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Differences in the Cellular Localization and Agonist-Mediated Internalization Properties of the α_1-Adrenoceptor Subtypes

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ABSTRACT

The cellular localization, agonist-mediated internalization, and desensitization properties of the α_1-adrenoceptor (α_1-AR) subtypes conjugated with green fluorescent protein (α_1-AR/GFP) were assessed using real-time imaging of living, transiently transfected human embryonic kidney (HEK) 293 cells. The α_{1B}-AR/GFP fluorescence was detected predominantly on the cell surface. Stimulation of the α_{1B}-AR with phenylephrine led to an increase in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and promoted rapid α_{1B}-AR/GFP internalization. Long-term exposure (15 h) to phenylephrine resulted in desensitization of the α_{1B}-AR-mediated activation of ERK1/2 phosphorylation. α_{1A}-AR/GFP fluorescence was detected not only on the cell surface but also intracellularly. The rate of internalization of the cell surface population α_{1A}-AR/GFPs was slower than that seen for the α_{1B}-AR. Agonist exposure also resulted in desensitization of the α_{1A}-AR-mediated increase in ERK1/2 phosphorylation. The α_{1D}-AR/GFP fluorescence was detected mainly intracellularly, and this localization was unaffected by exposure to phenylephrine. Phenylephrine treatment of α_{1D}-AR/GFP expressing cells increased ERK1/2 phosphorylation. However, this increase was not significant. Cotransfection with β-arrestin 1 did not increase the rate or extent of agonist-stimulated α_{1A}- or α_{1B}-AR/GFP internalization. However, a dominant-negative form of the β-arrestin 1, β-arrestin 1 (319–418), blocked agonist-mediated internalization of both the α_{1A}- and α_{1B}-ARs. These data show that transfected α_1-AR/GFP fusion proteins are functional, that there are differences in the cellular distribution and agonist-mediated internalization between the α_1-ARs, and that agonist-mediated α_1-AR internalization is dependent on arrestins and can be desensitized by long-term exposure to an agonist. These differences could contribute to the diversity in physiologic responses regulated by the α_1-ARs.

The α_1-ARs are members of the G-protein-coupled receptor (GPCR) family of receptors and are used by the sympathetic nervous system to regulate systemic arterial blood pressure and blood flow. The α_1-ARs also play a major role in mediating growth responses in cardiac and vascular smooth muscle cells (for recent reviews on all aspects of the α_1-ARs, see García-Sáinz et al., 1999; Graham et al., 1996; Schwinn and Price, 1999; Zhong and Minneman, 1999; Piascik and Perez, 2001). Three genes encoding unique receptor subtypes, the α_{1A}-, α_{1B}-, and α_{1D}-ARs, have been cloned and characterized. These subtypes use a variety of second messengers and G-proteins to modulate cellular processes. Alterations in normal α_1-AR function may contribute to the pathophysiology of diseases such as hypertension, congestive heart failure, and benign prostatic hyperplasia.

GPCR signaling is also tightly regulated by a series of cellular proteins that promote receptor desensitization and internalization (Krupnick and Benovic, 1998; Lefkowitz, 1998). Agonist occupation promotes receptor phosphorylation by a series of GPCR kinases (Hausdorff et al., 1990; Inglese et al., 1993; Premont et al., 1995). The phosphorylated receptor exhibits high affinity for the arrestins, which, in turn, prevent further interaction between the receptor and G-proteins (Wilden et al., 1986; Benovic et al., 1987). There are currently four known members of the arrestin family: visual arrestin (arrestin 1), β-arrestin 1 (arrestin 2), β-arrestin 2 (arrestin 3), and cone arrestin (arrestin 4) (Ferguson et al., 1996; Krupnick and Benovic, 1998). The β-arrestins promote internalization by binding to both the receptor and clathrin, thus, directing the receptor to coated pits (von Zastrow and

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ABBREVIATIONS: AR, adrenoceptor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; PMT, photomultiplier tube; BODIPY-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl ester.

Kobilka, 1992; Krupnick et al., 1997a; Gagnon et al., 1998). Oakley et al. (2000) demonstrated recently that GPCRs have different affinities for the different arrestins. Class A GPCRs, which include the β_2-AR, α_{1B}-AR, and β-opioid receptor, have high affinity for β-arrestin 2, whereas class B GPCRs, such as the angiotensin II type 1A receptor, neurotensin receptor 1, and vasopressin V2 receptor, exhibit high affinity for both β-arrestin 1 and 2 isoforms.

With regard to the α_1-AR subtypes, the desensitization, down-regulation, and internalization characteristics of the α_{1B}-AR have been most extensively examined. For example, agonist-mediated phosphorylation and internalization of the α_{1B}-AR have been demonstrated, and the domains of the receptor involved in internalization have been identified (Fonseca et al., 1995; Mhaouty-Kodja et al., 1999; Wang et al., 1997, 2000). We know much less regarding the molecular determinants of desensitization, down-regulation, and internalization for the α_{1A}- and α_{1D}-ARs. Vázquez-Prado et al. (2000) showed that the α_{1A}-AR could undergo agonist-mediated phosphorylation, albeit not to the same extent as the α_{1B}-AR. Yang and coworkers (1999) used fibroblasts stably transfected with each of the α_1-ARs to show that the increase in inositol phosphates mediated by the α_{1A}- and α_{1B}-ARs could be desensitized, whereas the increase mediated by the α_{1D}-AR was refractory to agonist-mediated desensitization. In contrast to this, García-Sáinz et al. (2001) showed that the α_{1D}-AR could be phosphorylated and desensitized.

In this report, we have examined subcellular distribution, agonist-mediated internalization and desensitization characteristics of green fluorescent protein (GFP)-tagged α_1-ARs using real-time imaging in transiently transfected human embryonic kidney (HEK) 293 cells. We show that there are significant differences in these parameters that could account for differences in the cellular signaling properties of the α_1-ARs.

Experimental Procedures

Materials. α_1-AR-green fluorescent protein (α_1-AR/GFP) vectors were constructed by ligating the coding region of the human α_{1A}-, α_{1B}-, and α_{1D}-AR into the *Eco*RI-*Kpn*I site of the basic pEGFP-N3 protein fusion vector (BD Biosciences Clontech, Palo Alto, CA) as described previously (Hirasawa et al., 1997; Awaji et al., 1998). The generation of wild-type β-arrestin and a dominant-negative β-arrestin 1 (319–418) in pcDNA3 has been reported previously (Krupnick et al., 1997b). Rabbit polyclonal antibodies targeted against β-arrestin 1 were generated as described by Orsini and Benovic (1998).

Cell Culture and Transient Transfection. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic cocktail [10,000 units/ml penicillin G sodium, 10,000 mg/ml streptomycin sulfate, and 25 mg/ml amphotericin B in 0.85% saline (Invitrogen, Carlsbad, CA)]. The cells were grown in T75 flasks in a 37°C cell culture incubator with a humidified atmosphere of 95% air and 5% CO_2 and were fed every 2 to 3 days. HEK cells used in immunocytochemistry protocols were grown on gelatin/laminin-treated coverslips in 35-mm tissue culture dishes (Corning Glassworks, Corning, NY), whereas cells for real-time studies were grown in culture dishes with a glass coverslip bottom (MatTek Co., Ashland, MA) that were also gelatin/laminin treated. Cells were grown to approximately 80% confluence and used for experimentation 3 days after being plated. HEK cells were transfected with cDNA encoding α_{1A}-, α_{1B}-, or α_{1D}-AR/GFP fusion protein using calcium phosphate precipitation. In certain studies, the receptor/GFP constructs were cotransfected with a cDNA encoding wild-type β-arrestin 1 or β-arrestin 1 (319–418). β-Arrestin 1 overexpression was confirmed using specific antibodies in immunocytochemistry protocols as we have described previously (Hrometz et al., 1999; McCune et al., 2000).

Activation of ERK1/2 Phosphorylation and Agonist-Mediated Desensitization. The coupling of the α_1-AR-/GFP constructs to functional responses and agonist-mediated desensitization was assessed by measuring the phosphorylation of ERK1/2. Cells were challenged with 100 μM phenylephrine for a period of 2 h. After the appropriate time, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min, and immunocytochemistry was performed as described previously by Hrometz et al. (1999) and McCune et al. (2000). In brief, cells were treated with mouse monoclonal IgG pERK (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution and then incubated with Rhodamine Red-X-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:100. The degree of ERK1/2 phosphorylation was assessed using laser scanning confocal microscopy as described below. Desensitization experiments were conducted on HEK 293 cells 72 h after transient transfection with cDNA encoding α_{1A}-, α_{1B}-, or α_{1D}-AR-/GFP. Cells were treated with 100 μM phenylephrine for 15 h. Vehicle-treated cells served as controls. After the incubation, cells were washed three times (30-min intervals between each wash) with Dulbecco's modified Eagle's medium, after which cells were rechallenged with phenylephrine for 2 h, and the effect on ERK1/2 phosphorylation was assessed.

Laser Scanning Confocal Microscopy. Transfected HEK 293 cells were imaged with a Spectra-Physics laser scanning confocal microscope attached to a TCS DM RXE microscope with a Plan-Apo 100x 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 for reduction and analysis with Adobe Photoshop version 4.0 (Adobe Systems, Mountain View, CA). The setting on the laser was constant for all experiments. However, both GFP and rhodamine signals were digitally enhanced by adjusting the photomultiplier tube (PMT). Initial adjustment of the PMT allowed us to minimize the background signal while maximizing the fluorescent signal(s) of interest. Because individual cells required a different PMT setting, the differences in intensity should not be construed as a measure of receptor expression levels.

Data and Image Analysis. The rate and extent to which the α_1-AR/GFP constructs were internalized after exposure to agonist were analyzed using the image analysis software NIH ImageJ 1.18x (http://rsb.info.nih.gov/ij/). The change in fluorescence intensity was measured in a rectangular area just below the cell surface before and during the internalization process. Data were normalized to a percentage of the fluorescence obtained before agonist treatment. The increase in fluorescence intensity above that observed in untreated cells is a measure of receptor internalization. A plot of the percentage increase in fluorescence intensity versus time after agonist treatment was then generated. The average phospho-ERK1/2 signal per determined area was quantitated using the same image analysis software. Only images acquired using exactly the same PMT settings were compared with each other. Treated cells were normalized to the control phospho-ERK1/2 activation signal. All data are reported as the mean ± S.E. Data were analyzed by analysis of variance followed by Student-Newman-Kuels analysis to determine where statistically significant differences existed. A P value of less than 0.05 was considered significant.

Results

Basal Cellular Localization. HEK 293 cells were transiently transfected with expression plasmids encoding α_1-AR/GFP fusion proteins and the living cells were visualized 72 h later. Transfection with a cDNA encoding the α_{1B}-AR/GFP

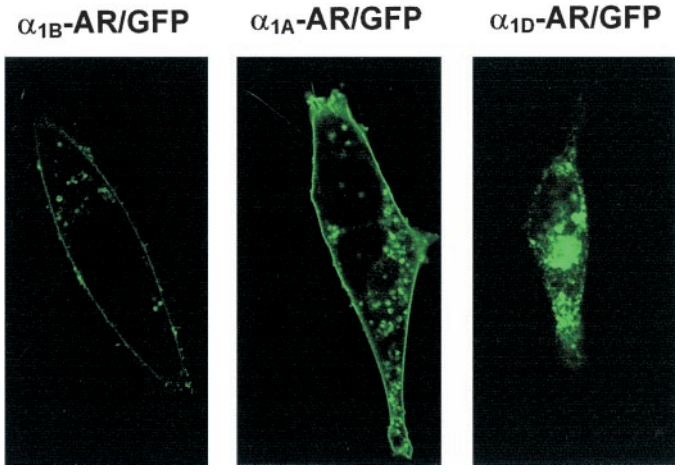

Fig. 1. Cellular localization of α_1-AR/GFP constructs in transiently transfected HEK-293 cells. Transient transfection of α_1-AR/GFP expression plasmids and laser scanning confocal microscopy were performed as described under *Experimental Procedures*. The images are representative of five to eight independent transfections.

resulted in a specific fluorescence that was detected predominantly on the margin of the cell, indicative of a cell surface localization (Fig. 1). Although there was cell surface expression, the majority of the α_{1D}-AR/GFP fluorescence was detected intracellularly in a perinuclear orientation (Fig. 1). Exhibiting localization properties of each of these subtypes, α_{1A}-AR/GFP fluorescence was observed on the cell surface and in a perinuclear orientation (Fig. 1).

Functional Responses Mediated by the α_1-AR/GFP Fusion Proteins. To demonstrate that the expressed α_1-AR/GFP fusion proteins were functional, transfected cells were stimulated with phenylephrine, and, after fixing of the cells, the effect on ERK1/2 phosphorylation was determined with a monoclonal antibody specific for phospho-ERK1/2. Treatment with phenylephrine resulted in a statistically significant increase in phospho-ERK1/2 immunoreactivity in cells transfected with either the α_{1A}- or the α_{1B}-AR/GFP constructs (Fig. 2, A, B, and D). This indicates that these GFP modified α_1-ARs are functional when expressed in HEK 293 cells. Phenylephrine treatment of cells transfected with the α_{1D}-AR also resulted in an increase in the level of ERK1/2 phosphorylation (Fig. 2, C and D). However, this increase was not significantly different compared with the untreated control (Fig. 2D). These findings could indicate that, although functional, the α_{1D}-AR is poorly coupled to second messenger pathways such that only a modest increase in ERK1/2 phosphorylation could be observed.

Effect of Agonist Stimulation on Receptor Localization. In addition to activating ERK1/2 phosphorylation, the ability of phenylephrine to promote changes in receptor localization was assessed in real-time. Addition of 100 μM phenylephrine to cells expressing the α_{1B}-AR/GFP resulted in a rapid translocation of the receptor from the cell surface to intracellular compartments (Fig. 3). The α_1-AR antagonist 1 μM prazosin blocked this internalization (data not shown).

The increase in intracellular fluorescence signal intensity, quantitated with image analysis software (as described un-

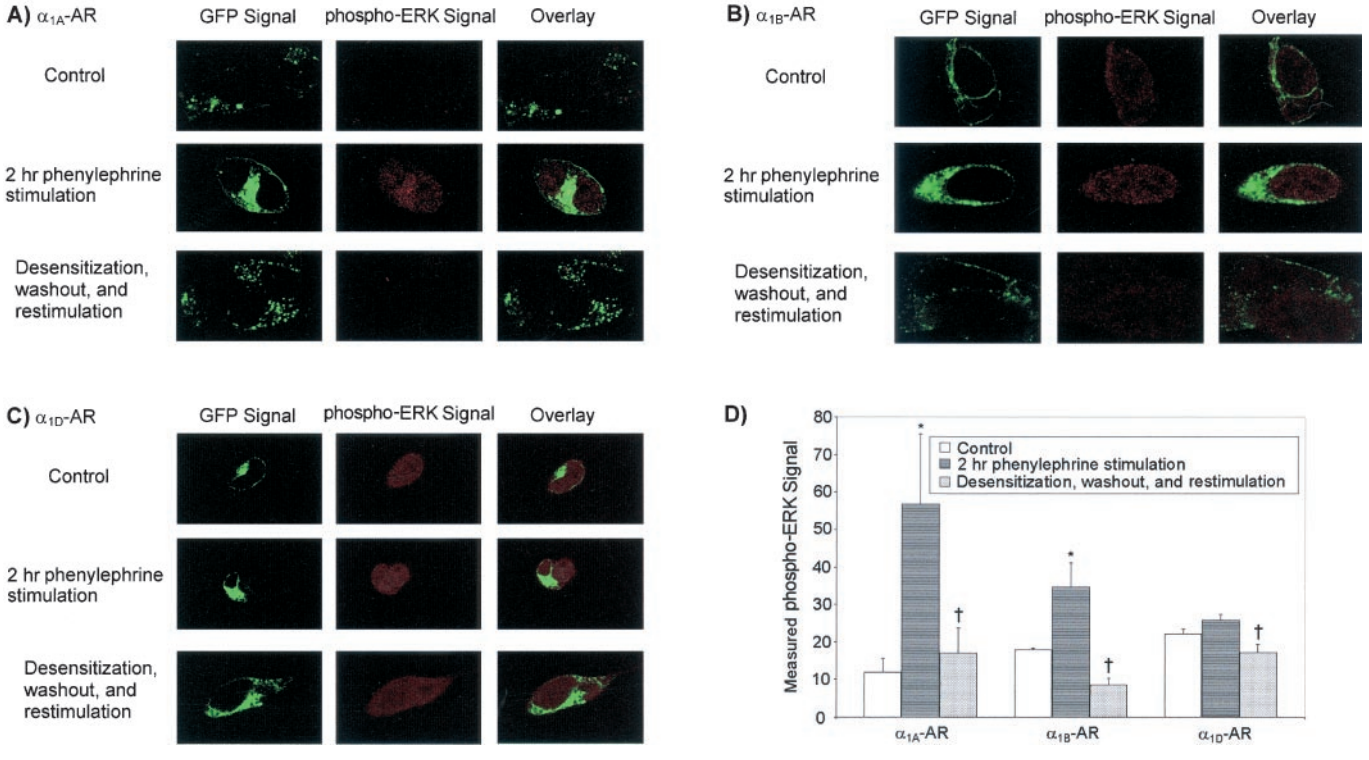

Fig. 2. The activation of ERK1/2 phosphorylation and agonist-mediated desensitization. Immunocytochemistry demonstrating receptor functionality and agonist-induced desensitization were performed as described under *Experimental Procedures*. Images displayed are for the α_1-AR/GFP signal, the phospho-ERK1/2 signal, and the overlay of these two images. Data presented are for the basal α_1-AR/GFP localization, the phenylephrine-stimulated ERK1/2 phosphorylation in naive cells, and the effect of 15 h of phenylephrine treatment on the subsequent ability of phenylephrine to activate ERK1/2 phosphorylation. Data are for the α_{1A}-AR/GFP (A), α_{1B}-AR/GFP (B), and α_{1D}-AR/GFP (C). The images are representative of three to seven independent transfections. D, bar graphs show the relative changes in the phospho-ERK1/2 signals for each receptor. ∗, significantly greater than the control level of ERK1/2 phosphorylation. †, statistically less than the phenylephrine stimulation of ERK1/2 phosphorylation seen in control cells.

der *Experimental Procedures*), was used to gain a measure of the rate of receptor internalization. A plot of the increase in intracellular fluorescence intensity versus time after phenylephrine administration is presented in Fig. 4A and shows that α_{1B}-AR internalization occurred in a very rapid fashion.

Receptor activation with phenylephrine also promoted the internalization of the cell surface population of α_{1A}-ARs (Figs. 3 and 4B). However, a significant increase in intracellular fluorescence was not detected until 50 min after agonist exposure. A plot of the increase in intracellular fluorescence intensity versus time revealed that the α_{1A}-AR internalization occurred at a slower rate than that seen for the α_{1B}-AR. Treatment of HEK cells expressing the α_{1D}-AR/GFP fusion protein with phenylephrine did not cause a translocation of the cell surface population of α_{1D}-ARs (Fig. 5).

Agonist-Mediated Receptor Desensitization. Transfected HEK 293 cells were incubated for 15 h with 100 μM phenylephrine, and the effect on α_1-AR/GFP localization and the ability of the α_1-ARs to stimulate ERK1/2 phosphorylation were assessed. Prolonged incubation with phenylephrine (followed by extensive washout) resulted in an internalization of α_{1A}- and α_{1B}-ARs (see GFP fluorescence in Fig. 2, A and B) in a fashion similar to that seen in untreated cells. Phenylephrine treatment for 15 h significantly decreased the ability of either the α_{1A}- or α_{1B}-AR to activate ERK1/2 phosphorylation in response to a second addition of phenylephrine (Fig. 2D). The long exposure to phenylephrine had no effect on the cellular localization of the α_{1D}-AR. Long-term exposure to phenylephrine significantly reduced the level of phospho-ERK1/2 seen after rechallenge with agonist in α_{1D}-AR expressing cells (Fig. 2D).

Effect of Arrestins on Agonist-Activated Receptor Internalization. HEK cells were cotransfected with α_1-AR/GFP constructs and an expression plasmid encoding β-arrestin 1. The overexpression of β-arrestin 1 was confirmed using immunocytochemical protocols with an antibody against β-arrestin 1 (Fig. 6). β-Arrestin 1 overexpression did not increase the rate or extent of α_{1A}- or α_{1B}-AR internalization after stimulation with phenylephrine (data not shown). In a similar fashion, cotransfection with β-arrestin 2 had no effect on agonist-mediated internalization (data not shown).

HEK 293 cells were cotransfected with a cDNA encoding the α_{1B}-AR/GFP construct and a dominant-negative form of β-arrestin 1, β-arrestin 1 (319–418). β-Arrestin 1 (319–418) had no effect on basal α_{1B}-AR cellular localization (Fig. 7A). However, the dominant-negative arrestin markedly decreased the ability of phenylephrine to promote α_{1B}-AR internalization (Fig. 7A). Analysis of these data revealed that the dominant-negative arrestin significantly reduced the rate of increase in intracellular fluorescence intensity seen after the addition of phenylephrine to HEK 293 cells (Fig. 4A). Similar to effects seen with the α_{1B}-AR, β-arrestin 1 (319–418) decreased the magnitude of the phenylephrine-induced α_{1A}-AR internalization (Figs. 4B and 7B).

Wild-type β-arrestin 1 did not affect the basal cellular localization or the ability of phenylephrine to promote

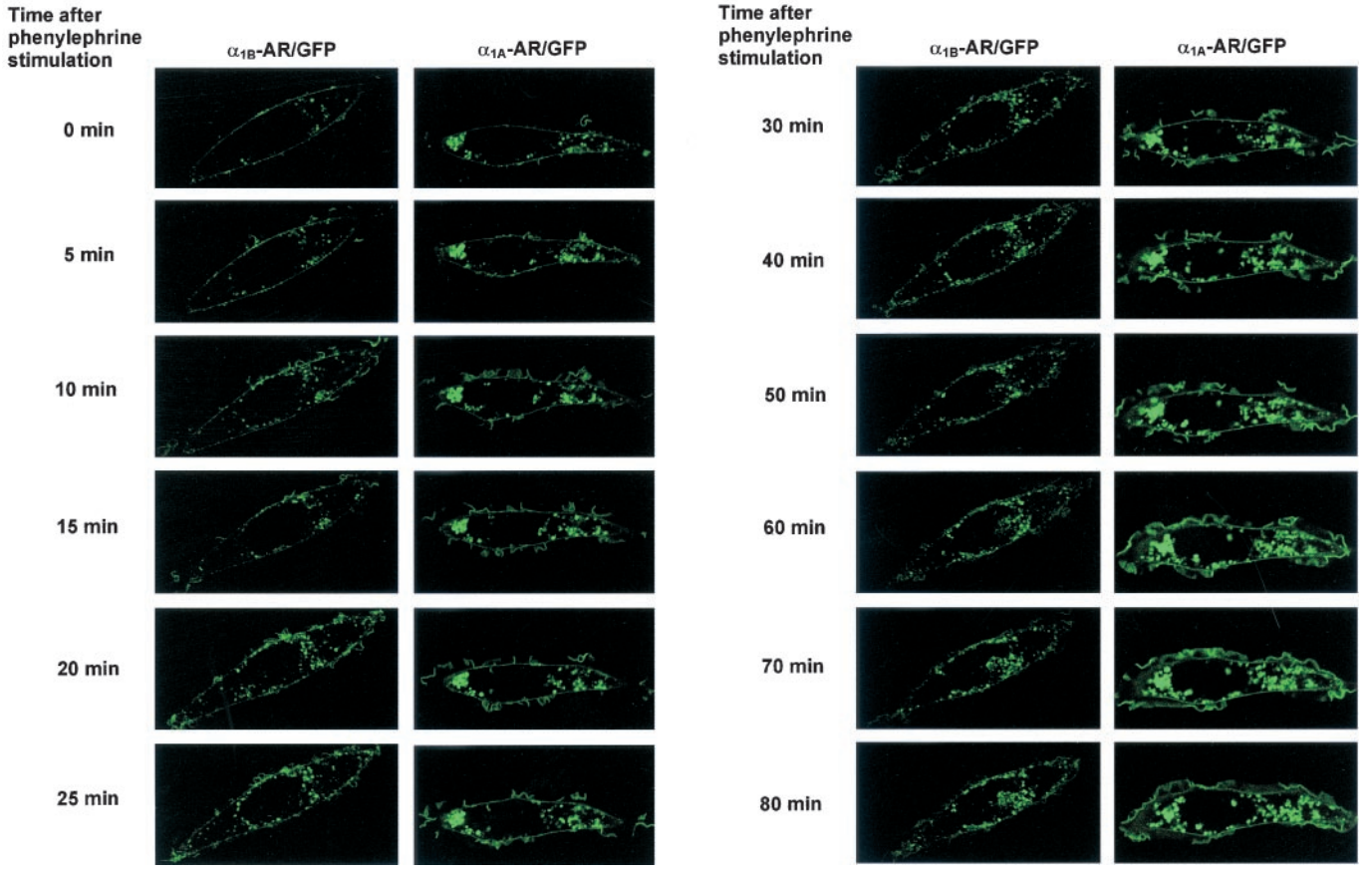

Fig. 3. Effects of 100 μM phenylephrine on the cellular localization of either the α_{1A}- or α_{1B}-ARs transiently transfected into HEK 293 cells. Real-time images were captured before and at the specified time points after phenylephrine addition as described under *Experimental Procedures*. The images are representative of five to eight independent transfections.

α_{1D}-AR internalization (Fig. 8A). Similarly, β-arrestin 1 (319–418) had no effect on the cellular localization of the α_{1D}-AR/GFP (Fig. 8B).

Discussion

In this communication, we have examined the cellular localization, agonist-mediated internalization, and desensitization properties of α_1-AR/GFP fusion proteins in transiently transfected HEK 293 cells. Previous studies with the α_{1B}-AR/GFP construct demonstrated that it is fully functional and internalizes in the same manner as a non-GFP tagged α_{1B}-AR construct (Awaji et al., 1998). In a similar fashion, previous studies showed that both the α_{1A}- and α_{1B}-ARs are coupled to the activation of ERK (for reviews, see García-Sáinz et al., 1999; Zhong and Minneman, 1999; Varma and Deng, 2000; Piascik and Perez 2001). In this report, we show that both the α_{1A}- and α_{1B}-ARs when coupled to GFP can promote an increase in ERK1/2 phosphorylation (Fig. 2). The phosphorylation of ERK1/2 is thought to mediate growth responses, at least in part. Demonstration of ERK1/2 phosphorylation in these studies is evidence that the α_1-ARs are functional and retain their ability to activate cellular signaling when conjugated to the GFP.

In this report, we also noted that, although phenylephrine could increase the level of phospho-ERK1/2 in α_{1D}-AR expressing cells, this increase was not statistically significant. This could indicate that the small population of cell surface α_{1D}-ARs is not efficiently coupled to ERK1/2 phosphorylation. This result is consistent with the observations of Theroux et al. (1996) who noted that the α_{1D}-AR was the most poorly coupled of the α_1-AR subtypes. In previous work with stably transfected fibroblasts, we showed that the α_{1D}-AR was constitutively active with regard to ERK activation (McCune et al., 2000). We also noted that there was a high basal level of ERK activity in these cells and that phenylephrine

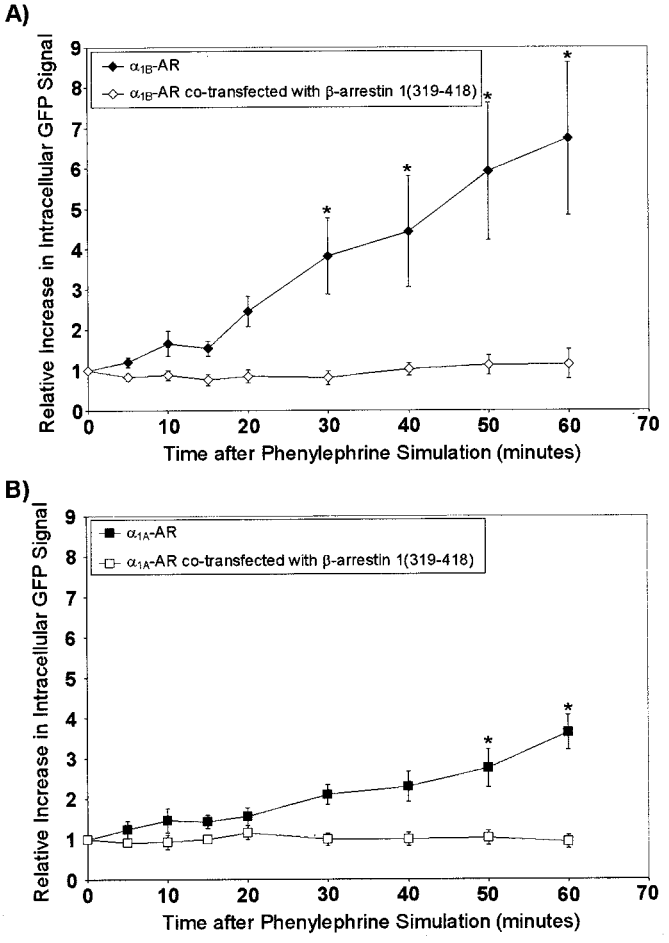

Fig. 4. Comparison of the effect of 100 μM phenylephrine on changes in intracellular fluorescence intensity in cells transfected with the α_{1A}- or α_{1B}-AR/GFP in the absence or presence of β-arrestin 1 (319–418). Relative intensity assessment were performed as described under *Experimental Procedures*. Data represent the mean and standard error of the mean values of four to eight independent transfections. *, values are significantly greater than the unstimulated control or cells cotransfected with β-arrestin 1 (319–418).

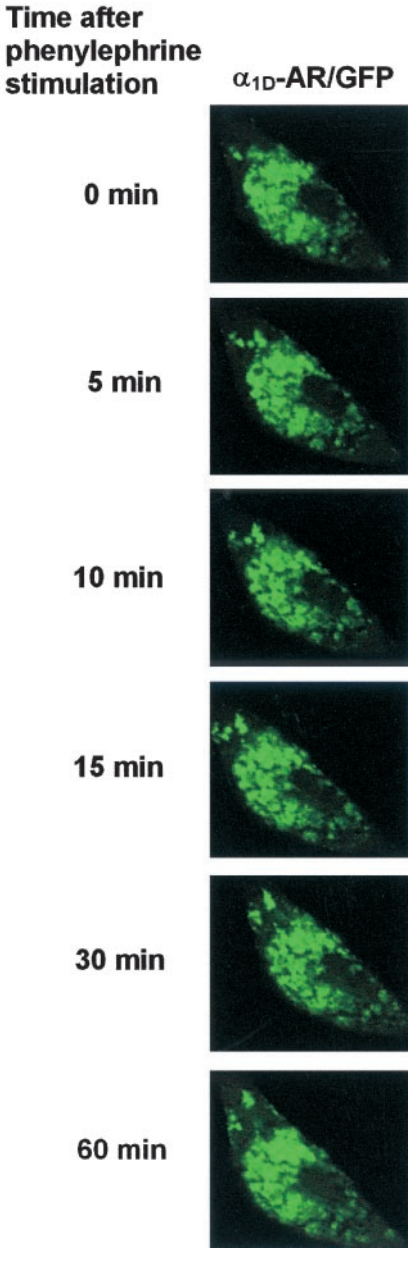

Fig. 5. Effects of 100 μM phenylephrine on the cellular localization of the α_{1D}-AR transiently transfected into HEK 293 cells. Real-time images at specific time points after phenylephrine treatment. Experiments were performed as described under *Experimental Procedures*. The images are representative of four independent transfections.

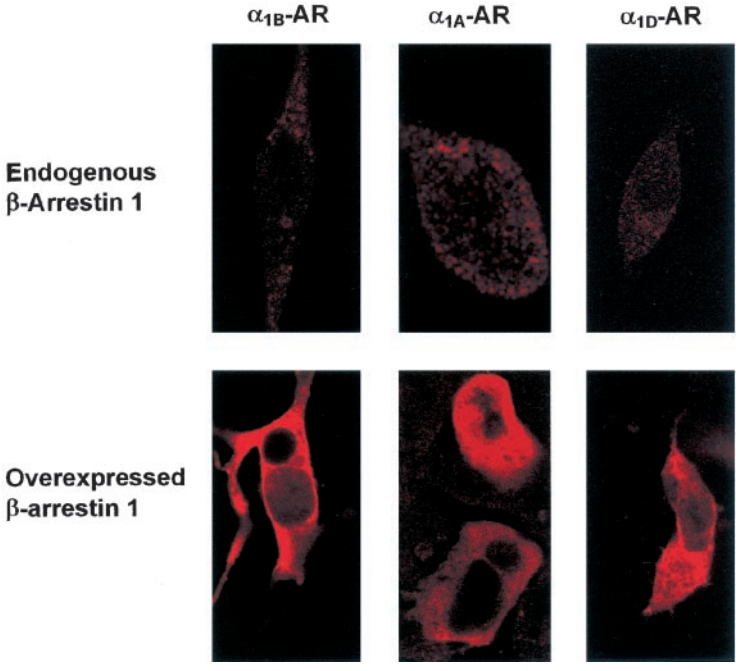

Fig. 6. Immunolocalization of endogenous β-arrestin 1 in native HEK 293 cells and cells transiently transfected with a cDNA encoding β-arrestin 1. The β-arrestin 1 immunofluorescence was detected with a specific antibody and a rhodamine-labeled secondary antibody as described under *Experimental Procedures*.

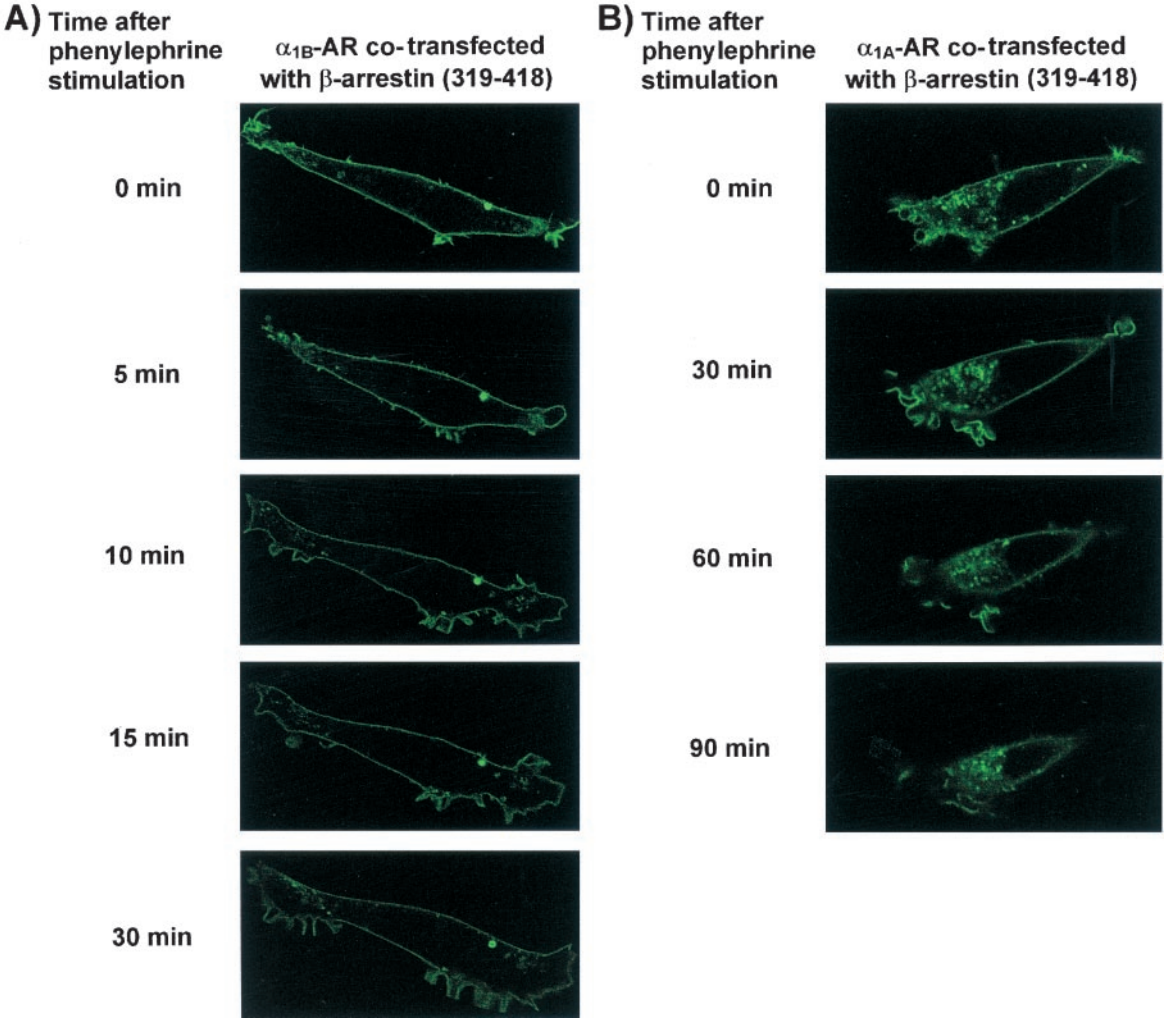

Fig. 7. The effect of cotransfection of β-arrestin 1 (319–418) on the ability of 100 μM phenylephrine to promote internalization of the α_{1A}- and α_{1B}-AR in HEK-293 cells. Drug administration and real-time visualization were performed as described under *Experimental Procedures*. Representative real-time images up to 30 and 90 min after agonist addition for α_{1B}- and α_{1A}-AR, respectively. The images are representative of four (α_{1A}-AR) or seven (α_{1B}-AR) independent transfections.

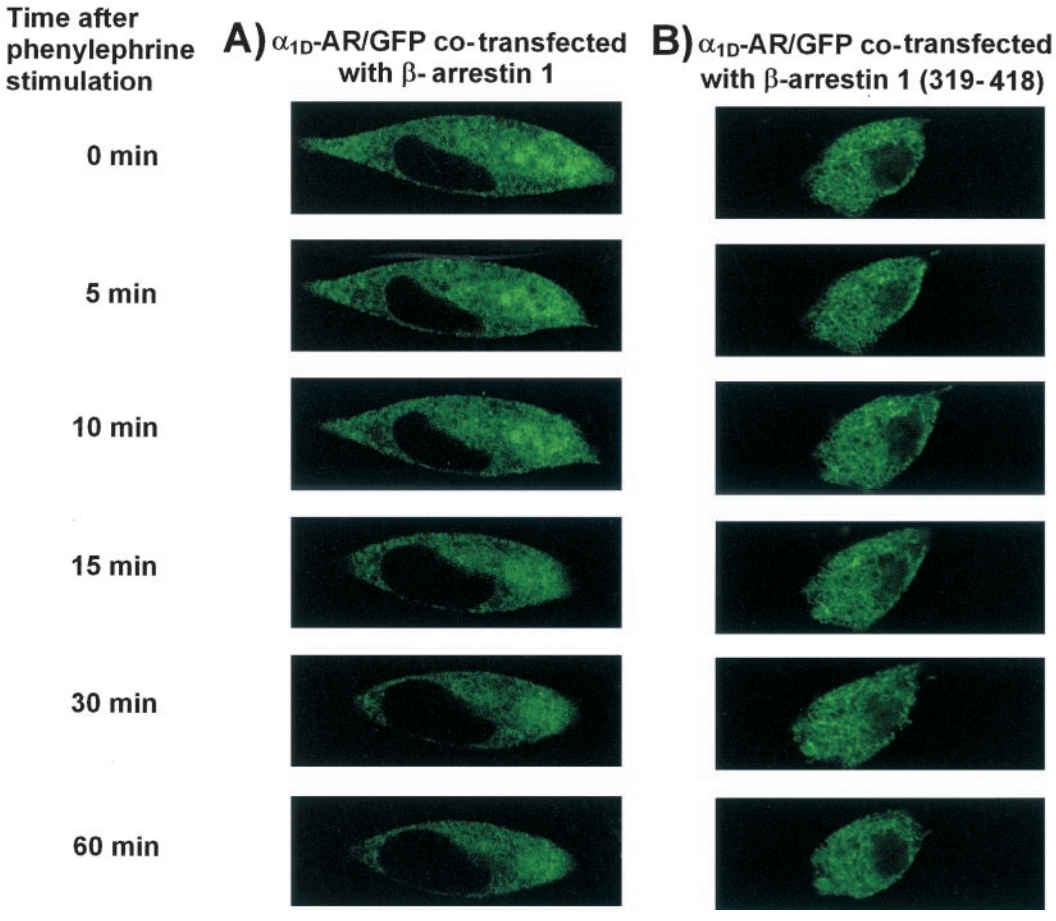

Fig. 8. The effect of cotransfection of β-arrestin 1 or β-arrestin 1 (319–418) on the ability of 100 μM phenylephrine to promote internalization of the α_{1D}-AR in HEK-293 cells. Drug administration and real-time visualization were performed as described under *Experimental Procedures*. Each transfection was repeated three to four times. A, representative real-time images of cell cotransfected with the wild-type β-arrestin 1. B, representative real-time images of cell cotransfected with β-arrestin 1 (319–418).

could not promote a further enhancement of kinase activity (McCune et al., 2000). Thus, the inability to detect a significant increase in ERK1/2 phosphorylation in this present study could also be due to a constitutively active α_{1D}-AR. Nonetheless, we must also accept the possibility that the α_{1D}-AR/GFP construct may not be functionally active (however, see the additional discussion below).

Laser scanning confocal microscopy revealed that the α_{1B}-AR was expressed predominantly on the cell surface (Fig. 1). Although there is some cell surface expression of the α_{1D}-AR, the majority of this receptor is expressed in intracellular compartments. The α_{1A}-AR has localization characteristics of both the α_{1B}- and α_{1D}-ARs, being expressed not only on the cellular surface but also intracellularly. Using immunocytochemistry with subtype selective antibodies, we previously observed a similar distribution pattern for the α_1-ARs in stably transfected fibroblasts and vascular smooth muscle cells (Hrometz et al., 1999; McCune et al., 2000). However, studies of the cellular localization of the α_1-ARs have been hampered by the low affinity of the commercially available antibodies. The present studies confirm and extend our initial findings with techniques that do not involve antibodies. Therefore, it seems likely that our observation of differential cellular localization of the α_1-AR accurately portrays the distribution pattern in vascular smooth muscle cells that normally express all three α_1-ARs. Indeed, an elegant series of studies using prazosin labeled with BODIPY-FL to image the α_1-AR subtypes noted an intracellular expression of these receptors in cultured prostate smooth muscle cells and stably transfected fibroblasts (MacKenzie et al., 2000). These authors estimated that in smooth muscle cells, 40% of the total α_1-AR population is expressed intracellularly.

Using real-time imaging of living cells, we observed differences in the agonist-mediated internalization properties of the α_1-ARs (Figs. 3–5). In agreement with previous work (Fonseca et al., 1995; Wang et al., 1997, 2000), we observed that the α_{1B}-AR undergoes rapid agonist-mediated internalization. However, there has been little investigation of the effect of agonist activation on the translocation of the other α_1-AR subtypes. We noted that the α_{1A}-AR also undergoes agonist-mediated internalization. Interestingly, this internalization occurs at a slower rate than for the α_{1B}-AR (Fig. 4, compare A and B). We were unable to detect any agonist-mediated internalization of the α_{1D}-AR (Fig. 5). We cannot discount the possibility that a small amount of receptor internalization did take place. However, this small increase in intracellular fluorescence could not be detected because of the considerable fluorescence produced by the intracellular population of α_{1D}-ARs.

We then assessed the extent to which the receptors could be desensitized after prolonged exposure to phenylephrine. Transfected HEK 293 cells were incubated with phenylephrine for 15 h and then extensively washed. This long incubation period resulted in redistribution of each of the α_1-AR/GFPs similar to that seen in nondesensitized cells (compare Fig. 2 with Figs. 1 and 3). After the washout period, the cells were rechallenged with phenylephrine. Using this protocol,

we demonstrated that prolonged exposure to agonist desensitizes the ability of the α_{1A}- and α_{1B}-ARs to promote ERK1/2 phosphorylation (Fig. 2). Interestingly, after a 15-h exposure to phenylephrine in α_{1D}-AR expressing cells, rechallenge with agonist could not promote any increase in the level of phospho-ERK1/2. Indeed, there was a statistically significant difference in the level of agonist-induced ERK1/2 phosphorylation in control HEK 293 cells and that seen after desensitization (Fig. 2). Thus even though phenylephrine could only promote a modest, nonsignificant increase in the level of phospho-ERK1/2 in control cells, this could be reduced by prolonged expose to agonist and supports the notion that the α_{1D}-AR/GFP construct is functional.

Our results are consistent with other studies that have examined the phosphorylation and desensitization of the α_1-ARs. Yang et al. (1999) noted that both the α_{1A}- and α_{1B}-ARs undergo agonist-mediated desensitization. However, these authors noted that the α_{1B}-AR was desensitized by lower concentrations of agonist. Similarly, Vázquez-Prado et al. (2000) found that the α_{1B}-AR underwent more extensive agonist-activated phosphorylation than did the α_{1A}-AR. The more rapid rate of α_{1B}-AR internalization noted here is also consistent with the observation that this receptor is more extensively phosphorylated and readily desensitized than the α_{1A}-AR. Yang et al. (1999) also observed that functional responses mediated by the α_{1D}-AR were not subject to desensitization. In contrast to the work of Yang et al. (1999), García-Sáinz et al. (2001) noted that in stably transfected fibroblasts, the α_{1D}-AR could be phosphorylated and desensitized. Therefore, a definitive answer regarding the desensitization characteristics of the α_{1D}-AR requires additional studies.

Arrestins have been implicated in mediating the internalization of a variety of GPCRs. There has been little work performed to determine the role of arrestins in agonist-mediated α_1-AR internalization. We were unable to observe any demonstrable effects of β-arrestin 1 overexpression to the degree to which agonist activation promotes the internalization of the α_{1A}- or α_{1B}-ARs. This probably reflects the fact that HEK 293 cells possess large amounts of β-arrestin 1 (Fig. 6). Therefore, overexpression of additional arrestin molecules would not be expected to have an effect on agonist-mediated receptor internalization. Similarly, overexpression of β-arrestin 2 had no effect on the degree of agonist-stimulated internalization of the α_{1A}- or α_{1B}-ARs (data not shown). A dominant-negative arrestin, β-arrestin 1 (319–418), completely blocked agonist-mediated internalization of both the α_{1A}- and the α_{1B}-ARs (Figs. 4 and 7). These data argue that agonist-activated internalization of the α_1-AR subtypes is mediated by arrestins. Although the dominant-negative arrestin confirms the role of arrestins in α_1-AR internalization, this reagent cannot be used to determine the specific role of β-arrestin 1 or 2 in the internalization process. This is because the dominant-negative arrestin binds to clathrin, thus preventing the binding of wild-type arrestin species. Therefore, the dominant-negative arrestin would be expected to block the actions of any wild-type arrestin.

The intracellular localization of α_{1D}-AR was not affected by overexpression of either wild-type β-arrestin 1 or β-arrestin 1 (319–418), arguing that the intracellular distribution of the α_{1D}-AR is not likely to be maintained by arrestin molecules (Fig. 8). The significance of the predominantly intracellular localization of the α_{1D}-AR is not clear. We do not know which of the α_{1D}-ARs, the small population of cell surface receptors or the large population of intracellularly expressed receptors, are signaling competent and responsible for the regulatory activity of this subtype. As noted above, data from several labs including ours show that the α_{1D}-AR is constitutively active (García-Sáinz and Torres-Padilla, 1999; McCune et al., 2000; Noguera et al., 1993). The observation of constitutive activity may shed some light on the relationship between cellular localization and functional responses. A constitutively active receptor assumes an activated conformation in the absence of agonist. The large degree of intracellular localization of the α_{1D}-AR may be due to continuous internalization of the receptor due to its constitutively active nature.

The three α_1-ARs are coexpressed on tissues and organs involved in cardiovascular regulation, yet these receptors modulate different physiological processes. We hypothesize that the observed differences in the cellular localization could contribute to the differences in the functional responses mediated by these receptors. We also propose that the α_{1B}-AR most approximates a prototypic GPCR in terms of cellular localization, agonist-mediated internalization, desensitization, and coupling to cellular signaling. In contrast, we postulate that the α_{1D}-AR is an atypical GPCR. Although the α_{1A}-AR is expressed intracellularly, it seems to have signaling properties expected of a GPCR.

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