

MOL 43422

**DIFFERENT INTERNALIZATION PROPERTIES OF THE  $\alpha$ 1a AND  $\alpha$ 1b-  
ADRENERGIC RECEPTOR SUBTYPES: THE POTENTIAL ROLE OF RECEPTOR  
INTERACTION WITH  $\beta$ ARRESTINS AND AP50**

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MOL 43422

Running title:  $\alpha$ 1-adrenergic receptors interaction with  $\beta$ arrestins and AP50

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text pages: 37

tables: none

figures: 10 (2 in supplemental data)

references: 25

Abstract: 245

Introduction: 501

Discussion: 1843

The abbreviations used are:

GPCR, G protein-coupled receptor; AR, adrenergic receptor; GRK, G protein-coupled receptor kinase; GST, glutathione S-transferase; HA, hemoagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffer saline; TBS, Tris-buffered saline; shRNA, short hairpin RNA; [<sup>125</sup>I]-HEAT, [<sup>125</sup>I]-iodo-2-[ $\beta$ -(4-hydroxyphenyl)-ethylaminomethyl]tetralone.

MOL 43422

## ABSTRACT

The internalization properties of the  $\alpha$ 1a and  $\alpha$ 1b-adrenergic receptors (AR) subtypes transiently expressed in HEK-293 cells were compared using biotinylation experiments and confocal microscopy. Whereas the  $\alpha$ 1b-AR displayed robust agonist-induced endocytosis, the  $\alpha$ 1a-AR did not. Constitutive internalization of the  $\alpha$ 1a-AR was negligible, whereas the  $\alpha$ 1b-AR displayed significant constitutive internalization and recycling. We investigated the interaction of the  $\alpha$ 1-AR subtypes with  $\beta$ arrestin 1 and 2 as well as with the AP50 subunit of the clathrin adaptor complex AP2. The results from both co-immunoprecipitation experiments and  $\beta$ arrestin translocation assays indicated that the agonist-induced interaction of the  $\alpha$ 1a-AR with  $\beta$ arrestins was much weaker than that of the  $\alpha$ 1b-AR. In addition, the  $\alpha$ 1a-AR did not bind AP50. The  $\alpha$ 1b-AR mutant M8, lacking the main phosphorylation sites in the receptor C-tail, was unable to undergo endocytosis and was profoundly impaired in binding  $\beta$ arrestins despite its binding to AP50. In contrast, the  $\alpha$ 1b-AR mutant  $\Delta$ R8, lacking AP50 binding, bound  $\beta$ arrestins efficiently and displayed delayed endocytosis. RNA interference showed that  $\beta$ arrestin 2 plays a prominent role in  $\alpha$ 1b-AR endocytosis. The findings of this study demonstrate differences in internalization between the  $\alpha$ 1a and  $\alpha$ 1b-AR and provide evidence that the lack of significant endocytosis of the  $\alpha$ 1a-AR is linked to its poor interaction with  $\beta$ arrestins as well as with AP50. We also provide evidence that the integrity of the phosphorylation sites in the C-tail of the  $\alpha$ 1b-AR is important for receptor/ $\beta$ arrestin interaction and that this interaction is the main event triggering receptor internalization.

## INTRODUCTION

The adrenergic receptors (AR) mediate the functional effects of epinephrine and norepinephrine in various tissues. The AR family includes nine different gene products: three  $\beta$  ( $\beta$ 1,  $\beta$ 2,  $\beta$ 3), three  $\alpha$ 2 ( $\alpha$ 2A,  $\alpha$ 2B,  $\alpha$ 2C) and three  $\alpha$ 1 ( $\alpha$ 1a,  $\alpha$ 1b,  $\alpha$ 1d) receptor subtypes. The  $\alpha$ 1-ARs are important regulators of cardiovascular physiology, glucose metabolism, genito-urinary functions and various behavioral responses, as demonstrated by recent studies on knock out mice lacking each of the three subtypes (Philipp and Hein, 2004). The  $\alpha$ 1-ARs have been extensively investigated with respect to their pharmacology and structure-function relationship (Cotecchia et al., 2004). In contrast, less is known about their regulatory properties, trafficking as well as about protein interactions involved in these processes.

We have previously reported that the  $\alpha$ 1b-AR in recombinant systems undergoes rapid desensitization and endocytosis upon exposure to the agonist and that the agonist-dependent receptor regulation involves G protein-coupled receptor kinases (GRKs) (Diviani et al., 1996). The structural determinants involved in agonist-induced regulation of the  $\alpha$ 1b-AR reside within the C-tail of the receptor as demonstrated by the fact that truncation of this region almost completely abolished receptor desensitization, internalization and phosphorylation (Lattion et al., 1994). The endocytosis of the  $\alpha$ 1b-AR occurs via clathrin-coated vesicles and seems to involve  $\beta$ arrestins as suggested by the fact that co-expression of a dominant negative  $\beta$ arrestin mutant could inhibit receptor internalization (Mhaouty-Kodja et al., 1999). We also demonstrated that the  $\alpha$ 1b-AR internalization involves, at least in part, direct binding to the receptor C-tail of the AP50 subunit of the AP2 clathrin-adaptor complex (Diviani et al., 2003).

Fewer studies have investigated the regulatory properties of the  $\alpha$ 1a-AR subtype. It has been clearly demonstrated that the bovine  $\alpha$ 1a-AR expressed in Rat-1 fibroblasts was poorly phosphorylated and desensitized compared to the  $\alpha$ 1b-AR (Vazquez-Pardo et al.,

MOL 43422

2000). Replacement of the  $\alpha$ 1a-AR C-tail with that of the  $\alpha$ 1b-AR significantly increased the ability of the receptor to undergo agonist-induced phosphorylation.

In one study (Chalothorn et al., 2002), the human  $\alpha$ 1a-AR fused to GFP and expressed in HEK-293 cells was found to internalize upon exposure to the agonist at slower rate compared to the GFP-tagged human  $\alpha$ 1b-AR. In another study (Morris et al., 2004), the human GFP-tagged human  $\alpha$ 1a-AR expressed in Rat-1 fibroblasts was also shown to undergo very modest agonist-induced endocytosis (20% maximal decrease of surface receptors). However, the  $\alpha$ 1a-AR, but not the  $\alpha$ 1b-AR, seemed to undergo constitutive internalization and recycling.

To better understand the mechanisms involved in  $\alpha$ 1-AR subtype trafficking, we performed a comparison between the  $\alpha$ 1a and  $\alpha$ 1b-AR with respect to their ability to undergo either constitutive or agonist-induced internalization as well as to bind  $\beta$ -arrestins and the AP50 subunit of the AP2 clathrin adaptor complex. Furthermore, we investigated the relative role of  $\beta$ -arrestins and AP50 in  $\alpha$ 1b-AR internalization. Finally, using RNA interference, we assessed the implication of  $\beta$ -arrestin 1 and 2 in  $\alpha$ 1b-AR endocytosis. Our findings indicate that the internalization properties of the  $\alpha$ 1a and  $\alpha$ 1b-AR are very different and suggest potential molecular mechanisms underlying these differences.

## MATERIALS AND METHODS

*Materials* - FLAG-tagged rat  $\beta$ arrestin 1 and 2 were the kind gift of Dr. R. J. Lefkowitz (Duke University, USA); rabbit polyclonal antibodies against  $\beta$ arrestin 1 were kindly provided by Dr. J.L. Benovic (Thomas Jefferson University, USA).

*Cell culture and transfections* - HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS) and gentamicin (100  $\mu$ g/ml) and transfected at 40-60% confluence in 100 mm dishes using the calcium phosphate method. After transfection, cells were grown for 48 h before harvesting. The total amount of transfected DNA was of 10-20  $\mu$ g/10 cm  $\Phi$  dish.

*DNA constructs* - HA tags (YPYDVPDYA) were introduced into the human  $\alpha$ 1a and hamster  $\alpha$ 1b-AR sequence at the N or C-terminus by PCR amplification and the tagged receptor cDNA were subcloned in pRK5 expression vector. The  $\alpha$ 1b-AR mutants  $\Delta$ R8 and M8 (S394-415A) were described elsewhere (Diviani et al., 2003 ; Diviani et al., 1997). The chimeric receptor  $\alpha$ 1aCtbHA was constructed by PCR amplification of a fragment encompassing aminoacids 369-515 and the C-terminal HA tag from the  $\alpha$ 1bHA-pRK5-pRK5 and subcloning it into the  $\alpha$ 1aHA-PRK5. The  $\alpha$ 1bCtaHA was similarly constructed by PCR amplifying the fragment encompassing aminoacids 343-466 and the HA tag from the  $\alpha$ 1aHA and subcloning it into the  $\alpha$ 1bHA-pRK5.

*Ligand binding and inositol phosphate accumulation* - [ $^{125}$ I]HEAT (DuPont) was used at a saturation concentration of 250 pM to measure receptor expression in cell membranes, as previously described (Lattion et al., 1994). Total inositol phosphates were measured in cells labeled for 15 hours with [ $^3$ H]inositol, as previously reported (Lattion et al., 1994). All receptors were expressed at comparable levels ranging 3-10 pmol/mg of protein and their

MOL 43422

stimulation with epinephrine resulted in a 3-5 fold increase of inositol phosphate accumulation above basal levels.

*GST pull-down and Immunoprecipitation* - GST pull-down experiments of endogenous AP50 were performed as previously described (Diviani et al., 2003). For immunoprecipitation, HEK-293 cells expressing the various constructs were grown in 100 mm dishes and harvested 48 h after transfection. Cells were incubated for 1h in serum-free DMEM, treated for various times with  $10^{-4}$  M epinephrine (Sigma) and then washed twice with PBS and lysed in 1 ml lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.5% digitonin (Sigma)), for 2 h at 4°C. For crosslinking experiments, 3.5 ml HEPES buffer (20 mM HEPES pH 7.4, 150 mM NaCl) containing 1 mM DSP (3,3'-dithio-bis(propionic acid N-hydroxysuccinimide ester)) were added to each plate. Crosslinking was allowed to proceed for 30 min at room temperature with gentle rocking. Cells were then washed twice with ice-cold DMEM supplemented with 10% FCS, twice with PBS and lysed in 1 ml lysis buffer for 2 h at 4°C. Cell lysates were centrifuged at 100,000 x g for 20 min and the protein content in the supernatants was measured using the Bradford assay. Equal amounts of total protein were then incubated with anti-HA polyclonal antibody (Sigma) and 20  $\mu$ l Protein A-Sepharose beads (Amersham) in a final volume of 1 ml lysis buffer. The beads were incubated at 4°C for 2 h, washed 3 times with 1 ml wash buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.2% digitonin) and resuspended in SDS-PAGE sample buffer. Eluted proteins were analyzed by SDS-PAGE and Western blotting. The amount of extract loaded on the gel was about 5% of that used for the immunoprecipitation.

*Receptor biotinylation* - To measure internalized biotinylated receptors, HEK-293 cells were transfected with the cDNAs encoding the HA-tagged receptors. After transfection,

MOL 43422

cells were incubated for 1 h in serum-free DMEM containing 10  $\mu$ M cycloheximide, then washed twice with cold PBS and biotinylated using 4 ml PBS containing 300  $\mu$ g/ml sulfo-NHS-SS-biotin, for 30 min at 4°C. The dishes were washed three times with cold TBS to quench unreacted biotin and fresh medium was added. After biotinylation, cells were treated with 10<sup>-4</sup> M epinephrine (Sigma) at 37°C for various times. To measure receptor recycling, after incubation with 10<sup>-4</sup> M epinephrine, the medium was removed, the cells were washed, fresh medium containing 10<sup>-7</sup> M prazosin was added and cells were incubated again at 37°C for various times. After incubations, cells were washed twice with cold PBS and biotin molecules covalently bound to cell surface proteins were cleaved off by incubation with 5 ml stripping solution (50 mM glutathione, 300 mM NaCl, 75 mM NaOH, 1% fetal calf serum) for 30 min at 4°C. Remaining glutathione was then quenched using 5 ml quenching solution (50 mM iodoacetamide, 1% bovine serum albumin) for 20 min at 4°C. Cells were then washed twice with PBS, lysed in lysis buffer for 3 h at 4°C and the cellular homogenate was centrifuged at 100,000 x g for 20 min. The supernatants were incubated overnight with 30  $\mu$ l streptavidin-Sepharose beads (Amersham Biosciences), pelleted by brief centrifugation and washed 4 times with 1 ml wash buffer supplemented with 0.5% Triton X-100. Biotinylated proteins were eluted from the beads by incubation with 40  $\mu$ l of 100 mM dithiothreitol (Sigma) for 1 h at 37°C; the samples were briefly centrifuged and SDS-PAGE sample buffer was added to the supernatants. Samples were analyzed by SDS-PAGE and Western blotting.

*SDS-PAGE and Western blotting* - Samples were denatured in SDS-PAGE sample buffer (65 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 5%  $\beta$ -mercaptoethanol, bromophenol blue) for 1 h at 37°C, separated on 10% acrylamide gels and electroblotted onto nitrocellulose membranes. The blots were incubated for 1 h in TBS-Tween (100 mM Tris, pH 7.4, 140 mM NaCl, 0.05% Tween 20) containing 5% (w/v) nonfat dry milk and then incubated with the specific



MOL 43422

primary antibody diluted in TBS-Tween for 2 h at room temperature. After washing, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1:5,000 in TBS-Tween for 1 h, washed three times with TBS-Tween and developed using the Enhanced Chemiluminescence detection system (Amersham Biosciences). The intensity of bands was quantified by densitometry of films exposed in the linear range and analyzed using ImageJ software. Different times of exposure were used to avoid saturation of the signal.

*Confocal microscopy* – For the experiments shown in Fig. 4 and 7, cells grown on glass coverslips, after the incubation with or without  $10^{-4}$  M epinephrine in presence of 10  $\mu$ M cycloheximide, were fixed in PBS containing 3.7% formaldehyde for 10 min and permeabilized for 5 min with PBS plus 0.2% (w/v) Triton X-100. After incubation in PBS plus 1% bovine serum albumin for 1 h, the primary antibodies in PBS plus 0.1% bovine serum albumin were added for 1 h. The coverslips were washed in PBS and incubated with the Texas Red- or FITC-conjugated secondary antibody (Jackson ImmunoResearch) diluted 1:100 in PBS plus 0.1% bovine serum albumin for 1 h. For the experiments shown in Fig. 2S (Supplementary materials), cells expressing HA- $\alpha$ 1a or HA- $\alpha$ 1b grown on glass coverslips were pre-labeled with monoclonal anti-HA antibody (Sigma) at 1:400 dilution for 30' at 4°C. The coverslips were then washed briefly 3 times in PBS and incubated with fresh medium at 37°C for different times. Cells were then fixed, permeabilized and incubated with the secondary fluorescent-labeled antibody as described above. After three washes in PBS and one wash in H<sub>2</sub>O, coverslips were mounted on glass slides using Prolong (Molecular Probes). The fluorescent staining was visualized on laser-scanning confocal microscope (Zeiss LSM 510 Meta).

*Antibodies* - The following primary antibodies were used for immunoprecipitation, immunoblotting or confocal microscopy: rabbit polyclonal anti-HA (1:2,000; Sigma);

MOL 43422

mouse monoclonal anti-HA (1:10,000; Sigma); mouse monoclonal anti-HA (1:1,000; Covance); mouse monoclonal anti-Flag (1:2,000; Roche Applied Science). The N-terminally HA-tagged receptors were only recognized by monoclonal anti-HA antibodies from Covance whereas the C-terminally HA-tagged receptors were recognized both by polyclonal and monoclonal antibodies. Thus, Western blots of biotinylated HA $\alpha$ 1a and HA $\alpha$ 1b were revealed using monoclonal anti-HA antibodies from Covance. Immunoprecipitation and Western blotting of C-terminally HA-tagged receptors were done using polyclonal and monoclonal anti-HA from Sigma, respectively. Important artifacts linked to the anti-HA antibodies were observed in confocal microscopy. Pre-labeling of cells with the Alexa595-coupled monoclonal anti-HA antibodies (Roche) induced a pronounced endocytosis of both the  $\alpha$ 1a and  $\alpha$ 1b-AR. Detection of N-terminally HA-tagged receptors in confocal microscopy was performed with the monoclonal anti-HA antibodies (Sigma). Monoclonal anti-FLAG antibodies were used to detect  $\beta$ arrestins by Western blotting.

*RNA interference* - Sequences used to design shRNA oligos directed against  $\beta$ arrestin1 and  $\beta$ arrestin2 were as published (Ahn et al., 2003). Namely, the sequence targeted in  $\beta$ arrestin1 was: AGCCTTCTGCGCGGAGAAT; the sequence targeted in  $\beta$ arrestin2 was: AAGGACCGCAAAGTGTTTGTG. The 19-nt oligos were cloned into the pSuper vector (Brummelkamp et al., 2002) using the BglIII-HindIII sites and the resulting plasmid was either transfected alone (17  $\mu$ g/10 cm  $\Phi$  dish) or cotransfected with  $\alpha$ 1aHA-pRK5 or  $\alpha$ 1bHA-pRK5 (17  $\mu$ g : 3  $\mu$ g /10 cm  $\Phi$  dish). Biotinylation experiments were carried out as described below.

*Statistical analysis* - Statistical significance of the data was assessed as indicated in the figure legends using GraphPad software (InStat 3).

## RESULTS

*Endocytosis and recycling of the  $\alpha1a$  and  $\alpha1b$ -AR subtypes.* To quantify receptor endocytosis, we used a biotinylation assay to selectively label internalized receptors, as previously described (Stanasila et al., 2006). The  $\alpha1a$  and  $\alpha1b$ -AR subtypes, carrying the HA-tag at their N-terminus (HA $\alpha1a$  and HA $\alpha1b$ ), were transiently expressed in HEK-293 cells. Cells were pretreated with cycloheximide to block protein synthesis and accumulation of newly synthesized receptor in the intracellular compartments, and incubated with biotin before treatment with epinephrine. After treatment, the biotin molecules covalently bound at the cell surface were cleaved off so that only internalized receptors were carrying the biotin moieties. Biotinylated internalized receptors were precipitated using streptavidin-sepharose beads and detected by Western blotting using anti-HA monoclonal antibodies. The internalized receptors were normalized to the total amount of biotinylation receptors in cells in which biotin molecules were not cleaved off.

As shown in Fig. 1 (A, C), epinephrine induced a robust internalization of the  $\alpha1b$ -AR. In agreement with our previous findings (Stanasila et al., 2006), about 60% of the  $\alpha1b$ -ARs were internalized after stimulation with epinephrine for 90 min and about 60% of the internalized receptors returned to the surface 90 min after removal of the agonist. In contrast, the  $\alpha1a$ -AR failed to display any significant agonist-induced endocytosis (Fig. 1, A, B). In preliminary experiments, we ruled out that receptor degradation was occurring in this interval of time since total receptor levels, as detected by Western blotting, remained stable for several hours in the presence of cycloheximide (results not shown).

MOL 43422

Biotinylation experiments performed on cells expressing the  $\alpha 1a$  and  $\alpha 1b$ -AR carrying the HA-tag at their C-terminus ( $\alpha 1a$ HA and  $\alpha 1b$ HA) gave similar results indicating that the HA sequence placed either before or after the receptor sequence did not change the regulatory properties of the receptors (results not shown).

To validate our results on the  $\alpha 1$ -AR subtypes, we determined the kinetics of endocytosis and recycling of a well-studied receptor, the  $\beta 2$  AR (Fig. 1S, Supplemental data). As previously reported (Kallal et al., 1998) the agonist-induced internalization of the  $\beta 2$  AR was rapid, reaching a maximum at 10 min and its recycling was also fast, displaying complete recovery of the receptors at the plasma membrane within 30 min after removal of the agonist.

The results from biotinylation experiments were confirmed using confocal microscopy on HEK293 cells expressing the HA-tagged  $\alpha 1$ -AR subtypes. As we previously reported (Stanasila et al., 2006), a punctate intracellular signal was observed in cells expressing the HA $\alpha 1b$ -AR already after 15 min of treatment with epinephrine. In contrast, the intracellular signal corresponding to internalized HA $\alpha 1a$ -ARs was faint and detectable only after 90 min of epinephrine stimulation (results not shown).

To further explore the differences in endocytosis between the  $\alpha 1a$  and  $\alpha 1b$ -AR subtypes, we compared the rate of constitutive internalization of the two receptors in the absence of the agonist (Fig. 2 A, B). The  $\alpha 1b$ -AR showed a small constitutive internalization of about 22% at 30 min remaining stable until 90 min (Fig. 2 A, B). This plateau might reflect an equilibrium between constitutive internalization and recycling of the receptor. The agonist-independent internalization of the  $\alpha 1b$ -AR was sensitive to sucrose, indicating that it involves a clathrin-dependent pathway (data not shown). The constitutive endocytosis of the  $\alpha 1a$ -AR was even smaller and slower than that of the  $\alpha 1b$ -AR reaching 12% only at 90 min (Fig. 2 A, B).

MOL 43422

One hypothesis that could explain the low extent of  $\alpha 1a$ -AR endocytosis was that receptor recycling to the plasma membrane is fast enough to compensate for its internalization. To test this hypothesis, biotinylation experiments of the HA $\alpha 1a$  and HA $\alpha 1b$ -AR were performed in the absence or presence of a Golgi-disrupting agent, monensin, which was shown to perturb recycling by interfering with the acidification of endosomes (Stein et al., 1984). As shown in Fig. 2 (C), treatment of cells with monensin increased two-fold the amount of the  $\alpha 1b$ -ARs constitutively internalized at 90 min, thus indicating that it impaired receptor recycling to the plasma membrane. However, monensin did not increase the amount of internalized  $\alpha 1a$ -AR, ruling out the possibility that the lack of  $\alpha 1a$ -AR endocytosis could be due to fast recycling of the receptor.

We devised a protocol for pulse labeling of the cells with an anti-HA antibody (see Experimental procedures) in order to follow the constitutive endocytosis of the receptors using confocal microscopy. In agreement with the results of the biotinylation experiments, we observed that the HA $\alpha 1b$ -AR underwent constitutive internalization (Fig. 2S, Supplemental data). The intracellular signal corresponding to the receptor became detectable after 15 min and reached a steady state after ~30 min. In contrast, cells expressing the HA $\alpha 1a$ -AR displayed little detectable intracellular signal within this time frame.

In conclusion, these findings indicate that in HEK-293 cells the  $\alpha 1a$ -AR, in contrast to the  $\alpha 1b$ -AR, lacks the ability to undergo endocytosis and this is not due to fast recycling of the receptor.

*$\beta$ arrestin interaction with the  $\alpha 1a$  and  $\alpha 1b$ -AR.* We have previously reported that the  $\alpha 1b$ -AR endocytosis is mediated by a clathrin and  $\beta$ -arrestin dependent mechanism (Mhaouty-Kodja et al., 1999). Thus, to elucidate the molecular basis underlying the different internalization behavior of the two  $\alpha 1$ -AR subtypes, we investigated their ability to interact with  $\beta$ arrestin 1 and 2. For this purpose, the  $\alpha 1a$  and  $\alpha 1b$ -AR were co-expressed in HEK-293

MOL 43422

cells with FLAG-tagged  $\beta$ arrestin 1 or 2. We could not use  $\beta$ arrestins tagged with GFP because GFP itself co-immunoprecipitates with the  $\alpha$ 1a-AR subtype (unpublished observation).

Co-immunoprecipitation experiments were performed from HEK-293 cells transiently co-expressing the HA-tagged receptors and FLAG-tagged  $\beta$ arrestins in the absence or presence of epinephrine. For quantification, the signals corresponding to the immunoprecipitated  $\beta$ arrestins were assessed by densitometry and normalized both to their signals in cell extracts and to that of the receptors in the immune complex. The ability of  $\beta$ arrestin to co-immunoprecipitate with the receptor was interpreted as the ability of the two proteins to interact inside the cell.

As shown in a representative SDS-PAGE in Fig. 3 (A), both  $\beta$ arrestin 1 and 2 co-immunoprecipitated with the receptors, even if to a different extent. To assess the agonist-induced interaction of each receptor with  $\beta$ arrestin 1 and 2, the amount of  $\beta$ arrestins co-immunoprecipitated after different times of epinephrine stimulation was expressed as percentage of that in the basal state (time 0'). As shown in Fig. 3 (B), epinephrine increased the association of the  $\alpha$ 1a-AR with either  $\beta$ arrestin1 or 2 by 50% above basal at 15 min, but this increase was not statistically significant. In contrast, the epinephrine-induced interaction of the  $\alpha$ 1b-AR with the two  $\beta$ arrestins was much greater and faster compared to that of the  $\alpha$ 1a-AR, reaching 180% above basal at 15 min (Fig. 3, C).

To compare the amount of each  $\beta$ arrestin co-immunoprecipitated with one or the other  $\alpha$ 1-AR subtype, the amount of bound  $\beta$ arrestin 2 at each time point was expressed as percentage of bound  $\beta$ arrestin 1 at the basal state (time 0') after normalizing for both  $\beta$ arrestin and receptor expression levels. As shown in Fig. 3 (D, E), for both the  $\alpha$ 1a and  $\alpha$ 1b-AR the

MOL 43422

amount of  $\beta$ arrestin 2 in the immune complex was three-fold greater than that of the  $\beta$ arrestin1 in the basal state.

When  $\beta$ arrestin1 or 2 co-immunoprecipitated with each receptor subtype were compared ( $\beta$ arrestin1/ $\alpha$ 1a *versus*  $\beta$ arrestin1/ $\alpha$ 1b and  $\beta$ arrestin2/ $\alpha$ 1a *versus*  $\beta$ arrestin2/ $\alpha$ 1b) after normalizing for both  $\beta$ arrestin and receptor expression levels, we observed that in the basal state the amount of each immunoprecipitated  $\beta$ arrestin did not differ between the two receptor subtypes (results not shown).

Altogether these findings indicate that both the  $\alpha$ 1a and  $\alpha$ 1b-AR can form a complex with  $\beta$ arrestin 1 and 2, but to a different extent. In fact, the amount of  $\beta$ arrestins associated the  $\alpha$ 1b-AR upon agonist stimulation is much greater than with the  $\alpha$ 1a-AR. In addition, the amount of  $\beta$ arrestin 2 bound to either the  $\alpha$ 1a or  $\alpha$ 1b-AR in the basal state is three-fold greater than that of the  $\beta$ arrestin 1 suggesting that  $\beta$ arrestin 2 has greater affinity for the receptor than  $\beta$ arrestin 1.

To confirm the results of the co-immunoprecipitation experiments, we used confocal microscopy to investigate the ability of the N-terminally HA-tagged  $\alpha$ 1-ARs to trigger the translocation of FLAG-tagged  $\beta$ arrestins in HEK-293 cells. As shown in Fig. 4, in the basal state both the  $\alpha$ 1a (panel A) and  $\alpha$ 1b-AR (panel B) were localized mainly at the plasma membrane. In cells expressing the  $\alpha$ 1b-AR (Fig. 4, B) epinephrine induced a pronounced translocation of both  $\beta$ arrestin 1 and 2, which was already evident at 5 min. In contrast, in cells expressing the  $\alpha$ 1a-AR (Fig. 4, A), stimulation with epinephrine for 15 min induced a modest translocation of  $\beta$ arrestin 2, but not of  $\beta$ arrestin 1, to the plasma membrane. Internalization of the  $\alpha$ 1a-AR was not observed whereas, after 15 min of exposure to the agonist, a substantial amount of  $\alpha$ 1b-AR immunofluorescence was localized in endocytic vesicles where it co-localized, at least in part, with  $\beta$ arrestin 2 (Fig. 4, B). Overlapping of the

MOL 43422

$\alpha$ 1b-AR signal with that of  $\beta$ arrestin 1 was not evident probably because the amount of  $\beta$ arrestin 1 interacting with the receptor is smaller than that of  $\beta$ arrestin 2 as observed in the co-immunoprecipitation experiments. Some co-localization of the  $\alpha$ 1b-AR and  $\beta$ arrestin signals in endocytic vesicles was also observed at time 0' in the absence of epinephrine (Fig. 4B) probably because of constitutive internalization of the receptor.

The results of confocal microscopy are coherent with those of the co-immunoprecipitation experiments indicating that, even if some translocation of  $\beta$ arrestin2 induced by the  $\alpha$ 1a-AR could be observed, it was weaker than that induced by the  $\alpha$ 1b-AR.

To gain further insight into the role played by each  $\beta$ arrestin in  $\alpha$ 1-AR endocytosis, we measured receptor internalization in cells co-expressing FLAG-tagged  $\beta$ arrestin 1 or 2 and receptors. Overexpression of  $\beta$ arrestins did not have any significant effect on the amount of internalized  $\alpha$ 1a or  $\alpha$ 1b-AR measured in biotinylation experiments after 30 min of epinephrine treatment (results not shown). This indicated that the amount of endogenous  $\beta$ arrestin 1 and 2 was not limiting in HEK-293 cells. In separate experiments receptors were also co-expressed with GRK2, but overexpression of the kinase did not modify the pattern of agonist-induced internalization (results not shown).

*Lack of interaction of AP50 with the  $\alpha$ 1a-AR subtype.* We have previously reported that the  $\alpha$ 1b-AR can directly bind the AP50 subunit of the clathrin adaptor AP-2 complex and that this interaction contributes to receptor endocytosis (Diviani et al., 2003). Therefore, we investigated whether a similar interaction of AP50 existed also with the  $\alpha$ 1a-AR subtype.

Pulldown experiments were performed by incubating the GST-fused C-tail of the  $\alpha$ 1a-AR ( $\alpha$ 1aCtail) or  $\alpha$ 1b-AR ( $\alpha$ 1bCtail) immobilized on Sepharose beads with lysates of HEK-293 cells. The results of these experiments indicated that, in contrast to the  $\alpha$ 1b Ctail, the C-tail of the  $\alpha$ 1a-AR did not bind endogenous AP50 (Fig. 5, B).



MOL 43422

Co-immunoprecipitation experiments were performed using HEK-293 cells expressing the HA-tagged  $\alpha 1a$  or  $\alpha 1b$ -AR. As shown in Fig. 5, the endogenous AP50 could co-immunoprecipitate with the  $\alpha 1b$ -AR and epinephrine increased this interaction, as previously reported (Diviani et al., 2003) (Fig. 6, B). In contrast, an association between the  $\alpha 1a$ -AR and AP50 was not detected. Interestingly, a mutant  $\alpha 1a$ -AR, carrying 8 additional arginines to reconstitute the AP50 binding site identified in the  $\alpha 1b$ -AR C-tail, failed to co-immunoprecipitate AP50 (unpublished observation) thus indicating that the eight arginines are not sufficient for receptor/AP50 interaction.

These findings make unlikely a direct association between the  $\alpha 1a$ -AR and the AP50 subunit of the AP2 complex, raising the possibility that the lack of this interaction might contribute to the poorly efficient endocytosis of the  $\alpha 1a$  subtype in HEK-293 cells.

*Role of the C-tail in receptor endocytosis.* We have previously demonstrated that the C-tail is an important regulatory domain of the  $\alpha 1b$ -AR. Its integrity is required for  $\alpha 1b$ -AR desensitization and endocytosis (Lattion et al., 1994). In addition, it contains the main phosphorylation sites of the receptor for GRKs and PKC (10). Therefore, to investigate the role of the receptor C-tail in the regulatory properties of the  $\alpha 1a$  and  $\alpha 1b$ -AR, we engineered  $\alpha 1a/\alpha 1b$  chimeras by replacing the C-terminus of one receptor subtype with that of the other and we monitored their endocytosis. As shown in Fig. 6 (B), the  $\alpha 1a/Ctb$  receptor chimera was able to internalize upon epinephrine treatment in a manner indistinguishable from the wild type  $\alpha 1b$ -AR. In contrast, substituting the C-tail of the  $\alpha 1b$  with the C-tail of the  $\alpha 1a$ -AR subtype almost entirely impaired receptor endocytosis (Fig. 6, A).

To investigate whether the internalization of the  $\alpha 1a/Ctb$  chimeric receptor was linked to its gain of interaction with regulatory proteins, we assessed its ability to co-immunoprecipitate with  $\beta$ arrestins and AP50. As shown in Fig. 6 (C), the  $\alpha 1a/Ctb$  chimeric

MOL 43422

receptor was able to co-immunoprecipitate both  $\beta$ arrestin2 and AP50 similarly to the wild type  $\alpha$ 1b-AR. Co-immunoprecipitation of the  $\alpha$ 1a/Ctb chimeric receptor with  $\beta$ arrestin1 was also observed (results not shown). Interestingly, the amount of  $\beta$ arrestin2 co-immunoprecipitating with the chimeric receptor in the absence of the agonist (time 0') was greater than that observed for the wild type  $\alpha$ 1b-AR. This higher basal receptor/ $\beta$ arrestin2 interaction might explain the observation that the  $\alpha$ 1a/Ctb chimeric receptor displays higher basal internalization compared to the wild type  $\alpha$ 1b-AR (Fig. 6, compare panels A and B). In contrast to the  $\alpha$ 1a/Ctb, the  $\alpha$ 1b/Cta chimeric receptor, carrying the C-tail of the  $\alpha$ 1a-AR, behaved like the wild type  $\alpha$ 1a-AR not being able to co-immunoprecipitate neither  $\beta$ arrestin2 nor AP50 (results not shown).

*The relative role of  $\beta$ arrestin and AP50 interaction in  $\alpha$ 1b-AR endocytosis.* Given that both  $\beta$ arrestins and the AP-2 complex serve as clathrin adaptors promoting receptor endocytosis (Goodman et al., 1996 ; Chang et al., 1993) and that the  $\alpha$ 1b-AR can directly interact with both classes of proteins, we wished to explore the relative role played by each in the internalization of the  $\alpha$ 1b-AR. To do so, we used two previously described receptor mutants: the  $\Delta$ R8, lacking the stretch of eight arginines in the C-tail that are responsible for direct binding of AP50, and the M8, lacking eight serines in the C-tail that represent the phosphorylation sites for protein kinase C (PKC) and G protein-coupled receptor kinases (GRK). We have previously reported that the  $\Delta$ R8 mutant was delayed in agonist-induced internalization as compared to the wild type  $\alpha$ 1b-AR (Diviani et al., 2003) and that the M8 was defective in GRK2-mediated phosphorylation and desensitization (Diviani et al., 1997).

We used confocal microscopy on HEK-293 cells expressing the C-terminally HA-tagged M8 receptor mutant to test whether the receptor mutant was able to internalize. As shown in Fig. 7 (A), the M8 receptor did not undergo significant agonist-induced

MOL 43422

endocytosis. Similar findings were obtained in cell surface biotinylation experiments (Fig. 7, B).

We performed co-immunoprecipitation experiments to assess the ability of the internalization deficient M8 mutant to interact with either  $\beta$ arrestins or AP50, and compared its properties with those of the  $\Delta$ R8 mutant that is only partially impaired in endocytosis. Co-immunoprecipitation experiments were performed from HEK-293 cells transiently co-expressing the HA-tagged receptors and FLAG-tagged  $\beta$ arrestin2 in the absence or presence of epinephrine. A representative SDS-PAGE (Fig. 8, A) shows that  $\beta$ arrestin 2 could co-immunoprecipitate with both the wild type  $\alpha$ 1b-AR and its  $\Delta$ R8 mutant, whereas the amount of  $\beta$ arrestin 2 bound to the M8 mutant was very low both in the basal state and after stimulation with epinephrine. The results from several experiments in which the amount of bound  $\beta$ arrestin 2 was expressed as percentage of that bound to the wild type  $\alpha$ 1b-AR in the basal state (time 0') showed that the amount of  $\beta$ arrestin 2 interacting with the  $\Delta$ R8 was similar to that of the wild type receptor and that this interaction was significantly increased by epinephrine (Fig. 8, B). In contrast,  $\beta$ arrestin 2 binding to the M8 was only 20% of that to the wild type receptor and, even if epinephrine could slightly increase this interaction, the total amount of bound  $\beta$ arrestin 2 was very low (Fig. 8, B). Similar results were obtained with  $\beta$ arrestin 1 (data not shown).

To assess the receptor/AP50 interaction, the HA-tagged receptors were expressed in HEK-293 cells and tested for their ability to co-immunoprecipitate the endogenous AP50. The M8 receptor mutant, like the wild type  $\alpha$ 1b-AR, was able to co-immunoprecipitate the endogenous AP50 and this interaction was significantly increased by epinephrine (Fig. 9). As we previously reported (Diviani et al., 2003), stimulation with epinephrine failed to increase the AP50 interaction with the  $\Delta$ R8 mutant (Fig. 9).

MOL 43422

Altogether these findings indicate that the phosphorylation-deficient M8 receptor mutant is almost totally impaired in its ability to bind  $\beta$ arrestins and to undergo endocytosis, despite the fact that its direct binding to AP50 is preserved. The  $\Delta$ R8 receptor mutant, which lacks direct binding to AP50, can efficiently bind  $\beta$ arrestins and it is only partially impaired in its ability to internalize.

*The relative role of  $\beta$ arrestin1 and 2 in the internalization of the  $\alpha$ 1b-AR.* To elucidate the relative role played by  $\beta$ arrestin1 and 2 in the internalization of the  $\alpha$ 1b-AR, we silenced the expression of  $\beta$ arrestins using RNA interference, as previously described (Ahn et al., 2003). As shown in Fig. 10, transfection of cells with plasmids encoding  $\beta$ arrestin 1 and 2 specific shRNAs significantly decreased the expression of the targeted  $\beta$ arrestin by 45% and 60%, respectively, compared to mock transfected cells. Both shRNAs displayed significant isoform specificity (Fig. 10) even if specificity was not absolute. Our findings are in agreement with those reported in the study based on which the shRNA oligos were designed (Ahn et al., PNAS 2003). Interestingly, in cells transfected with  $\beta$ arrestin 2 specific shRNA the epinephrine-induced internalization of the  $\alpha$ 1b-AR at 45' was entirely impaired (Fig. 10, D) thus demonstrating the important role of  $\beta$ arrestin 2 in endocytosis of the  $\alpha$ 1b-AR. However, since the real stoichiometry of the two  $\beta$ arrestins in HEK293 cells is unknown and the gene silencing effect was partial, the lack of effect of  $\beta$ arrestin 1 silencing on receptor internalization does not rule out a role of  $\beta$ arrestin 1 in  $\alpha$ 1b-AR endocytosis.

## DISCUSSION

In this study we demonstrate that the human  $\alpha 1a$ -AR subtype, in contrast to the  $\alpha 1b$ -AR, does not undergo significant endocytosis and we provide strong evidence that this property is linked to its poor interaction with  $\beta$ arrestins as well as with the AP50 subunit of the AP2 complex. We assess the relative contribution of  $\alpha 1b$ -AR interaction with  $\beta$ arrestins versus AP50 in receptor endocytosis and demonstrate that  $\beta$ arrestin binding is the *conditio sine qua non* for receptor internalization. Finally, using RNA interference we provide evidence that  $\alpha 1b$ -AR endocytosis involves  $\beta$ arrestin 2.

The results of our study show that the  $\alpha 1a$  and  $\alpha 1b$ -AR subtypes display striking differences in their internalization properties. Whereas the  $\alpha 1b$ -AR undergoes robust agonist-induced endocytosis, the  $\alpha 1a$ -AR does not. Our findings on the  $\alpha 1a$ -AR subtype seem to disagree with the conclusions of two previous studies in which a modest degree of  $\alpha 1a$ -AR internalization was reported. The study of Chalothorn et al. (2002) reported that the  $\alpha 1a$ -AR was internalized at 50 minutes after exposure to the agonist, but to a smaller extent compared to the  $\alpha 1b$ -AR. Morris et al. (2004) reported that the  $\alpha 1a$ -AR could undergo a modest (~20%) internalization after 60 min of exposure to the agonist. Agonist-induced internalization of the  $\alpha 1b$ -AR was not measured for comparison in the same study. In addition, the  $\alpha 1a$ -AR, but not the  $\alpha 1b$ -AR, displayed some constitutive internalization and it was shown to recycle fast to the plasma membrane.

MOL 43422

Three main factors might explain the discrepancies observed for the  $\alpha$ 1a-AR subtype between our study and the two others: a) the receptor constructs, b) the expression systems, and c) the methods used to measure endocytosis.

Whereas in our study the  $\alpha$ 1a-AR was HA-tagged (either at the N- or C-terminus), both Chalothorn et al. (2002) and Morris et al. (2004) used EGFP-tagged receptor. We cannot exclude that different tags (HA *versus* EGFP) might result in small differences in receptor behavior.

In our study the receptors were transiently expressed in HEK-293 cells whereas both Chalothorn et al. (2002) and Morris et al. (2004) used cells permanently expressing the  $\alpha$ 1-AR subtypes (HEK-293 cells in Chalothorn et al., Rat fibroblasts in Morris et al.). It is possible that the high level of receptor expression occurring in transient transfection systems might not allow to detect small receptor movements.

With respect to the methods used to measure receptor endocytosis, in both Chalothorn et al. (2002) and Morris et al. (2004) receptor internalization was mainly assessed using different fluorescence imaging techniques, whereas we have quantified it using biotinylation experiments. We consider that receptor biotinylation is an accurate method to quantify receptors. However, it might be less sensitive than fluorescence imaging techniques and not detect small receptor movements that have been observed for the  $\alpha$ 1a-AR in previous studies. Nevertheless, these small discrepancies do not hamper the main message of our study which is that large differences do exist between the regulatory properties of the  $\alpha$ 1a and  $\alpha$ 1b-AR subtypes in recombinant systems.

The results from both co-immunoprecipitation experiments (Fig. 3) and  $\beta$ arrestin translocation assays (Fig. 4) indicated that the agonist-induced interaction of the  $\alpha$ 1a-AR with  $\beta$ arrestin 1 and 2 was much weaker than that of the  $\alpha$ 1b-AR. In addition, the  $\alpha$ 1a-AR displayed almost no interaction with the AP50 subunit of the AP2 complex, which was

MOL 43422

previously shown to directly bind  $\alpha 1b$ -AR (6) (Fig. 5). Our hypothesis is that the lack of internalization of the  $\alpha 1a$ -AR is mainly linked to its poor interaction with  $\beta$ arrestins as well as with AP50. In support of this hypothesis is the fact that replacement of  $\alpha 1a$ -AR C-tail with that of the  $\alpha 1b$ -AR, which contains the structural determinants for binding  $\beta$ arrestins and AP50, confers to the  $\alpha 1a$ -AR the ability to internalize and to interact with both regulatory proteins (Fig. 6). This also agrees with findings from a previous study (Vazquez-Prado et al., 2000) showing that, when the two receptor subtypes were compared in the same study, the  $\alpha 1a$ -AR was poorly phosphorylated and desensitized compared to the  $\alpha 1b$ -AR. Replacement of the  $\alpha 1a$ -AR C-tail with that of the  $\alpha 1b$ -AR markedly increased the ability of the receptor to undergo agonist-induced phosphorylation, but the internalization properties of the chimeric receptor were not investigated.

A recent study (Morris et al., 2007) has provided some evidence that the  $\alpha 1a$ -AR is localized in membrane rafts, but exits from rafts following stimulation with agonists. One hypothesis could be that biotinylation of receptors interferes with the exit of the  $\alpha 1a$ -AR from rafts thus impairing endocytosis. However, this hypothesis can be excluded based on findings obtained from experiments in which  $\alpha 1$ -AR internalization was measured using a different biotinylation protocol in which surface (and not internalized) receptors were detected, as previously described (Stanasila et al., 2006). In this protocol, cells were treated for different times with the agonist to induce receptor internalization and receptors remaining at the cell surface were biotinylated after that endocytosis had occurred. Since using this protocol we failed to observe any significant  $\alpha 1a$ -AR endocytosis (results not shown), it is unlikely that the lack of significant  $\alpha 1a$ -AR internalization found in our study is an artifact of biotinylation.

MOL 43422

The differences in internalization observed between the  $\alpha 1a$  and  $\alpha 1b$ -AR subtypes are very similar to those between the  $\beta 1$  and  $\beta 2$ -AR reported in a study in which receptor/ $\beta$ arrestin interaction was investigated using different approaches (Shiina et al., 2000). The  $\beta 1$ -AR expressed in HEK-293 cells showed resistance to agonist-induced internalization as well as weak interaction with  $\beta$ arrestins compared to the  $\beta 2$ -AR subtype. These findings strongly support the hypothesis that the interaction of GPCRs with  $\beta$ arrestins as well as with a number of other regulatory proteins determine the regulatory properties of the receptors.

The findings of our study provide further insight into the molecular mechanisms underlying the endocytosis of the  $\alpha 1b$ -AR. This study indicates that the interaction of the  $\alpha 1b$ -AR with  $\beta$ arrestins requires the integrity of the phosphorylation sites in the receptor C-tail and it is crucial for receptor endocytosis. This was mainly demonstrated by the results obtained with the  $\alpha 1b$ -AR mutant, M8, lacking eight serines in the C-tail which include the main phosphorylation sites for GRKs. The M8 receptor, previously shown to be profoundly impaired in agonist-induced phosphorylation and desensitization (Diviani et al., 1997), was shown in this study to be unable to undergo epinephrine-induced endocytosis (Fig. 7) and to bind  $\beta$ arrestins (Fig. 8). However, the M8 receptor was still able to interact with the AP50 subunit of the AP2 clathrin-adaptor complex upon exposure to epinephrine (Fig. 9). Altogether these findings strongly suggest that  $\alpha 1b$ -AR endocytosis cannot occur in the absence of  $\beta$ arrestin binding and that the direct interaction with the AP50 is not sufficient for receptor internalization. This conclusion is in agreement with the results previously obtained with the  $\alpha 1b$ -AR mutant,  $\Delta R8$ , lacking the binding site for AP50 (Diviani et al., 2003) which could undergo agonist-induced internalization, albeit delayed as compared to wild type  $\alpha 1b$ -AR.



MOL 43422

Our findings identify  $\beta$ arrestin 2 as an important player in the internalization process of  $\alpha$ 1b-AR. This is supported by several lines of evidence: i) the decrease by 60% of endogenous levels of  $\beta$ arrestin 2 using RNA interference almost entirely impaired  $\alpha$ 1b-AR internalization (Fig. 10); ii) the amount of  $\beta$ arrestin 2 forming a complex with the  $\alpha$ 1b-AR was greater as compared to  $\beta$ arrestin 1 suggesting a greater affinity of the receptor for  $\beta$ arrestin 2 than 1 (Fig. 3, E); iii) the  $\alpha$ 1b-AR was found to colocalize, at least in part, with  $\beta$ arrestin 2, but much less with  $\beta$ arrestin 1, in endocytic vesicles after exposure to the agonist (Fig. 4, B). Further studies using cells devoid of either one of the two  $\beta$ arrestins (Conner et al., 1997; Bohn et al., 1999) should help to unequivocally determine the role played by each  $\beta$ arrestin in  $\alpha$ 1b-AR internalization.

Two classes of GPCRs, designated as class A and B, have been identified based on their pattern of interaction with  $\beta$ arrestins (Oakley et al., 2000). Class A receptors, such as the  $\beta$ 2-AR, bind  $\beta$ arrestin 2 with greater affinity than  $\beta$ arrestin 1 forming a short-lived receptor/ $\beta$ arrestin complex. In contrast, class B receptors, such as the angiotensin II type 1A receptor, display similar affinity for both  $\beta$ arrestin isoforms establishing a stable receptor/ $\beta$ arrestin complex. Our findings indicate that the  $\alpha$ 1b-AR, previously reported as belonging to class A (Oakley et al., 2000), displays indeed some clear features of class A receptors such as its greater affinity for  $\beta$ arrestin 2 than for  $\beta$ arrestin 1. However, the pattern of  $\alpha$ 1b-AR internalization is quite different from that observed for the  $\beta$ 2-AR, a prototypic class A receptor. In fact, the kinetics of both internalization and recycling of the  $\alpha$ 1b-AR is much slower than that of the  $\beta$ 2-AR (compare Fig.1 and Fig.1S). In addition, co-localization of the  $\alpha$ 1b-AR with  $\beta$ arrestin 2 was observed in endocytic vesicles, suggesting that the receptor can form a stable complex with  $\beta$ arrestin within the cells, a feature considered to be a hallmark of class B GPCRs. This suggests that the classification of GPCRs properties into

MOL 43422

major classes might be sometimes reductive if it concerns complex regulatory phenomena which should be investigated using different approaches.

The implication of different  $\beta$ arrestin isoforms in GPCR endocytosis has been investigated in a large number of studies. However, in most cases GPCR/ $\beta$ arrestin interaction has been followed in intact cells by monitoring  $\beta$ arrestin translocation and receptor/ $\beta$ arrestin co-localization using confocal microscopy. Few studies have measured GPCR/ $\beta$ arrestin co-immunoprecipitation (Chen et al., 2002 ; Kishi et al., 2002) and even fewer have performed *in vitro* biochemical measurements of  $\beta$ arrestin binding to receptors (reviewed in Gurevich and Gurevich, 2006). Therefore, despite the large number of studies on the subject, there is probably still more to learn about the specificity of GPCR/ $\beta$ arrestin interaction.

Altogether, the findings of this study demonstrate important regulatory differences between the  $\alpha$ 1a and  $\alpha$ 1b-AR in a recombinant system, based on the fact that the properties of the two receptor subtypes were directly compared using different experimental approaches. We believe that, at least in part, these differences are linked to the different interaction pattern of the receptors with  $\beta$ arrestins and AP50. However, a number of important questions can be raised: are these differences occurring in native tissues where the  $\alpha$ 1a and  $\alpha$ 1b-AR are expressed? what might be the functional implications of these differences in native tissues? Since GPCR regulation depends on a variety of protein interactions and these differ among tissues, its pattern might be highly dependent on the cell system in which the receptors are expressed. It is, therefore, possible that HEK-293 cells do not contain all the components necessary for  $\alpha$ 1a-AR endocytosis. For this reason, future studies will aim at expressing the  $\alpha$ 1-AR subtypes and investigating their properties in cell systems more closely related to their native environment (vascular smooth muscle cells, cardiomyocytes). At present, it is difficult to speculate on how the different regulatory properties of the  $\alpha$ 1a and  $\alpha$ 1b-AR subtypes might be linked to their physiological functions.

MOL 43422

Studies on cells devoid of either receptor subtype might provide a useful contribution in this respect.

### Acknowledgements

We are grateful to Monique Nenniger-Tosato for excellent technical assistance and to Dr. Dario Diviani for helpful discussions.

MOL 43422

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MOL 43422

Footnotes

This work was supported by the Fonds National Suisse de la Recherche Scientifique (grant n.3100A0-100703).



## FIGURE LEGENDS

Fig. 1. Agonist-induced internalization and recycling of the  $\alpha 1a$  and  $\alpha 1b$ -AR monitored by receptor biotinylation. The internalized biotinylated HA-tagged receptors were measured in the absence of agonist for 90 min (B=basal), 30 (+30') and 90 (+90') min after stimulation with  $10^{-4}$  M epinephrine, or 60 (60'Ø) and 90 (90'Ø) min after removing epinephrine. (A) Representative Western blots of biotinylated  $\alpha 1a$  and  $\alpha 1b$ -AR tagged with HA at their-N-terminus. Western blots of biotinylated receptors in the cell extract (Ext.) and precipitate (Prec.) were revealed using monoclonal anti-HA antibodies (Covance). (B) and (C) The internalized biotinylated HA-tagged receptors are expressed as percentage of the total biotinylated receptors (Tot). Results are the mean $\pm$ S.E. of eight independent experiments.

\*  $p < 0.05$ , \*\* $p < 0.01$  one-way ANOVA compared to basal (B).

Fig. 2. Constitutive receptor internalization the  $\alpha 1a$  and  $\alpha 1b$ -AR monitored by receptor biotinylation. In (A) and (B), the internalized biotinylated receptors tagged with HA at their N-terminus were measured after incubating the cells in the absence of epinephrine at the indicated times. In (A), representative Western blots of biotinylated receptors, revealed as in Fig. 1, are shown. In (B), the internalized biotinylated HA-tagged receptors are expressed as percentage of the total biotinylated receptors. Results are the mean $\pm$ S.E. of three independent experiments. \* $p < 0.05$  Kruskal-Wallis test compared to 0'. In (C), constitutive internalization was measured after 90 min incubation in the absence or presence of monensin (+Mon). Results are the mean $\pm$ S.E. of three independent experiments. \* $p < 0.05$  Student's t test compared to 90' (in the absence of monensin).

MOL 43422

Fig. 3. Co-immunoprecipitation of  $\beta$ arrestins with the  $\alpha$ 1a and  $\alpha$ 1b-AR. FLAG-tagged  $\beta$ arrestin 1 and 2 were co-expressed with the C-terminally HA-tagged receptors in cells and co-immunoprecipitated from cells incubated in the absence (0') or presence of  $10^{-4}$  M epinephrine for the indicated times. (A) Western blots from a representative experiment are shown. Cell lysates (Ext.) were immunoprecipitated using polyclonal anti-HA antibodies. Western blots of immunoprecipitates (IP) were revealed using monoclonal anti-FLAG for  $\beta$ arrestins and monoclonal anti-HA antibodies (Sigma) for the receptors. The blots shown were overexposed (15 min) to show low  $\beta$ arrestin signals. In (B) and (C),  $\beta$ arrestin 1 and 2 co-immoprecipitated with each receptor are expressed as percentage of the protein co-immunoprecipitated in the absence of epinephrine (0'). Results are mean $\pm$ S.E. of five independent experiments. \* $p$ <0.05, \*\* $p$ <0.01 Kruskal-Wallis test compared to time 0'. In (D) and (E),  $\beta$ arrestin 1 and 2 co-immunoprecipitated with each receptor are expressed as percentage of  $\beta$ arrestin 1 in the absence of epinephrine (0'). Results are the mean $\pm$ S.E. of five independent experiments. \* $p$ <0.05, \*\* $p$ <0.01 Kruskal-Wallis test compared to  $\beta$ arrestin 1 at each time.

Fig. 4. Cellular localization of the  $\beta$ arrestins and the  $\alpha$ 1-AR subtypes monitored by confocal microscopy. Confocal microscopy was performed on cells co-expressing the C-terminally HA-tagged  $\alpha$ 1a (A) and  $\alpha$ 1b-AR (B) with FLAG-tagged  $\beta$ arrestin 1 or 2 in the absence (0') or presence of  $10^{-4}$  M epinephrine at different times. Cells were fixed, permeabilized and stained using polyclonal anti-HA and secondary rhodamine-coupled antibodies to detect the receptor, and monoclonal anti-FLAG and secondary FITC-coupled antibodies, to detect  $\beta$ arrestins. The images are representative of four independent experiments.

MOL 43422

Fig. 5. Interaction of AP50 with the  $\alpha$ 1a and  $\alpha$ 1b-AR. (A) Pulldown of endogenous AP50 from cell lysates using purified GST or GST-fused to the C-tail of the  $\alpha$ 1a or  $\alpha$ 1b-AR. (B) Co-immunoprecipitation of endogenous AP50 from cells expressing the  $\alpha$ 1a and  $\alpha$ 1b-AR tagged with HA at their C-tail in the absence (0') or presence of  $10^{-4}$  M epinephrine for 15 min. Cell lysates (Ext.) were immunoprecipitated using polyclonal anti-HA antibodies. Western blots of immunoprecipitates (IP) were revealed using monoclonal anti-HA antibodies (Sigma) for the receptors and specific monoclonal antibodies against AP50. The Western blots are representative of three experiments.

Fig. 6. Regulatory properties of  $\alpha$ 1a/ $\alpha$ 1b chimeric ARs. (A) and (B) The internalized biotinylated receptors tagged with HA at their C-tail were measured in the absence of agonist for 60 min (B=basal), 30 (+30') and 60 (+60') min after stimulation with  $10^{-4}$  M epinephrine. The internalized biotinylated HA-tagged receptors were detected as in Fig. 1 and are expressed as percentage of the total biotinylated receptors. Results are the mean $\pm$ S.E. of three independent experiments. \* $p < 0.05$  Student's t test compared to  $\alpha$ 1bHA (A) and  $\alpha$ 1aHA (B) at each time point. (C) Co-immunoprecipitation of FLAG-tagged  $\beta$ arrestin2 and endogenous AP50 with the  $\alpha$ 1b-AR and  $\alpha$ 1a/Ctb chimeric receptor tagged with HA at their C-tail. Cell lysates (Ext.) were immunoprecipitated using polyclonal anti-HA antibodies. Western blots of immunoprecipitates (IP) were revealed as in Fig. 3 and 5. The Western blots are representative of two experiments.

Fig. 7. Internalization of the  $\alpha$ 1b-AR and its mutant M8 monitored by confocal microscopy (A) and biotinylation (B). (A) Confocal microscopy was performed on cells expressing the C-terminally HA-tagged receptors in the absence (0') or presence of  $10^{-4}$  M epinephrine at different times. Cells were fixed, permeabilized and stained using polyclonal

MOL 43422

anti-HA and secondary rhodamine-coupled antibodies to detect the receptors. The images are representative of three independent experiments in which very little variability was observed among cells. (B) The internalized biotinylated HA-tagged receptors were measured in the absence of agonist at time 0' (B=basal) or in the presence of  $10^{-4}$  M epinephrine for 90 min. Western blots of biotinylated receptors in the cell extract (Ext.) and precipitate (Prec.) were revealed using monoclonal anti-HA antibodies (Sigma).

Fig. 8. Co-immunoprecipitation of  $\beta$ arrestin 2 with the  $\alpha$ 1b-AR and its mutants  $\Delta$ R8 and M8. FLAG-tagged  $\beta$ arrestin 2 was co-expressed with the receptors tagged with HA at their C-tail in cells and co-immunoprecipitated in the absence (0') or presence of  $10^{-4}$  M epinephrine for different times. (A) Western blots from a representative experiment are shown. Cell lysates (Ext.) were immunoprecipitated using polyclonal anti-HA antibodies. Western blots of immunoprecipitates (IP) were revealed using monoclonal anti-FLAG for  $\beta$ arrestin and monoclonal anti-HA antibodies (Sigma) for the receptors. The blots shown were overexposed (15 min) to show low  $\beta$ arrestin signals. In (B),  $\beta$ arrestin 2 co-immoprecipitated with the each receptor is expressed as percentage of the  $\beta$ arrestin 2 co-immunoprecipitated with the  $\alpha$ 1b-AR in the absence of epinephrine (0'). Results are mean $\pm$ S.E. of four independent experiments. \* $p$ <0.05 Kruskal-Wallis test compared to the  $\alpha$ 1b-AR for each time.

Fig. 9. Co-immunoprecipitation of endogenous AP50 with the  $\alpha$ 1b-AR and its mutants  $\Delta$ R8 and M8. Endogenous AP50 was co-immunoprecipitated from cells expressing the receptors tagged with HA at their C-tail in the absence (0') or presence of  $10^{-4}$  M epinephrine for different times. (A) Western blots from a representative experiment are shown. Cell lysates (Ext.) were immunoprecipitated using polyclonal anti-HA antibodies. Western blots of

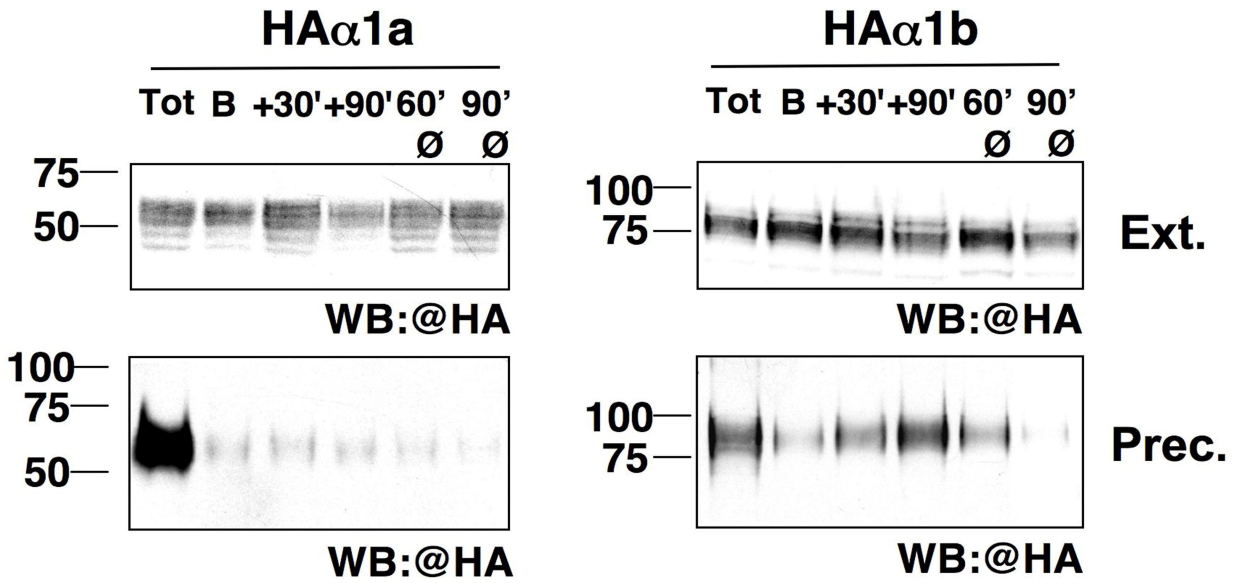
MOL 43422

immunoprecipitates (IP) were revealed using specific monoclonal antibodies (Sigma) against AP50 or monoclonal anti-HA antibodies (Sigma) for the receptors. The blots shown were overexposed (15 min) to show low AP50 signals. In (B), AP50 co-immoprecipitated with each receptor is expressed as percentage of the protein co-immunoprecipitated in the absence of epinephrine (0'). Results are the mean±S.E. of four independent experiments. \* $p < 0.05$  Kruskal-Wallis test compared to time 0'.

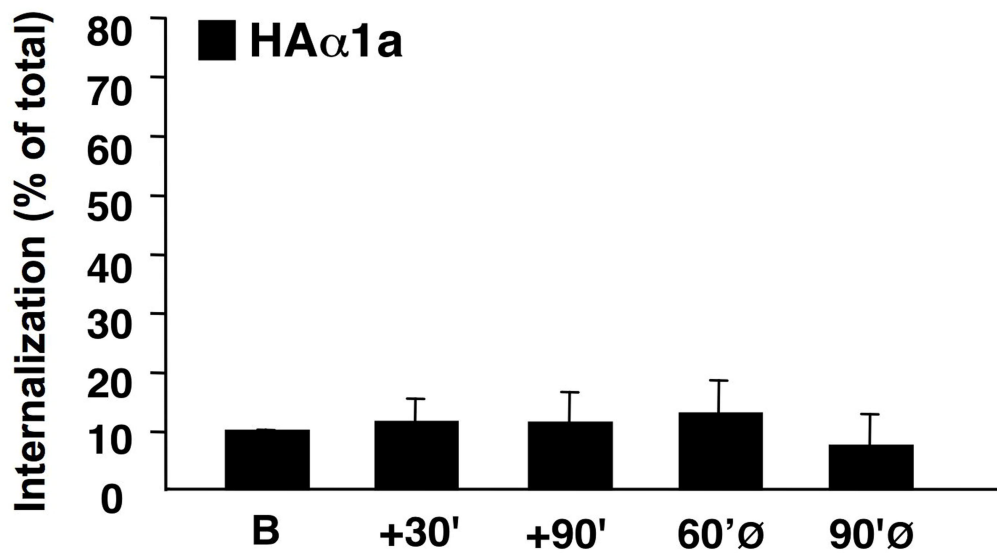
Fig. 10. Effect of silencing  $\beta$ arrestins expression on  $\alpha 1b$ -AR internalization. Cells were co-transfected with the plasmid encoding the C-terminally HA-tagged  $\alpha 1b$ -AR and pSuper encoding shRNA for  $\beta$ arrestin 1 or 2. (A) and (B) Endogenous  $\beta$ arrestin 1 (upper band) or 2 (lower band) were detected by Western blot using polyclonal antibodies against rat  $\beta$ arrestin 1. In (A) are shown representative Western blots of extracts from cells transfected with the wild type  $\alpha 1b$ -AR cDNA together with empty pSuper (wt) or with the shRNA for  $\beta$ arrestin 1 or 2. In (B) the levels of  $\beta$ arrestins in cells transfected with the shRNA for  $\beta$ arrestin 1 or 2 are expressed as percentage of those in cells expressing the wild type  $\alpha 1b$ -AR alone. Results are the mean±S.E. of six independent experiments. \*\* $p < 0.01$ , \* $p < 0.05$  Student's t test compared to wild type  $\alpha 1b$ -AR in the absence of shRNA. (C) and (D) Internalization of the  $\alpha 1b$ -AR was monitored by receptor biotinylation, as described in Fig. 1, in cells expressing the wild type  $\alpha 1b$ -AR alone (wt) or co-expressing the shRNA for  $\beta$ arrestin 1 or 2. Results are the mean±S.E. of six independent experiments. \* $p < 0.05$  one-way ANOVA compared to  $\alpha 1b$ -AR at 45'.

Fig. 1

A)



B)



C)

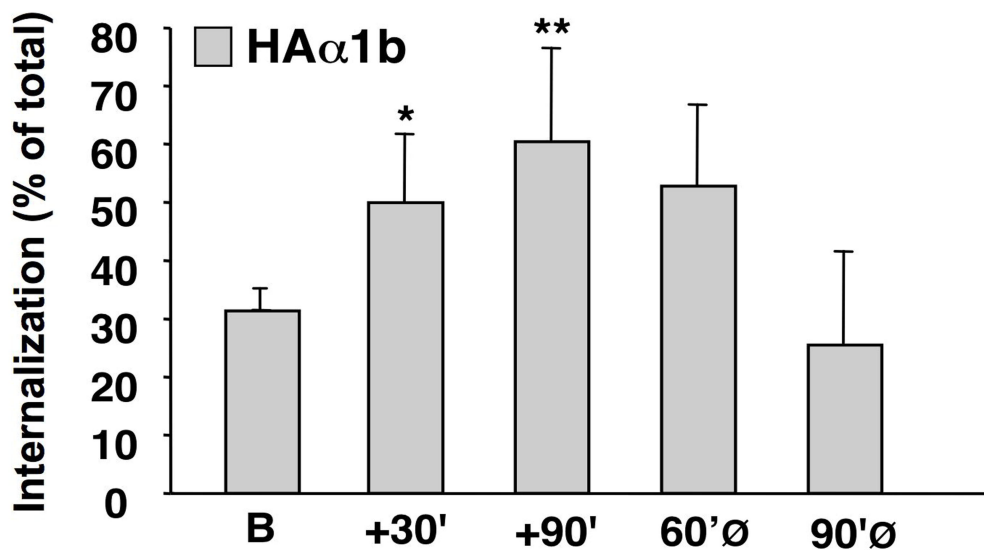
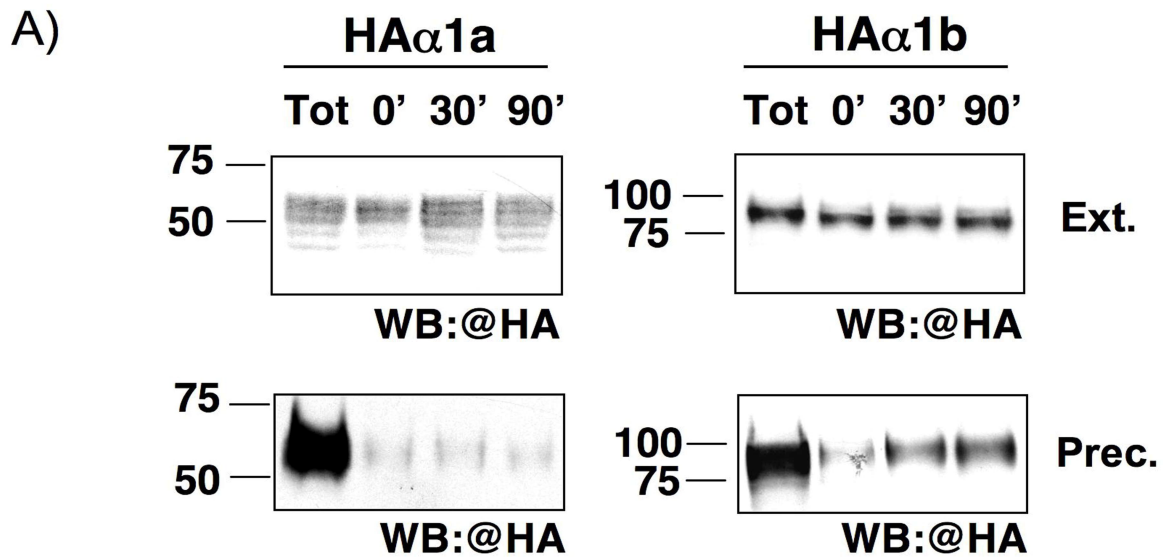
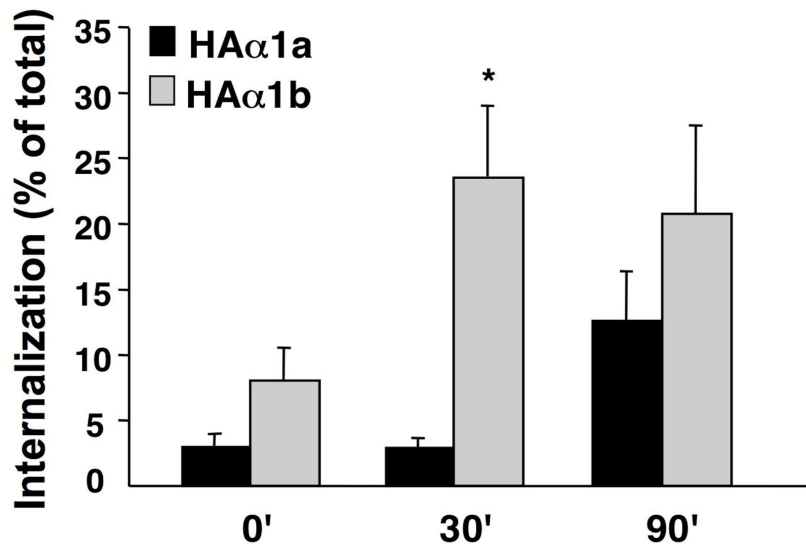


Fig. 2



B)



C)

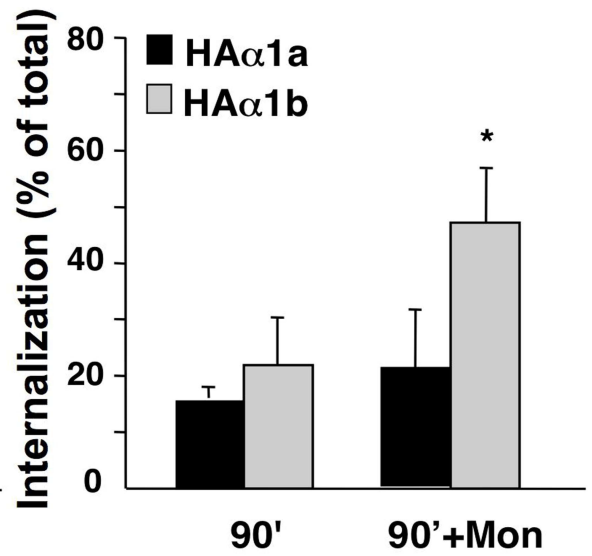
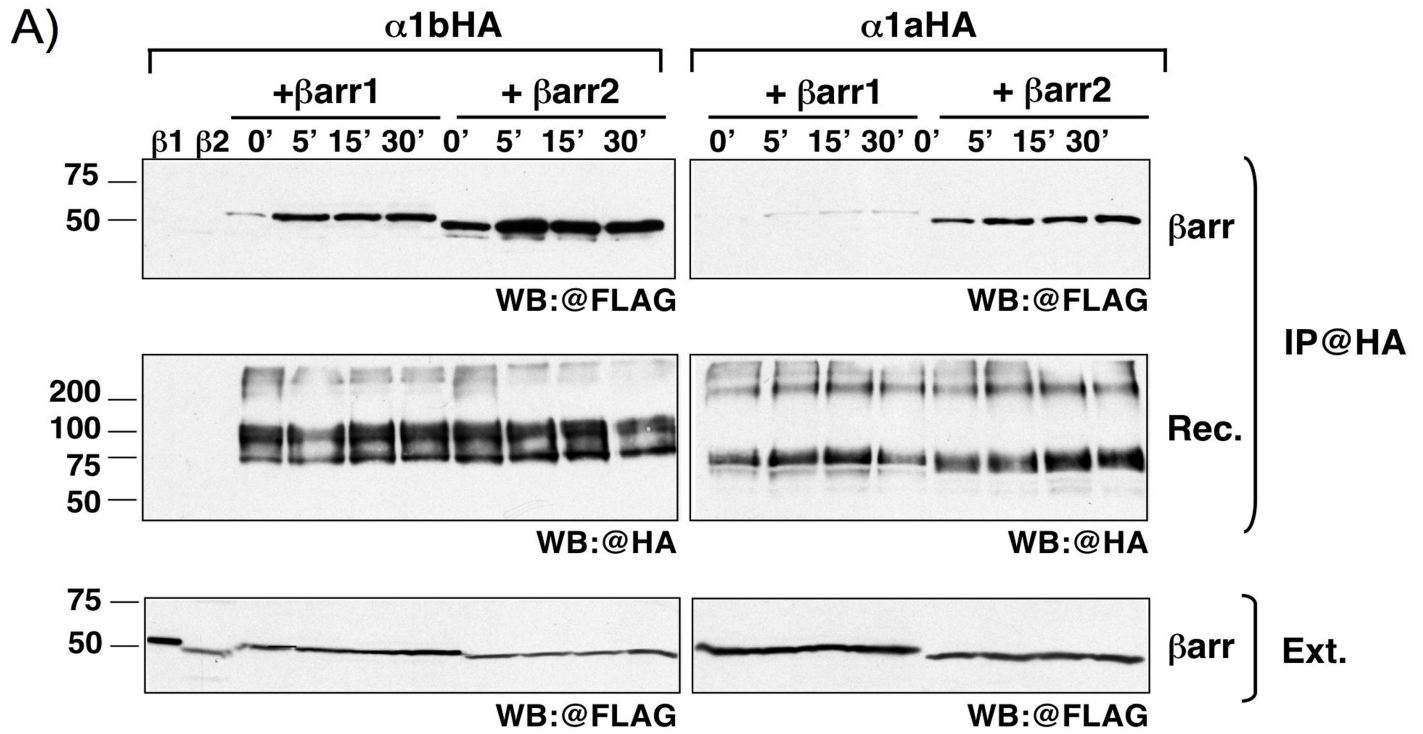
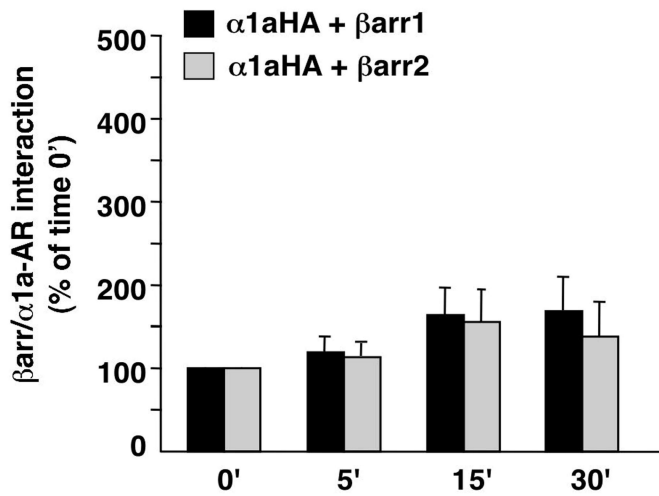


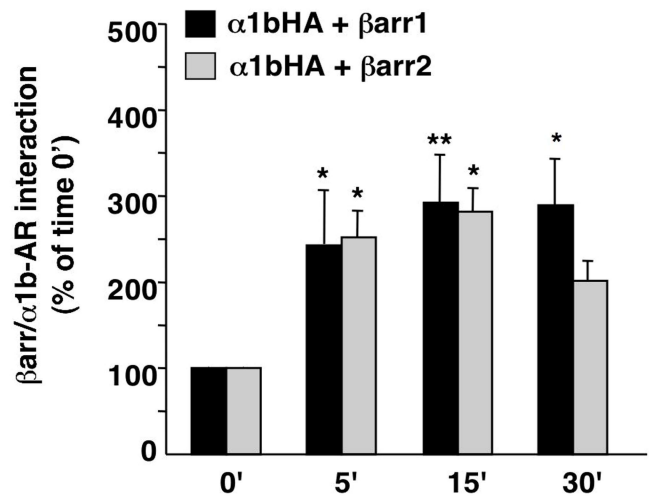
Fig. 3



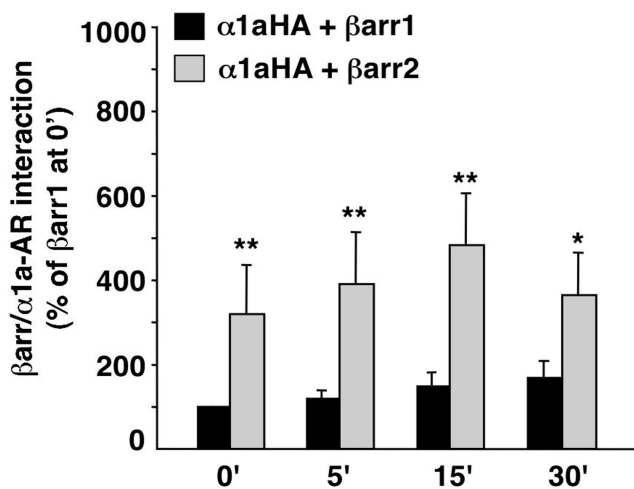
**B)**



**C)**



**D)**



**E)**

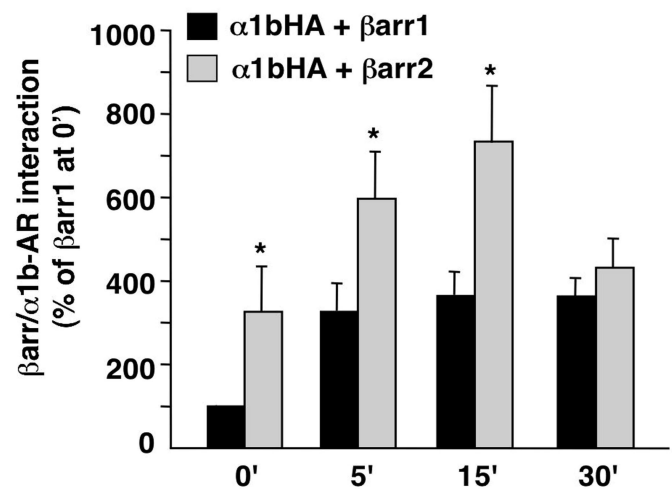




Fig. 4

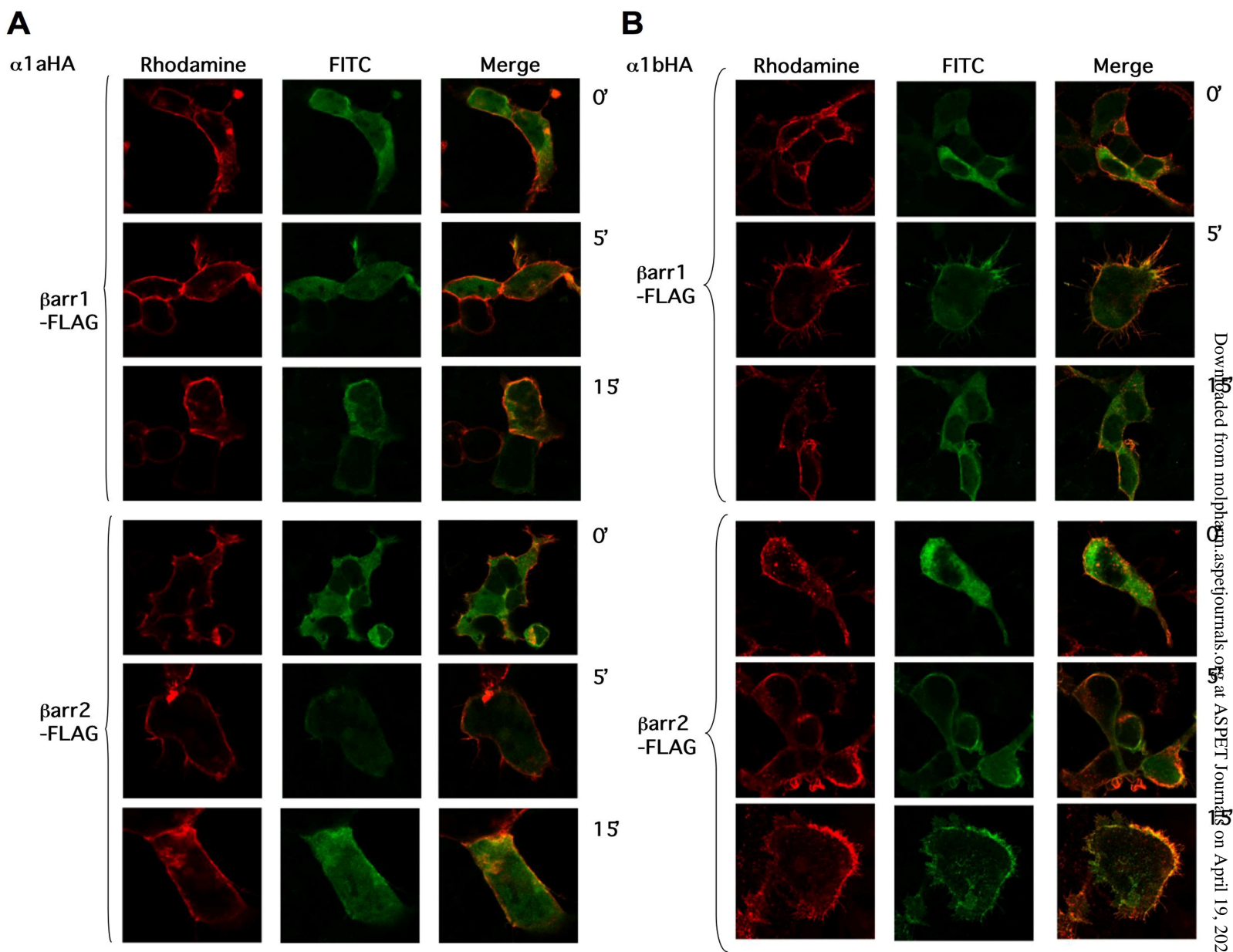


Fig. 5

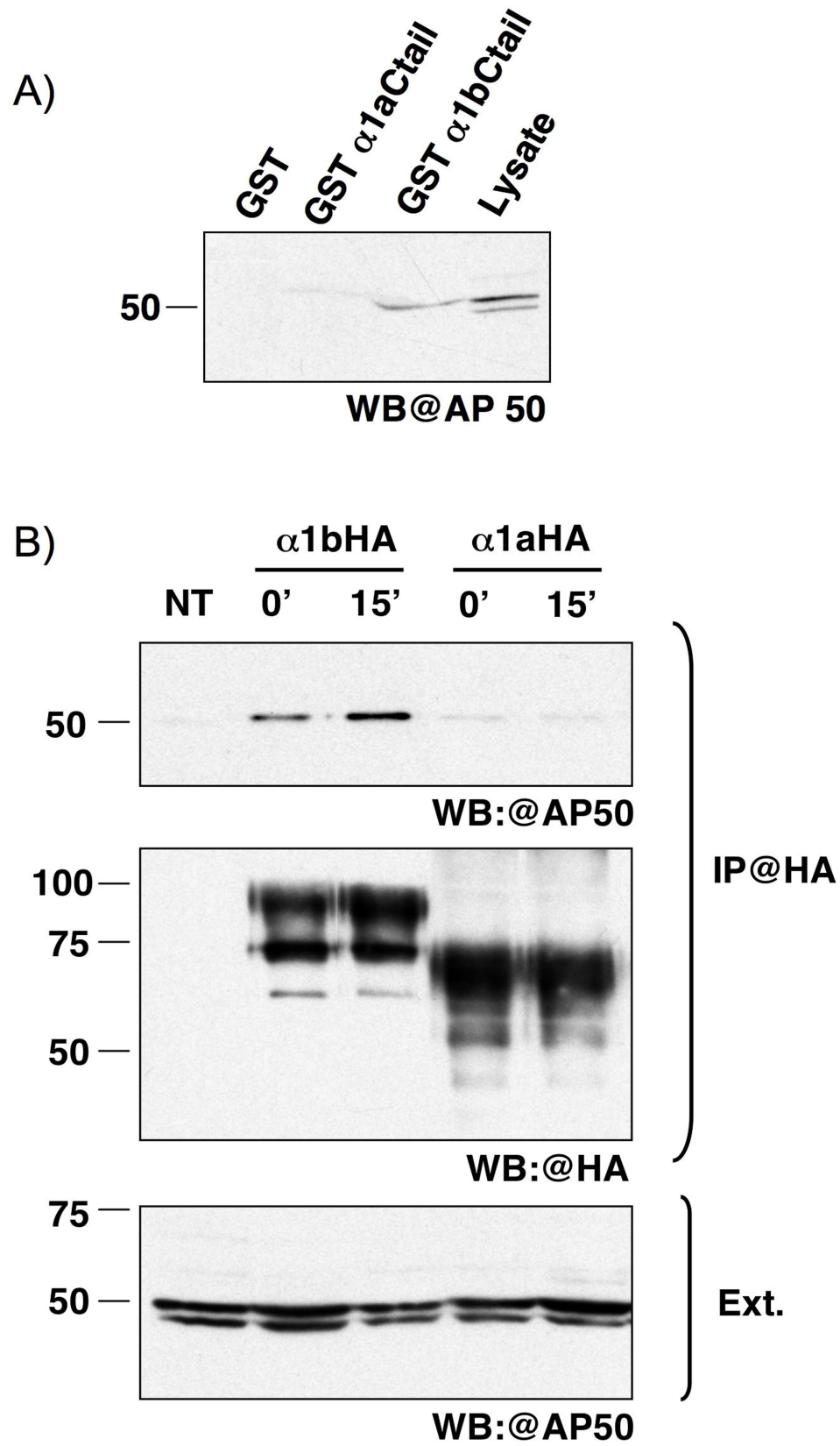


Fig. 6

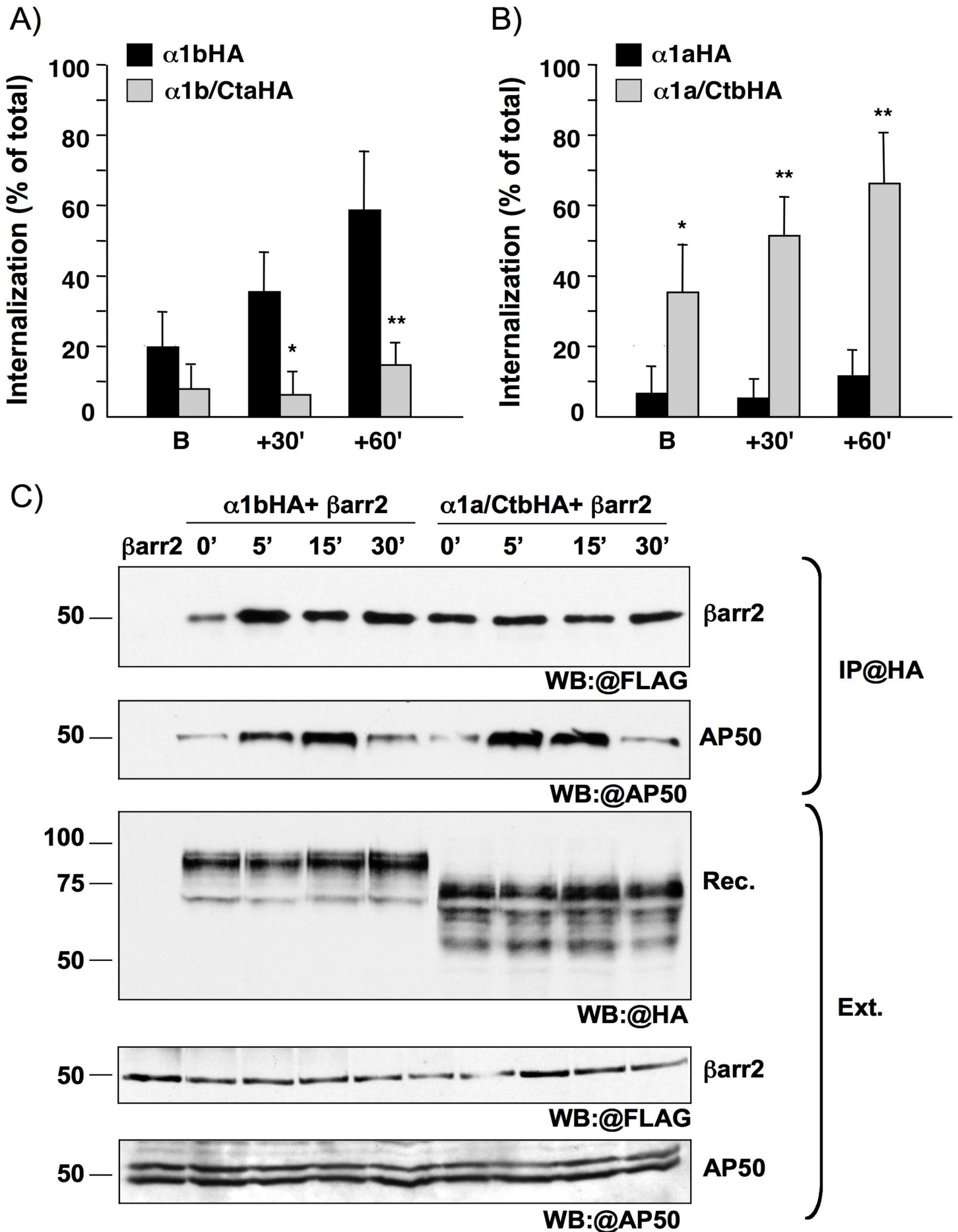


Fig. 7

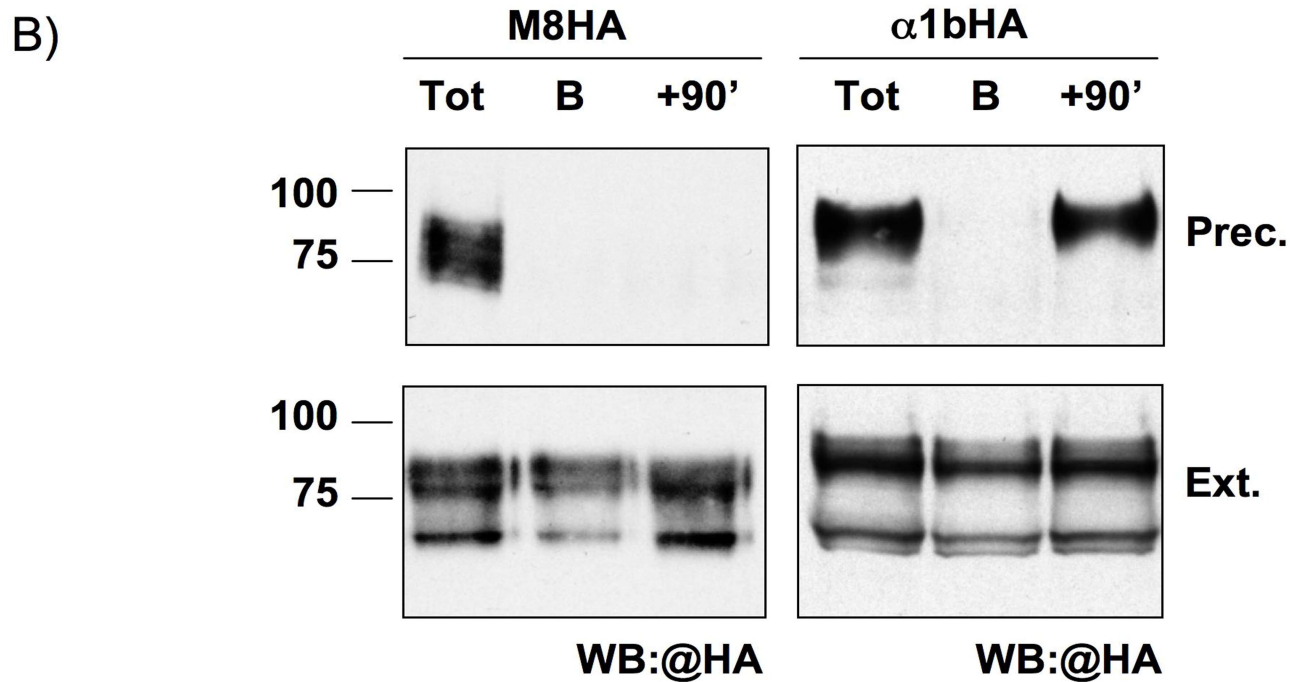
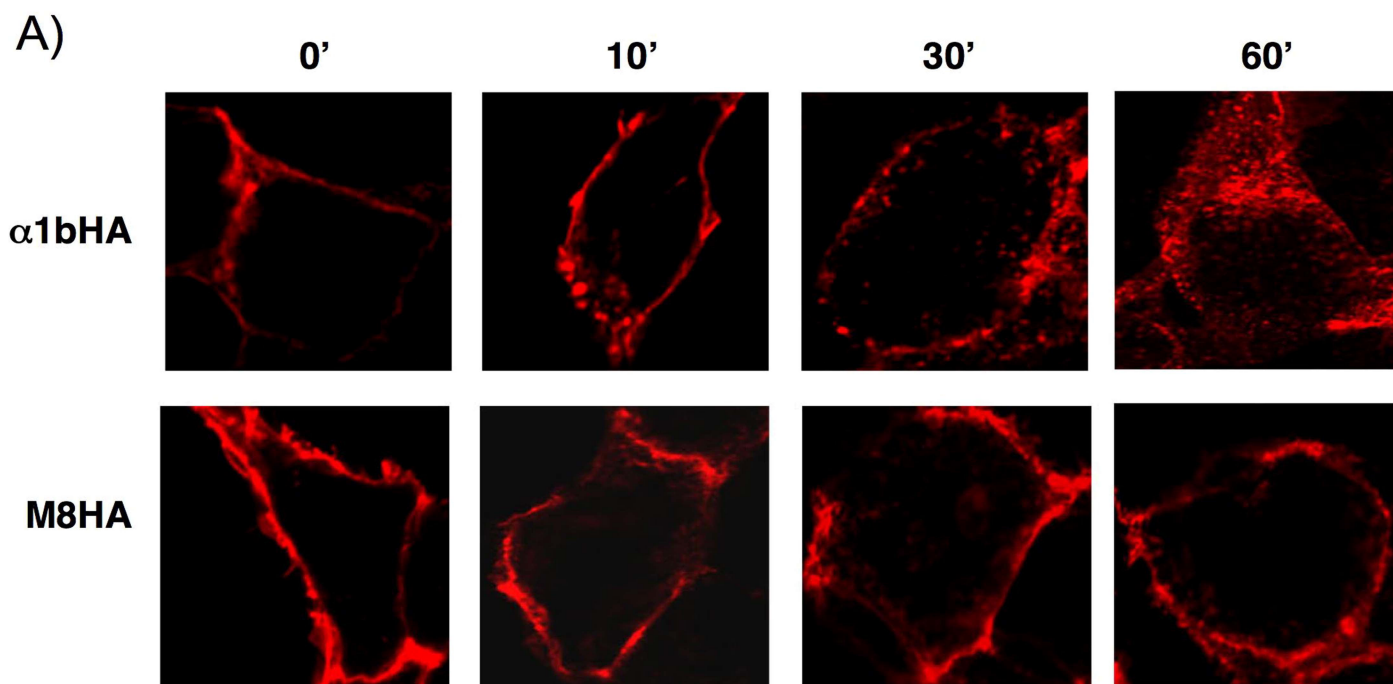
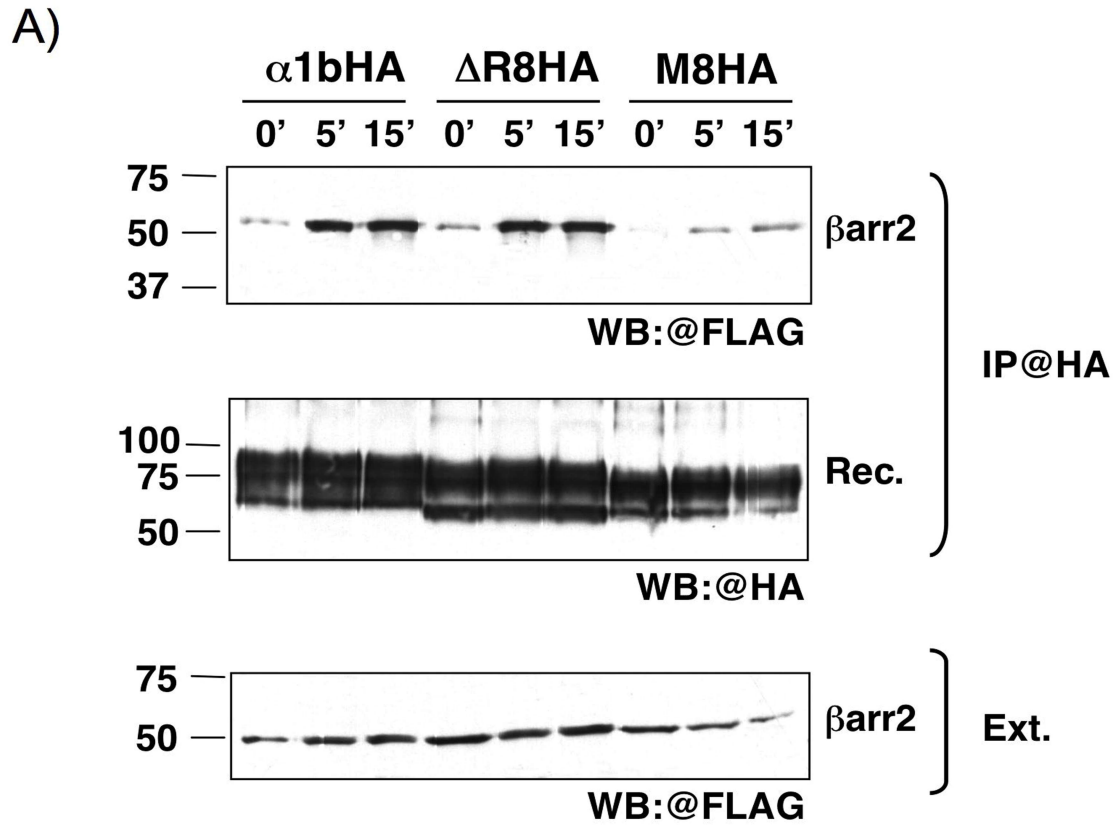


Fig. 8



B)

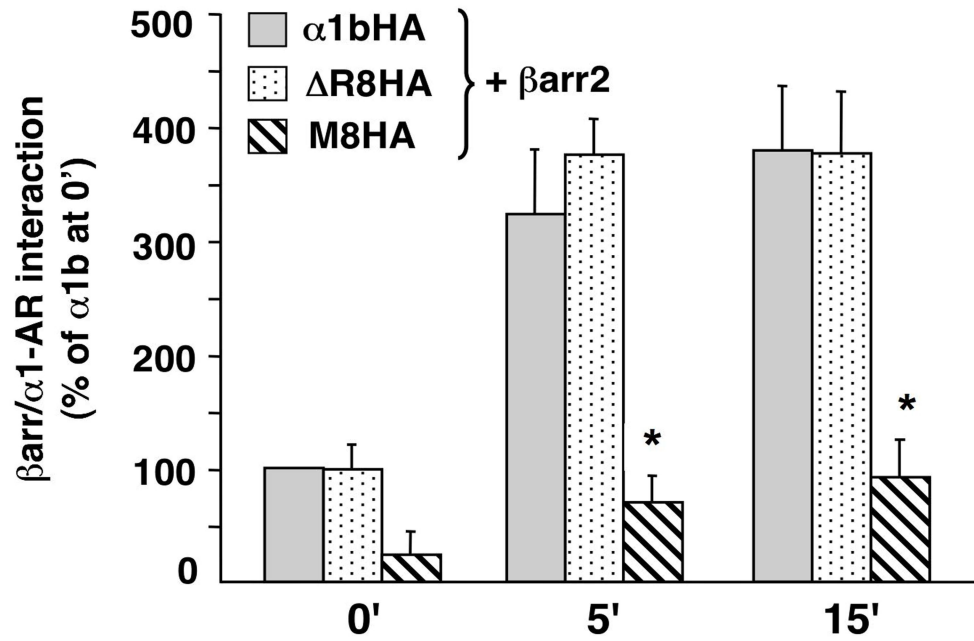
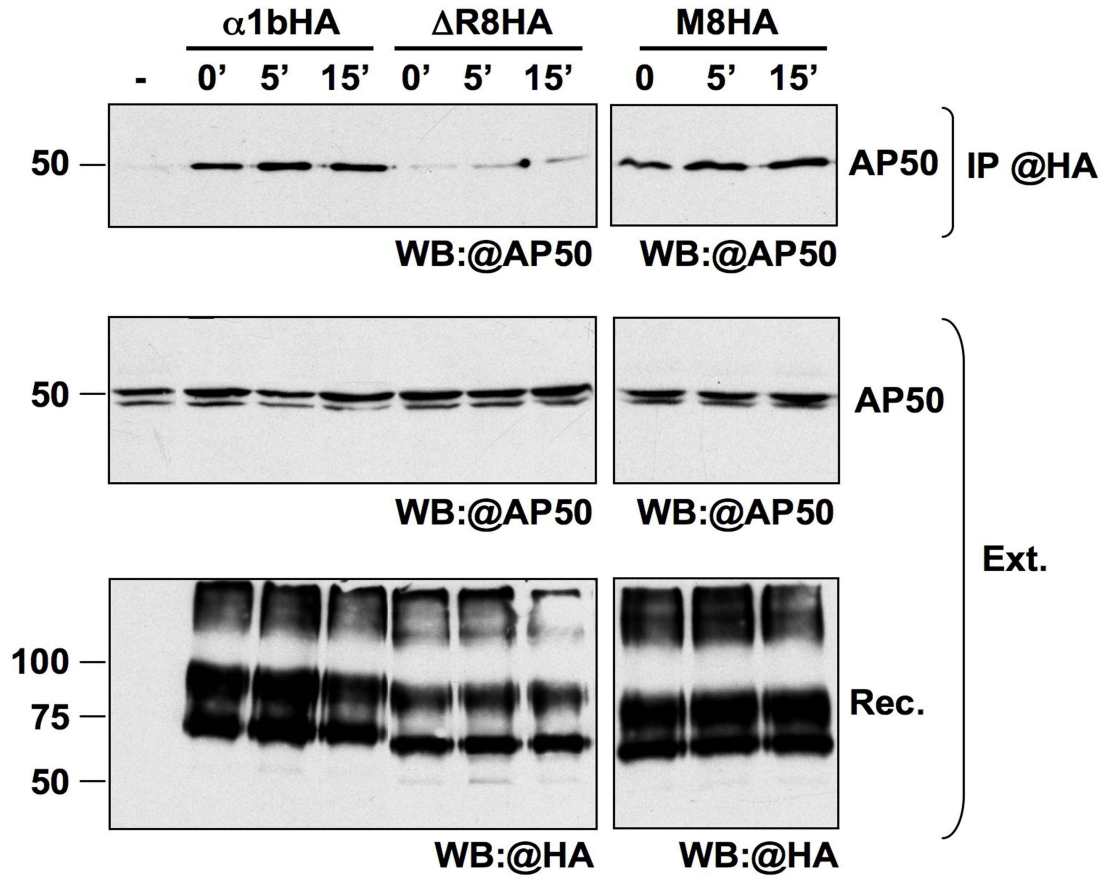


Fig. 9

A)



B)

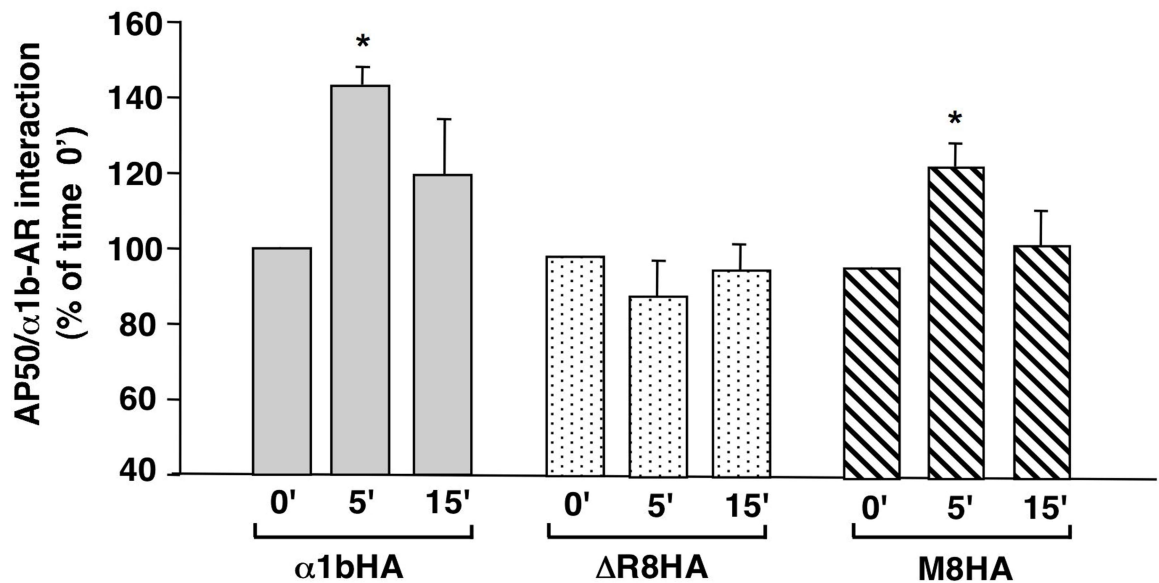


Fig. 10

