1b}$S298/294-AR subtypes in another type of cells in which these receptor subtypes are normally expressed. For this purpose, we selected neonatal...
Comparison of the Signaling Properties of the Constitutively Active α1a-S290/293-AR and α1b-S288–294-AR Subtypes in Rat Cardiac Myocytes—In a previous study, we showed that both the α1a- and α1b-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 α1 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), which signaling pathways were activated in cardiac myocytes expressing either the constitutively active α1a-S290/293-AR and α1b-S288–294-AR subtypes. To control for endogenous and mutant receptor expression levels, both the endogenous and mutant receptor subtypes were measured simultaneously using a whole cell binding assay. Receptor expression levels were obtained using 2.6 μg/ml cDNA transfection for the α1a-S290/293-AR and 0.008 and 0.2 μg/ml cDNA transfection for the α1b-S288–294-AR subtypes. Each value represents the mean ± S.E. of at least four individual experiments performed in duplicate. Data shown have had subtracted the total inositol phosphate accumulation measured in cells transfected in the presence of vector alone (28,854 ± 6,160 dpm/mg protein; n = 4). ##, p < 0.01 compared with corresponding α1a-S290/293-AR value.

Whereas standard gene transfer methods were effective for introducing luciferase reporter plasmids into cardiac myocytes, they were ineffective for expression of the constitutively active α1-AR subtypes in myocytes. However, we recently demonstrated the first successful use of recombinant Sindbis viruses to express G protein β 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 α1a-S290/293-AR and α1b-S288–294-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 α1a-S290/293-AR and α1b-S288–294-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 α1-ARs (Bmax = 270 and 178 ± 16 fmol/mg protein for control and LacZ virus-infected cells, respectively). By contrast, cardiac myocytes infected with either the α1a-S290/293-AR Sindbis virus or the α1b-S288–294-AR Sindbis virus expressed approximately 10-fold higher receptor densities than myocytes infected with the LacZ Sindbis virus (Bmax = 2120 ± 219 and 1518 ± 350 fmol/mg protein for α1a-S290/293-AR virus-infected and α1b-S288–294-AR virus-infected cells, respectively).

By having confirmed receptor expression, we examined which signaling pathways were activated in cardiac myocytes expressing either the constitutively active α1a-S290/293-AR subtype or the α1b-S288–294-AR subtype. Fig. 7 shows a comparison of basal and phenylephrine-stimulated phosphatidylinositol hydrolysis in cardiac myocytes expressing similar levels of constitutively activated α1a-S290/293-AR and α1b-S288–294-AR subtypes. Under the basal condition (30 min with 10 mM LiCl in the absence of agonist), cells expressing the constitutively activated α1a-S290/293-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 α1b-S288–294-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 α1a-S290/293-AR subtype showed a further 2-fold increase in phenylephrine-stimulated inositol phosphate accumulation compared with the LacZ cells, whereas the phenylephrine-stimulated activity in the cells expressing the constitutively activated α1b-S288–294-AR subtype was indistinguishable from that observed in the LacZ cells (Fig. 7B). These data clearly demonstrate that the constitutively activated α1a-S290/293-AR
Data shown have had subtracted the total inositol phosphate accumulation in un-stimulated (basal) LacZ-transfected cells incubated in the and total inositol phosphates measured as described under "Experimental Procedures." Each value represents mean

Next, we examined the MAPK signal-

Comparison of the MAPK Signaling Pathways of the Constitutively Active $\alpha_{1a}$-S<sup>290/293</sup>-AR and $\alpha_{1b}$-S<sup>288/294</sup>-AR Subtypes in Rat Cardiac Myocytes—Next, we examined the MAPK signaling pathway. Fig. 8 shows a comparison of basal and phe-

Comparison of Gene Activation by the Signaling Pathways of the Constitutively Active $\alpha_{1a}$-S<sup>290/1293</sup> and $\alpha_{1b}$-S<sup>288/294</sup>-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 cardiac gene transcription. Both of these genes are activated during norepinephrine-induced cardiac hypertrophy in the heart, and as such represent important targets for the $\alpha_{1A}$-AR and $\alpha_{1B}$-AR subtype signaling pathways. The ANF gene is activated by the $\alpha_{1}$-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 $\alpha_{1A}$-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 $\alpha_{1}$-ARs (36, 38–43). The c-fos gene is up-regulated during cardiac hypertrophy of myocytes (44–45). Therefore, we investigated the effect of the constitutively activated $\alpha_{1a}$-S<sup>290/293</sup>-AR and $\alpha_{1b}$-S<sup>288/294</sup>-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<sup>290/293</sup>-AR and $\alpha_{1b}$-S<sup>288/294</sup>-AR subtypes. Fig. 9 shows a comparison of luciferase gene activity in transfected cardiac myocytes expressing similar levels of the activated $\alpha_{1a}$-S<sup>290/293</sup>-AR and $\alpha_{1b}$-S<sup>288/294</sup>-AR subtypes. In the absence of agonist, cells expressing the constitutively activated $\alpha_{1a}$-S<sup>290/293</sup>-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<sup>290/293</sup>-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<sup>288/294</sup>-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<sup>290/293</sup>-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<sup>290/293</sup>-AR subtype preferentially activates ANF-luciferase activity compared with SRE/c-fos-
the effect of phenylephrine on the level of active MAPK in LacZ, as described under “Experimental Procedures.”

S290/293-AR and S288–294-AR subtype stimulation of ANF-luciferase activity is consistent with the ability of the S288–294-AR subtype to activate PI hydrolysis (Fig. 7). Third, the a1a-S290/293-AR mutant and/or in the a1b-S288–294-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 probed 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 α1-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 α1b-S288–294-AR subtype by making the analogous mutations in the COOH-terminal end of the third cytoplasmic loop that produced the previously described constitutively active α1b-S288–294-AR subtype by Dr. Cotecchia (9). We did not characterize the properties of the constitutively active α1b-S288–294-AR subtype as it has already been reported (9). Characterization of the functional properties of this mutant α1b-S288–294-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. These data confirm and extend previous pharmacological results that reached a similar conclusion (7).
Future studies will be necessary to elucidate the mechanism by which various antagonists can increase receptor density.

The Mutant $\alpha_{1A}$-$S^{290/293}$-AR and $\alpha_{1B}$-$S^{288/294}$-AR Subtypes Coupled to Different Signaling Pathways and Gene Expression in Cardiac Myocytes—With the development of constitutively activated $\alpha_{1A}$-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 $\alpha_{1A}$-AR subtypes. Rat cardiac myocytes express comparable levels of the endogenous $\alpha_{1A}$-AR and $\alpha_{1B}$-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 $\alpha_{1A}$-AR stimulus-response pathways. Thus, following the introduction of either the constitutively activated $\alpha_{1A}$-$S^{290/293}$-AR or $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1A}$-$S^{290/293}$-AR and $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1A}$-$S^{290/293}$-AR or $\alpha_{1B}$-$S^{288/294}$-AR subtype confirms our recent pharmacological results (7) but provides definitive molecular evidence that the $\alpha_{1A}$-$S^{290/293}$-AR subtype is preferentially coupled to the phosphatidylinositol (PI) hydrolysis signaling pathway and ANF-luciferase gene expression. The ANF gene is activated by the $\alpha_{1A}$-AR receptor agonist, phenylephrine, through multiple promoter elements (AP-1, SP1, AT, 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 $\alpha_{1A}$-$S^{290/293}$-AR subtype couples to ANF gene expression is consistent with previous reports, which suggested that the $\alpha_{1A}$-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-K-NF signaling pathway (46).

A novel finding of this study is that the $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1A}$-$S^{290/293}$-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 $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1A}$-AR subtype agonist and antagonists are not sufficiently selective.

These data, as well as previously published pharmacological data (7), indicate that the $\alpha_{1B}$-$S^{288/294}$-AR subtype couples to the MAPK/SRE/c-fos signaling pathway in rat cardiac myocytes. In Fig. 8, we observed the $\alpha_{1B}$-$S^{288/294}$-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 $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_{1A}$- and $\alpha_{1B}$-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 $\alpha_{1A}$-AR subtype with a different heterotrimeric G protein to produce distinct bifurcating signals in the form of Gs 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 $\alpha_{1A}$-AR subtype couples through 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 $\alpha$ or $\beta$ subunits of the Gq11 Protein family in vitro (66). Whether the $\alpha$ or the $\beta$ 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 $\alpha_{1B}$-AR subtype associates with a member of the Gq11 or G12-13 Protein family rather than the Gs protein family. However, other than the activation of phospholipase C-β, the downstream components regulated by the $\alpha$ or $\beta$ 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 $\alpha_{1A}$-$S^{288/294}$-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 Gα subunit exhibited marked stimulation of the PI hydrolysis pathway but no activation of the MAPK pathway also argues against this mechanism (73). Alternatively, the $\beta$γ subunits rather than the $\alpha$ subunits of Gq11 or G12-13 could be involved in stimulation of the MAPK pathway, since it has been shown that the $\beta$γ subunits released from Gs can activate a Ras-dependent pathway leading to stimulation of Raf-1 kinase (74–76). Whether the $\beta$γ subunits released from Gq11 or G12-13 can similarly activate a Ras-dependent pathway in cardiac myocytes is in contrast with the previous report of Deng et al. (40). They demonstrated, using pharmacological methods, that the $\alpha_{1A}$-$S^{290/293}$-AR subtype was responsible for c-fos gene activation in cardiac myocytes (40).
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The Mutant α1-S290/293-AR and α1b-S288/294-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 Gαs 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 Gαs 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-S290/293-AR subtype through its activation of the PI hydrolysis pathway should 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, Miyano and colleagues (77) reported that transgenic hearts overexpressing the constitutively activated Gαs subunit in the heart caused cardiac hypertrophy, which was followed by apoptosis of myocytes through an increase in p38 and JNK activities (76).

The demonstration that the α1a-S290/293-AR and α1b-S288/294-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 Gαs 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 Gαs subunit in the heart caused cardiac hypertrophy, which was followed by apoptosis of myocytes through an increase in p38 and JNK activities (76).

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Differential Coupling of Active α₁-AR Subtypes

Constitutively Active Mutants of the α1a- and the α1b-Adrenergic Receptor Subtypes Reveal Coupling to Different Signaling Pathways and Physiological Responses in Rat Cardiac Myocytes

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