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DIFFERENT INTERNALIZATION PROPERTIES OF THE α1a AND α1b-ADRENERGIC RECEPTOR SUBYTPES: THE POTENTIAL ROLE OF RECEPTOR INTERACTION WITH βARRESTINS AND AP50

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Running title: α1-adrenergic receptors interaction with βarrestins and AP50

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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; [^{125}I]-HEAT, [^{125}I]-iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone.

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ABSTRACT

The internalization properties of the α1a and α1b-adrenergic receptors (AR) subtypes transiently expressed in HEK-293 cells were compared using biotinylation experiments and confocal microscopy. Whereas the α1b-AR displayed robust agonist-induced endocytosis, the α1a-AR did not. Constitutive internalization of the α1a-AR was negligible, whereas the α1b-AR displayed significant constitutive internalization and recycling. We investigated the interaction of the α1-AR subtypes with βarrestin 1 and 2 as well as with the AP50 subunit of the clathrin adaptor complex AP2. The results from both co-immunoprecipitation experiments and βarrestin translocation assays indicated that the agonist-induced interaction of the α1a-AR with βarrestins was much weaker than that of the α1b-AR. In addition, the α1a-AR did not bind AP50. The α1b-AR mutant M8, lacking the main phosphorylation sites in the receptor C-tail, was unable to undergo endocytosis and was profoundly impaired in binding βarrestins despite its binding to AP50. In contrast, the α1b-AR mutant ΔR8, lacking AP50 binding, bound βarrestins efficiently and displayed delayed endocytosis. RNA interference showed that βarrestin 2 plays a prominent role in α1b-AR endocytosis. The findings of this study demonstrate differences in internalization between the α1a and α1b-AR and provide evidence that the lack of significant endocytosis of the α1a-AR is linked to its poor interaction with βarrestins as well as with AP50. We also provide evidence that the integrity of the phosphorylation sites in the C-tail of the α1b-AR is important for receptor/βarrestin interaction and that this interaction is the main event triggering receptor internalization.

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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 β (β1, β2, β3), three α2 (α2A, α2B, α2C) and three α1 (α1a, α1b, α1d) receptor subtypes. The α1-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 α1-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 α1b-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 α1b-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 α1b-AR occurs via clathrin-coated vesicles and seems to involve βarrestins as suggested by the fact that co-expression of a dominant negative βarrestin mutant could inhibit receptor internalization (Mhaouty-Kodja et al., 1999). We also demonstrated that the α1b-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 α1a-AR subtype. It has been clearly demonstrated that the bovine α1a-AR expressed in Rat-1 fibroblasts was poorly phosphorylated and desensitized compared to the α1b-AR (Vazquez-Pardo et al.,

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2000). Replacement of the α1a-AR C-tail with that of the α1b-AR significantly increased the ability of the receptor to undergo agonist-induced phosphorylation.

In one study (Chalothorn et al., 2002), the human α1a-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 α1b-AR. In another study (Morris et al., 2004), the human GFP-tagged human α1a-AR expressed in Rat-1 fibroblasts was also shown to undergo very modest agonist-induced endocytosis (20% maximal decrease of surface receptors). However, the α1a-AR, but not the α1b-AR, seemed to undergo constitutive internalization and recycling.

To better understand the mechanisms involved in α1-AR subtype trafficking, we performed a comparison between the α1a and α1b-AR with respect to their ability to undergo either constitutive or agonist-induced internalization as well as to bind β-arrestins and the AP50 subunit of the AP2 clathrin adaptor complex. Furthermore, we investigated the relative role of β-arrestins and AP50 in α1b-AR internalization. Finally, using RNA interference, we assessed the implication of β-arrestin 1 and 2 in α1b-AR endocytosis. Our findings indicate that the internalization properties of the α1a and α1b-AR are very different and suggest potential molecular mechanisms underlying these differences.

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MATERIALS AND METHODS

Materials - FLAG-tagged rat βarrestin 1 and 2 were the kind gift of Dr. R. J. Lefkowitz (Duke University, USA); rabbit polyclonal antibodies against βarrestin 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 μg/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 μg/10 cm Φ dish.

DNA constructs - HA tags (YPYDVPDYA) were introduced into the human α1a and hamster α1b-AR sequence at the N or C-terminus by PCR amplification and the tagged receptor cDNA were subcloned in pRK5 expression vector. The α1b-AR mutants ΔR8 and M8 (S394-415A) were described elsewhere (Diviani et al., 2003 ; Diviani et al., 1997).The chimeric receptor α1aCtbHA was constructed by PCR amplification of a fragment encompassing aminoacids 369-515 and the C-terminal HA tag from the α1bHA-pRK5-pRK5 and subcloning it into the α1aHA-PRK5. The α1bCtaHA was similarly constructed by PCR amplifying the fragment encompassing aminoacids 343-466 and the HA tag from the α1aHA and subcloning it into the α1bHA-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

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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 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/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 μl 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,

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cells were incubated for 1 h in serum-free DMEM containing 10 μM cycloheximide, then washed twice with cold PBS and biotinylated using 4 ml PBS containing 300 μg/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^{-4} M epinephrine (Sigma) at 37°C for various times. To measure receptor recycling, after incubation with 10^{-4} M epinephrine, the medium was removed, the cells were washed, fresh medium containing 10^{-7} 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 μl 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 μl of 100 mM dithiotreitol (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% β-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

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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 μM 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-α1a or HA-α1b 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_2O, 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);

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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 HAα1a and HAα1b 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 α1a and α1b-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 βarrestins by Western blotting.

RNA interference - Sequences used to design shRNA oligos directed against βarrestin1 and βarrestin2 were as published (Ahn et al., 2003). Namely, the sequence targeted in βarrestin1 was: AGCCTTCTGCGCGGAGAAT; the sequence targeted in βarrestin2 was: AAGGACCGCAAAGTGTTTGTG. The 19-nt oligos were cloned into the pSuper vector (Brummelkamp et al., 2002) using the BglII-HindIII sites and the resulting plasmid was either transfected alone (17 μg/10 cm Φ dish) or cotransfected with α1aHA-pRK5 or α1bHA-pRK5 (17 μg : 3 μg /10 cm Φ 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).

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RESULTS

Endocytosis and recycling of the α1a and α1b-AR subtypes. To quantify receptor endocytosis, we used a biotinylation assay to selectively label internalized receptors, as previously described (Stanasila et al., 2006). The α1a and α1b-AR subtypes, carrying the HA-tag at their N-terminus (HAα1a and HAα1b), 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 biotinylated 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 α1b-AR. In agreement with our previous findings (Stanasila et al., 2006), about 60% of the α1b-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 α1a-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).

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Biotinylation experiments performed on cells expressing the α1a and α1b-AR carrying the HA-tag at their C-terminus (α1aHA and α1bHA) 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 α1-AR subtypes, we determined the kinetics of endocytosis and recycling of a well-studied receptor, the β2 AR (Fig. 1S, Supplemental data). As previously reported (Kallal et al., 1998) the agonist-induced internalization of the β2 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 α1-AR subtypes. As we previously reported (Stanasila et al., 2006), a punctate intracellular signal was observed in cells expressing the HAα1b-AR already after 15 min of treatment with epinephrine. In contrast, the intracellular signal corresponding to internalized HAα1a-ARs was faint and detectable only after 90 min of epinephrine stimulation (results not shown).

To further explore the differences in endocytosis between the α1a and α1b-AR subtypes, we compared the rate of constitutive internalization of the two receptors in the absence of the agonist (Fig. 2 A, B). The α1b-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 α1b-AR was sensitive to sucrose, indicating that it involves a clathrin-dependent pathway (data not shown). The constitutive endocytosis of the α1a-AR was even smaller and slower than that of the α1b-AR reaching 12% only at 90 min (Fig. 2 A, B).

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One hypothesis that could explain the low extent of α1a-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 HAα1a and HAα1b-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 α1b-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 α1a-AR, ruling out the possibility that the lack of α1a-AR endocytosis could be due to fast recyling 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 HAα1b-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 HAα1a-AR displayed little detectable intracellular signal within this time frame.

In conclusion, these findings indicate that in HEK-293 cells the α1a-AR, in contrast to the α1b-AR, lacks the ability to undergo endocytosis and this is not due to fast recycling of the receptor.

βarrestin interaction with the α1a and α1b-AR. We have previously reported that the α1b-AR endocytosis is mediated by a clathrin and β-arrestin dependent mechanism (Mhaouty-Kodja et al., 1999). Thus, to elucidate the molecular basis underlying the different internalization behavior of the two α1-AR subtypes, we investigated their ability to interact with βarrestin 1 and 2. For this purpose, the α1a and α1b-AR were co-expressed in HEK-293

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cells with FLAG-tagged βarrestin 1 or 2. We could not use βarrestins tagged with GFP because GFP itself co-immunoprecipitates with the α1a-AR subtype (unpublished observation).

Co-immunoprecipitation experiments were performed from HEK-293 cells transiently co-expressing the HA-tagged receptors and FLAG-tagged βarrestins in the absence or presence of epinephrine. For quantification, the signals corresponding to the immunoprecipitated βarrestins 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 βarrestin 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 βarrestin 1 and 2 co-immunoprecipitated with the receptors, even if to a different extent. To assess the agonist-induced interaction of each receptor with βarrestin 1 and 2, the amount of βarrestins 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 α1a-AR with either βarrestin1 or 2 by 50% above basal at 15 min, but this increase was not statistically significant. In contrast, the epinephrine-induced interaction of the α1b-AR with the two βarrestins was much greater and faster compared to that of the α1a-AR, reaching 180% above basal at 15 min (Fig. 3, C).

To compare the amount of each βarrestin co-immunoprecipitated with one or the other α1-AR subtype, the amount of bound βarrestin 2 at each time point was expressed as percentage of bound βarrestin 1 at the basal state (time 0') after normalizing for both βarrestin and receptor expression levels. As shown in Fig. 3 (D, E), for both the α1a and α1b-AR the

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amount of βarrestin 2 in the immune complex was three-fold greater than that of the βarrestin1 in the basal state.

When βarrestin1 or 2 co-immunoprecipitated with each receptor subtype were compared (βarrestin1/α1a *versus* βarrestin1/α1b and βarrestin2/α1a *versus* βarrestin2/α1b) after normalizing for both βarrestin and receptor expression levels, we observed that in the basal state the amount of each immunoprecipitated βarrestin did not differ between the two receptor subtypes (results not shown).

Altogether these findings indicate that both the α1a and α1b-AR can form a complex with βarrestin 1 and 2, but to a different extent. In fact, the amount of βarrestins associated the α1b-AR upon agonist stimulation is much greater than with the α1a-AR. In addition, the amount of βarrestin 2 bound to either the α1a or α1b-AR in the basal state is three-fold greater than that of the βarrestin 1 suggesting that βarrestin 2 has greater affinity for the receptor than βarrestin 1.

To confirm the results of the co-immunoprecipitation experiments, we used confocal microscopy to investigate the ability of the N-terminally HA-tagged α1-ARs to trigger the translocation of FLAG-tagged βarrestins in HEK-293 cells. As shown in Fig. 4, in the basal state both the α1a (panel A) and α1b-AR (panel B) were localized mainly at the plasma membrane. In cells expressing the α1b-AR (Fig. 4, B) epinephrine induced a pronounced translocation of both βarrestin 1 and 2, which was already evident at 5 min. In contrast, in cells expressing the α1a-AR (Fig. 4, A), stimulation with epinephrine for 15 min induced a modest translocation of βarrestin 2, but not of βarrestin 1, to the plasma membrane. Internalization of the α1a-AR was not observed whereas, after 15 min of exposure to the agonist, a substantial amount of α1b-AR immunofluorescence was localized in endocytic vesicles where it co-localized, at least in part, with βarrestin 2 (Fig. 4, *B*). Overlapping of the

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α1b-AR signal with that of βarrestin 1 was not evident probably because the amount of βarrestin 1 interacting with the receptor is smaller than that of βarrestin 2 as observed in the co-immunoprecipitation experiments. Some co-localization of the α1b-AR and βarrestin 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 βarrestin2 induced by the α1a-AR could be observed, it was weaker than that induced by the α1b-AR.

To gain further insight into the role played by each βarrestin in α1-AR endocytosis, we measured receptor internalization in cells co-expressing FLAG-tagged βarrestin 1 or 2 and receptors. Overexpression of βarrestins did not have any significant effect on the amount of internalized α1a or α1b-AR measured in biotinylation experiments after 30 min of epinephrine treatment (results not shown). This indicated that the amount of endogenous βarrestin 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 α1a-AR subtype. We have previously reported that the α1b-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 α1a-AR subtype.

Pulldown experiments were performed by incubating the GST-fused C-tail of the α1a-AR (α1aCtail) or α1b-AR (α1bCtail) immobilized on Sepharose beads with lysates of HEK-293 cells. The results of these experiments indicated that, in contrast to the α1b Ctail, the C-tail of the α1a-AR did not bind endogenous AP50 (Fig. 5, B).

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Co-immunoprecipitation experiments were performed using HEK-293 cells expressing the HA-tagged α1a or α1b-AR. As shown in Fig. 5, the endogenous AP50 could co-immunoprecipitate with the α1b-AR and epinephrine increased this interaction, as previously reported (Diviani et al., 2003) (Fig. 6, B). In contrast, an association between the α1a-AR and AP50 was not detected). Interestingly, a mutant α1a-AR, carrying 8 additional arginines to reconstitute the AP50 binding site identified in the α1b-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 α1a-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 α1a 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 α1b-AR. Its integrity is required for α1b-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 α1a and α1b-AR, we engineered α1a/α1b 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 α1a/Ctb receptor chimera was able to internalize upon epinephrine treatment in a manner indistinguishable from the wild type α1b-AR. In contrast, substituting the C-tail of the α1b with the C-tail of the α1a-AR subtype almost entirely impaired receptor endocytosis (Fig. 6, A).

To investigate whether the internalization of the α1a/Ctb chimeric receptor was linked to its gain of interaction with regulatory proteins, we assessed its ability to co-immunoprecipitate with βarrestins and AP50. As shown in Fig. 6 (C), the α1a/Ctb chimeric

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receptor was able to co-immunoprecipitate both βarrestin2 and AP50 similarly to the wild type α1b-AR. Co-immunoprecipitation of the α1a/Ctb chimeric receptor with βarrestin1 was also observed (results not shown). Interestingly, the amount of βarrestin2 co-immunoprecipitating with the chimeric receptor in the absence of the agonist (time 0') was greater than that observed for the wild type α1b-AR. This higher basal receptor/βarrestin2 interaction might explain the observation that the α1a/Ctb chimeric receptor displays higher basal internalization compared to the wild type α1b-AR (Fig. 6, compare panels A and B). In contrast to the α1a/Ctb, the α1b/Cta chimeric receptor, carrying the C-tail of the α1a-AR, behaved like the wild type α1a-AR not being able to co-immunoprecipitate neither βarrestin2 nor AP50 (results not shown).

The relative role of βarrestin and AP50 interaction in α1b-AR endocytosis. Given that both βarrestins and the AP-2 complex serve as clathrin adaptors promoting receptor endocytosis (Goodman et al., 1996 ; Chang et al., 1993) and that the α1b-AR can directly interact with both classes of proteins, we wished to explore the relative role played by each in the internalization of the α1b-AR. To do so, we used two previously described receptor mutants: the ΔR8, 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 ΔR8 mutant was delayed in agonist-induced internalization as compared to the wild type α1b-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

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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 βarrestins or AP50, and compared its properties with those of the ΔR8 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 βarrestin2 in the absence or presence of epinephrine. A representative SDS-PAGE (Fig. 8, A) shows that βarrestin 2 could co-immunoprecipitate with both the wild type α1b-AR and its ΔR8 mutant, whereas the amount of βarrestin 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 βarrestin 2 was expressed as percentage of that bound to the wild type α1b-AR in the basal state (time 0') showed that the amount of βarrestin 2 interacting with the ΔR8 was similar to that of the wild type receptor and that this interaction was significantly increased by epinephrine (Fig. 8, B). In contrast, βarrestin 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 βarrestin 2 was very low (Fig. 8, B). Similar results were obtained with βarrestin 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 α1b-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 ΔR8 mutant (Fig. 9).

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Altogether these findings indicate that the phosphorylation-deficient M8 receptor mutant is almost totally impaired in its ability to bind βarrestins and to undergo endocytosis, despite the fact that its direct binding to AP50 is preserved. The ΔR8 receptor mutant, which lacks direct binding to AP50, can efficiently bind βarrestins and it is only partially impaired in its ability to internalize.

The relative role of βarrestin1 and 2 in the internalization of the α1b-AR. To elucidate the relative role played by βarrestin1 and 2 in the internalization of the α1b-AR, we silenced the expression of βarrestins using RNA interference, as previously described (Ahn et al., 2003). As shown in Fig. 10, transfection of cells with plasmids encoding βarrestin 1 and 2 specific shRNAs significantly decreased the expression of the targeted βarrestin 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 βarrestin 2 specific shRNA the epinephrine-induced internalization of the α1b-AR at 45' was entirely impaired (Fig. 10, D) thus demonstrating the important role of βarrestin 2 in endocytosis of the α1b-AR. However, since the real stoichiometry of the two βarrestins in HEK293 cells is unknown and the gene silencing effect was partial, the lack of effect of βarrestin 1 silencing on receptor internalization does not rule out a role of βarrestin 1 in α1b-AR endocytosis.

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DISCUSSION

In this study we demonstrate that the human α1a-AR subtype, in contrast to the α1b-AR, does not undergo significant endocytosis and we provide strong evidence that this property is linked to its poor interaction with βarrestins as well as with the AP50 subunit of the AP2 complex. We assess the relative contribution of α1b-AR interaction with βarrestins *versus* AP50 in receptor endocytosis and demonstrate that βarrestin binding is the *conditio sine qua non* for receptor internalization. Finally, using RNA interference we provide evidence that α1b-AR endocytosis involves βarrestin 2.

The results of our study show that the α1a and α1b-AR subtypes display striking differences in their internalization properties. Whereas the α1b-AR undergoes robust agonist-induced endocytosis, the α1a-AR does not. Our findings on the α1a-AR subtype seem to disagree with the conclusions of two previous studies in which a modest degree of α1a-AR internalization was reported. The study of Chalothorn et al. (2002) reported that the α1a-AR was internalized at 50 minutes after exposure to the agonist, but to a smaller extent compared to the α1b-AR. Morris et al. (2004) reported that the α1a-AR could undergo a modest (~20%) internalization after 60 min of exposure to the agonist. Agonist-induced internalization of the α1b-AR was not measured for comparison in the same study. In addition, the α1a-AR, but not the α1b-AR, displayed some constitutive internalization and it was shown to recycle fast to the plasma membrane.

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Three main factors might explain the discrepancies observed for the α1a-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 α1a-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 α1-AR subytpes (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 α1a-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 α1a and α1b-AR subtypes in recombinant systems.

The results from both co-immunoprecipitation experiments (Fig. 3) and βarrestin translocation assays (Fig. 4) indicated that the agonist-induced interaction of the α1a-AR with βarrestin 1 and 2 was much weaker that that of the α1b-AR. In addition, the α1a-AR displayed almost no interaction with the AP50 subunit of the AP2 complex, which was

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previously shown to directly bind α1b-AR (6) (Fig. 5). Our hypothesis is that the lack of internalization of the α1a-AR is mainly linked to its poor interaction with βarrestins as well as with AP50. In support of this hypothesis is the fact that replacement of α1a-AR C-tail with that of the α1b-AR, which contains the structural determinants for binding βarrestins and AP50, confers to the α1a-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 α1a-AR was poorly phosphorylated and desensitized compared to the α1b-AR. Replacement of the α1a-AR C-tail with that of the α1b-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 α1a-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 α1a-AR from rafts thus impairing endocytosis. However, this hypothesis can be excluded based on findings obtained from experiments in which α1-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 α1a-AR endocytosis (results not shown), it is unlikely that the lack of significant α1a-AR internalization found in our study is an artifact of biotinylation.

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The differences in internalization observed between the α1a and α1b-AR subtypes are very similar to those between the β1 and β2-AR reported in a study in which receptor/βarrestin interaction was investigated using different approaches (Shiina et al., 2000). The β1-AR expressed in HEK-293 cells showed resistance to agonist-induced internalization as well as weak interaction with βarrestins compared to the β2-AR subtype. These findings strongly support the hypothesis that the interaction of GPCRs with βarrestins 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 α1b-AR. This study indicates that the interaction of the α1b-AR with βarrestins 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 α1b-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 βarrestins (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 α1b-AR endocytosis cannot occur in the absence of βarrestin 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 α1b-AR mutant, ΔR8, lacking the binding site for AP50 (Diviani et al., 2003) which could undergo agonist-induced internalization , albeit delayed as compared to wild type α1b-AR.

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Our findings identify βarrestin 2 as an important player in the internalization process of α1b-AR. This is supported by several lines of evidence: i) the decrease by 60% of endogenous levels of βarrestin 2 using RNA interference almost entirely impaired α1b-AR internalization (Fig. 10); ii) the amount of βarrestin 2 forming a complex with the α1b-AR was greater as compared to βarrestin 1 suggesting a greater affinity of the receptor for βarrestin 2 than 1 (Fig. 3, E); iii) the α1b-AR was found to colocalize, at least in part, with βarrestin 2, but much less with βarrestin 1, in endocytic vesicles after exposure to the agonist (Fig. 4, B). Further studies using cells devoid of either one of the two βarrestins (Conner et al., 1997; Bohn et al., 1999) should help to unequivocally determine the role played by each βarrestin in α1b-AR internalization.

Two classes of GPCRs, designated as class A and B, have been identified based on their pattern of interaction with βarrestins (Oakley et al., 2000). Class A receptors, such as the β2-AR, bind βarrestin 2 with greater affinity than βarrestin 1 forming a short-lived receptor/βarrestin complex. In contrast, class B receptors, such as the angiotensin II type 1A receptor, display similar affinity for both βarrestin isoforms establishing a stable receptor/βarrestin complex. Our findings indicate that the α1b-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 βarrestin 2 than for βarrestin 1. However, the pattern of α1b-AR internalization is quite different from that observed for the β2-AR, a prototypic class A receptor. In fact, the kinetics of both internalization and recycling of the α1b-AR is much slower than that of the β2-AR (compare Fig.1 and Fig.1S). In addition, co-localization of the α1b-AR with βarrestin 2 was observed in endocytic vesicles, suggesting that the receptor can form a stable complex with βarrestin within the cells, a feature considered to be a hallmark of class B GPCRs. This suggests that the classification of GPCRs properties into

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

The implication of different βarrestin isoforms in GPCR endocytosis has been investigated in a large number of studies. However, in most cases GPCR/βarrestin interaction has been followed in intact cells by monitoring βarrestin translocation and receptor/βarrestin co-localization using confocal microscopy. Few studies have measured GPCR/βarrestin co-immunoprecipitation (Chen et al., 2002 ; Kishi et al., 2002) and even fewer have performed *in vitro* biochemical measurements of βarrestin 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/βarrestin interaction.

Altogether, the findings of this study demonstrate important regulatory differences between the α1a and α1b-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 βarrestins and AP50. However, a number of important questions can be raised: are these differences occurring in native tissues where the α1a and α1b-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 α1a-AR endocytosis. For this reason, future studies will aim at expressing the α1-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 α1a and α1b-AR subytpes might be linked to their physiological functions.

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

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Footnotes

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FIGURE LEGENDS

Fig. 1. Agonist-induced internalization and recycling of the α1a and α1b-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 α1a and α1b-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±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 α1a and α1b-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±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±S.E. of three independent experiments. *$p<0.05$ Student's t test compared to 90' (in the absence of monensin).

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Fig. 3. Co-immunoprecipitation of βarrestins with the α1a and α1b-AR. FLAG-tagged βarrestin 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 βarrestins and monoclonal anti-HA antibodies (Sigma) for the receptors. The blots shown were overexposed (15 min) to show low βarrestin signals. In (B) and (C), βarrestin 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±S.E. of five independent experiments. *$p<0.05$, **$p<0.01$ Kruskal-Wallis test compared to time 0'. In (D) and (E), βarrestin 1 and 2 co-immunoprecipitated with each receptor are expressed as percentage of βarrestin 1 in the absence of epinephrine (0'). Results are the mean±S.E. of five independent experiments. *$p<0.05$, **$p<0.01$ Kruskal-Wallis test compared to βarrestin 1 at each time.

Fig. 4. Cellular localization of the βarrestins and the α1-AR subtypes monitored by confocal microscopy. Confocal microscopy was performed on cells co-expressing the C-terminally HA-tagged α1a (A) and α1b-AR (B) with FLAG-tagged βarrestin 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 βarrestins. The images are representative of four independent experiments.

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Fig. 5. Interaction of AP50 with the α1a and α1b-AR. (A) Pulldown of endogenous AP50 from cell lysates using purified GST or GST-fused to the C-tail of the α1a or α1b-AR. (B) Co-immunoprecipitation of endogenous AP50 from cells expressing the α1a and α1b-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 α1a/α1b 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±S.E. of three independent experiments. *$p<0.05$ Student's t test compared to α1bHA (A) and α1aHA (B) at each time point. (C) Co-immunoprecipitation of FLAG-tagged βarrestin2 and endogenous AP50 with the α1b-AR and α1a/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 α1b-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

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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 βarrestin 2 with the α1b-AR and its mutants ΔR8 and M8. FLAG-tagged βarrestin 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 βarrestin and monoclonal anti-HA antibodies (Sigma) for the receptors. The blots shown were overexposed (15 min) to show low βarrestin signals. In (B), βarrestin 2 co-immoprecipitated with the each receptor is expressed as percentage of the βarrestin 2 co-immunoprecipitated with the α1b-AR in the absence of epinephrine (0'). Results are mean±S.E. of four independent experiments. *$p<0.05$ Kruskal-Wallis test compared to the α1b-AR for each time.

Fig. 9. Co-immunoprecipitation of endogenous AP50 with the α1b-AR and its mutants ΔR8 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

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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 βarrestins expression on α1b-AR internalization. Cells were co-transfected with the plasmid encoding the C-terminally HA-tagged α1b-AR and pSuper encoding shRNA for βarrestin 1 or 2. (A) and (B) Endogenous βarrestin 1 (upper band) or 2 (lower band) were detected by Western blot using polyclonal antibodies against rat βarrestin 1. In (A) are shown representative Western blots of extracts from cells transfected with the wild type α1b-AR cDNA together with empty pSuper (wt) or with the shRNA for βarrestin 1 or 2. In (B) the levels of βarrestins in cells transfected with the shRNA for βarrestin 1 or 2 are expressed as percentage of those in cells expressing the wild type α1b-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 α1b-AR in the absence of shRNA. (C) and (D) Internalization of the α1b-AR was monitored by receptor biotinylation, as described in Fig. 1, in cells expressing the wild type α1b-AR alone (wt) or co-expressing the shRNA for βarrestin 1 or 2. Results are the mean±S.E. of six independent experiments. *$p<0.05$ one-way ANOVA compared to α1b-AR at 45'.

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Fig. 1

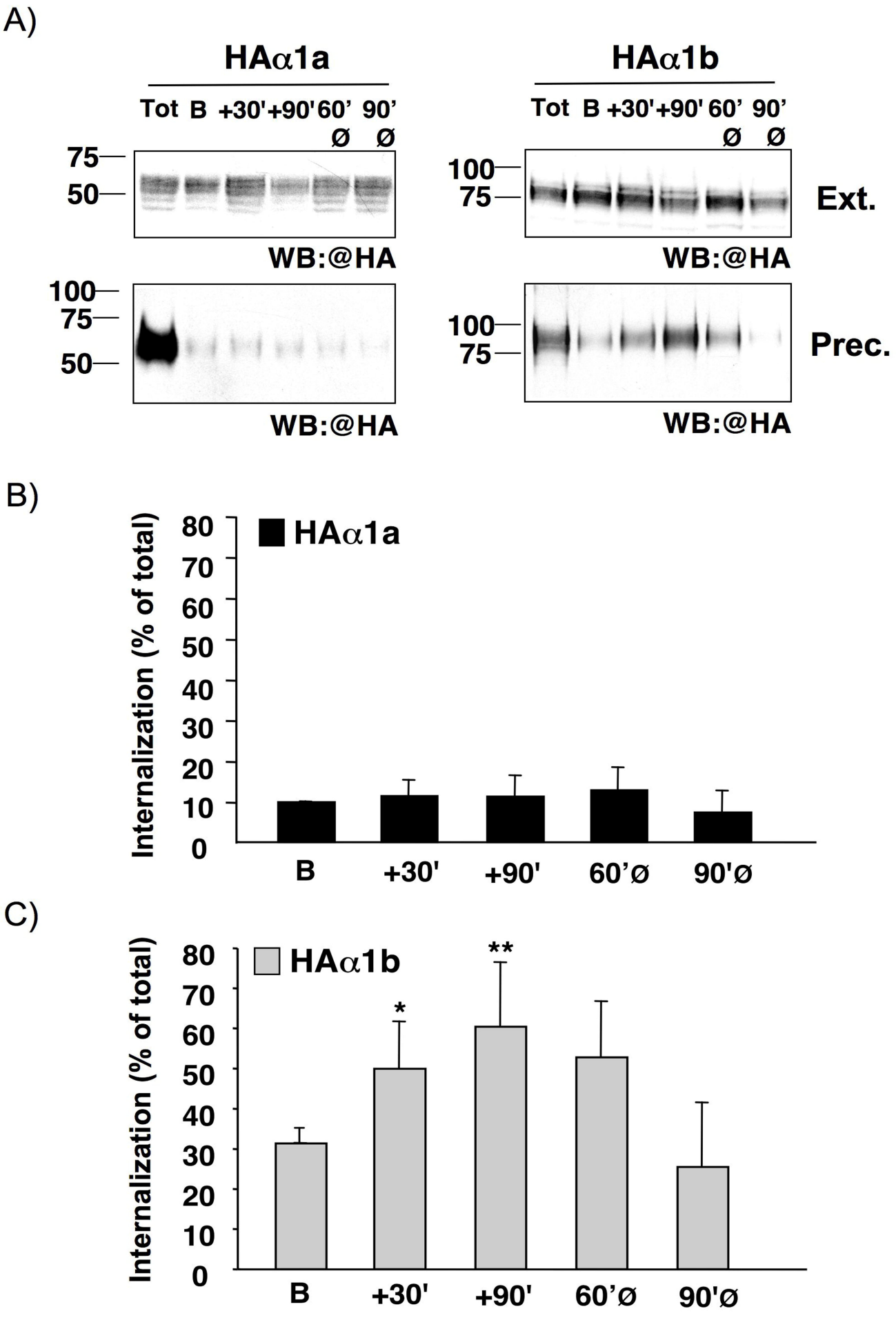

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Fig. 2

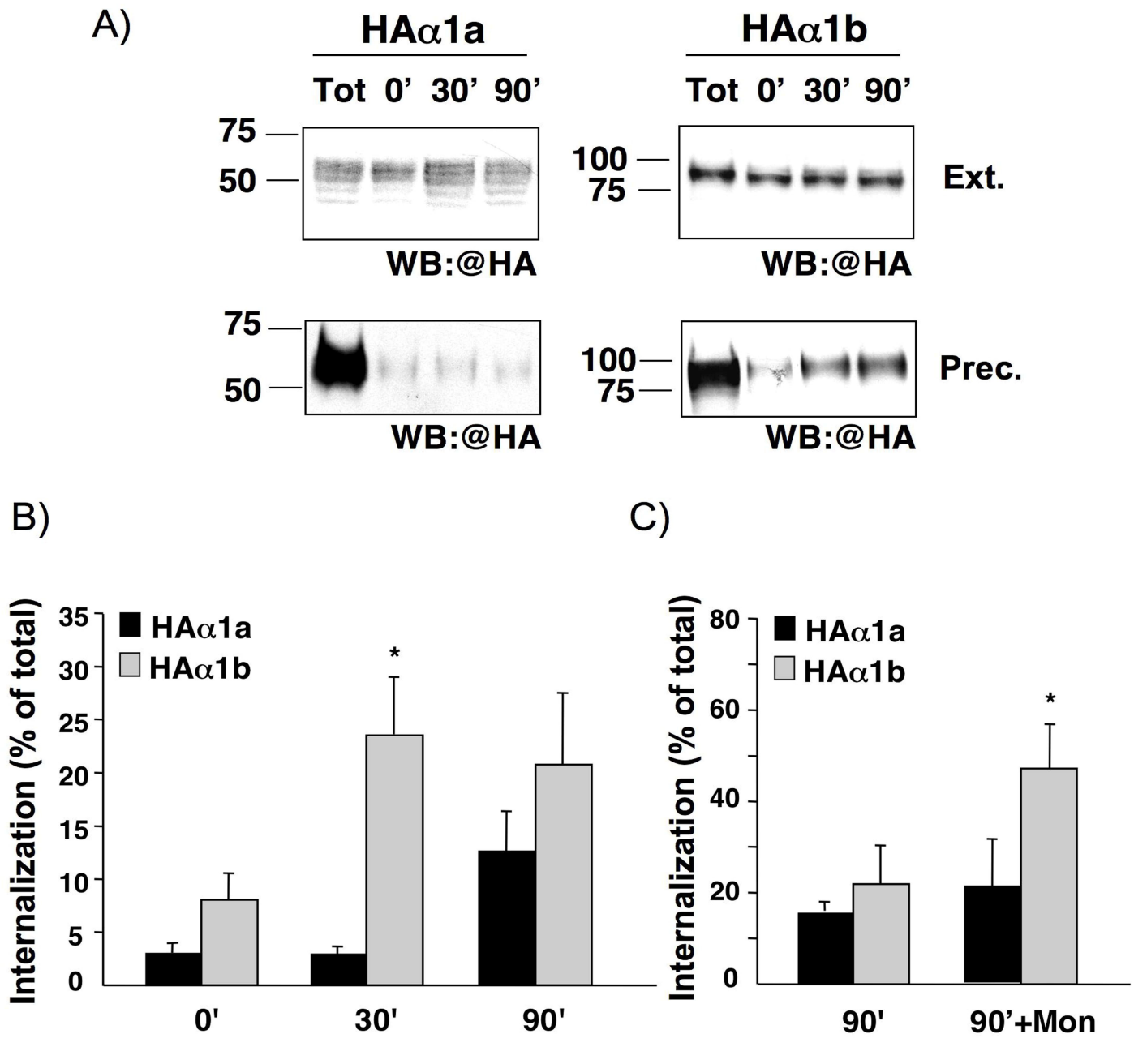

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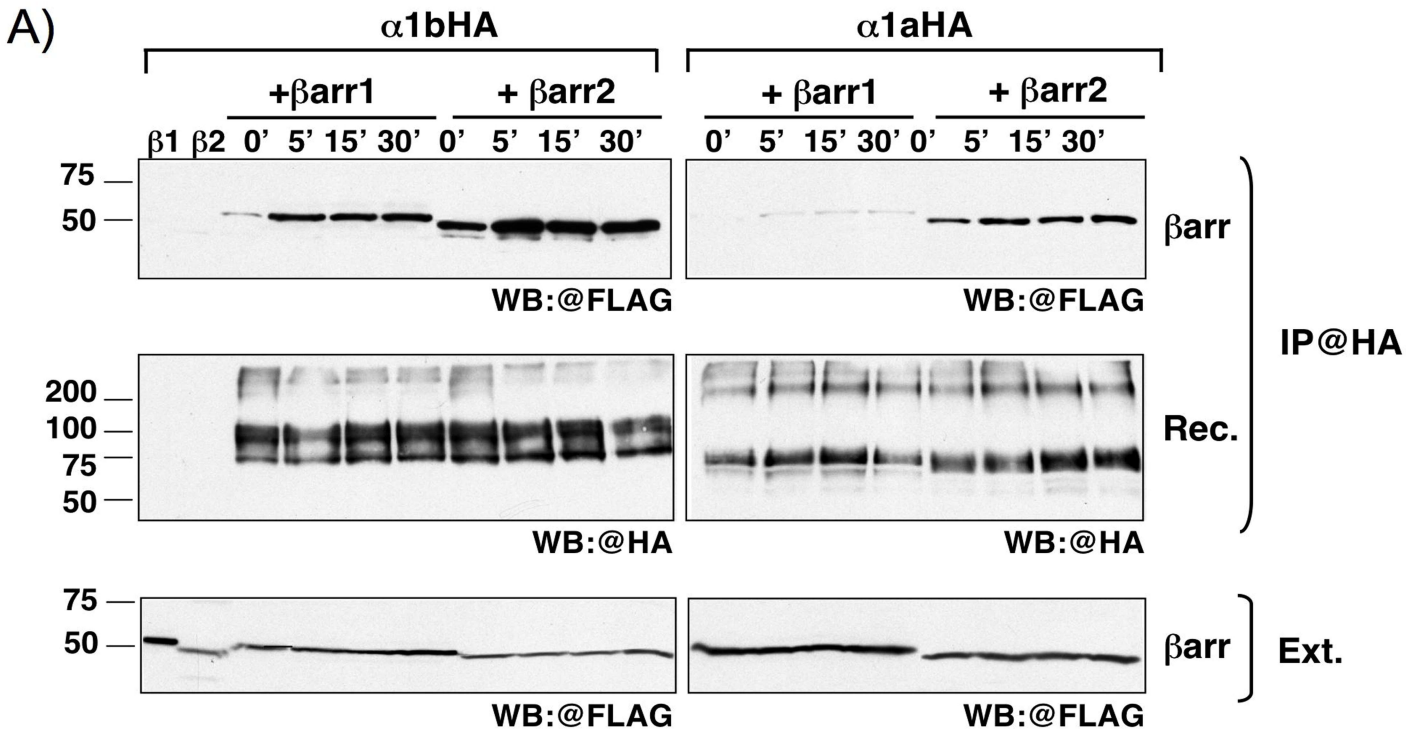
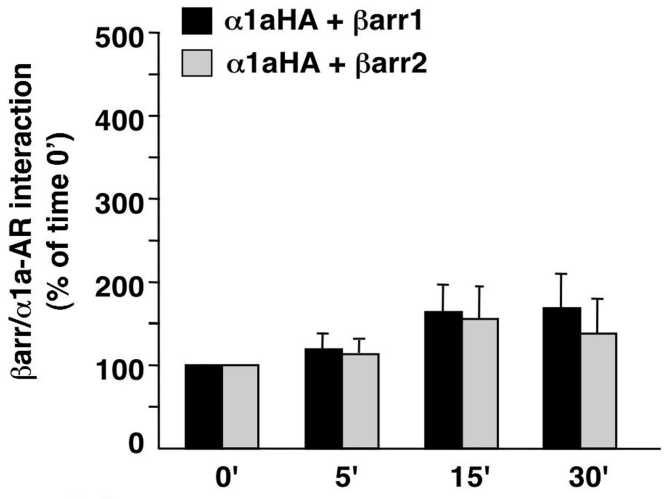
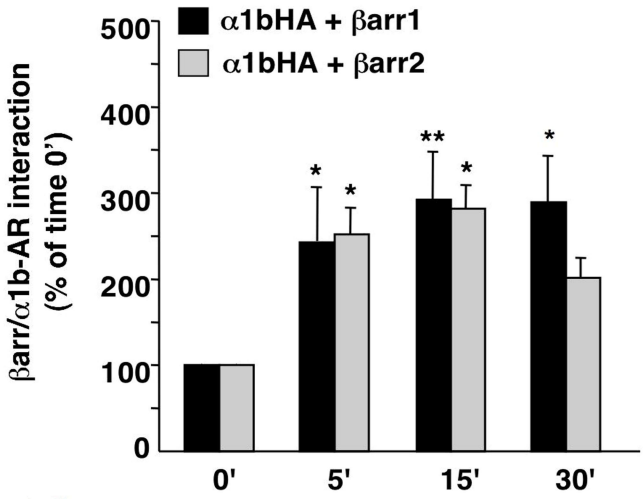
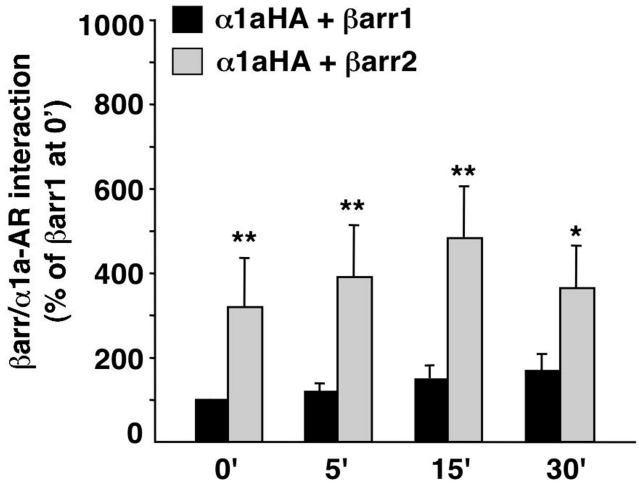
Fig. 3









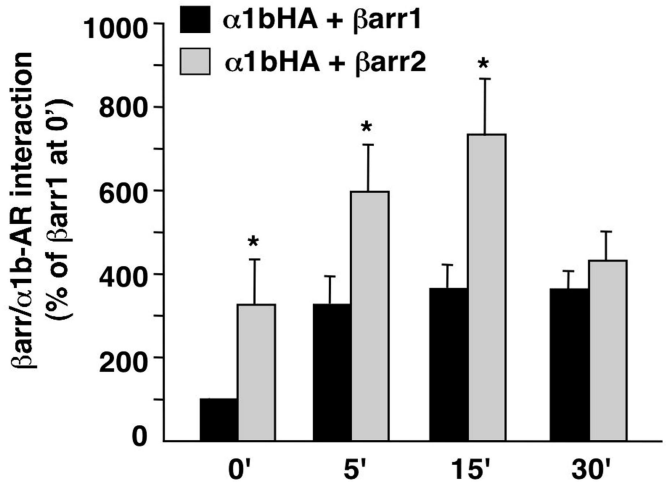


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Fig. 4

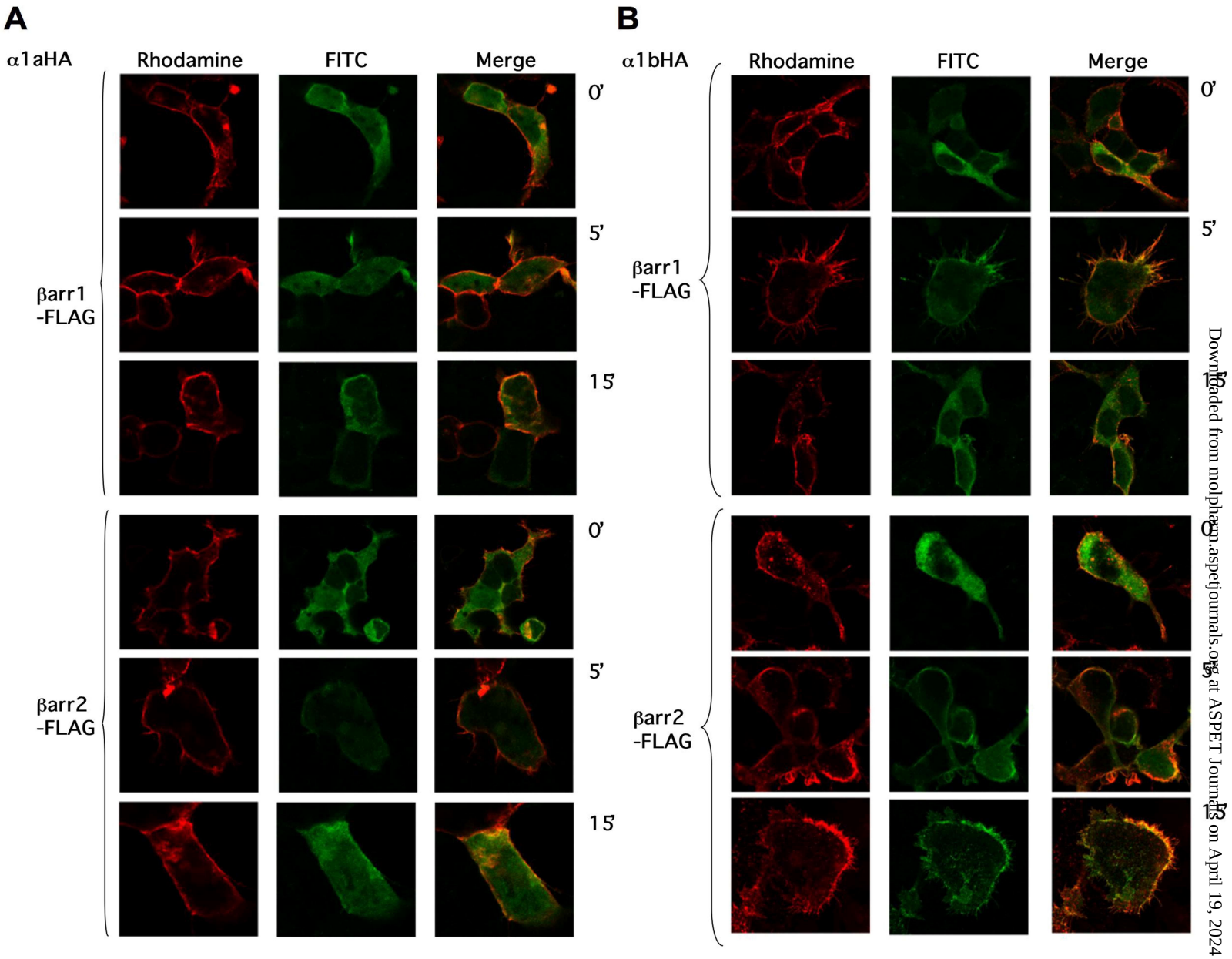

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Fig. 5

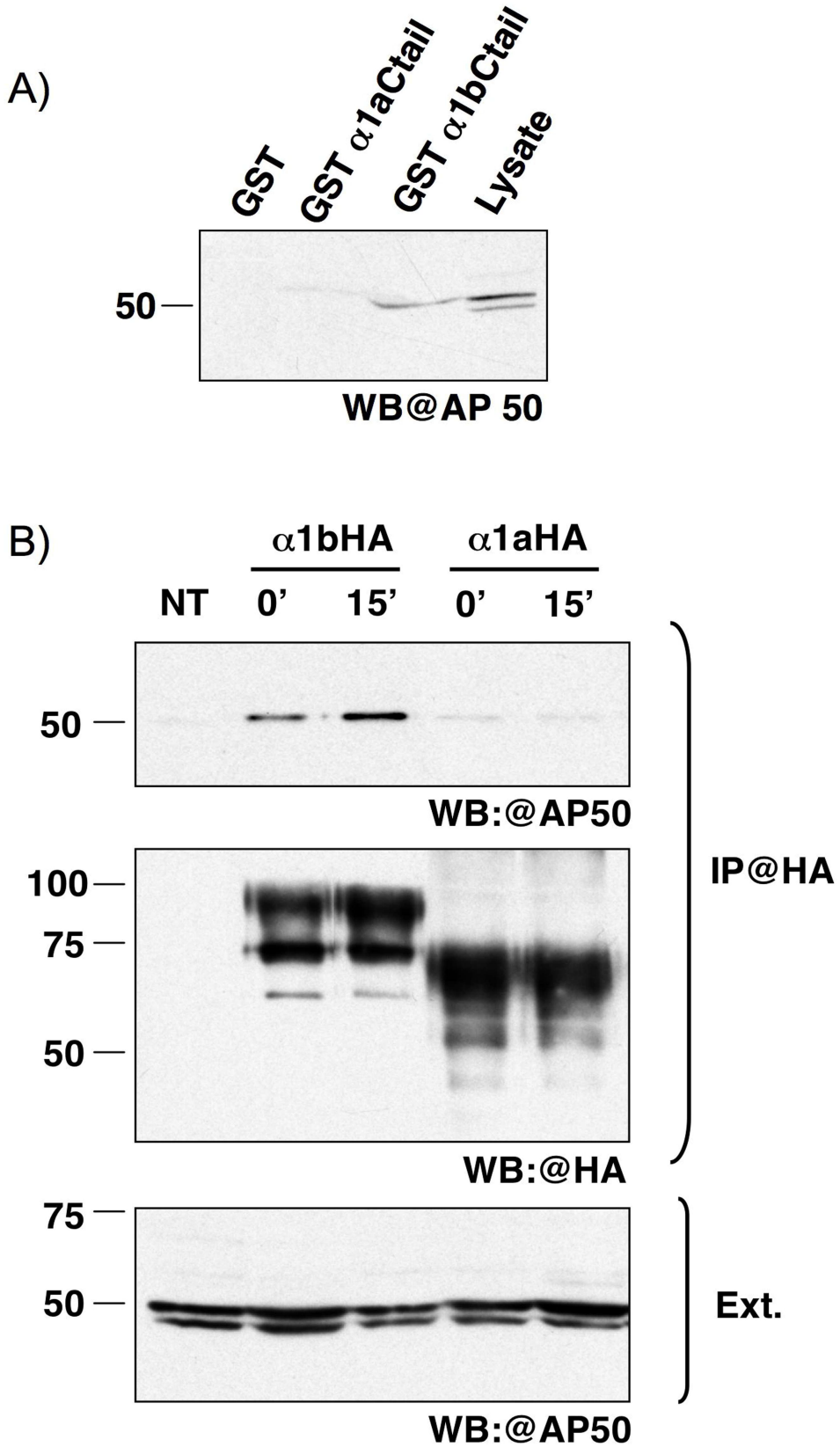

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Fig. 6

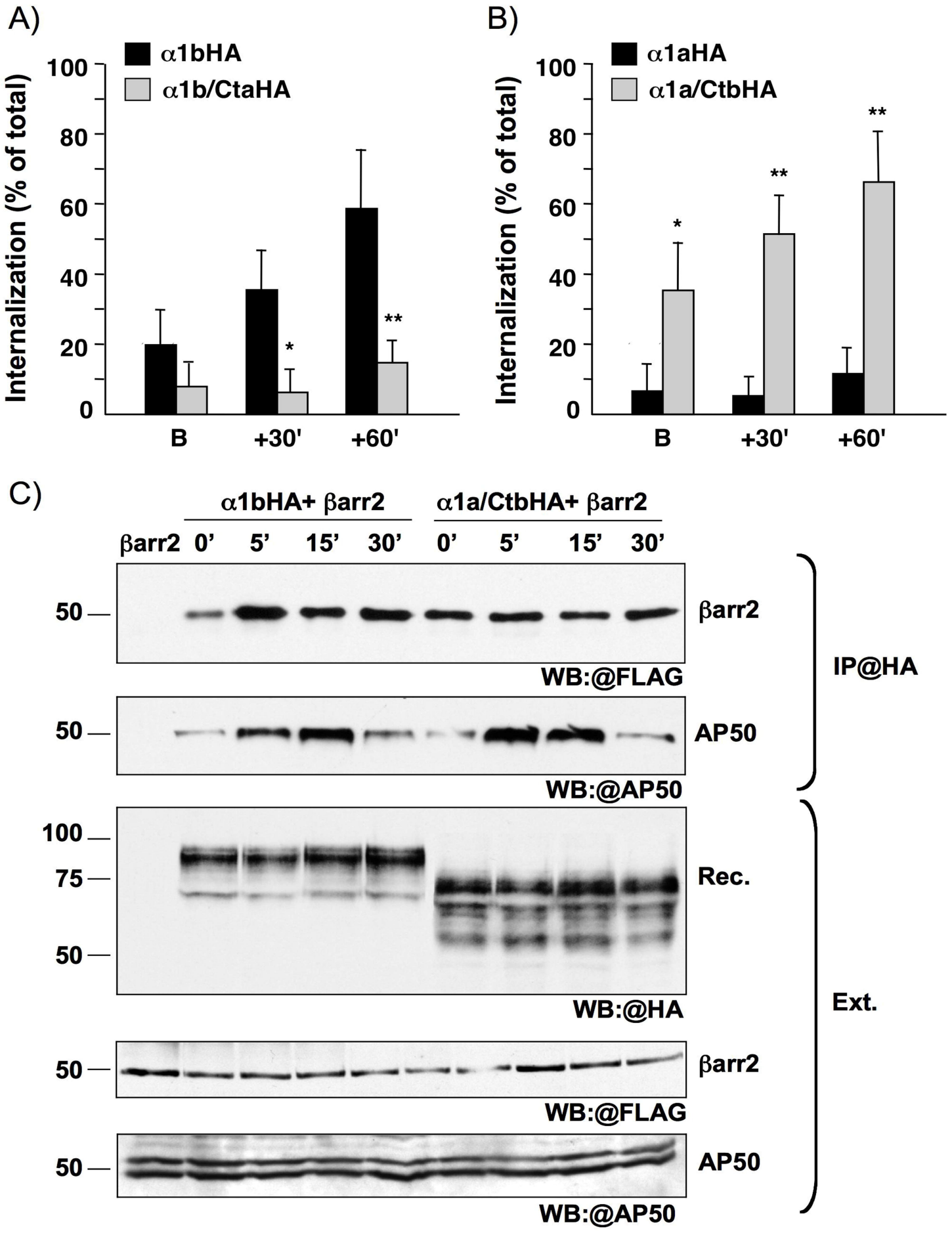

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Fig. 7

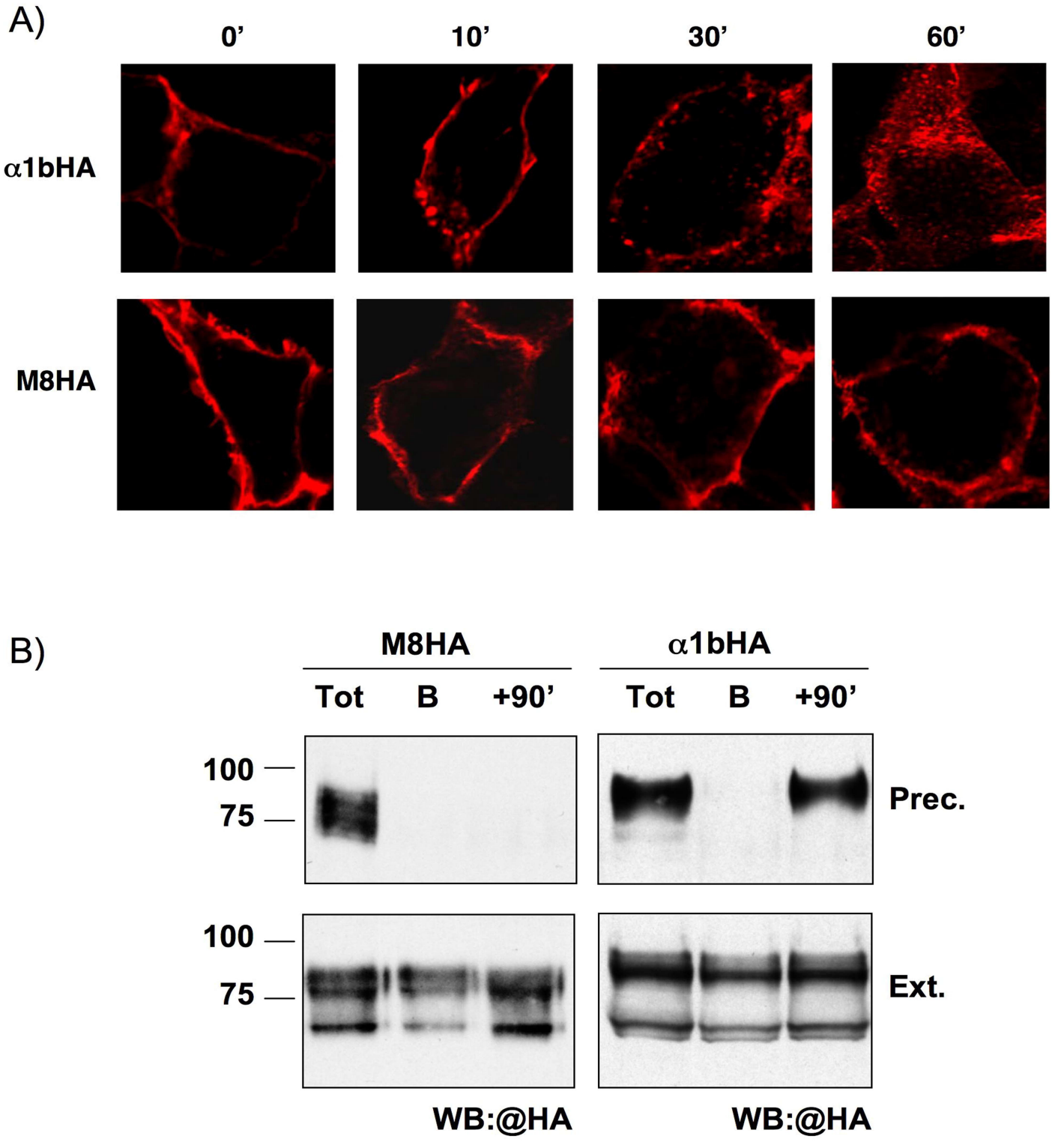

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Fig. 8

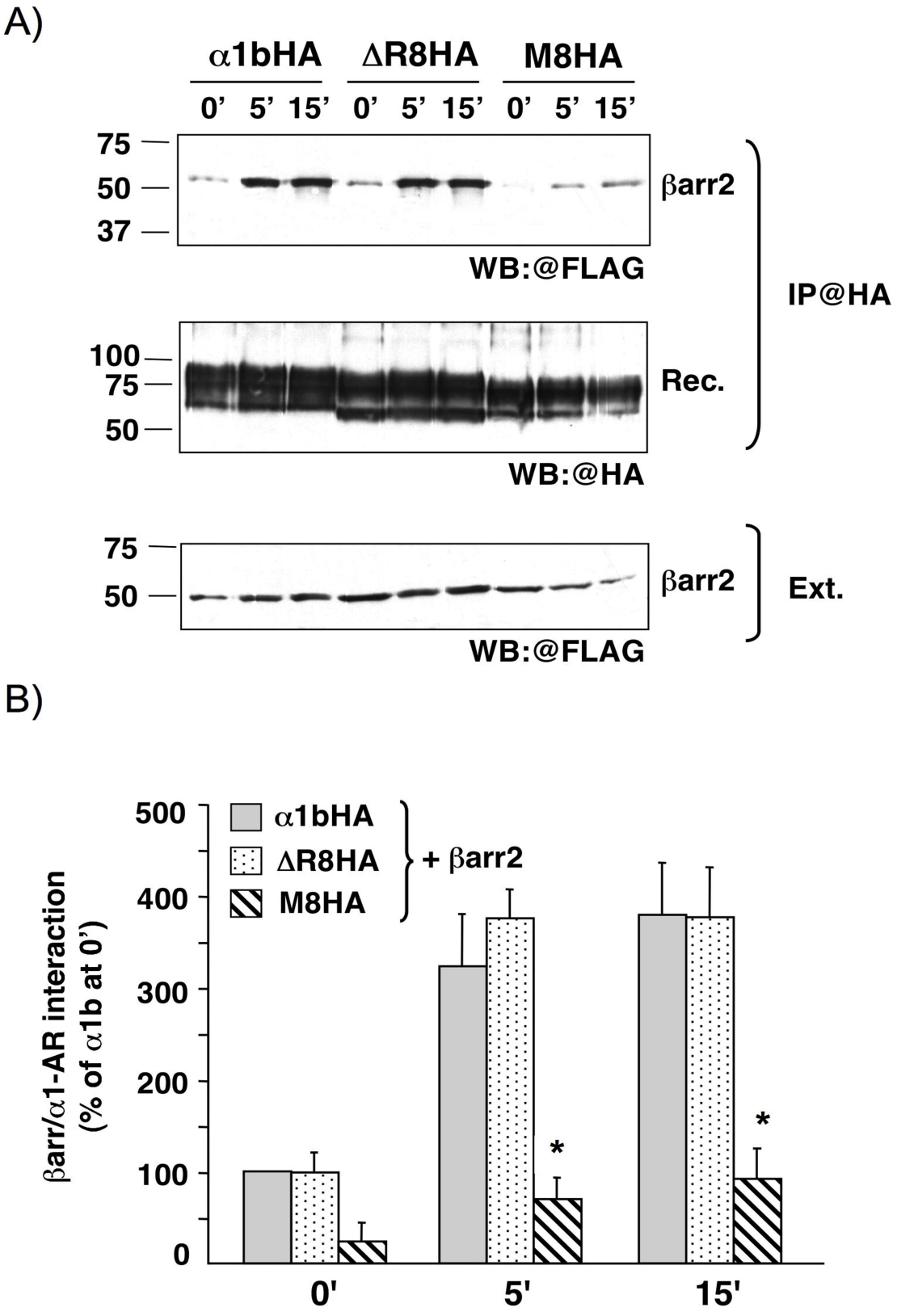

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Fig. 9

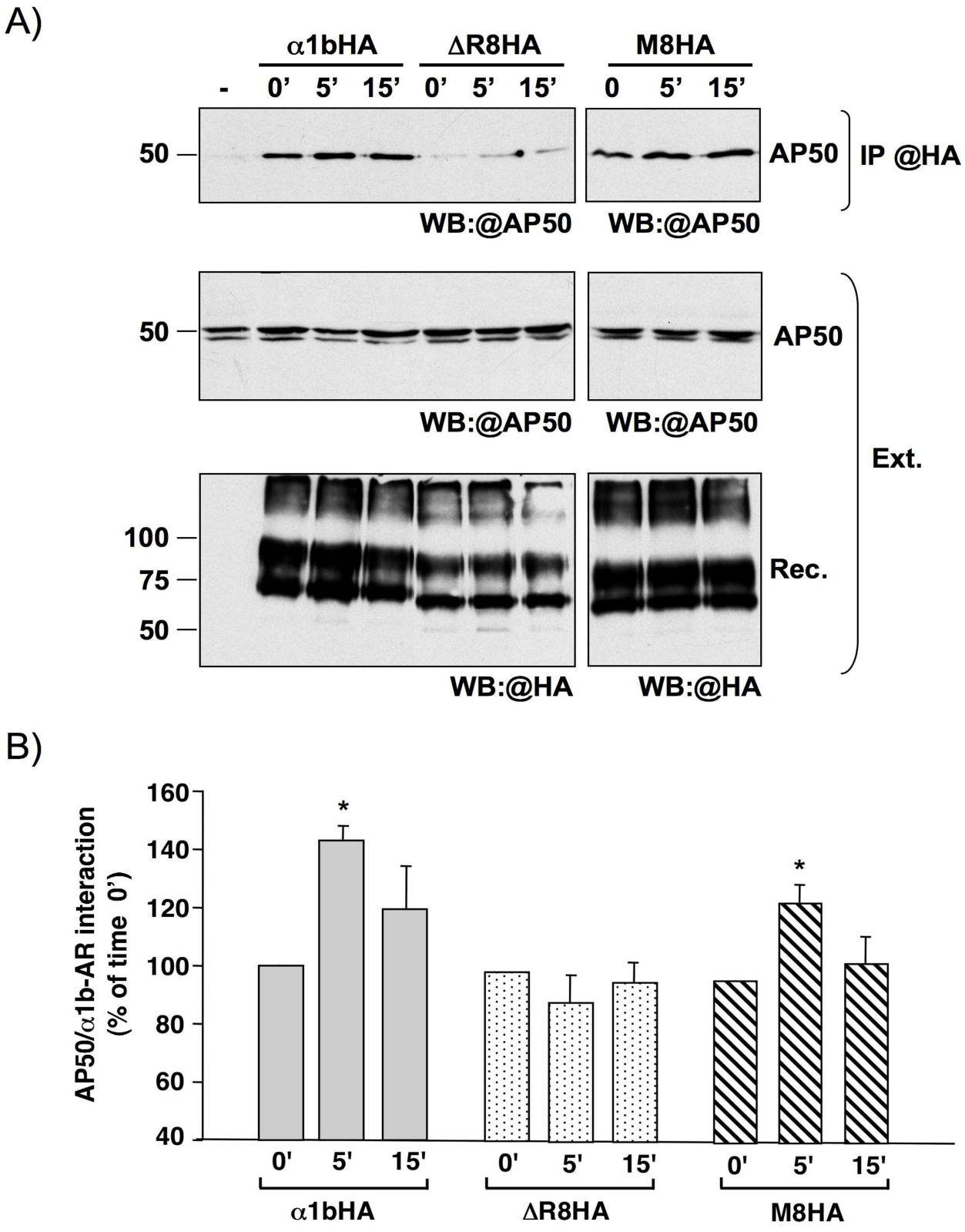

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Fig. 10