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Differential coupling of α_1-adrenoreceptor subtypes to phospholipase C and mitogen activated protein kinase in neonatal rat cardiac myocytes

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Abstract

Activation of cardiac α_1-adrenoreceptors has a number of physiological effects. Ascribing these effects to a specific α_1-adrenoreceptor subtype first requires the elucidation of the subtypes that are present in the tissue of interest. In the present study, mRNA transcripts for the α_{1A}, α_{1B} and α_{1D}-adrenoreceptor subtypes were detected in cultured neonatal rat cardiac myocytes, using reverse transcriptase–polymerase chain reaction analysis. However, binding sites for only the α_{1A} and α_{1B}-adrenoreceptor subtypes were detected in cultured neonatal rat cardiac myocytes, using competition binding analysis with a variety of α_1 selective receptor antagonists. Phenylephrine-stimulated phosphatidylinositol hydrolysis was inhibited by α_1 selective receptor antagonists with affinities consistent with the α_{1A}-adrenoreceptor subtype, whereas phenylephrine-induced activation of the mitogen activated protein kinase cascade was inhibited by these same antagonists with affinities more closely resembling the α_{1B}-adrenoreceptor subtype. In the case of both signaling pathways, the α_{1D} selective receptor antagonist, BMY 7378, exhibited affinities suggestive of the relative absence of a α_{1D}-adrenoreceptor subtype. Thus, despite the presence of mRNA transcripts for all three α_1-adrenoreceptor subtypes, only the α_{1A} and α_{1B}-adrenoreceptor subtypes were expressed and functionally coupled at detectable levels in neonatal rat cardiac myocytes. Of particular interest, phenylephrine-induced activation of the mitogen activated protein kinase cascade appears to be mediated by a subtype resembling most closely the pharmacological profile of the α_{1B}-adrenoreceptor subtype. © 1997 Elsevier Science B.V.

Keywords: α_1-Adrenoreceptor subtype; Cardiac myocyte, rat, neonatal; Phosphatidylinositol hydrolysis; Mitogen activated protein kinase

1. Introduction

In the heart, activation of α_1-adrenoreceptors has a number of physiological effects. These include rapid regulation of contractile activity through changes in inotropy and chronotropy and long term maintenance of cardiac function through regulation of gene expression and cell growth (Morgan and Baker, 1991; Terzic et al., 1993). Three α_1-adrenoreceptor subtypes [1] have recently been identified by molecular cloning techniques: the α_{1a} (Schwinn et al., 1990; Stewart et al., 1994), the α_{1b} (Cotecchia et al., 1988; Voigt et al., 1990) and the α_{1d} (Lomasney et al., 1991; Perez et al., 1991)-adrenoreceptor subtypes. Based on the analysis of their pharmacological properties, the cloned α_{1a} and α_{1b}-adrenoreceptor subtypes are thought to represent the pharmacologically-identified α_{1A} and α_{1B}-adrenoreceptor subtypes, respectively, whereas the cloned α_{1d}-adrenoreceptor subtype appears to represent a novel subtype (Laz et al., 1994; Michel and Insel, 1994a). With the recognition that multiple α_1-adrenoreceptor subtypes exist, the identification of the specific α_1-adrenoreceptor subtype responsible for the various physiological effects in the heart are not known (Terzic et al., 1993). At the RNA level, all three subtypes appear to be present in the heart (Stewart et al., 1994; Rokosh et al., 1996). At the protein level, both the α_{1A} and α_{1B}-adrenoreceptor subtypes have been reported to be present in cardiac tissue, using selective receptor antagonists (Hanft and Gross, 1989; Lazou et al., 1994; Michel et al., 1994b). However, evidence for the existence of a

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[1] Nomenclature used for the α_1-adrenoreceptors is that recommended by the International Union of Pharmacology (Hieble et al., 1995) where native α_1-adrenoreceptor subtypes are denoted by uppercase subscripts (e.g. α_{1A}) and cloned subtypes denoted by lower case subscripts (e.g. α_{1a}). Also, in accordance with these recommendations, the recombinant α_{1c}-subtype is renamed α_{1a} and is believed to represent the pharmacological α_{1A}-subtype.

α_{1D}-adrenoreceptor binding site in cardiac tissue has been hindered by the lack of a receptor antagonist selective for this subtype. Recently, the 5-HT_{1A} partial receptor agonist, BMY 7378 (8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-ethyl)-8azaspiro(4,5)decane-7,9-dione dihydrochloride), has been found to exhibit a 100-fold greater selectivity for the cloned α_{1d}-adrenoreceptor subtype over the other cloned α_1-adrenoreceptor subtypes (Goetz et al., 1995).

In the present study, the α_1-adrenoreceptor subtypes present in neonatal rat cardiac myocytes have been identified in terms of their coupling to the phosphatidylinositol hydrolysis and the mitogen-activated protein kinase cascades, two pathways demonstrated to be activated following the stimulation of cardiac α_1-adrenoreceptors (Sugden and Bogoyevitch, 1995). While both the α_{1A} and α_{1B}-adrenoreceptor subtypes were identified in rat neonatal cardiac myocyte, the α_{1D}-adrenoreceptor subtype either was not present or present in insufficient amount to be detected with the α_{1d} selective receptor antagonist, BMY-7378. Both the α_{1A} and α_{1B}-adrenoreceptor subtypes were functionally coupled in neonatal cardiac myocytes, with the α_{1A}-adrenoreceptor subtype appearing to preferentially couple to stimulation of phosphatidylinositol hydrolysis and a receptor whose pharmacology most closely resembled that of the α_{1B} adrenoreceptor subtype appearing to preferentially couple to activation of the mitogen activated protein kinase cascade.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP, [^{32}P]dCTP and [^{125}I]HEAT (2,[β-(4-hydroxyl-3-[^{125}I]iodophenylaminomethyl]tetralone) were obtained from DuPont-New England Nuclear; *myo*-[^{3}H]inositol from Amersham; CEC (chlorethylclonidine), BMY 7378 (8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-ethyl)-8-azaspiro(4,5)decane-7,9-dione dihydrochloride), prazosin, 5-methyl-urapidil, (+)-niguldipine and WB-4101 ((2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride) from Research Biochemicals International; 5′-guanylimidodiphosphate (Gpp[NH]p), phenylephrine, phentolamine and propranolol from Sigma and myelin basic protein from Gibco BRL.

2.2. Myocyte preparation and tissue culture

Hearts from 1 to 2 day-old Sprague–Dawley rats were obtained and the ventricles were digested with a mixture of trypsin, chymotrypsin and elastase in a Celstir apparatus at 37°C. To obtain myocytes, the dissociated cells were centrifuged through Percoll step gradients (Hansen et al., 1994) and then suspended in Modified Eagle's Medium (MEM) containing essential and non-essential amino acids, vitamins, 2 mM glutamine, 7.5% heat-inactivated newborn calf serum, 100 μM 5-bromo-2′-deoxyuridine, 50 U/ml penicillin and 50 μg/ml streptomycin. Myocytes were plated at a density of 8.0×10^4 cells/cm^2 on dishes pre-coated with 0.1% gelatin. Following an overnight incubation, the cells were washed with serum-free MEM and then incubated in a defined serum-substitute media as described previously (Hansen et al., 1994). The cells were then maintained for 3 days in culture before being processed as detailed below.

2.3. Reverse transcriptase–polymerase chain reaction

Analysis of mRNA transcripts for the α_1-adrenoreceptor subtypes in neonatal cardiac myocytes was performed by sequentially combining the techniques of reverse transcription and polymerase chain reaction (RT-PCR), as described by Dostal et al. (Dostal et al., 1994). Primers were designed to amplify products from extensively mismatched sequence regions of the rat α_{1a}, α_{1b} and α_{1d}-adrenoreceptor cDNAs. Primer pairs used were from bases 708–727 (5′-GACAAGTCAGACTCGGAGCA-3′) and 1384–1403 (5′-CGACCATTAAGATCCACACC-3′) of the rat α_{1a}-adrenoreceptor subtype (Stewart et al., 1994); from bases 323–342 (5′-CCAACCAGACCTCGAGCAAC-3′) and 815–834 (5′-ACAAGGAATGCGGAGTCACC-3′) of the rat α_{1b}-adrenoreceptor subtype (Voigt et al., 1990) and from bases 997–1017 (5′-GCTGCACTGCCTCCATC-CTTA-3′) and 1525–1545 (5′-GTGTCTTCGTC-CTGTGCTGGT-3′) of the rat α_{1d}-adrenoreceptor subtype (Lomasney et al., 1991). These primer pairs generated products of 696, 512 and 549 basepairs, respectively. In addition, primers derived from bases 635–654 and 982–963 of the rat elongation factor-1α (Dostal et al., 1994) were also synthesized to generate a 347 bp product for use as a standard to correct for differences in amplification efficiency.

Reverse transcription reactions were performed with 5 μg of total RNA from 4 day old cultures of rat neonatal cardiac myocytes in a buffer consisting of 10 mM Tris, pH 8.3, 4 mM $MgCl_2$, 75 mM KCL, 250 mM of each dNTP and 7.5 U/ml of reverse transcriptase. After incubation at 35°C for 1 h, reactions were terminated by heating at 98°C for 5 min. To ensure that there was no genomic DNA contamination, reactions were also run in the absence of reverse transcriptase. Competitive polymerase chain reactions were performed with 5 μl of reverse transcription mix and 35 μl of buffer containing 11 mM Tris, pH 8.3, 3.25 mM $MgCl_2$, 55 mM KCl, 150 μM of each dNTP, 150 pmol of each primer, 2.5 μCi [^{32}P]dCTP and 2.5 μl of AmpliTaq polymerase. The thermal cycle reactions were carried out as follows: denaturation at 96°C for 30 s, annealing at 64°C for 1 min and extension at 72°C for 1 min. Following three cycles of amplification, 10 μl of buffer containing 150 pmol of EF-1α primers were added to each tube. Reactions were then carried out for an additional 32 cycles, with a final extension at 72°C for 10

min. The resulting PCR products were separated by electrophoresis on 8% polyacrylamide gels and autoradiographs of the gels were quantitated using a Molecular Dynamics Phosphoimager.

2.4. Membrane preparation

Neonatal cardiac myocytes grown on 100 mm dishes were washed with ice-cold phosphate buffered saline and collected by scraping on ice in homogenization buffer consisting of 20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 1 mM AEBSF (4-(-2-aminoethyl-benzenesulfonylfluoride) and 10 μg/ml each of aprotinin, leupeptin and pepstatin A. The cells were homogenized by passing the cell suspension 12 times through a 25 gauge needle. The particulate fraction was collected by centrifugation at 285,000 $\times g$ for 30 min at 4°C, resuspended in incubation buffer consisting of 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1% bovine serum albumin at a protein concentration of approximately 1–3 mg/ml and stored at −70°C. Protein determinations were performed with a Coomassie Protein Assay Kit (Pierce), using bovine serum albumin as a standard.

2.5. [^{125}I]HEAT ligand binding studies

[^{125}I]HEAT binding incubations were carried out at room temperature for 45 min with 0.02 mg of membrane protein in a total volume of 90 μl of incubation buffer. Incubations were terminated by the addition of 1 ml ice-cold incubation buffer followed by rapid filtration over Whatman GF/C glass fiber filters that had been presoaked for 1 h in ice-cold incubation buffer. The tubes and filters were washed 3 times with 1 ml ice-cold incubation buffer and the filters were counted in a gamma counter at 70% efficiency. In competition studies, membranes were incubated with 200 pM [^{125}I]HEAT in the presence or absence of varying concentrations of α_1 selective receptor antagonists. When phenylephrine was used as a competing ligand, 100 μM Gpp[NH]p was included in the incubation. Non-specific binding was defined as binding in the presence of 1 μM prazosin, where non-specific binding typically represented 30% of the total binding at 200 pM [^{125}I]HEAT. In saturation experiments, membranes were incubated in the presence or absence of 1 μM prazosin and a range of 10–12 concentrations of [^{125}I]HEAT (10 to 4000 pM).

2.6. Measurement of phosphatidylinositol hydrolysis

Neonatal cardiac myocytes growing on 18 mm wells were labeled for 48 h with *myo*-[^{3}H]inositol (2 μCi/ml). After labeling, cells were pre-incubated with 1 μM propranolol and varying concentrations of α_1 selective receptor antagonists or vehicle for 50 min. After addition of 10 mM LiCl, cells were stimulated with 3 μM phenylephrine for 30 min and then stopped by addition of ice-cold 6% trichloroacetic acid. Following removal of precipitated proteins by centrifugation, the supernatants were extracted 3 times with 3 volumes of diethyl ether. The neutralized aqueous supernatants were applied to 1 ml columns of Dowex AG-1-X8, formate form. Total inositol phosphates were eluted using a standard procedure (Berridge et al., 1983) and counted by liquid scintillation spectrometry.

2.7. Measurement of mitogen activated protein kinase activated by 'in gel' phosphorylation of myelin basic protein

Neonatal rat cardiac myocytes grown on 18 mm wells were incubated with varying concentrations of α_1 selective receptor antagonists or vehicle for 60 min and then stimulated with 3 μM phenylephrine for 5 min. Reactions were stopped by two rapid washes with ice-cold phosphate buffered saline followed by addition of ice-cold extraction buffer (20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 1 mM EGTA, 0.2 mM Na-vanadate, 10 mM benzamidine, 1 mM AEBSF, 0.5 mM dithiothreitol and 1% Triton X-100). Cells were collected by scraping, transferred to Eppendorf tubes, and extracted by rotation for 30 min at 4°C. Cell extracts were centrifuged at 16,000 $\times g$ for 10 min at 4°C. The supernatant fractions were collected and protein concentrations were measured by an amido black assay (Schaffner and Weissmann, 1973). Mitogen activated protein kinase activity was monitored by 'in gel' phosphorylation of myelin basic protein, as described by Kameshita and Fujisawa (1989). Briefly, 10 μg of protein from the various supernatant fractions were resolved on 11% SDS-polyacrylamide gels containing 0.1 mg/ml myelin basic protein. After electrophoresis, gels were washed twice in buffer A (50 mM Tris/HCl, pH 8.0) containing 20% isopropanol for 20 min at room temperature; incubated for 1 h at room temperature in buffer A containing 5 mM 2-mercaptoethanol and then incubated twice for 30 min at room temperature in buffer A containing 6 M guanidine chloride. After renaturation of resolved proteins in buffer A containing 0.04% Tween 20 (5 washes over 16 h at 4°C), the mitogen activated protein kinase assays were performed, as described previously (Bogoyevitch et al., 1994), with the exception that the concentration of myelin basic protein was reduced to 0.1 mg/ml and protein kinase inhibitor was eliminated from these assays. Briefly, gels containing the resolved and renatured proteins were equilibrated for 1 h in buffer B (40 mM Na-HEPES, pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$, and 1 mM dithiothreitol). Kinase assays were initiated by changing to buffer B containing 0.5 mM EGTA, 0.1 mM ATP and 5 μCi/ml [γ-^{32}P]-ATP, and then incubating for 1 h at 30°C. The assays were stopped by transferring the gels into 9% trichloracetic acid containing 1 mM Na-pyrophosphate and then removing free radioactivity from the gels by six subsequent washes with 9% trichloracetic acid containing

1 mM Na-pyrophosphate at room temperature. Autoradiographs of dried gels were quantified with a Molecular Devices Phosphoimager, using ImageQuant software.

2.8. Data analysis

All experiments were performed in triplicate and all values represent the mean ± S.E.M. Curve fitting was performed using the non-linear, least-squares regression analysis program GraphPad Prizm (GraphPad Software). Competition binding data was analyzed using either single- or two-site models. Two-site data were only accepted if a statistically better fit was obtained compared to a single-site model as assessed by use of the *F*-test ($p < 0.05$). Estimates of ligand binding affinity (K_D) 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_i values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [A]/K_D)$$

where [A] is the concentration of ligand used and K_D was determined from binding studies. IC_{50} values from functional inhibition curves were converted to K_b values using a functional equivalent of the Cheng–Prusoff equation (Lazareno and Birdsall, 1993; Leff and Dougall, 1993):

$$K_b = IC_{50}/\left(2 + \left([A_f]/EC_{50}\right)^b\right)^{1/b} - 1$$

where $[A_f]$ is the fixed agonist concentration, EC_{50} is the agonist concentration causing half maximal effect and *b* is the slope factor of the agonist dose response curve.

3. Results

3.1. Expression of α_1-adrenoreceptor subtypes in neonatal cardiac myocytes

As shown in Fig. 1, competitive RT-PCR studies revealed that mRNA transcripts for three α_1-adrenoreceptor subtypes were present in cultured rat neonatal cardiac myocytes consistent with previous results (Stewart et al., 1994). Radioligand binding studies have previously demonstrated two binding sites in these cells (Knowlton et al., 1993), although the exact identity of the subtypes corresponding to these binding sites was unclear due to the lack of suitable antagonists for the α_{1D}-adrenoreceptor subtype. Therefore, in the present study, a full characterization of the binding sites present in rat neonatal cardiac myocytes was performed using a range of selective receptor antagonists, including BMY 7378, which exhibited a 100-fold greater selectivity for the α_{1d}-adrenoreceptor subtype over the α_{1a} and α_{1b}-adrenoreceptor subtypes (Goetz et al., 1995).

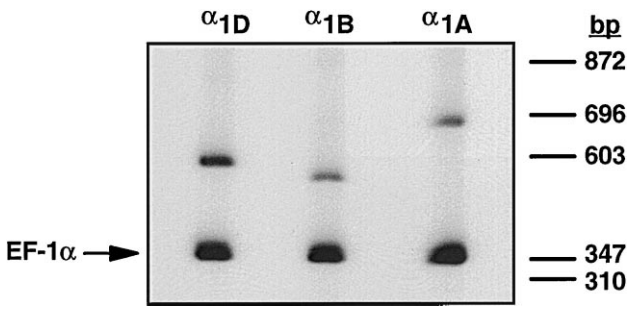


Fig. 1. Competitive RT-PCR assay of α_1 adrenoreceptor mRNAs in cultured neonatal cardiac myocytes. A representative autoradiograph from a competitive RT-PCR assay showing the presence of all three mRNAs. The expected sizes of the PCR products for the α_{1d}-, α_{1b}- and α_{1a}-adrenoreceptors were 549, 512 and 696 bp, respectively. The expected size of the PCR product for the EF-1α was 347 bp.

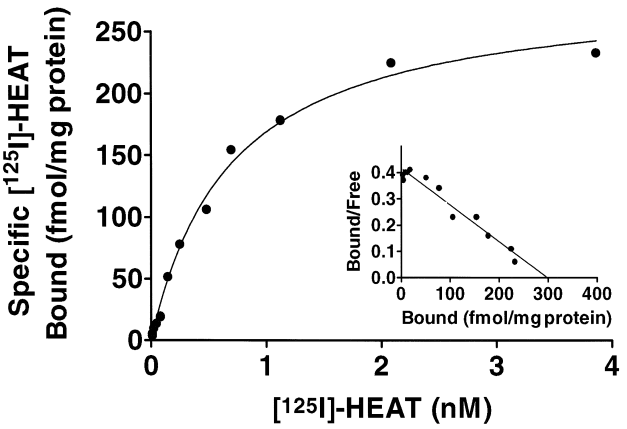


Fig. 2. [^{125}I]HEAT binding to cardiac myocyte membranes. Saturation binding studies were performed using 0.02 mg of membrane protein and a range of [^{125}I]HEAT concentrations as described in Section 2. Data shown is from a representative saturation study, showing a specific binding isotherm. Each point is the mean of triplicate observations. Inset: Scatchard analysis of the data.

As shown in Fig. 2, saturation binding studies demonstrated that [^{125}I]HEAT bound to a single saturable site in membranes prepared from rat neonatal cardiac myocytes. Analysis of four saturation curves revealed a K_D of 767 ± 39 pM and a B_{MAX} of 286 ± 2 fmol/mg protein. Competition studies showed increasing concentrations of BMY 7378, a α_{1d} selective receptor antagonist, displaced [^{125}I]HEAT binding with a steep monophasic curve (Fig. 3). Curve analysis revealed a low affinity constant consistent with the presence of the α_{1A} and α_{1B}-adrenoreceptor subtypes but failed to detect a high affinity constant indicative of the presence of the α_{1D}-adrenoreceptor subtype (Table 1). Likewise, phenylephrine, a α_{1d} selective receptor agonist, displaced [^{125}I]HEAT binding with a monophasic curve. Again, curve analysis yielded an affinity constant consistent with the presence of the α_{1A} and α_{1B}-adrenoreceptor subtypes but not the α_{1D}-adrenoreceptor subtype (Fig. 3; Table 1). Hence, despite the presence of mRNA for the α_{1D}-adrenoreceptor subtype, radioligand binding studies with BMY 7378 and phenylephrine were not able to detect the α_{1D}-adrenoreceptor subtype at the protein level in cardiac myocytes. These results suggest

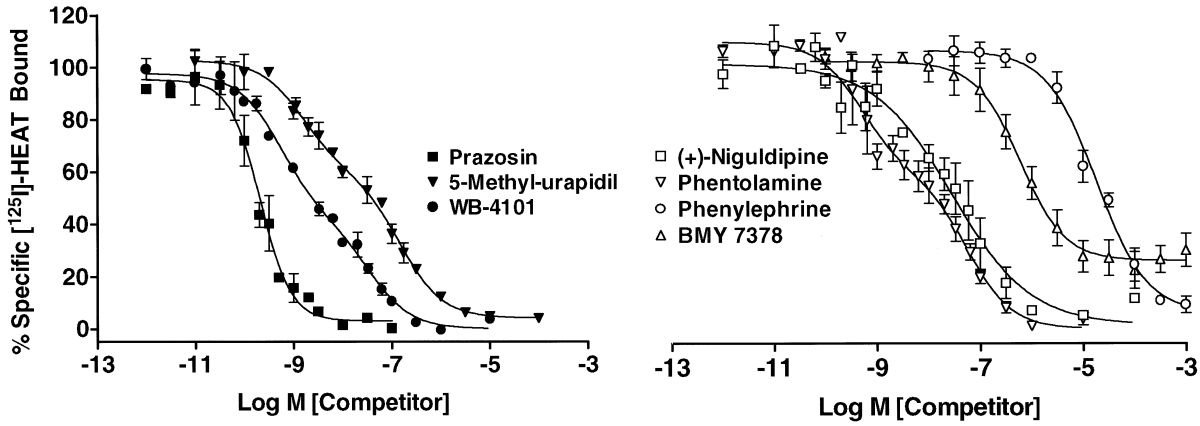


Fig. 3. Inhibition of [^{125}I]HEAT binding from cardiac myocyte membranes by a range of α_1-adrenoreceptor ligands. Competition curves showing the inhibition of [^{125}I]HEAT binding by $\alpha_1$adrenoreceptor ligands were performed on membranes prepared from cultured neonatal cardiac myocytes as described in METHODS. The competition curves for prazosin ($n = 3$) and phenylephrine ($n = 3$) and BMY 7378 ($n = 4$) were best fitted to a single-site model and those for 5-methyl-urapidil ($n = 4$), WB-4101 ($n = 3$), (+)-niguldipine ($n = 3$) and phentolamine ($n = 3$) were fitted better to a two-site model (see Table 1). Data represent the mean ± S.E.M. of separate membrane preparations where assays were performed in triplicate.

that the α_{1D}-adrenoreceptor subtype either was not expressed or expressed at a very low level compared to the α_{1A} and α_{1B}-adrenoreceptor subtypes in cardiac myocytes. Clarification on this point will require the development of high affinity antibodies with the requisite specificity for the various α_1-adrenoreceptor subtypes.

To further characterize the α_1-adrenoreceptor subtypes present in cardiac myocytes, a variety of additional α_1 selective receptor antagonists were employed. 5-Methyl-urapidil, WB-4101, and (+)-niguldipine displaced [^{125}I]HEAT with shallow slopes that were best fit as biphasic curves (Fig. 3). Analysis of these curves yielded two binding sites. By comparison with the values obtained for the cloned α_1-adrenoreceptor subtypes, the high affinity binding sites obtained for 5-methyl-urapidil and (+)-niguldipine were consistent with the presence of the α_{1A}-adrenoreceptor subtype, whereas the low affinity binding sites were consistent with the presence of the α_{1B} or α_{1D}-adrenoreceptor subtypes (Table 1). Since the α_{1D}-adrenoreceptor subtype was not detected by BMY 7378 or phenylephrine, this suggests that the low affinity binding sites observed with 5-methyl-urapidil and (+)-niguldipine most likely reflects the α_{1B}-adrenoreceptor subtype (Table 1). In a similar way, the low affinity binding site obtained for WB-4101 was consistent with the presence of the α_{1B}-adrenoreceptor subtype and the high affinity binding site was consistent with the presence of either the α_{1A} or α_{1D}-adrenoreceptor subtypes (Table 1). However, again, since the α_{1D}-adrenoreceptor subtype was not detected by BMY 7378 or phenylephrine, this suggests that the high affinity binding site observed with WB4101 most likely reflects the α_{1A}-adrenoreceptor subtype. Finally, phentolamine displaced [^{125}I]HEAT with a shallow slope that was best fit as a biphasic curve (Fig. 3). While the low affinity binding site was consistent with the presence of the α_{1B}-adrenoreceptor subtype, the high affinity binding site ex-

Table 1
Pharmacological profile of [^{125}I]-HEAT binding sites in membranes prepared from neonatal rat cardiac myocytes

Competitor	Neonatal rat cardiac myocyte membranes					Cloned α_1 adrenoceptors		
	one-site fit		two-site fit			log K_i		
	log K_i	nH	log K_H	log K_L	% K_H	α_{1a}	α_{1b}	α_{1d}
Phenylephrine	−4.8 ± 0.1	0.9 ± 0.1				−4.8 (1,3,4,5)	−4.9 (1,3,4,5)	−5.7 (1,4,5)
BMY 7378	−6.3 ± 0.2	1.0 ± 0.1				−6.1 (8)	−6.4 (8)	−8.2 (8)
5-Me-Urapidil	−7.8 ± 0.1	0.4 ± 0.1	−8.9 ± 0.1	−6.9 ± 0.1	47.7 ± 6.2	−8.5 (3,6,7,9)	−6.8 (3,6,7,9)	−7.5 (2,6,9)
WB-4101	−8.4 ± 0.2	0.5 ± 0.1	−9.6 ± 0.3	−7.6 ± 0.1	49.3 ± 6.5	−9.1 (3,6,9)	−7.8 (3,6,9)	−9.0 (2,6,9)
Prazosin	−9.7 ± 0.1	1.2 ± 0.1				−9.1 (2,6,7,9)	−9.5 (3,6,7,9)	−9.3 (3,6,7,9)
(+)-Niguldipine	−7.6 ± 0.4	0.5 ± 0.1	−9.5 ± 0.3	−7.1 ± 0.1	34.6 ± 4.7	−9.0 (4,6)	−7.1 (4,6)	−6.8 (4,6)
Phentolamine	−8.2 ± 0.1	0.4 ± 0.1	−9.6 ± 0.2	−7.4 ± 0.2	43.0 ± 5.8	−7.8 (1,6,7,9)	−6.9 (1,6,7,9)	−7.1 (1,6,7,9)

Competition curves were performed as described in Fig. 3 and the data were analysed for best fit to either a one- or two-site model as described in Section 2.8. Log K_i values represent the mean ± S.E.M. of 3–5 separate experiments, where each competition curve was fitted separately. nH corresponds to the Hill coefficient when data was fitted to a one-site model. K_H and K_L are high and low affinity binding sites, respectively, obtained from two-site analysis. Affinity constants for the cloned mammalian α_1-adrenoceptors are shown for comparison where the values represent means from the following species (rat, bovine and human α_{1a}-adrenoceptors; rat, hamster and human α_{1b}-adrenoceptors; rat and human α_{1d}-adrenoceptors) obtained from the following reports ((1) Lomasney et al., 1991; (2) Perez et al., 1991; (3) Schwinn et al., 1991; (4) Forray et al., 1994; (5) Goetz et al., 1994; (6) Laz et al., 1994; (7) Michel and Insel, 1994a; (8) Goetz et al., 1995; (9) Saussy et al., 1994).

Table 2
Pharmacological characterization of phenylephrine induced total inositol phosphate accumulation and MAP kinase activation in neonatal rat cardiac myocytes

Antagonist	Total inositol phosphate accumulation (Log K_b)	MAP kinase activation (Log K_b)	
		ERK1	ERK2
BMY 7378	-6.2 ± 0.3	-5.7 ± 0.1	-5.5 ± 0.1
5-Me-Urapidil	-8.0 ± 0.2	-7.1 ± 0.3[a]	-7.0 ± 0.2[a]
WB-4101	-8.9 ± 0.1	-7.8 ± 0.05[a]	-7.9 ± 0.03[a]
Phentolamine	-8.0 ± 0.2	ND	ND
Prazosin	-8.7 ± 0.1	-8.4 ± 0.1	-8.5 ± 0.3

Functional inhibition curves of phenylephrine (3 μM) induced total inositol phosphate accumulation and MAP kinase activation were performed as described in Fig. 4 and the data were analysed as described in Section 2.8. Log K_b values represent the mean ± S.E.M. of 4 separate experiments.
[a]Represents a significant difference ($P < 0.05$; Students unpaired t-test) compared with the corresponding total inositol phosphate accumulation log K_b value.
ND = not determined.

hibited an affinity constant 60-fold greater than that previously reported for any of the α_1-adrenoreceptor subtypes (Table 1). Although the reason for this is not clear, phentolamine has been shown previously to exhibit a higher affinity for the native α_1-adrenoreceptor subtypes (eg. log K_i's of -9.6 in the rat aorta (Piascik et al., 1994), -9.2 in rat brain (Terman et al., 1990) and -8.9 in rat submaxillary gland (Michel et al., 1989)) than for any of the cloned $\alpha_1$adrenoreceptor subtypes.

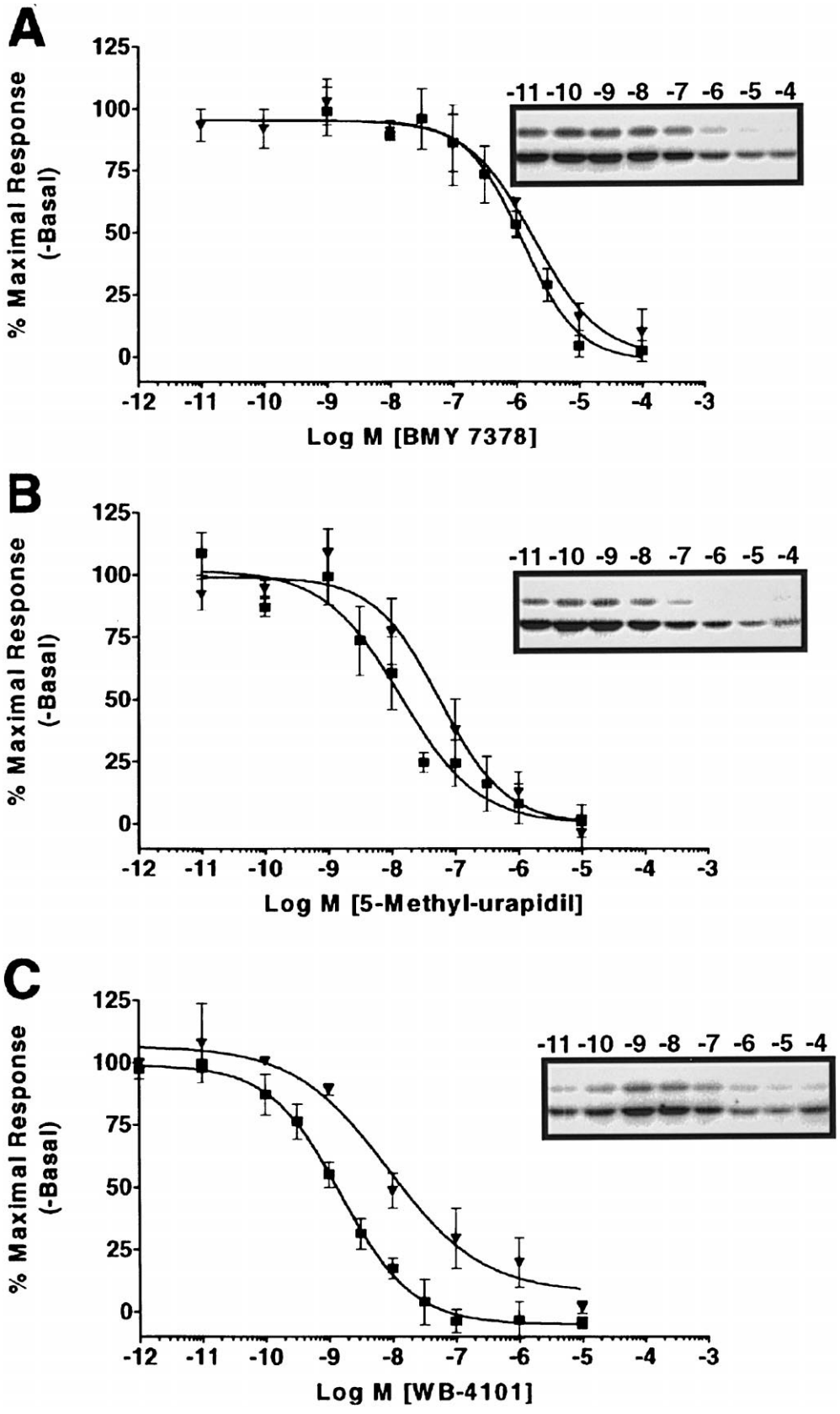
3.2. Stimulation of phosphatidylinositol hydrolysis by α_1-adrenoreceptor subtypes

Previous studies with recombinant α_1-adrenoreceptor subtypes have shown that all three subtypes are capable of activating phosphatidylinositol hydrolysis in response to phenylephrine (Schwinn et al., 1991; Perez et al., 1993; Minneman et al., 1994). In the present study, the ability of the native α_1-adrenoreceptor subtypes to activate phosphatidylinositol hydrolysis in cardiac myocytes was examined. Phenylephrine was shown to stimulate phosphatidylinositol hydrolysis in a concentration dependent manner in cardiac myocytes with a pD_2 ($-\log EC_{50}$) of 5.6 ± 0.01 and a slope of 1.6 ± 0.07 ($n = 3$). At a dose of 3 μM phenylephrine, phosphatidylinositol hydrolysis was stimulated 4.49 ± 1.72 fold over control ($n = 14$). BMY 7378, the α_{1d} selective receptor antagonist, was found to inhibit phenylephrine-stimulated phosphatidylinositol hydrolysis (Fig. 4) with an affinity constant (log K_B) similar to that observed in the radioligand binding studies (Table 2), suggesting the relative absence of a α_{1D}-adrenoreceptor subtype coupled to phospholipase C in cardiac myocytes. 5-Methyl-urapidil and WB-4101 both inhibited phenylephrine-induced phosphatidylinositol hydrolysis (Fig. 4) with affinity constants consistent with either the α_{1A} or α_{1D}-adrenoreceptor subtypes but not the α_{1B}-adrenoreceptor subtype (Table 2 versus Table 1). Since BMY 7378 exhibited a low affinity constant inconsistent with the presence of an α_{1d}-adrenoreceptor subtype in these studies, this response appears to be mediated primarily via the α_{1A}-adrenoreceptor subtype. Finally, phentolamine inhibited phenylephrine-induced phosphatidylinositol hydrolysis with an affinity consistent with that of the α_{1A}-adrenoreceptor subtype, although lower than that observed for the high affinity binding site observed in the radioligand binding studies above (Table 2 versus Table 1).

3.3. Stimulation of MAP kinase activity by α_1-adrenoreceptor subtypes

In addition to enhanced phosphatidylinositol hydrolysis, activation of α_1-adrenoreceptors has been reported to result in the stimulation of p44 and p42 mitogen activated protein kinases (ERK1 and ERK2, respectively) (Clerk et al., 1994; Bogoyevitch et al., 1995). Accordingly, we examined the time course of mitogen activated protein kinase activation by phenylephrine in cardiac myocytes. Similar to that reported by others (Clerk et al., 1994), phenylephrine stimulated mitogen activated protein kinase activity in a concentration dependent manner, where pD_2 values were 4.9 ± 0.1 and 5.0 ± 0.1 ($n = 3$) for ERK1 and ERK2, respectively, with corresponding slope values of 1.7 ± 0.2 and 1.6 ± 0.1 ($n = 3$), respectively. At a dose of 3 mM phenylephrine, mitogen activated protein kinase

Fig. 4. Inhibition of phenylephrine-stimulated phosphatidylinositol hydrolysis and mitogen activated protein kinase activation by $\alpha_1$adrenoreceptor subtype selective antagonists in cardiac myocytes. For determination of phosphatidylinositol hydrolysis, rat neonatal cardiac myocytes were cultured for 48 h in the presence of [^{3}H]-inositol (2 μCi/ml). Cells were preincubated for 1 h with 1 μM propranolol and various concentrations of the specified antagonist or vehicle control (basal). Following the addition of 10 mM LiCl, cells were stimulated with 3 μM phenylephrine for 30 min, stopped and total [^{3}H]-inositol phosphates were determined as described in Section 2. For mitogen activated protein kinase assays, a similar protocol was used except cells were stimulated for 5 min with 3 μM phenylephrine in the absence of LiCl. The data displayed are curves for BMY 7378 (A), 5-methyl-urapidil (B) and WB-4101 (C) inhibition of phenylephrine stimulated total inositol phosphate accumulation (■) and ERK2 (42 kDa mitogen activated protein kinase) activation (▼). Data represent the mean ± S.E.M. of 3–5 experiments performed in triplicate.


A
% Maximal Response (-Basal)
125
100
75
50
25
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
Log M [BMY 7378]
-11 -10 -9 -8 -7 -6 -5 -4
B
% Maximal Response (-Basal)
125
100
75
50
25
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
Log M [5-Methyl-urapidil]
-11 -10 -9 -8 -7 -6 -5 -4
C
% Maximal Response (-Basal)
125
100
75
50
25
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
Log M [WB-4101]
-11 -10 -9 -8 -7 -6 -5 -4

activity was stimulated 3.1 ± 0.3 and 2.5 ± 0.2 fold above controls for ERK1 and ERK2-mediated phosphorylation of myelin basic protein, respectively ($n = 10$). To determine the α_1-adrenoreceptor subtypes responsible for activation of the mitogen activated protein kinase cascade, antagonist inhibition curves of phenylephrine-stimulated mitogen activated protein kinase activity were constructed. BMY 7378 inhibited phenylephrine-stimulated ERK1 and ERK2 activities with an affinity constant much lower than that expected for the α_{1D}-adrenoreceptor subtype but consistent with that expected for either the α_{1A} or α_{1B}-adrenoreceptors (Fig. 4; Table 2). Of particular interest, 5-methyl-urapidil and WB-4101 inhibited phenylephrine-stimulated ERK1 and ERK2 phophorylating activities with affinity constants significantly lower than those obtained for inhibition of phosphatidylinositol hydrolysis (Fig. 4; Table 2). Thus, unlike phosphatidylinositol hydrolysis that appears to be mediated primarily through the α_{1A} adrenoreceptor subtype, the activation of the mitogen activated protein kinase cascade appears to be mediated by a subtype with affinity constants most consistent with the α_{1B} adrenoreceptor subtype.

4. Discussion

Ascribing physiological effects to individual α_1-adrenoreceptor subtypes first requires the identification of which subtypes are present in the tissue of interest. In the present study, we have examined which α_1-adrenoreceptor subtypes are present in cultured rat neonatal cardiac myocytes. In agreement with a previous study (Stewart et al., 1994), mRNA transcripts corresponding to the cloned α_{1a}, α_{1b}, and α_{1d}-adrenoreceptor subtypes were readily detected by RT-PCR analysis. Since specific antibodies of sufficiently high titer for each of the α_1-adrenoreceptor subtypes have yet to be developed, identification of proteins corresponding to the cloned α_{1a}, α_{1b} and α_{1d}-adrenoreceptor subtypes were determined by radioligand binding analysis with a wide range of selective receptor antagonists. While the majority of selective receptor antagonists currently available show selectivity for the α_{1A}-adrenoreceptor subtype (see Table 1), the recent discovery of BMY 7378 that exhibits 100-fold higher selectivity for the α_{1D}-adrenoreceptor subtype (Goetz et al., 1995) has allowed the pharmacological separation of all three α_1-adrenoreceptor subtypes. The three α_{1a} selective receptor antagonists, 5-methyl-urapidil, (+)-niguldipine and WB 4101, revealed the presence of two binding sites with affinity constants consistent with the expected properties of the α_{1A} and α_{1B}-adrenoreceptor subtypes. Surprisingly, the α_{1d} selective receptor antagonist, BMY 7378, failed to detect the presence of a binding site with the expected properties of the α_{1D}-adrenoreceptor subtype despite the existence of mRNA for this subtype in cardiac myocytes. A determination of whether the α_{1D}-adrenoreceptor subtype is expressed at a very low level in cardiac myocytes will need to await the development of antibodies of the requisite affinity and specificity.

Functionally, the α_{1A} and α_{1B}-adrenoreceptor subtypes appear to couple differentially to the signaling pathways in cardiac myocytes, since the inhibition constants for the phenylephrine-induced stimulation of phosphatidylinositol hydrolysis and mitogen activated protein kinase pathways by α_1 selective receptor antagonists were found to be different. The α_{1a} selective receptor antagonists, 5-methyl-urapidil and WB 4101, inhibited phenylephrine-induced activation of phosphatidylinositol hydrolysis with high affinity constants suggestive of a predominantly α_{1A}-adrenoreceptor subtype response. This result confirms and extends a previous study (Knowlton et al., 1993). On the other hand, inhibition of phenylephrine-induced activation of the mitogen activated protein kinase pathway by these same antagonists yielded low affinity constants indicative of a predominantly α_{1B}-adrenoreceptor subtype response. This finding has important implications in view of a recently proposed role for the α_{1B}-adrenoreceptor subtype in the regulation of cardiac hypertrophy (Milano et al., 1994). Finally, the α_{1d} selective receptor antagonist, BMY 7378, inhibited phenylephrine-induced activation of both phosphatidylinositol hydrolysis and mitogen activated protein kinase pathways with low affinity constants indicative of a α_{1A} or α_{1B}-adrenoreceptor subtype response but no evidence for any high affinity constants suggestive of a α_{1D}-adrenoreceptor subtype involvement.

The demonstration that specific α_1-adrenoreceptor subtypes couple to different signaling pathways may explain the wide variety of physiological effects observed in cardiac myocytes. The finding that the α_{1A}-adrenoreceptor subtype couples preferentially to the phosphatidylinositol hydrolysis pathway and the α_{1B}-adrenoreceptor subtype couples preferentially to the mitogen activated protein kinase pathway suggests that these two receptor subtypes must possess differences in their abilities to interact with downstream components of these pathways. It may be that each α_1-adrenoreceptor subtype interacts with a different heterotrimeric G protein to produce bifurcating signals in the form of G_{α} and $G_{\beta\gamma}$ subunits (Gilman, 1995; Neer, 1995). Since phenylephrine stimulation of the phosphatidylinositol hydrolysis pathway is insensitive to pertussis toxin (Schwinn et al., 1995), this suggests that the α_{1A}-adrenoreceptor subtype interacts with a member of the $G_{q/11}$ protein family to regulate phospholipase C-β. The predominant phospholipase C-β isoform in rat neonatal cardiac myocytes is phospholipase C-β3 (Hansen et al., 1995), which can be regulated by either the α or the $\beta\gamma$ subunits of the $G_{q/11}$ protein family in vitro (Smrcka and Sternweis, 1993). Whether the α or the $\beta\gamma$ subunits of the $G_{q/11}$ protein family are responsible for in vivo regulation will be the subject of future investigations.

The underlying mechanism for activation of the mitogen activated protein kinase pathway by the α_{1B}-adrenoreceptor subtype in neonatal cardiac myocytes is not

presently clear. Since phenylephrine stimulation of the mitogen activated protein kinase pathway is insensitive to pertussis toxin (Bogoyevitch et al., 1995), this suggests that the α_{1B}-adrenoreceptor subtype interacts with a member of the $G_{q/11}$ protein family rather than the $G_{i/o}$ protein family. However, the downstream components regulated by $G_{q/11}$ have yet to be conclusively identified. On the one hand, Thorburn and colleagues have provided evidence that phenylephrine-induced activation of the mitogen activated protein kinase pathway is mediated by Raf-1 kinase in cardiac myocytes (Thorburn et al., 1994). Since Raf-1 kinase can be activated by protein kinase C (Downward et al., 1990; Nakafuku et al., 1992; Kolch et al., 1993; Sugden and Bogoyevitch, 1995), which, in turn, can be activated by products of the phosphatidylinositol hydrolysis pathway (Harrington and Ware, 1995), this could provide a mechansim for activation of the mitogen activated protein kinase pathway by the α_{1B}-adrenoreceptor subtype. However, this mechanism is difficult to reconcile with the lower potency of the α_{1B}-adrenoreceptor subtype compared to the α_{1A}-adrenoreceptor subtype in stimulating the phosphatidylinositol pathway. Also, arguing against this mechanism is the recent finding that transgenic hearts overexpressing the G_q α subunit exhibited marked stimulation of the phosphatidylinositol hydrolysis pathway but no activation of the mitogen activated protein kinase pathway (D'Angelo et al., 1997). This suggests that the $\beta\gamma$ subunits rather than the α subunits of $G_{q/11}$ could be involved in stimulation of the mitogen activated pathway. In this regard, the $\beta\gamma$ subunits released from G_i have been shown to activate a Ras-dependent pathway for stimulation of Raf-1 kinase (Koch et al., 1994; van Biesen et al., 1995). Whether the $\beta\gamma$ subunits released from $G_{q/11}$ are similarly able to activate a Ras-dependent pathway in cardiac myocytes will be the subject of future investigations.

In conclusion, the present study demonstrates that even though all three α_1-adrenoreceptor subtypes are present in rat neonatal cardiac myocytes at the mRNA level, only the α_{1A} and α_{1B}-adrenoreceptor subtypes could be detected at the protein level on the basis of radioligand binding analysis. Moreover, the α_{1A}-adrenoreceptor subtype appeared to couple preferentially to phosphatidylinositol turnover, whereas activation of the mitogen activated protein kinase cascade appeared to occur via a subtype whose pharmacology most closely resembled that of the α_{1B}-adrenoreceptor subtype. This is the first demonstration of differences between native and/or recombinant α_1-adrenoreceptor subtypes in terms of their coupling to the mitogen activated protein kinase pathway although differences between recombinant α_1-adrenoreceptor subtypes in terms of their coupling to the phosphatidylinositol hydrolysis pathway have been noted previously (Schwinn et al., 1991). The demonstration that specific α_1-adrenoreceptor subtypes couple to different signaling pathways may explain the wide variety of contractile and cell growth processes that are altered upon addition of α_1 agonists to cardiac myocytes. Future studies employing antisense knockout of individual receptor subtypes or expression of constitutively active receptor subtypes will further test this idea.

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