

## Full-length article

# Internalization and distribution of three $\alpha_1$ -adrenoceptor subtypes in HEK293A cells before and after agonist stimulation<sup>1</sup>

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## Key words

adrenoceptor; agonist stimulation; confocal image; enzyme-linked immunosorbent assay; [<sup>3</sup>H]-prazosin binding assay; internalization; subcellular distribution; subtype

<sup>1</sup> Project supported by the National Key Basic Research Program of the People's Republic of China (No. G2000056906) and the National Natural Science Foundation of China (No. 30490172, 30171083).

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Received 2006-08-11

Accepted 2006-10-13

doi:10.1111/j.1745-7254.2007.00509.x

## Abstract

**Aim:** To examine the subcellular distribution of the 3  $\alpha_1$ -adrenoceptor ( $\alpha_1$ -AR) subtypes and their internalization and trafficking upon agonist stimulation in human embryonic kidney 293A cells. **Methods:** Confocal real-time imaging, enzyme linked immunosorbent assay (ELISA) and whole cell [<sup>3</sup>H]-prazosin binding assay were applied to detect the distribution and localization of the 3  $\alpha_1$ -AR subtypes. **Results:**  $\alpha_{1A}$ -AR was found both on the cell surface and in the cytoplasm;  $\alpha_{1B}$ -AR, however, was predominantly detected on the cell surface, while  $\alpha_{1D}$ -AR was detected mainly in the intracellular compartments. After stimulation with phenylephrine, localization changes were detected by confocal microscopy for  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR, but the localization of  $\alpha_{1D}$ -AR were unaffected. Phenylephrine stimulation promoted a more rapid internalization of  $\alpha_{1B}$ -AR than  $\alpha_{1A}$ -AR.  $\alpha_{1D}$ -AR internalization was detected only by ELISA. Whole cell [<sup>3</sup>H]-prazosin binding assay showed that  $\alpha_{1A}$ -AR functional receptors were detected both on the cell surface and in the cytoplasm;  $\alpha_{1B}$ -AR, however, were detected predominantly on the cell surface, while  $\alpha_{1D}$ -AR were detected mainly in intracellular compartments. Phenylephrine stimulation promoted internalization of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR. **Conclusion:** Phenylephrine stimulation induced changes in the localization of the 3  $\alpha_1$ -AR.

## Introduction

$\alpha_1$ -adrenoceptors ( $\alpha_1$ -AR) are G<sub>q/11</sub>-coupled receptors that respond to the neurotransmitters and hormones norepinephrine and epinephrine to mediate physiological effects such as prostate smooth muscle contraction and myocardial hypertrophy by activating phospholipase C and generating second messengers that release stored intracellular Ca<sup>2+</sup> and stimulate protein kinase C<sup>[1,2]</sup>.  $\alpha_1$ -AR are also involved in the regulation of growth-promoting responses via the mitogen-activated protein kinase family<sup>[3]</sup>.

Three human  $\alpha_1$ -AR subtypes have been cloned:  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR. Although  $\alpha_1$ -AR family members are highly homologous (eg 75% amino acid identity in transmembrane domains), they share little homology at their amino and carboxyl termini<sup>[3,4]</sup>, suggesting that  $\alpha_1$ -AR subtypes have different expression, function, and subcellular distribution.

$\alpha_1$ -AR expression was found in many human tissues such as the brain, heart, and vascular smooth muscles<sup>[5]</sup>. Several reports have shown that  $\alpha_{1B}$ -AR was mainly localized on the cell surface<sup>[6-9]</sup>, and agonists induced its phosphorylation and internalization. G-protein-coupled receptors (GPCR), kinases 2 and 3, seem to be involved in the phosphorylation of agonist-bound  $\alpha_{1B}$ -AR during homologous desensitization<sup>[10,11]</sup>. The phosphorylation sites involved have been located at Ser404, Ser408, and Ser440 in the receptor C terminus<sup>[11]</sup>. Several researchers found that  $\alpha_{1A}$ -AR was mainly located on the cell surface and the intracellular compartments<sup>[9]</sup>. However,  $\alpha_{1D}$ -AR was mainly localized in the intracellular compartments<sup>[12]</sup>, but could be induced to translocate to the cell surface by co-expressed  $\alpha_{1B}$ -AR<sup>[13]</sup>. Unfortunately, the molecular determinants of desensitization and internalization for  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR were largely unknown. Vazquez-Prado *et al*<sup>[14]</sup> showed that  $\alpha_{1A}$ -AR could undergo

agonist-mediated phosphorylation, but not to the same extent as  $\alpha_{1B}$ -AR. Yang and colleagues<sup>[15]</sup> stably transfected fibroblasts with each  $\alpha_1$ -AR and observed that increases in inositol phosphates mediated by  $\alpha_{1A}$  and  $\alpha_{1B}$ -AR could be desensitized, whereas the increase mediated by  $\alpha_{1D}$ -AR was refractory to agonist-mediated desensitization. In contrast, Garcia-Sainz *et al*<sup>[16]</sup> found that  $\alpha_{1D}$ -AR could be phosphorylated and desensitized. Recent reports showed that cell trafficking (desensitization and redistribution) was an important step in the regulation of GPCR, particularly in response to receptor stimulation by agonists. The ultimate effect on receptor signaling and the fate of sequestered receptors varies with receptor type, duration of agonist exposure, and cellular environment<sup>[17]</sup>. In this study, we examined subcellular distribution of the 3  $\alpha_1$ -AR subtypes and their internalization and trafficking upon agonist stimulation in human embryonic kidney (HEK) 293A cells. We also compared the distribution and trafficking property between total receptors and functional receptors.

## Materials and methods

Flag-tagged human  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR were gifts from Prof KP MINNEMAN (Emory University, Atlanta, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Rockville, MD, USA). Geneticin, an anti-flag antibody, and phenylephrine were obtained from Sigma-Aldrich (St Louis, MO, USA). [<sup>125</sup>I]-BE2254 and [<sup>3</sup>H]-prazosin were obtained from Amersham Biosciences (Buckinghamshire, UK).  $\alpha_1$ -AR green fluorescent protein (GFP) vectors were constructed by ligating the coding region of human  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR into the *EcoR* I–*BamH* I site of the basic pEGFP-C2 protein fusion vector (Clontech, USA) as described previously<sup>[7]</sup>.

**Cell culture and transfection** HEK293A cells were cultured in DMEM with sodium pyruvate supplemented with 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere with 5% CO<sub>2</sub>. Cells for transient transfection were grown in culture dishes with a glass coverslip. When grown to approximately 60% confluence, cells were transfected with 5  $\mu$ g cDNA encoding  $\alpha_{1A}$ -,  $\alpha_{1B}$ -,  $\alpha_{1D}$ -AR/GFP fusion protein using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. Cell lines stably transfected with  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR/Flag, which were cloned and established before<sup>[18]</sup>, were grown on 6-well plates and maintained with geneticin (200 mg/mL). As previously described, the  $B_{max}$  of  $\alpha_{1A}$ -AR/Flag,  $\alpha_{1B}$ -AR/Flag, and  $\alpha_{1D}$ -AR/Flag were 4.8, 20.8, and 0.24 pmol/mg protein,

respectively, on cell membranes as determined by [<sup>125</sup>I]-BE2254 binding assay<sup>[18]</sup>.

**Western blot assay** HEK293A cells transiently expressing  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, or  $\alpha_{1D}$ -AR were treated with phenylephrine (10  $\mu$ mol/L) or prazosin and phenylephrine or vehicle. Cells were lysed with ice in cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L edetic acid, 50 mmol/L NaF, 0.1 mmol/L Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1% TritonX-100, 10% glycerol, 0.1% SDS, 1% deoxycholic acid, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mg/mL aprotinin for 20 min, sonicated for 20 s, and centrifuged at 12 870×g for 15 min. The supernatant is the whole cell protein. The lysate [30  $\mu$ g, extracellular signal-related kinase (ERK) assay] was separated by electrophoresis using 10% SDS-PAGE and transferred onto nitrocellulose membrane or polyvinylidene difluoride membrane. Nonspecific IgG was blocked with 5% fat-free milk, and the membrane was incubated with the antibody to phospho-ERK (Cell Signaling Technology, Inc, Beverly, MA, USA); horseradish peroxidase-conjugated anti-mouse IgG was used as a second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The peroxidase reaction products were visualized by LumiGLO Chemiluminescent Substrate (New England Biolabs, Beverly, MA, USA). The same membrane was then stripped and reprobed with anti-ERK to determine the total protein.

**Confocal microscopy and image quantification** HEK293A cells transiently transfected with GFP-tagged construct were grown on sterile coverslips, fixed for 30 min with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and rinsed 3 times with PBS. Cells were imaged with a Spectra-Physics laser scanning confocal microscope with a Plan-Apo 40× oil immersion objective lens (Leica, Wetzlar, Germany). The software used to collect the images was the Leica TCS NT, version 1.6.587. The images were transferred to a computer and analyzed with Adobe Photoshop version 5.0 (Adobe Systems, Mountain View, CA, USA). The setting on the laser was constant for all experiments. Fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected at 510–520 nm. The extent to which the  $\alpha_1$ -AR/GFP was internalized after exposure to agonists was quantified by the intracellular immunofluorescence intensity. The extent of  $\alpha_1$ -AR/GFP receptor internalization was defined as the rate of the fluorescence intensity before and after exposure to agonists.

**Internalization assay** The HEK293A cells stably expressing  $\alpha_1$ -AR were seeded at a density of 2×10<sup>5</sup>/well onto poly-L-lysine-coated 24-well plates. The next day, the cells were pre-incubated with 1  $\mu$ g of mouse anti-flag antibody for 1 h

in DMEM free of serum at 37 °C and washed twice using serum-free DMEM. Cells were then treated with 10 μmol/L phenylephrine for different time periods in the serum-free DMEM. Subsequently, the cells were fixed and incubated with peroxidase-conjugated anti-mouse antibody (1:1000, Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the plates were developed with 250 μL of o-phenylenediamine dihydrochloride (OPD) solution (4 mg OPD in 10 mL PBS, 15 μL 30% H<sub>2</sub>O<sub>2</sub>). After 10–30 min, 200 μL of the substrate solution from each well was transferred to 96-well plates and analyzed at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

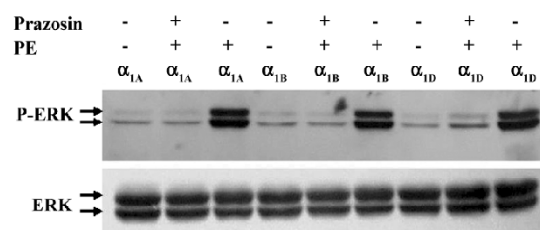
**Whole cell binding assay** To further determine the sub-cellular distribution of the 3 α<sub>1</sub>-AR subtypes prior to, or after incubation with phenylephrine for 1 h, radioligand binding was performed on whole cells stably transfected with the flag-tagged 3 α<sub>1</sub>-AR constructs as described by Eason MG et al<sup>[19]</sup>. The cells were re-suspended in ice-cold DMEM medium, then incubated with increasing concentrations (0.01–2 nmol/L) of [<sup>3</sup>H]-prazosin alone, or in the presence of the lipophilic competitor, phentolamine (10 μmol/L), or in the presence of the hydrophilic competitor, adrenaline (100 mmol/L), for 4 h at 4 °C. With the intact cells, specific binding, as defined with phentolamine, represented the total cellular amount of α<sub>1</sub>-AR, because it is able to penetrate the plasma membrane and scatter about the whole cell, whereas specific binding, as defined with adrenaline, represents only cell surface receptors owing to its hydrophilic properties, that is, adrenaline is unable to penetrate the plasma membrane, especially at 4 °C. The intracellular pool of receptors is defined as the total number of receptors minus those on the cell surface. Saturation and displacement curves were analyzed by non-linear regression analysis with Prism (GraphPad Software, San Diego, CA, USA).

**Statistical analysis** Data from confocal quantity were analyzed using the unpaired t-test with statistical significance defined as P<0.05. Data from Western and internalization assays were analyzed by GraphPad Prism using the 2-tailed paired t-test. Significant differences were defined as P<0.05.

## Results

**ERK 1/2 were activated by phenylephrine in the HEK293A cells transiently transfected with α<sub>1</sub>-AR/GFP fusion protein** To demonstrate that the α<sub>1</sub>-AR/GFP fusion proteins expressed in HEK293A cells were functional, the HEK293A cells transfected with α<sub>1A</sub>-, α<sub>1B</sub>-, and α<sub>1D</sub>-AR/GFP, respectively, were treated with phenylephrine, and the

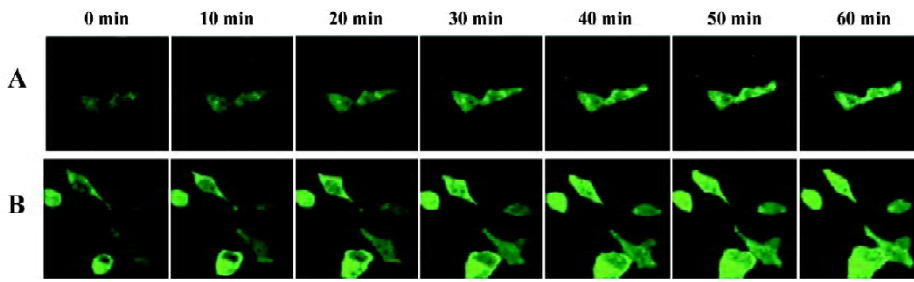
phosphorylation of ERK 1/2 was measured by Western blot. The levels of the phospho-ERK1/2 in HEK293A cells transfected with α<sub>1A</sub>-, α<sub>1B</sub>-, or α<sub>1D</sub>-AR/GFP significantly increased after treatment with phenylephrine (PE, 10 μmol/L), an agonist of α<sub>1</sub>-AR, for 30 min. Pretreatment with prazosin (1 μmol/L), an antagonist of α<sub>1</sub>-AR, however, greatly attenuated the effects of phenylephrine on the activation of ERK1/2 in the transfected HEK293A cells. These results suggested that the α<sub>1</sub>-AR/GFP fusion proteins expressed in the HEK293A cells were biologically active (Figure 1).



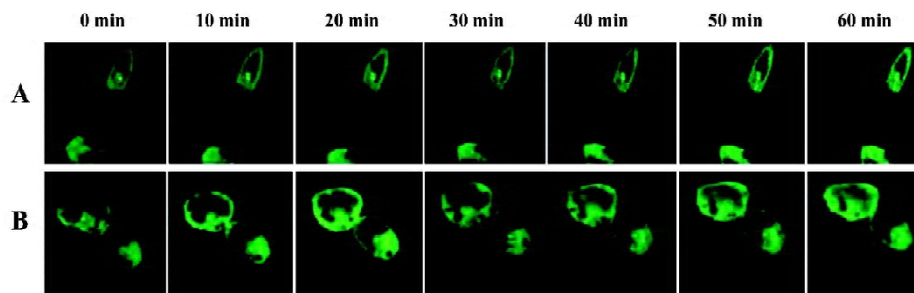
**Figure 1.** Activation of ERK1/2 phosphorylation. HEK293A cells transiently expressing the 3 α<sub>1</sub>-AR subtypes were pretreated with or without 1 μmol/L prazosin for 30 min and stimulated with phenylephrine (PE) for 30 min. Whole cell lysates were immunoblotted with anti-phospho-ERK. Membranes were stripped and reprobed using anti-ERK antibody. Three separate experiments showed similar results. <sup>b</sup>P<0.05 vs control.

**Agonist-induced localization changes of α<sub>1</sub>-AR subtypes in HEK293A cells** By real-time confocal microscopy, we found that the incubation of HEK293A cells transiently expressing α<sub>1A</sub>- or α<sub>1B</sub>-AR/GFP with 10 μmol/L phenylephrine caused changes of receptor localization (Figures 2, 3). An accumulation of α<sub>1</sub>-AR in vesicle-like structures was found in the cytoplasm, and the membrane receptors became uneven. Because coated vesicles are the prelude of GPCR internalization by agonist stimulation, these results suggested that agonist stimulation could induce α<sub>1A</sub>- and α<sub>1B</sub>-AR internalization. The receptor localization in cells expressing α<sub>1D</sub>-AR, however, was unchanged after phenylephrine stimulation (Figure 4).

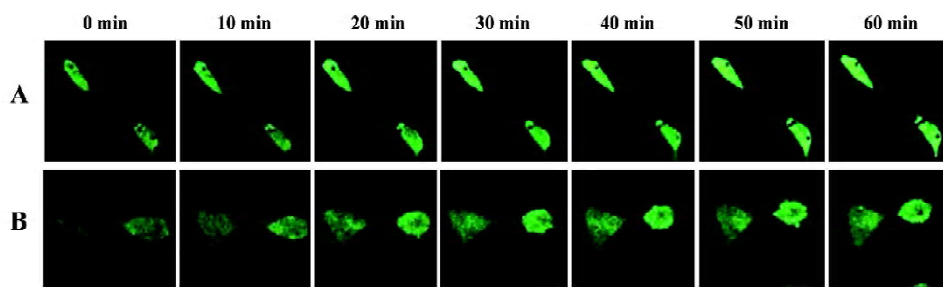
We then measured the signal intensity of intracellular fluorescence to estimate the rate of receptor internalization. The fluorescent intensity of α<sub>1A</sub>- and α<sub>1B</sub>-AR was significantly increased in the cytoplasm after phenylephrine stimulation, and α<sub>1B</sub>-AR occurred in a very rapid manner (20 min after phenylephrine stimulation, Figure 5A), while α<sub>1A</sub>-AR occurred in a slower manner (25 min, Figure 5B). HEK293A cells expressing α<sub>1D</sub>-AR, however, showed no translocation of the receptors on the cell surface after phenylephrine stimulation (Figure 5C). These results indicated that the location



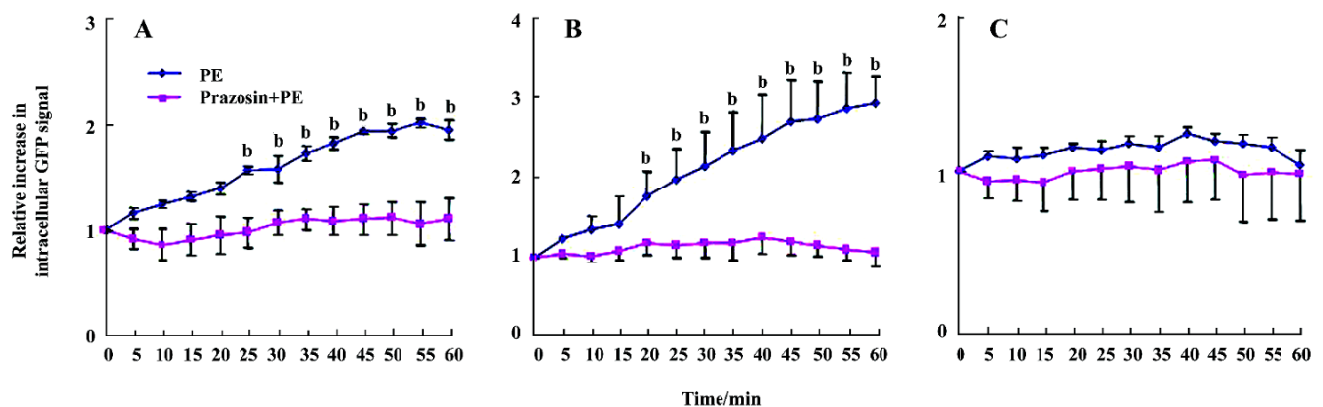
**Figure 2.** Effects of 10  $\mu\text{mol/L}$  phenylephrine (PE) on the cellular localization of  $\alpha_{1A}$ -AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) prazosin+PE; (B) PE alone. Magnification $\times 600$ .



**Figure 3.** Effects of 10  $\mu\text{mol/L}$  phenylephrine (PE) on the cellular localization of  $\alpha_{1B}$ -AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at the different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) prazosin+PE; (B) PE alone. Magnification $\times 600$ .



**Figure 4.** Effects of 10  $\mu\text{mol/L}$  phenylephrine (PE) on the cellular localization of  $\alpha_{1D}$ -AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) Prazosin+PE; (B) PE alone. Magnification  $\times 600$ .



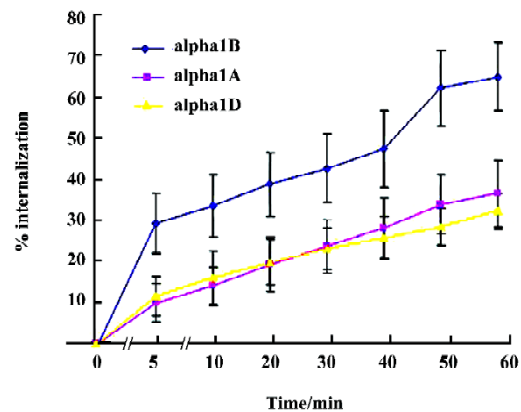
**Figure 5.** Comparison of the effect of 10  $\mu\text{mol/L}$  phenylephrine (PE) on changes of intracellular fluorescence intensity in cells transiently transfected with the  $\alpha_{1A}$ -AR/GFP or  $\alpha_{1B}$ -AR/GFP or  $\alpha_{1D}$ -AR/GFP with or without prazosin. (A) relative fluorescence intensity of  $\alpha_{1A}$ -AR; (B) relative fluorescence intensity of  $\alpha_{1B}$ -AR; (C) relative fluorescence intensity of  $\alpha_{1D}$ -AR. Data represent the mean and standard error of the mean values of 3–5 independent experiments. <sup>b</sup> $P < 0.05$  vs prazosin and PE cotreatment.

changes of the receptors after phenylephrine stimulation were due to receptor internalization.

Pretreatment with prazosin prior to phenylephrine stimulation inhibited  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR translocation and decreased the fluorescent intensity of cytoplasmic receptors in the transfected HEK293A cells (Figure 5), suggesting that phenylephrine acted directly on  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR to induce receptor translocation, a phenomenon called homologous desensitization or internalization.

**$\alpha_1$ -AR internalization induced by phenylephrine in HEK293A cells** We next examined the effects of phenylephrine stimulation on cell surface receptor internalization using HEK293A cells stably expressing  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, or  $\alpha_{1D}$ -AR by intact cell enzyme-linked immunosorbent assay (ELISA). Five minutes after phenylephrine stimulation, the amounts of  $\alpha_{1B}$ -,  $\alpha_{1A}$ -, and  $\alpha_{1D}$ -AR on the cell surface decreased by about 29.2%, 9.7%, and 11.3%, respectively. One hour after the stimulation, they further decreased by about 64.8%, 36.4%, and 32.1%. These results indicated that phenylephrine could induce the internalization of  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR, and the internalization of  $\alpha_{1B}$ -AR occurred faster than that of  $\alpha_{1A}$ - and  $\alpha_{1D}$ -AR (Figure 6).

**Agonist-induced distribution changes in binding of functional  $\alpha_1$ -AR subtypes in HEK293A cells** To test whether membrane and cytoplasm functional receptors changed under phenylephrine stimulation, whole cell [<sup>3</sup>H]-prazosin binding assay was used. Functional  $\alpha_{1B}$ -AR was mainly expressed on the surface of transfected HEK293A cells, which was about 92.4% of the total receptor.  $\alpha_{1D}$ -AR was mainly expressed in the cytoplasm, about 69.5% of total receptor, and  $\alpha_{1A}$ -AR was expressed both on the cell surface and in the cytoplasm, which was consistent with the results of the confocal analysis. One hour after phenylephrine stimulation, functional  $\alpha_{1D}$ -AR did not change compared with the control.  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR on the cell surface, however, decreased significantly:  $\alpha_{1A}$ -AR from 53.7% to 35.5% of the total receptor, and  $\alpha_{1B}$ -AR from 92.4% to 30.2% of the total receptor, which suggested that phenylephrine stimulation



**Figure 6.** Quantitative analysis of agonist-induced internalization of  $\alpha_{1A}$ -AR or  $\alpha_{1B}$ -AR or  $\alpha_{1D}$ -AR stably expressed in HEK293A cells by ELISA. Percentage of loss of cell surface receptor at different times in phenylephrine (PE)-treated cells. *n*=4 independent experiments. Mean±SEM.

could induce the internalization of functional  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR proteins (Table 1).

**Discussion**

In this report, we investigated the trafficking profile of the 3  $\alpha_1$ -AR. We created a fusion construct consisting of N-terminal GFP-tagged  $\alpha_1$ -AR and transfected this construct into HEK293A cells, which allowed us not only to identify the 3 subtype receptors by confocal microscopy, but also to compare the surface and total receptor by intact cell ELISA and [<sup>3</sup>H]-prazosin binding assay before and after phenylephrine stimulation.

As described in the Methods and in our previous work, the *B*<sub>max</sub> of  $\alpha_{1B}$ -AR on the membrane was found to be the highest, followed by  $\alpha_{1A}$ -AR and  $\alpha_{1D}$ -AR, respectively<sup>[18]</sup>. These results are consistent with the previous report in which the functional binding of wild type  $\alpha_1$ -AR was examined<sup>[20]</sup>.

Previous studies with the  $\alpha_{1B}$ -AR/GFP construct demonstrated that it was fully functional and internalized in the

**Table 1.** Effect of phenylephrine (PE) on the localization of functional  $\alpha_1$ -AR subtypes in whole cell, cell surface, and cytoplasm of transfected HEK293A using [<sup>3</sup>H]-prazosin binding assay. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05 compared with control.

	Total receptors		<i>B</i> <sub>max</sub> (fmol per 1×10 <sup>6</sup> cells)		Intracellular receptor	
	Control	PE	Control	PE	Control	PE
$\alpha_{1A}$	763±239	2251±26	1483±300	798±133 <sup>b</sup>	1280±72	1453±108
$\alpha_{1B}$	3232±391	3781±434	2986±396	1140±325 <sup>b</sup>	246±141	2641±511
$\alpha_{1D}$	3324±513	3165±286	1048±358	1091±108	2276±160	2074±370

same manner as a non-GFP tagged  $\alpha_{1B}$ -AR construct<sup>[21]</sup>. Both the  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR were associated with the activation of ERK<sup>[22]</sup>. In this study, all the  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -AR, when coupled to GFP, promoted the increase in ERK1/2 phosphorylation, suggesting that the  $\alpha_1$ -AR were functional and retained their ability to activate cellular signaling when conjugated to the GFP.

Cellular localization and trafficking is very important for  $\alpha_1$ -AR to accomplish their physiological functions. Results obtained over the past years have shown that there is a significant difference in the subcellular distribution among the  $\alpha_1$ -AR subtypes. Fonseca *et al*<sup>[6]</sup> observed that  $\alpha_{1B}$ -AR was expressed predominantly on the cell membrane and  $\alpha_{1A}$ -AR was expressed both on the membrane and cytoplasm in the stably-transfected fibroblasts by immunocytochemistry. This was confirmed by Awaji *et al*<sup>[21]</sup> who used  $\alpha_{1B}$ -AR/GFP fusion proteins to verify cell membrane localization of  $\alpha_{1B}$ -AR in COS-7 cells. McGrath *et al*<sup>[23]</sup> used BODIPY-FL-labeled prazosin to image  $\alpha_1$ -AR subtypes in cultured prostate smooth muscle cells and fibroblasts stably transfected with each subtype and found that the 3  $\alpha_1$ -AR subtypes were expressed in the cytoplasm of fibroblasts. Very little  $\alpha_{1D}$ -AR expression was detected on the cell surface. In this study, we transfected HEK293A cells with cDNA encoding  $\alpha_1$ -AR/GFP. Living cells were then visualized by real-time laser scanning confocal microscopy. We found that  $\alpha_{1B}$ -AR/GFP fluorescence was predominant on the cell surface, whereas the  $\alpha_{1A}$ -AR expression was detected both on the cell surface and in the cytoplasm.  $\alpha_{1D}$ -AR was mainly localized in intracellular compartments, suggesting that the localization of the 3  $\alpha_1$ -AR subtypes was different in the transfected HEK293A cells.

$\alpha_1$ -AR are subject to dynamic regulation by a variety of mechanisms, including phosphorylation, protein-protein interactions, protein trafficking, and transcription. One of the most intensive studies of these mechanisms is internalization and desensitization, a general phenomenon in which the intensity of a biological response wanes over time, despite continued stimulus. Desensitization can be further characterized as either homologous, where receptor response wanes upon continuous exposure to its agonist, or heterologous, where agonist-mediated stimulation of a receptor can attenuate the response by other receptors mediating similar cellular events<sup>[24]</sup>. With regard to the  $\alpha_1$ -AR subtypes, the desensitization, downregulation, and internalization characteristics of the  $\alpha_{1B}$ -AR have been extensively examined. For example, agonist-mediated phosphorylation and internalization of the  $\alpha_{1B}$ -AR have been demonstrated, and the domains of the receptors involved in internalization have

been identified<sup>[6,10,11]</sup>. We know much less about the molecular determinants of desensitization, downregulation, and internalization for  $\alpha_{1A}$ - and  $\alpha_{1D}$ -AR. Here, we transfected HEK293A cells with cDNA encoding  $\alpha$ -AR/GFP. Living cells were then visualized by real time laser scanning confocal microscopy under phenylephrine stimulation. Stimulation of  $\alpha_1$ -AR with phenylephrine induced the changes of localization of  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR, but not the localization of  $\alpha_{1D}$ -AR.

Confocal image quantification analysis found that stimulation of  $\alpha_{1B}$ -AR with phenylephrine promoted a rapid internalization of  $\alpha_{1B}$ -AR, which began at about 20 min, and a slower internalization of  $\alpha_{1A}$ -AR, which began at about 25 min. However,  $\alpha_{1D}$ -AR internalization did not occur after phenylephrine stimulation. We only assayed the changes of fluorescence intensity of the plasma, but  $\alpha_{1D}$ -AR was mainly localized in intracellular compartments. Therefore, this method can not determine whether  $\alpha_{1D}$ -AR was internalized under phenylephrine stimulation.

To confirm the results of confocal microscopy, the internalization of membrane receptors was detected by intact cell ELISA. This does not change the permeability of cell membrane, so the antibodies to  $\alpha_1$ -AR subtypes only bind to the membrane surface of stably-transfected HEK293A cells. The internalization percentage of  $\alpha_{1A}$ -AR was about 9.7% at 5 min and about 36.4% at 1 h after phenylephrine stimulation. The internalization percentage of  $\alpha_{1B}$ -AR was about 29.2% at 5 min and about 64.8% at 1 h after phenylephrine stimulation. Intact cell ELISA assay also demonstrated  $\alpha_{1D}$ -AR internalization after phenylephrine stimulation. The internalization percentage of  $\alpha_{1D}$ -AR was about 11.3% at 5 min and about 32.1% at 1 h after phenylephrine stimulation. Although Garcia-Sainz *et al*<sup>[22]</sup> showed that  $\alpha_{1D}$ -AR could be phosphorylated and internalized, many other studies reported that  $\alpha_{1D}$ -AR could not be internalized under agonist stimulation. Our results by ELISA are the same as Garcia-Sainz *et al*. As mentioned earlier, cell-intact ELISA only detects the receptors located on the outside surface of the membrane, but confocal imaging analysis mainly assesses the changes of fluorescence intensity of the plasma. When internalization occurs,  $\alpha_{1D}$ -AR may just move from the outside to the inside of the membrane. Although some  $\alpha_{1D}$ -AR may translocate from the membrane to the plasma, the internalized  $\alpha_{1D}$ -AR are negligible compared with the total  $\alpha_{1D}$ -AR because most  $\alpha_{1D}$ -AR are expressed in the plasma. This may explain why  $\alpha_{1D}$ -AR internalization can be detected by ELISA rather than confocal imaging analysis. However, further studies are needed to elucidate this discrepancy.

The confocal imaging and ELISA analyses demonstrated

the localization changes and internalization of the 3  $\alpha_1$ -AR subtypes. Receptor proteins include receptors with binding activity and receptors without binding activity. Past studies generally did not differentiate these 2 categories. In this study, we detected the  $B_{\max}$  of the total cell surface and intracellular functional  $\alpha_1$ -AR by whole cell [ $^3\text{H}$ ]-prazosin binding assay. We found that the total  $B_{\max}$  were the same in the 3 subtypes, and not different before and after phenylephrine stimulation.  $\alpha_{1A}$  functional receptors were not only on the cell surface, but also in intracellular compartments.  $\alpha_{1B}$  functional receptors were predominantly on the cell surface;  $\alpha_{1D}$  functional receptors were mainly in intracellular compartments. In general, AR are G-protein-coupled membrane receptors. From the previous point of view, only the receptors on the membrane have the binding function, whereas the receptors in the cytoplasm are immature and do not have binding ability. However, the present study demonstrated that there were functional receptors in the cytoplasm. Stimulation of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR with phenylephrine for 1 h promoted internalization of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR, but not  $\alpha_{1D}$ -AR. This apparent discrepancy in  $\alpha_{1D}$ -AR internalization, as determined by different methods, may be partly explained by the different observed parameters. Both confocal imaging and internalization assay determine receptor protein, whereas whole cell binding assay determines functional receptors. Since only a few  $\alpha_{1D}$ -AR are located on the cell surface, in order to maintain the reactivity as strongly as possible, most of them are probably functional receptors. On the contrary, since both  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR on the cell surface are quite abundant, the amount of their functional receptors would be far more than that of  $\alpha_{1D}$ -AR, even though their functional receptors may only account for a small fraction of the whole receptors. It is supposed that when internalization occurs, there would be as many mature functional  $\alpha_{1D}$ -AR as possible sorted on the cell surface to complement the internalized ones in order to maintain the maximal reactivity. However, even if quite a lot of functional  $\alpha_{1A}$ - or  $\alpha_{1B}$ -AR were internalized upon phenylephrine stimulation, the residual functional receptors on the cell surface would be enough to maintain the same reactivity as before, so it would not be necessary to sort the mature functional receptors on the cell membrane. Therefore, internalization could only be observed on  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR, but not  $\alpha_{1D}$ -AR by confocal imaging. However, the exact mechanism remains to be explored.

These results also explain why the total amount of the 3  $\alpha_1$ -AR subtypes was equal by whole cell [ $^3\text{H}$ ]-prazosin binding assay, while their densities on the membrane were quite different when detected by traditional [ $^{125}\text{I}$ ]-BE2254 binding assay. Because [ $^{125}\text{I}$ ]-BE2254 binding assay only reflected

the receptors on the membrane, a part of the whole cell receptors, [ $^3\text{H}$ ]-prazosin binding assay reflected the total amount of receptors, including both receptors on the cell membrane and those in the cytoplasm. The discrepancy between the 2 methods offers evidence that the 3  $\alpha_1$ -AR subtypes have different distribution, localization, and trafficking characteristics, which is consistent with that examined by other methods in this study.

In summary, in transfected HEK293A cells,  $\alpha_{1A}$ -AR evenly distribute in the cytoplasm and membranes, but phenylephrine stimulation causes their internalization.  $\alpha_{1B}$ -AR are mainly located on the membrane, however, phenylephrine can result in rapid internalization, and the number of internalized  $\alpha_{1B}$ -AR is more than  $\alpha_{1A}$ -AR.  $\alpha_{1D}$ -AR predominantly exist in the cytoplasm, and their internalization upon phenylephrine stimulation could be detected only by intact-cell ELISA, but not by other methods. Therefore, a definitive answer regarding the internalization characteristics of  $\alpha_{1D}$ -AR requires additional study. Furthermore, we compared the distribution between functional receptors and total receptor proteins. Because there were functional receptors in the cytoplasm, we suggested that the receptors in the plasma act as a receptor's storage pool.

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