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Regulation of the Cellular Localization and Signaling Properties of the α_{1B}- and α_{1D}-Adrenoceptors by Agonists and Inverse Agonists

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ABSTRACT

The regulation of the cellular distribution and intracellular signaling properties of the α_{1B}- and α_{1D}- adrenoceptor (α_1-AR) subtypes was examined in stably transfected Rat 1 fibroblasts. In unstimulated cells, α_{1B}-AR expression was noted primarily on the cell surface. Treatment with phenylephrine induced internalization of the α_{1B}-AR and promoted association with arrestin 2. The internalized α_{1B}-AR colocalized with the transferrin receptor, an endosomal marker. In unstimulated fibroblasts, the α_{1D}-AR was detected in a perinuclear orientation and was colocalized with arrestin 2 in a compartment also containing the transferrin receptor. After treatment with prazosin, which exhibits inverse agonist properties, the α_{1D}-AR was redistributed from intracellular sites to the cellular periphery and was no longer associated with the transferrin receptor or arrestin 2. α_{1D}-AR-expressing cells exhibited a high degree of basal activity for both inositol phosphate formation and extracellular signal regulated kinase (ERK), which was reduced by treatment with prazosin. In these cells, phenylephrine induced a dose-dependent increase in inositol phosphate formation but had no effect on ERK activity. In α_{1B} -AR-expressing cells, phenylephrine stimulated both inositol phosphate formation and ERK activity. These data show that: 1) there are differences in the cellular localization of the α_1-AR subtypes; 2) the α_{1B}-AR exhibits expected G protein-coupled receptor activity regarding cellular localization, agonist-mediated internalization, and coupling to second messengers; and 3) the α_{1D}-AR is constitutively active and, as a result, is localized to intracellular compartments involved in receptor recycling.

Three subtypes of the α_1-adrenoceptor (AR), the α_{1A}, α_{1B}, and α_{1D}, have thus far been identified, cloned, and characterized (Cotecchia et al., 1988; Schwinn et al., 1990; Lomasney et al., 1991; Perez et al., 1991). Our understanding of the complexity of the α_1-ARs and their interplay with other signaling systems has steadily increased. The α_1-ARs are major effectors used by the sympathetic nervous system to regulate systemic arterial blood pressure, blood flow, and cell growth. Previous work by us and others has shown that the mRNA encoding each of these receptors is widely distributed in the peripheral vasculature (Ping and Faber, 1993; Piascik et al., 1994, 1995; Eckhart et al., 1996; Guarino et al., 1996) and that the α_1-ARs are also expressed as protein in all of the peripheral arteries thus far examined (Piascik et al., 1997; Hrometz et al., 1999). Using both pharmacological approaches and antisense oligonucleotide technology, we have found evidence that the activation of smooth muscle contraction is caused by a single receptor in any given artery and that the α_1-AR subtype that mediates contraction varies throughout the vasculature (Piascik et al., 1995, 1997; Hrometz et al., 1999).

These findings have lead us to explore possible mechanisms to account for the observation that expression in blood vessels is not sufficient to link an α_1-AR to contraction. We have focused on the subcellular localization of the α_1-ARs as a factor that modifies the ability of these receptors to regulate cellular function. In models of cellular signaling, the heptahelical G protein-coupled receptors (GPCRs) are postulated to reside in the cell membrane where they transduce a variety of intracellular signals initiated by binding of neurotransmitters or exogenously administered pharmacologic agents. Recent evidence has indicated that the α-ARs are not exclusively localized with the cell membrane. Using green

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ABBREVIATIONS: AR, adrenoceptor; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GPCR, G protein-coupled receptor; MBP, myelin basic protein.

fluorescent protein/α_1-AR fusion proteins, Hirasawa et al. (1997) showed that the α_{1B}-AR was expressed on the cell surface whereas the α_{1A}-AR was expressed in intracellular compartments. In transfected fibroblasts, the α_{2C}-AR was detected in intracellular compartments as well as on the cell surface, whereas the α_{2A}-AR was found exclusively on the cell membrane (von Zastrow et al., 1993; Daunt et al., 1997).

Internalization of GPCR after activation by agonists is a well known phenomenon. Agonist-induced internalization of the GPCR regulates signal transduction by this class of transmembrane receptors. Like constitutively recycling receptors for transferrin and the low-density lipoprotein, GPCRs are internalized via clathrin-coated pits to endosomes (von Zastrow and Kobilka, 1992; reviewed by Krupnick and Benovic, 1998). In addition to activation of cellular signaling cascades, agonist occupancy of GPCR results in the recruitment of a GPCR kinase to the membrane, receptor phosphorylation, and promotion of arrestin binding to the receptor (Krupnick and Benovic, 1998). Arrestin binding is thought to uncouple the receptor from G proteins and initiate the process of internalization by directing the receptor to the clathrin-coated pits. This process has traditionally been thought of as a way of terminating cellular signaling. However, studies using transfected β_2-ARs or endogenous opioid receptors indicate that internalization of certain GPCR also activates cellular signaling. Daaka et al. (1998) and Ignatova et al. (1999) showed that receptor internalization was necessary to activate extracellular signal-regulated kinase (ERK). In these studies, expression of dominant negative mutants of β-arrestin or dynamin prevented receptor internalization and receptor-stimulated ERK activity.

In this report we have examined the regulation of the cellular localization and signaling properties of the α_{1B}- and α_{1D}-ARs. We show that the α_{1B}-AR is expressed primarily on the cell surface, is internalized by agonist occupation, and is coupled to inositol phosphate formation and ERK activity, consistent with traditional views of GPCR. The α_{1D}-AR exhibits behavior atypical of a GPCR, and we postulate that this is due to the constitutive activity exhibited by this receptor.

Materials and Methods

Cell Culture Conditions. Rat 1 fibroblasts stably transfected with cDNA for the human α_{1B}-AR or α_{1D}-AR (a gift from Glaxo Wellcome) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies), a 1% antibiotic/antimycotic mixture (Life Technologies), and 500 μg/ml geneticin (Life Technologies). Cells were maintained in cell culture flasks at 37°C in 5% CO_2, fed every 2 to 3 days with supplemented DMEM, and trypsinized every 5 to 7 days. After the fibroblasts reached confluency, they were plated out on sterile 20-mm × 20-mm glass coverslips and returned to the cell culture incubator for 72 h to allow attachment. Cells were deprived of serum overnight (18–24 h) before experimentation.

Immunocytochemistry and Laser Scanning Confocal Microscopy. Cells were fixed with 3.7% formaldehyde in PBS for 10 min, washed with PBS containing 0.05% BSA, and permeabilized with 0.1% Triton in PBS for 5 min. Cells were incubated in blocking solution containing 10% fetal bovine serum in PBS for 1 h at room temperature, then washed and incubated with primary antibody for 2 h. The primary antibodies (against the α_1-ARs, the transferrin receptor, and arrestin 2) are described in a section below. Fibroblasts were then incubated with either a fluorescein-conjugated (donkey anti-goat IgG, for α_1-AR visualization) or a rhodamine-conjugated (donkey anti-rabbit or -mouse IgG, for arrestin or transferrin receptor visualization) secondary antibody. Dual-label immunocytochemistry was used to simultaneously assess the localization of the α_1-ARs, arrestin, or transferrin. In certain experiments, the effect of phenylephrine or a series of antagonists on the cellular location of proteins of interest was assessed. We also examined α_{1B}-AR immunoreactivity in COS 1 cells transiently transfected with the wild-type α_{1B}-AR or a constitutively active mutant in which alanine 293 was mutated to lysine (A293K, Kjelsberg et al., 1992). Images were analyzed using a laser-scanning confocal microscope (Leica TCS, Exton, PA) with a 63× Water Immersion objective. Fluorescein isothiocyanate (FITC) fluorescence was excited by an argon laser at 488 nm and monitored at a wavelength no greater than 545 nm. Rhodamine fluorescence was excited using a 568-nm helium-neon laser and monitored at a wavelength no greater than 690 nm.

Quantitation of Intracellular Inositol Phosphates. Rat 1 fibroblasts plated on 60-mm culture plates were grown in DMEM (Bio-Whittaker, Walkersville, MD) supplemented with fetal bovine serum. On reaching 90% confluency, 3 μCi of ^{3}H-myo-inositol (DuPont-NEN, Boston, MA) was added 24 h before use in experiments. Measurement of intracellular inositol phosphates was performed in serum-free DMEM after several changes of media. Assays were conducted in the presence of LiCl (10 mM) in a total assay volume of 3 ml. Cells were incubated at 37°C for 60 min in a 5% CO_2 atmosphere in the presence or absence of increasing concentrations of phenylephrine. The effect of 1 μM prazosin, an inverse agonist, which was added 24 h before experimentation was also determined. Incubations were terminated by removal of the media and addition of 1 ml of a 0.4 M perchloric acid solution. The cell lysates were scraped, collected, and neutralized by the addition of 0.5 ml of a 0.72 N KOH/0.6 M $KHCO_3$ solution. Soluble inositol phosphates in the cell lysates were isolated by passage through a Bio-Rad AG 1X-8 resin column that was buffered with a 0.1 M formic acid solution. After washing the column with 0.1 M formic acid, bound ^{3}H-IPs were displaced from the column by eluting with a 0.1 M formic acid solution containing 1 M ammonium formate and the radioactivity was detected by liquid scintillation counting. Data were analyzed using a one-tailed t test analysis (GraphPad Prism).

Assays for ERK Activity. ERK activity was determined using in-gel kinase assays as we have described previously (Post et al., 1996). Briefly, fibroblasts were incubated with phenylephrine in the presence or absence of prazosin for the indicated times, washed with ice-cold PBS, and scraped into 1 ml of ice-cold buffered sucrose. The cell suspension was centrifuged (5 min, 1000g, 4°C), and the pellet was resuspended in 20 μl of lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, 20 mM β-glycerophosphate, 0.5% NP-40, 100 μM Na_3VO_4, 5 μM AEBSF (Calbiochem, La Jolla, CA), 1.5 nM aprotinin, 10 nM E-64, 10 nM leupeptin, pH 7.4). After incubation on ice for 30 min, the lysate was centrifuged (15 min, 15,000g, 4°C), the supernatant was collected, and protein content was determined. Protein was resolved on 10% SDS-polyacrylamide gels containing 0.5 mg/ml myelin basic protein (MBP). After electrophoresis, gels were first washed with 20% 2-propanol in 50 mM HEPES, pH 7.6 and then with 5 mM β-mercaptoethanol in HEPES buffer. Proteins were denatured by washing gels in 6 M urea and then renatured in HEPES buffer containing 0.05% (v/v) Tween-20 (renaturation buffer) at 4°C. After overnight incubation in renaturation buffer at 4°C, gels were preincubated in 25 ml of cold kinase buffer (20 mM HEPES, 20 mM $MgCl_2$, 2 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4) for 30 min. Phosphorylation of MBP was performed in situ by incubating gels in kinase buffer containing 20 μM ATP and 150 to 160 μCi [γ^{32}P]ATP for 90 to 120 min at 30°C. After extensive washing in 5% trichloroacetic acid/1% sodium pyrophosphate, gels were dried and exposed to film. ^{32}P incorporation into MBP was quantified by densitometry. The ERK activity reported in Table 1 is in integrated optical density units, whereas those

TABLE 1
Effect of prazosin on basal levels of inositol phosphate formation and ERK activity in fibroblasts expressing either the α_{1B}- or α_{1D}-AR
Each point represents the mean ± S.E. for four (inositol phosphate) or five (ERK) independent experiments.

Receptor	Basal IP Levels	Basal IP Levels in the Presence of Prazosin[a]	Prazosin Effect as % of Basal IP Levels	Basal ERK Activity[b]	Basal ERK Levels in the Presence of Prazosin	Prazosin Effect as % of Basal ERK Levels
	cpm	*cpm*		*IOD*	*IOD*	
α_{1B}-AR	587.4 ± 37.2	645.6 ± 57.4	109.9	1.736 ± 0.755	2.653 ± 1.074	152.8
α_{1D}-AR	1069 ± 163.5	876.4 ± 128.9	81.98[c]	3.466 ± 0.943	1.824 ± 0.728	52.6[c]

[a] Basal inositol phosphates were measured as described in *Materials and Methods*.
[b] Basal ERK activity is expressed as integrated optical density (IOD) units. IOD values were obtained from densitometer scans of the in gel-kinase assays as described in *Materials and Methods*.
[c] Significantly ($P < .5$) different from basal activity obtained in the absence of prazosin.

presented in Fig. 4 are normalized to a percentage of the control level of ERK activity obtained in each cell line. Data were analyzed by a one-way ANOVA.

Antibodies and Reagents. The primary antibodies used were as follows: goat anti-human α_{1B} IgG at 1:100 to 1:200, goat anti-rat α_{1D} IgG at 1:25 to 1:50, (Santa Cruz Biotech, Santa Cruz, CA) and mouse anti-rat CD71, a marker for the transferrin receptor, at 1:100 (Research Diagnostics, Flanders, NJ). Arrestin antibodies were provided by Dr. Jeffrey Benovic (Thomas Jefferson University). All secondary antibodies were used at a dilution of 1:100, which included fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG, rhodamine-conjugated donkey anti-rabbit IgG, and rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). Prazosin and phenylephrine were purchased from Sigma (St. Louis, MO).

Results and Discussion

In this report we have examined the regulation of the cellular distribution of the α_{1B}- and α_{1D}-ARs in Rat 1 fibroblasts stably transfected with either subtype. We have previously used antibodies to examine the distribution of the α_1-ARs in blood vessels, vascular smooth muscle cells, and stably transfected fibroblasts (Hrometz et al., 1999). In these studies we showed that the antibodies are specific and stain only those cells expressing the receptor against which the antibody was raised. Additionally, we show that preabsorption of the antibody with its immunizing peptide significantly reduces immunostaining.

In radioligand binding studies we determined that the α_{1B}- and α_{1D}-AR were expressed at similar levels in the Rat 1 fibroblast cell lines used in these studies (between 5.5 and 10 pmol/mg protein; data not shown). In α_{1B}-AR-expressing fibroblasts, receptor immunoreactivity was detected on the margin of the cell, indicating that, as expected, this receptor was localized to the cell membrane (Fig. 1). In contrast, although there was α_{1D}-AR immunoreactivity on the boundary of the cell, a significant degree of immunostaining was also detected in a perinuclear orientation (Fig. 1).

Figure 2 shows the immunostaining pattern obtained in fibroblasts expressing the α_{1B}-AR after treatment with the α_1-AR selective agonist phenylephrine. Agonist activation promotes a significant degree of α_{1B}-AR internalization as evidenced by the enhanced FITC fluorescence detected in a perinuclear orientation (Fig. 2, panel 1). Internalization can be detected within 5 min of receptor activation (see Fig. 2, panel 4 for time course) and is blocked by prior treatment of the cells with prazosin. Isoproterenol activation of the β_2-AR promotes receptor internalization to endosomes (von Zastrow and Kobilka, 1992). We determined whether this is also true for the α_{1B}-AR by performing dual-label immunofluorescence studies using an antibody against the transferrin receptor (CD71 in the figures), an endosomal marker. In these photomicrographs, the receptor is detected by a FITC-labeled secondary antibody, which emits a green color. The transferrin receptor is detected with a rhodamine-labeled secondary antibody that appears red (Fig. 2, panel 5). When these secondary antibodies are colocalized, the resulting fluorescent image appears yellow. Dual antibody labeling after activation of the α_{1B}-AR with phenylephrine yields a perinuclear yellow fluorescence, indicating that the internalized α_{1B}-AR is localized to endosomes (Fig. 2, panel 2). This colocalization and color change were prevented by pretreatment with prazosin.

Arrestin binding to phosphorylated GPCR is thought to uncouple the receptor from its cognate G protein(s) and participate in the process of internalization by directing the receptor to clathrin-coated pits. Dual-label immunocytochemistry shows that in unstimulated Rat 1 fibroblasts, there is little colocalization of the α_{1B}-AR with arrestin 2 (detected with a rhodamine-labeled secondary antibody, see Fig. 2, panels 3 and 5). Agonist mediated α_{1B}-AR internalization promotes the association of the receptor with arrestin 2 (see Fig. 2, panel 3). Receptor internalization and arrestin colocalization were blocked by prior treatment with prazosin. Detection of agonist-stimulated internalization of the α_{1B}-AR into endosomes by confocal microscopy has been reported previously in stably transfected HEK 293 cells (Fonseca et al., 1995) and cells expressing an α_{1B}-AR/green fluorescent protein fusion protein (Awaji et al., 1998). Association of constitutively active α_{1B}-AR mutants with arrestin 2 has also been demonstrated (Mhaouty-Kodja et al., 1999).

The functional response to α_{1B}-AR activation was assessed by measuring inositol phosphate formation and ERK activity

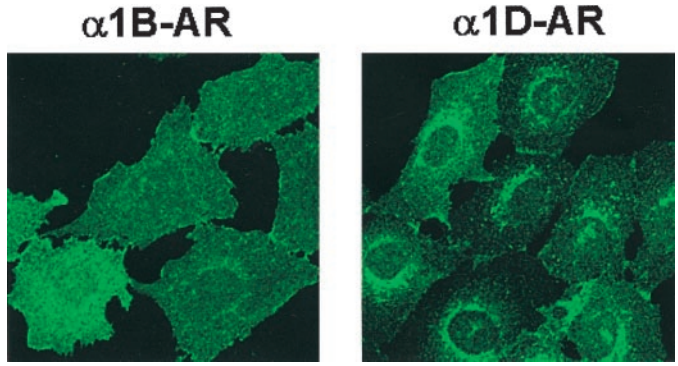

Fig. 1. Immunolocalization of the α_{1B}- and α_{1D}-ARs in stably transfected fibroblasts. Immunocytochemistry was carried out as described in *Materials and Methods*.

in stably transfected fibroblasts. Phenylephrine stimulated the formation of inositol phosphates and ERK activity in cells expressing the α_{1B}-AR (see Figs. 3 and 4).

Immunocytochemical analysis and cell signaling studies in Rat 1 fibroblasts expressing the α_{1D}-AR revealed a distinctly different cellular localization pattern and functional responses than those observed for the α_{1B}-AR. Although some α_{1D}-AR immunoreactivity was noted on the boundary of the cell, a significant degree of immunostaining was detected in a perinuclear orientation (see Fig. 1). The α_{1D}-AR colocalizes with the transferrin receptor, indicating that in unstimulated fibroblasts, the α_{1D}-AR is localized to endosomes (see Fig. 5, panel 2). In unstimulated fibroblasts, the α_{1D}-AR was also localized with arrestin 2 (see Fig. 5, panel 3). The control

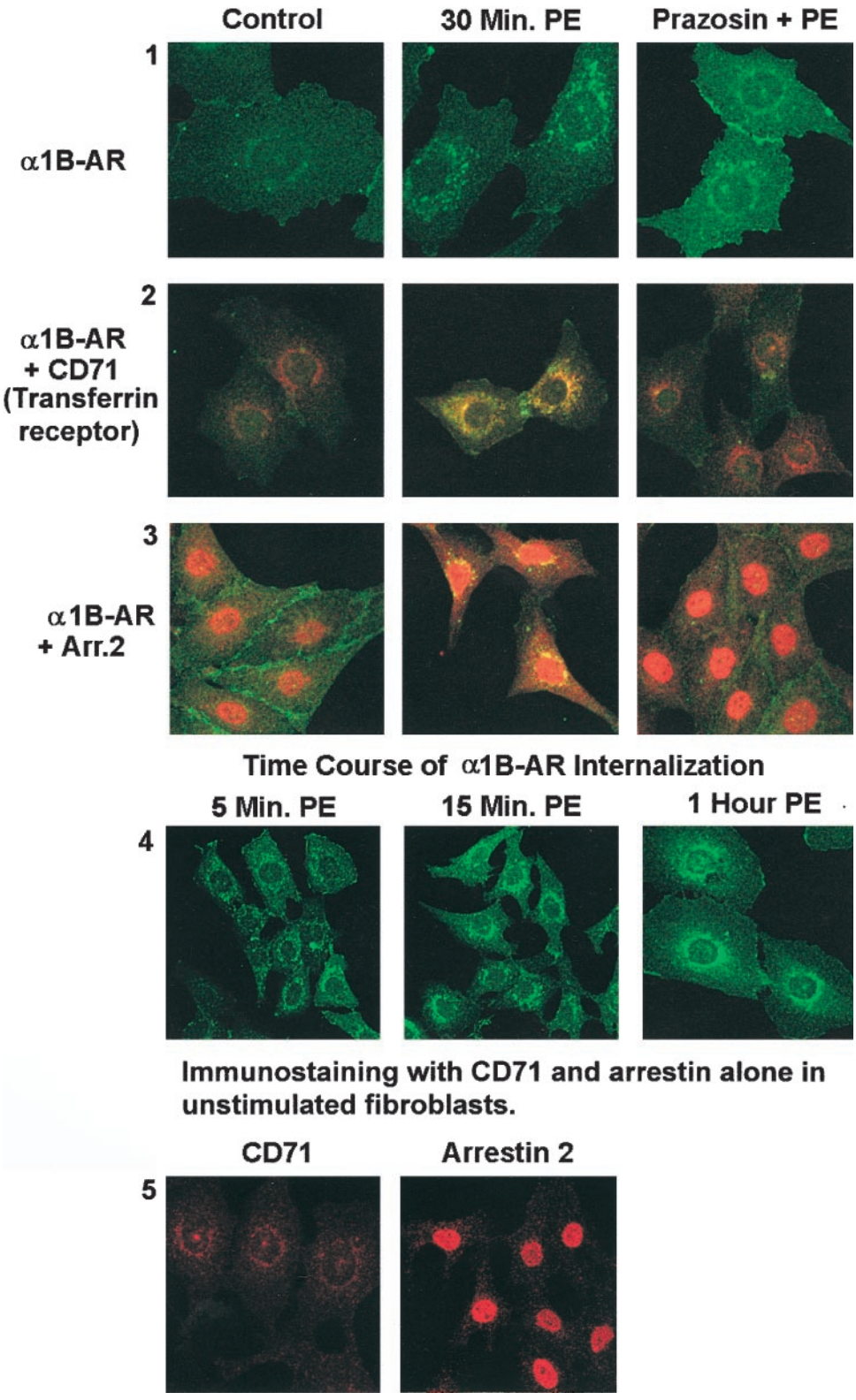

Fig. 2. Effect of 100 μM phenylephrine on the cellular localization of the α_{1B}-AR. Also presented is the colocalization of the receptor with the transferrin receptor (CD 71 antibody, panel 2) and arrestin 2 (panel 3). The receptor was detected with a FITC-labeled secondary antibody. The transferrin receptor and arrestin 2 were detected with rhodamine-labeled secondary antibody. When the two proteins colocalize, the fluorescence is detected as yellow.

transferrin and arrestin immunoreactivities in α_{1D}-AR-expressing fibroblasts were not different from those obtained in α_{1B}-AR-expressing cells as shown in Fig. 2.

Fibroblasts expressing the α_{1D}-AR exhibited a high degree of basal inositol phosphate formation compared with α_{1B}-AR-expressing cells (see Table 1). Phenylephrine was capable of inducing an additional dose-dependent increase in inositol phosphate formation in α_{1D}-AR-expressing fibroblasts (Fig. 3). Basal ERK activity in α_{1D}-AR-expressing fibroblasts was

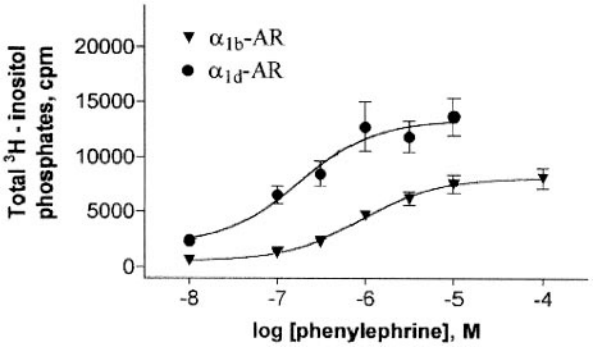

Fig. 3. Phenylephrine stimulation of inositol phosphate formation in fibroblasts expressing the α_{1B}- and α_{1D}-ARs. Experiments were carried out as described in *Materials and Methods*. Each point represents the mean and S.E. of four independent experiments from each cell line.

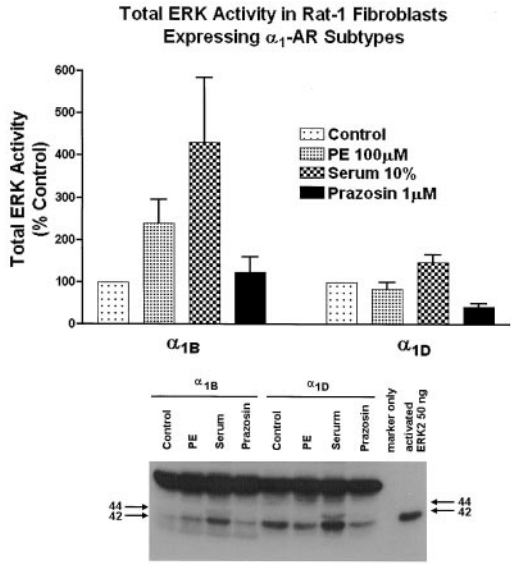

Fig. 4. Phenylephrine-mediated ERK activity in fibroblasts expressing the α_{1B}- and α_{1D}-ARs. Experiments were carried out as described in *Materials and Methods*. Data from each cell line is normalized to the percentage of the control optical density obtained from densitometric scans of the autoradiographs generated by the in-gel kinase assays. A representative autoradiograph is also presented. Each point represents the mean and S.E. of five independent experiments from each cell line.

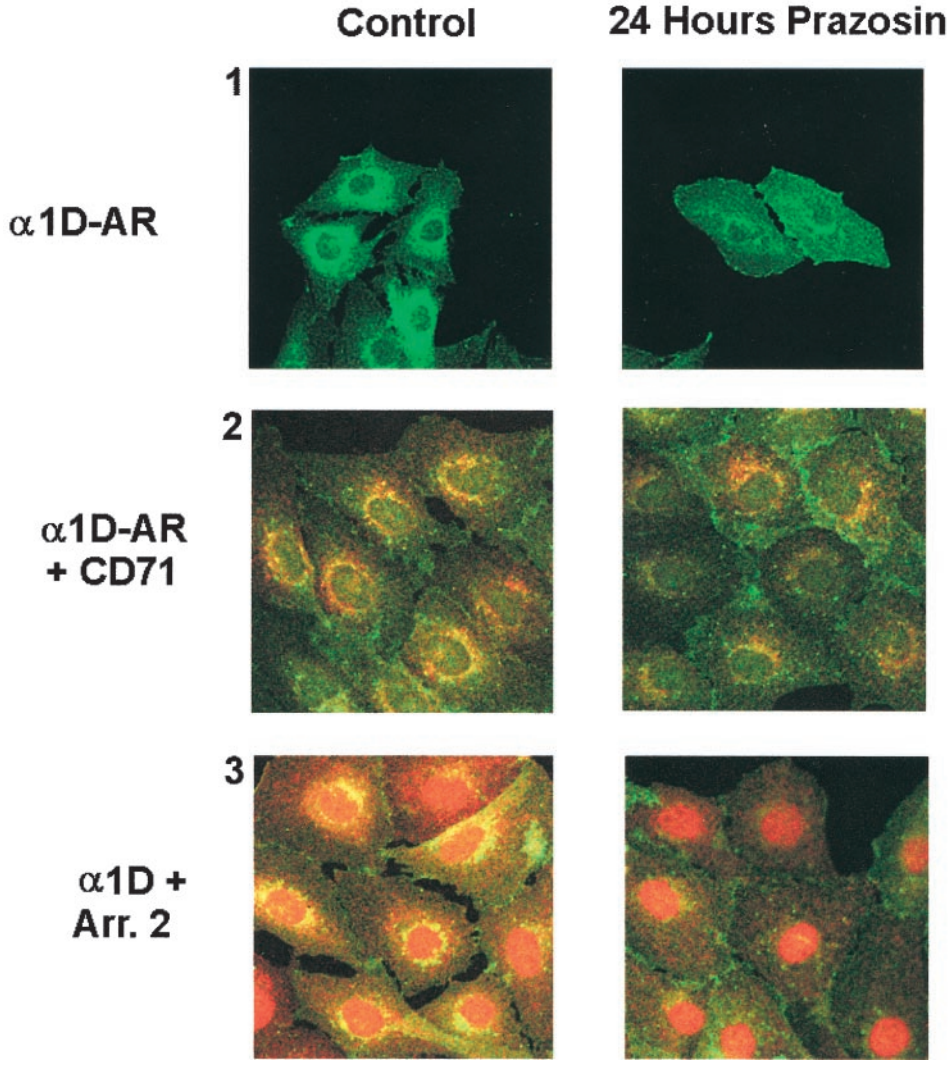

Fig. 5. Cellular localization of the α_{1D}-AR in stably transfected fibroblasts cultured in the absence and presence of prazosin. Also presented are dual-label immunocytochemistry with CD 71, an antibody against the transferrin receptor, and antibodies against arrestin 2. The receptor was detected with a FITC-labeled secondary antibody. The transferrin receptor and arrestin 2 were detected with rhodamine. When the two proteins colocalize, the fluorescence is detected as yellow.

also greater than that seen in α_{1B}-AR-expressing cells. However, we did not detect phenylephrine-induced increases in ERK activity above basal levels in α_{1D}-AR-expressing cells (Fig. 4). Interestingly, basal ERK activity was similar in magnitude to serum-stimulated levels in α_{1D}-AR-expressing cells. It may be that the high basal levels of kinase activity we observed precluded any additional agonist-induced increases in the α_{1D}-AR-expressing fibroblasts.

In unstimulated cells, we noted that the α_{1D}-AR is localized with arrestin and endosomes, the same cellular locale as the α_{1B}-AR after agonist activation. In addition, fibroblasts expressing the α_{1D}-AR exhibit a high level of basal phospholipase C and ERK activity compared with the α_{1B}-AR. These data argue that the α_{1D}-AR is constitutively active in Rat 1 fibroblasts. To more fully substantiate that the α_{1D}-AR is constitutively active, we performed immunocytochemistry, inositol phosphate, and ERK assays after culturing α_{1B}- or α_{1D}-AR-expressing fibroblasts in the presence of prazosin for 24 h. Prazosin has recently been shown to have inverse agonist properties (Scheer et al., 1997). Treatment of α_{1B}-AR expressing fibroblasts with prazosin had no effect on the cellular localization of the α_{1B}-AR (data not shown). Likewise, prazosin did not decrease either basal inositol phosphate formation or ERK activity (see Table 1) in α_{1B}-AR

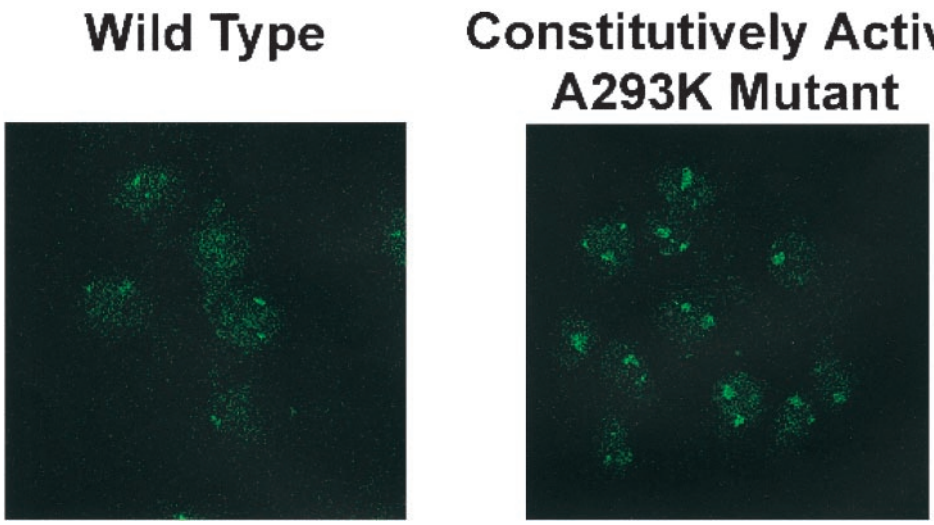

Fig. 6. Immunolocation of the wild-type α_{1B}-AR and a constitutively active mutant (A293K, Kjelsberg et al., 1992) of the α_{1B}-AR in transiently transfected COS 1 cells. Immunocytochemistry and laser scanning confocal microscopy were carried out as described in *Materials and Methods*.

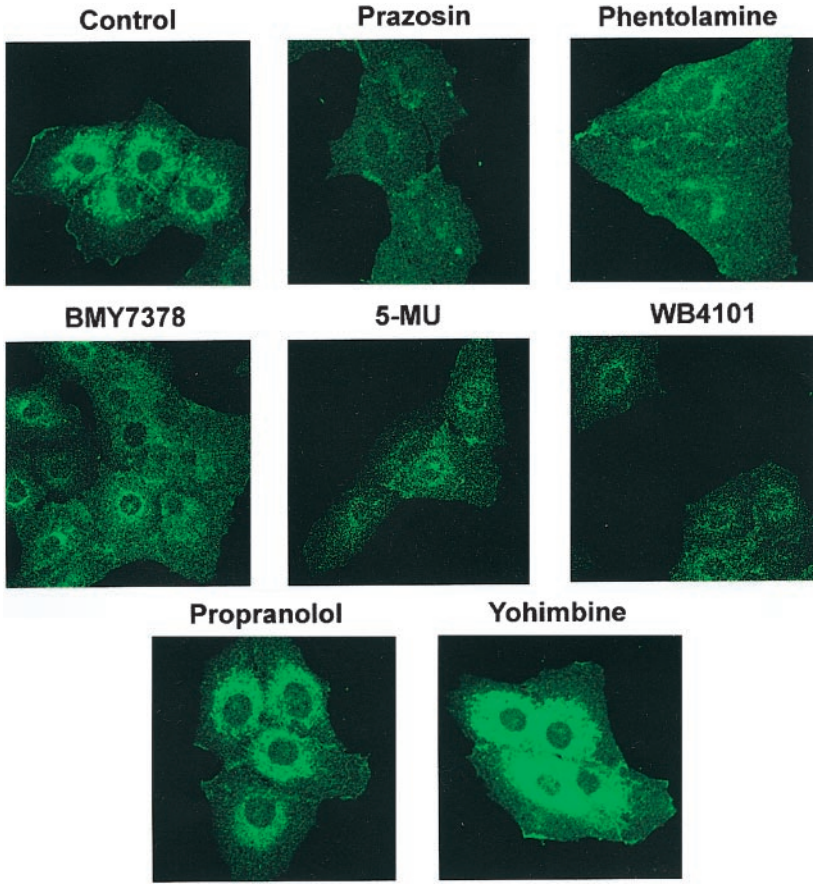

Fig. 7. Effect of antagonist compounds (1 μM) on the cellular localization of the α_{1D}-AR in stably transfected fibroblasts. Drug incubation, immunocytochemistry, and laser scanning confocal microscopy were performed as described in *Materials and Methods*.

expressing cells. Treatment of α_{1D}-AR-expressing fibroblasts with prazosin caused a significant degree of redistribution of the α_{1D}-AR from a perinuclear orientation to the cell periphery (Fig. 5, panel 1). After prazosin treatment, the α_{1D}-AR was no longer colocalized with either the transferrin receptor or arrestin 2 (see Fig. 5, panels 2 and 3). Treatment of fibroblasts expressing the α_{1D}-AR with prazosin also decreased basal levels of inositol phosphate formation and ERK activity (see Table 1). The cellular localization of a constitutively active mutant (A293K, Kjelsberg et al., 1992) of the α_{1B}-AR was assessed after transient transfection in COS 1 cells. Like the α_{1D}-AR, we detect a significant degree of α_{1B}-AR internalization in cells expressing the constitutively active mutant (Fig. 6). Therefore, receptor internalization in the absence of agonist is a property common to constitutively active α_1-ARs.

To determine whether the effects of prazosin on α_{1D}-AR immunolocalization were unique to this compound, we examined the effect of a series of antagonists on α_{1D} -AR expression. Rat 1 fibroblasts expressing the α_{1D}-AR were cultured for 24 h in the presence of phentolamine, BMY 7378, 5-methylurapidil, or WB 4101 (α_1-AR antagonists), the α_2-AR antagonist yohimbine, or the β-AR antagonist propranolol (see Fig. 7). Yohimbine and propranolol had no effect on the cellular localization of the α_{1D}-AR. Phentolamine and BMY 7378, which have been reported to have inverse agonist properties (Scheer et al., 1997; Garcia-Sainz and Torres-Padilla, 1999), induced redistribution of the α_{1D}-AR from internal compartments to the cell membrane. Phentolamine was as effective as prazosin in promoting α_{1D}-AR redistribution, whereas BMY 7378, WB 4101, and 5-methylurapidil were less effective than either phentolamine or prazosin at promoting α_{1D}-AR redistribution. Therefore, the ability to promote α_{1D}-AR redistribution is not unique to prazosin but is also manifested by other ligands that interact with this receptor. These data also support the notion that inverse agonists, like traditional agonists, differ in their intrinsic activity.

Taken together, these data are strong evidence that the α_{1D}-AR is constitutively active in fibroblasts. However, the data obtained in a stably transfected cell line may not accurately represent the localization and signaling characteristics of the α_{1D}-AR in cells that natively express these receptors such as in vascular smooth muscle cells. Figure 8 shows immunoreactivity of femoral artery smooth muscle cells immunostained with α_{1B}- and α_{1D}-AR antibodies. Similar to the immunoreactivity seen in fibroblasts, the α_{1D}-AR is localized in a perinuclear fashion in vascular smooth muscle cells. This similarity in immunolocalization suggests that the α_{1D}-AR may be constitutively active in cells that natively express all three α_1-AR subtypes, an area of investigation that we are actively pursuing.

The α_{1D}-AR has been somewhat of an enigma. It is not as well studied as the other α_1-AR subtypes. Theroux et al. (1996) showed that this receptor was weakly coupled to second messenger pathways. Yang et al. (1997) were unable to detect a significant degree of expression of the α_{1D}-AR in a variety of rat tissues. These findings are consistent with our observations that the receptor is predominantly expressed in intracellular compartments due to constitutive activity. As a result, the α_{1D}-AR is not as responsive to agonist activation as the other subtypes of the α_1-AR. We noted that phenyl-

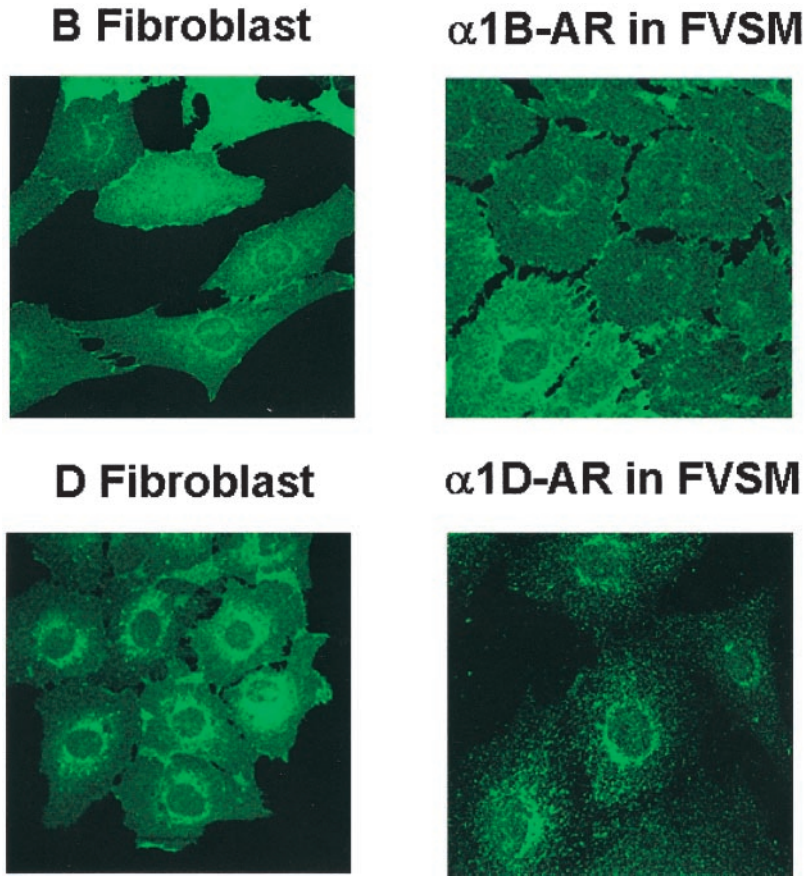

Fig. 8. Immunolocalization of the α_{1B}- and α_{1D}-ARs in cultured femoral artery smooth muscle cells (FVSM) and fibroblasts expressing either receptor subtype. Immunocytochemistry was carried out as described in *Materials and Methods*.

ephrine stimulated inositol phosphate accumulation but had no effect on ERK activity in α_{1D}-AR-expressing cells. This suggests that the effect of constitutive activity on functional responses differs depending on the second messenger/kinase being studied.

Studies with either transfected HEK 293 or SK-N-MC cells failed to demonstrate any constitutive function of the α_{1D}-AR (Theroux et al., 1996). The constitutive activity of the α_{1D}-AR we observe may be related to the level of α_{1D}-AR expression. In our studies, we note differences in cellular localization and signaling of the α_{1B}- and the α_{1D}-AR, even though these receptors are expressed at similar levels in the Rat 1 fibroblasts (5–10 pmol/mg protein), suggesting that the level of receptor expression is not sufficient to account for our results. Previous studies also using the stably transfected α_{1D}-AR subtype in Rat 1 fibroblasts showed constitutive activation of the calcium response (Garcia-Sainz and Torres-Padilla, 1999), a function linked to the production of IP_3.

The α_{1D}-AR also binds most agonists but not antagonists with higher affinity than the other two α_1-AR subtypes. This phenotype is a hallmark of constitutive activity based on the revised ternary complex model (Samama et al., 1993). Constitutive activity is also associated with constitutive phosphorylation and, therefore, desensitization. Current models of GPCR signaling indicate that the receptor is dephosphorylated and recycled back to the cell surface. The internalized pool of α_{1D}-ARs may represent the equilibrium component of the recycling process due to its constitutive nature.

In summary, we have shown that there are significant differences in the cellular expression of the α_1-ARs. The α_{1B}-AR exhibits characteristics of a typical GPCR as it is expressed on the cell surface, is subject to agonist-mediated internalization, and couples to second messenger/kinase activation. The results with the α_{1D}-AR are not consistent with traditional models of GPCR. This receptor is expressed mainly in intracellular compartments. The unstimulated α_{1D}-AR appears to be constitutively active and is subject to regulation by inverse agonists. In preliminary studies in vascular smooth muscle cells, we have observed that the α_{1D}-AR is localized in a perinuclear orientation similar to that seen in fibroblasts. These data argue that the cellular localization we report in this paper is not an epiphenomenon of experiments in fibroblasts but rather is a characteristic of this receptor. We are currently investigating the possibility that the α_{1D}-AR is constitutively active in cells natively expressing the receptor.

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References

Awaji T, Hirasawa A, Kataoka M, Shinoura H, Nakayama Y, Sugawara T, Izumi S and Tsujimoto G (1998) Real-time optical monitoring of ligand-mediated internalization of α_{1b}-adrenoceptor with green fluorescent protein. *Mol Endocrinol* **12:** 1099–1111.

Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG and Kobika BK (1988) Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc Natl Acad Sci USA* **85:**7159–7163.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG and Lefkowitz RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* **273:**685–688.

Daunt DA, Hurt C, Hein L, Kallio J, Feng F and Kobilka BK (1997) Subtype-specific intracellular trafficking of α2-adrenergic receptors. *Mol Pharmacol* **51:**711–720.

Eckhart AD, Zhu Z, Arendshorst WJ and Faber JE (1996) Oxygen modulates α_{1B}-adrenergic receptor gene expression by arterial but not venous vascular smooth muscle. *Am J Physiol* **271:**H1599-H1608.

Fonseca MI, Button DC and Brown RD (1995) Agonist regulation of α_{1B}-adrenergic receptor subcellular distribution and function. *J Biol Chem* **270:**8902–8909.

Garcia-Sainz JA and Torres-Padilla ME (1999) Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblasts stably expressing α_{1d}-adrenoceptors. *FEBS Lett* **443:**277–281.

Guarino RD, Perez DM and Piascik MT (1996) The α_1-adrenergic receptors. *Cell Signal* **8:**323–333.

Hirasawa A, Sugawara T, Awaji T, Tsumaya K, Ito H and Tsujimoto G (1997) Subtype-specific differences in subcellular localization of α1-adrenoceptors (ARs); Chloroethylclonidine preferentially alkylates the accessible surface α1-ARs irrespective of subtype. *Mol Pharmacol* **52:**764–770.

Hrometz SL, Edelmann SE, McCune DF, Olges JR, Hadley RW, Perez DM and Piascik MT (1999) Expression of multiple alpha1-adrenergic receptors on vascular smooth muscle: Correlation with the regulation of contraction. *J Pharmacol Exp Ther* **290:**452–463.

Ignatova EG, Belcheva MM, Bohn LM, Neuman MC and Cosica CJ (1999) Requirement of receptor internalization for opioid stimulation of mitogen-activated protein kinase: Biochemical and immunofluorescence confocal microscopic evidence. *J Neurosci* **19:**56–63.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Lefkowitz RJ (1992) Constitutive activation of the α1b-adrenergic receptor by all amino acid substitutions at a single site. *J Biol Chem* **267:**1430–1433.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.

Lomasney JW, Cotecchia S, Lorenz W, Leung W-Y, Schwinn DA, Yang-Feng TL, Brownstein M, Lefkowitz RJ and Caron MG (1991) Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor. *J Biol Chem* **266:**6365–6369.

Mhaouty-Kodja S, Barak LS, Scheer A, Abuin L, Diviani D, Caron MG and Cotecchia S (1999) Constitutively active α-1b adrenergic receptor mutants display different phosphorylation and internalization features. *Mol Pharmacol* **55:**339–347.

Perez DM, Piascik MT and Graham RM (1991) Solution-phase library screening for the identification of rare clones: Isolation of an α_{1D}-adrenergic receptor cDNA. *Mol Pharmacol* **40:**876–883.

Piascik MT, Guarino RD, Smith MS, Soltis EE, Saussy DL and Perez DM (1995) The specific contribution of the novel α-1D adrenoceptor to the contraction of vascular smooth muscle. *J Pharmacol Exp Ther* **275:**1583–1589.

Piascik MT, Hrometz SL, Edelmann SE, Guarino RD and Brown RD (1997) Immunocytochemical localization and contribution to vascular smooth muscle contraction of the α_{1B}-adrenergic receptor: Analysis with selective ligands and antisense oligonucleotides. *J Pharmacol Exp Ther* **283:**854–868.

Piascik MT, Smith MS, Soltis EE and Perez DM (1994) Identification of the mRNA for the novel α_{1D} and two other α_1-adrenoceptors in vascular smooth muscle. *Mol Pharmacol* **46:**30–40.

Ping P and Faber JE (1993) Characterization of α-adrenoceptor gene expression in arterial and venous smooth muscle. *Am J Physiol* **265:**H1501-H1509.

Post GR, Goldstein D, Thuerauf DJ, Glembotski CC and Brown JH (1996) Dissociation of p44 and p42 mitogen-activated protein kinase activation from receptor-induced hypertrophy in neonatal rat ventricular myocytes. *J Biol Chem* **271:**8452–8457.

Samama P, Cotecchia S, Costa T and Lefkowitz R (1993) A mutation-induced activated state of the β_2-adrenergic receptor. *J Biol Chem* **268:**4625–4636.

Scheer A, Fanelli F, Costa T, De Benedetti PG and Cotecchia S (1997) The activation process of the α_{1B}-adrenergic receptor: Potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc Natl Acad Sci USA* **94:**808–813.

Schwinn DA, Lomasney JN, Loreng W, Szklut PJ, Fremeau RT, Yang-Feng TL, Caron MG, Lefkowitz RJ and Cotecchia S (1990) Molecular cloning and expression of cDNA for a novel α-adrenergic receptor subtype. *J Biol Chem* **265:**8183–8189.

Theroux TL, Esbenshade TA, Peavy RD and Minneman KP (1996) Coupling efficiencies of human α_1-adrenergic receptor subtypes: Titration of receptor density and responsiveness with inducible and repressible expression vectors. *Mol Pharmacol* **50:**1376–1387.

von Zastrow M and Kobilka BK (1992) Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J Biol Chem* **267:**3530–3538.

von Zastrow M, Link R, Daunt D, Barsh G and Kobilka B (1993) Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. *J Biol Chem* **268:**763–766.

Yang M, Verfurth F, Buscher R and Michel MC (1997) Is alpha1D-adrenoceptor protein detectable in rat tissue? *Naunyn Schmiedebergs Arch Pharmacol* **355**:438–446.

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