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Histamine H2 Receptor Trafficking: Role of Arrestin, Dynamin, and Clathrin in Histamine H2 Receptor Internalization

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ABSTRACT

Agonist-induced internalization of G protein-coupled receptors (GPCRs) has been implicated in receptor desensitization, resensitization, and down-regulation. In the present study, we sought to establish whether the histamine H2 receptor (H2r) agonist amthamine, besides promoting receptor desensitization, induced H2r internalization. We further studied the mechanisms involved and its potential role in receptor resensitization. In COS7 transfected cells, amthamine induced H2r time-dependent internalization, showing 70% of receptor endocytosis after 60-min exposure to amthamine. Agonist removal led to the rapid recovery of resensitized receptors to the cell surface. Similar results were obtained in the presence of cycloheximide, an inhibitor of protein synthesis. Treatment with okadaic acid, an inhibitor of the protein phosphatase 2A (PP2A) family of phosphatases, reduced the recovery of both H2r membrane sites and cAMP response. Arrestin 3 but not arrestin 2 overexpression reduced both H2r membrane sites and H2r-evoked cAMP response. Receptor cotransfection with dominant-negative mutants for arrestin, dynamin, Eps15 (a component of the clathrin-mediated endocytosis machinery), or RNA interference against arrestin 3 abolished both H2r internalization and resensitization. Similar results were obtained in U937 cells endogenously expressing H2r. Our findings suggest that amthamine-induced H2r internalization is crucial for H2r resensitization, processes independent of H2r de novo synthesis but dependent on PP2A-mediated dephosphorylation. Although we do not provide direct evidence for H2r interaction with β-arrestin, dynamin, and/or clathrin, our results support their involvement in H2r endocytosis. The rapid receptor recycling to the cell surface and the specific involvement of arrestin 3 in receptor internalization further suggest that the H2r belongs to class A GPCRs.

Histamine is a natural widely distributed body constituent that mediates numerous functions, especially in the central nervous system, mast cells, gastric mucosa, parietal cells, and basophils. Four distinct receptor subtypes (H1, H2, H3, and H4) belonging to the large family of G protein-coupled receptors (GPCRs) mediate histamine biological effects (Simons, 2004). The histamine H2 receptor (H2r) subserves hypotension, flushing, headache, increased gastric acid production, and enhanced vascular permeability (Sachs et al., 2002; Spitaler et al., 2002). By coupling to Gs, H2r triggers adenylyl cyclase activation and subsequent rapid cAMP accumulation (Hill, 1990). The activity of GPCRs results from a coordinated balance among the diverse mechanisms that govern receptor signaling at the different levels of signal propagation, and H2r-coupled signaling is not an exception. cAMP response to H2 agonists is attenuated within minutes of the onset of agonist stimulation by a process termed desensitization (Lemos Legnazzi et al., 2000). The desensitization of GPCRs is a physiologically adaptive mechanism triggered by continuous or repeated stimuli, which protects the cell from both short- and long-term receptor overstimulation. The underlying mechanisms are complex and involve receptor phosphorylation, uncoupling from G proteins, internalization, and ultimately receptor down-regulation (Zhang et al., 1997). These mechanisms have been extensively studied for the β2 adrenoreceptor, and based on these investigations, a general pathway for agonist-mediated receptor desensitization and internalization has been outlined (Zhang et al., 1997). In this canonical model, agonist-induced activation of

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; H2r, histamine H2 receptor; IBMX, isobutylmethylxanthine; amthamine, 2-amino-4-methylthiazole-5-ethanamine; RNAi, RNA interference; EGFP, enhanced green fluorescent protein; AP-2, activator protein 2.

GPCRs leads to receptor phosphorylation by second messenger-dependent kinases and/or specific G protein-coupled receptor kinases (GRKs) (Pitcher et al., 1998). In turn, GRK-mediated phosphorylation facilitates the binding of the third intracellular loop and the carboxyl-terminal tail of the receptor to cytoplasmic accessory proteins called arrestins, which physically uncouple the receptor from the G protein. In addition, the interaction of GPCRs with arrestins targets the phosphorylated GPCR to clathrin-coated pits and initiates internalization by the interaction of the carboxyl-terminal of arrestin with both the clathrin heavy chain and the β2-adaptin subunit of AP-2 complex (Laporte et al., 1999). After pinching off the vesicles from plasma membrane by dynamin (Sever, 2002), the receptor is sequestered into intracellular vesicular compartments (endosomes) (von Zastrow, 2003). Based on their aptitude to bind β-arrestin, GPCRs are divided into A and B classes. Class A GPCRs are dephosphorylated in the endosomal compartment after internalization and are recycled rapidly to the cell surface (rapid resensitization). Class B GPCRs are retained in the endosomal compartment and slowly recycled to the plasma membrane (slow resensitization) or targeted to lysosomes for degradation (down-regulation) (Oakley et al., 2000; Prossnitz, 2004).

However, other mechanisms for GPCR internalization have also been described. Some GPCRs are internalized by clathrin-independent endocytic mechanisms via caveola and lipid rafts (Ginés et al., 2001). The latter are small clathrin-free microdomains of the cell membrane enriched in cholesterol and sphingolipids that can present the addition of members of the caveolin family of scaffolding proteins (Anderson and Jacobson, 2002). Clathrin-independent internalization has been described in some cell types for β2-adrenoreceptors (Raposo et al., 1989). The internalization of AT1A angiotensin receptor and M2 muscarinic receptor is mediated by a dynamin-independent mechanism (Pals-Rylaarsdam et al., 1997; Zhang et al., 1997), whereas that of the 5-hydroxytryptamine-2A receptor is mediated by an arrestin-independent pathway (Bhatnagar et al., 2001). These findings clearly indicate that the mechanisms underlying receptor internalization is by no means universal for all GPCRs, thus supporting the complexity of GPCR signaling, desensitization, and internalization. This spatial and temporal control determines the specificity of receptor-mediated signal transduction among the distinct downstream effectors and the ultimate cellular response.

Despite the wide therapeutic use of H2 ligands for gastric ulcers, their cardioprotective effects in patients with chronic heart failure (Asanuma et al., 2006; Kim et al., 2006), and their implication in HL-60 and U937 leukemic cell maturation (Tasaka et al., 1994; Fernández et al., 2002), little is known about H2r regulation. H2r internalization was first reported in human embryonic kidney 293 cells in which the authors showed that histamine treatment induces loss of H2r membrane immunoreactivity (Smit et al., 1995). However, the molecular mechanism underlying H2r desensitization, internalization, and H2r fate after endocytosis still remain uncertain.

The purpose of the present study was to investigate agonist-induced H2r internalization in COS7 and U937 leukemic cells and to determine the role of β-arrestins, dynamin, and clathrin in this process as well as in H2r response resensitization. Our findings show that agonist-induced H2r internalization is crucial for the rapid recovery of H2r-mediated cAMP response, which is independent of de novo H2r synthesis. Furthermore, arrestin 3, dynamin, and clathrin are involved in both the internalization and resensitization of the H2r.

Materials and Methods

Materials. U937 and COS7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture medium, antibiotics, isobutylmethylxanthine (IBMX), cAMP, cycloheximide, okadaic acid, G418, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Amthamine and tiotidine were from Tocris Cookson Inc. (Ballwin, MO). [^{3}H]cAMP ($\cong$31 Ci/mmol) and [^{3}H]tiotidine ($\cong$75 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Other chemicals used were of analytical grade. pcDNA3-β1arrestin (arrestin 2), pcDNA3-β2arrestin (arrestin 3), pcDNA3-HA-dynaminK44A, and pcDNA3-β1-arrestin(319–418) were generous gifts from Dr. J. Benovic (Thomas Jefferson University, Microbiology and Immunology Department, Kimmel Cancer Center, Philadelphia, PA). pEGFP-C2-Eps15 EH29, pEGFP-C2-Eps15 DIII, and pEGFP-C2-Eps15 D3Δ2 constructs were generous gifts from Dr. Benmerah (Université Paris 5, Institut Cochin, Departement de Maladies Infectieuses, Paris, France). The RNAi sequences targeting arrestin 2 (5′-CCAAUCUCAUAGAACUUGACACAAA-3′) or arrestin 3 (5′-GGGUCUUCAAGAAGUCGAGCCCUAA-3′ and 5′-GCCACAGAUGAUGACAUUGUGUUUG-3′) and the RNAi control duplexes were Stealth RNAi purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Transfection. COS7 cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. For transient transfection, COS7 cells were grown to 80 to 90% confluence. cDNA constructs were transfected into cells using LipofectAmine 2000. The transfection protocol was optimized as recommended by the supplier (Invitrogen). Assays were performed 48 h after transfection. The expression of the EGFP-Eps construct was confirmed by fluorescence microscopy and the presence of wild-type and/or dominant-negative mutants for arrestin and dynamin by immunoblotting using specific antibodies. For RNA interference experiments, COS7 cells split 24 h before transfection were cotransfected at 80% confluence with H2r and Stealth RNAi negative control or directed against arrestin 2 (100 nM) or arrestin 3 (100 nM) using LipofectAmine 2000, following the manufacturer's instruction (Invitrogen). Assays were performed 72 h after transfection.

U937 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. For stable transfection, U937 cells were harvested by centrifugation from cultures in exponential growth phase, washed in phosphate-buffered saline, and resuspended at a density of 2×10^7 cells/ml in fresh RPMI medium on ice. pcDNA3-β1arrestin(319–418) or pcDNA3-HA-dynaminK44A (10 μg) was linearized with SalI and then added to the cell suspension (250 μl) and kept on ice for 10 min. Cells and DNA were then subjected to a pulse of 200 V at a capacitance of 950 μF using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA), returned to ice for 10 min, and incubated overnight in a nonselective medium. Cells were then plated in a 48-well culture plate in 0.5 ml/well RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin containing 0.8 mg/ml G-418. After 2 to 3 weeks, the surviving clones were amplified. The expression of the constructs was verified by reverse transcription-polymerase chain reaction using the following primers: forward, 5′-CGACATTGTATTTGAGG-3′, and reverse, 5′-ATTTAGGTGACACTATAG-3′ for Arr(319–418); and forward, 5′-TACCCGTATGATGTTCCG-3′, and reverse 5′-TCGGTGAATTTCTTTCCC-3′ for HA-dynaminK44A.

Western Blots. Cells were resuspended in lysis buffer (5 mM Tris-HCl, pH 8, 5 mM EDTA, 1% Triton X-100, 0.1% dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 10 μM leupeptin, 5 μM pepstatin, and 1 mM sodium vanadate). Samples were resolved by SDS-polyacrylamide gel electrophoresis (12 or 15% gel) and transferred to nitrocellulose for immunoblotting. The membranes were probed with 1 μg/ml rabbit anti-β-arrestins or goat β-arrestin 1-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-β-arrestin monoclonal antibody (BD Biosciences PharMingen, San Diego, CA).

cAMP Assays. Concentration-response assays were performed by incubating the cells for 3 min in culture medium supplemented with 1 mM IBMX at 37°C followed by 9-min exposure to different concentrations of amthamine. For desensitization assays, cells were pretreated with 10 μM amthamine in the absence of IBMX for periods ranging from 1 to 240 min. Cells were then washed and resuspended in fresh medium containing 1 mM IBMX, incubated for 3 min, and exposed to 10 μM amthamine for 9 min to determine whether they were able to generate cAMP. For resensitization assays, cells were first treated with 10 μM amthamine for 60 min, washed, and incubated in fresh medium for different periods of time to evaluate the recovery of H2r active sites after the desensitizing stimulus. Assays were also performed in the presence of 50 μM cycloheximide or 0.5 μM okadaic acid, which were added 30 min before amthamine and with fresh medium after washing the cells.

In all experiments, the reaction was stopped by ethanol addition followed by centrifugation at 2000*g* for 5 min. The ethanolic phase was then dried, and the residue was resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. cAMP content was determined by competition of [^{3}H]cAMP for protein kinase A, as described previously (Davio et al., 1995).

Radioligand Binding Assay. Triplicate assays were performed in 50 mM Tris-HCl, pH 7.4. For saturation studies, 10^6 U937 cell/tube or 10^4 COS7 cell/96-well were incubated for 40 min at 4°C with increasing concentrations of [^{3}H]tiotidine, ranging from 0.4 to 240 nM in the absence or in the presence of 1 μM unlabeled tiotidine. The incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4. For U937 cells or derived clones, rapid filtration under reduced pressure onto Whatman GF/B glass fibers filters followed by three washes with 3 ml of ice-cold buffer were performed. For COS7 cells, after three washes with 3 ml of ice-cold buffer, the bound fraction was collected in 200 μl of ethanol. Experiments with intact cells were performed at 4°C to avoid ligand internalization. The kinetic studies performed with 2 nM [^{3}H]tiotidine at 4°C showed that the equilibrium was reached at 30 min and sustained for 4 h (data not shown).

Receptor Internalization and Recovery. COS7, U937 cells, or derived clones were incubated at different times with 10 μM amthamine, and the number of receptor sites was analyzed by radioligand binding assay. The recovery of binding sites was evaluated by radioligand binding assay at different time points after washing the cells treated with 10 μM amthamine for 60 min. In assays performed in the presence of 50 μM cycloheximide or 0.5 μM okadaic acid, the inhibitors were added 30 min before amthamine treatment and with fresh medium after cell wash.

Statistical Analysis. Binding data, sigmoidal dose-response, and desensitization fittings were performed with Prism 4.00 for Windows (GraphPad Software, San Diego, CA). One-way analysis of variance followed by the Dunnett's post test was performed using GraphPad InStat version 3.01. Specific binding was calculated by the subtraction of nonspecific binding from total binding.

Results

H2r Desensitization and Internalization. We reported previously that a rapid homologous desensitization of the H2r is observed in transfected COS7 cells and U937 cells (Lemos Legnazzi et al., 2000; Shayo et al., 2001). In addition, Smit and coworkers (1995) showed the loss of H2r membrane immunofluorescence after 1-h exposure to histamine. We evaluated the relationship between the loss of H2r response and the number of membrane sites in H2r-transfected COS7 cells in an attempt to further understand the underlying molecular mechanisms involved. The number of H2r membrane sites was assessed by [^{3}H]tiotidine saturation binding assays, whereas cAMP response was determined after cell exposure to 10 μM amthamine (0–240 min). H2r desensitization and internalization exhibited similar kinetic profiles, reaching minimal values after agonist treatment for 60 min (Fig. 1A). However, H2r desensitization was faster than receptor internalization, suggesting that receptor desensitization is not dependent on internalization.

Recovery of H2r Sites and cAMP Response. Because maximal H2r internalization was achieved at 60 min (Fig. 1A), cells were exposed to amthamine for 60 min to assess cAMP response and H2r sites after cell washing and incubation at different time points. The removal of the stimulus led to a rapid recovery of H2r sites, whereas H2r response resensitized slower (Fig. 1B), suggesting that H2r membrane localization was not sufficient to achieve H2r-evoked cAMP response.

To determine whether H2r sites and response recovery was mediated by de novo protein synthesis, cells were treated with the well-characterized inhibitor of protein synthesis, cycloheximide. Figure 2A shows that the degree of H2r in-

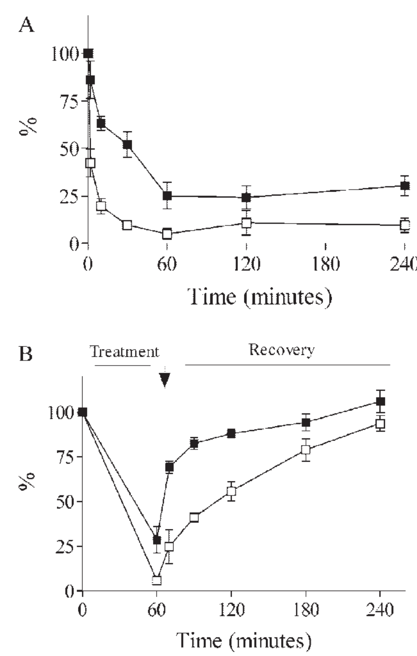

Fig. 1. H2r desensitization, internalization, and recovery. A, H2r desensitization and internalization. H2r-transfected COS7 cells were incubated with 10 μM amthamine at different time points and washed. H2r binding sites (■) and cAMP response (□) were evaluated as described under *Materials and Methods*. B, recovery of H2r sites and cAMP response. H2r-transfected COS7 cells were treated for 60 min with 10 μM amthamine, washed (↓), and the recovery of H2r binding sites (■) and cAMP response (□) were determined at different time points. A and B, H2r sites were evaluated by saturation binding assays with [^{3}H]tiotidine and the B_{max} value was fitted by nonlinear regression. Response to cAMP was measured after stimulation with 10 μM amthamine in the presence of 1 mM IBMX. Data represent the percentage with respect to untreated cells and were calculated as the means ± S.E.M. (n = 3).

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ternalization and recovery resulted similarly in both cycloheximide-treated and untreated cells. Furthermore, cAMP response to 10 μM amthamine showed no significant differences between control and cycloheximide-treated cells (Fig. 2B).

Based on these findings, it can be assumed that the H2r is recycled to the cell surface once internalized. To determine whether H2r rapid resensitization involved receptor dephosphorylation, the recovery of H2r sites and coupled signaling was assessed in the presence of okadaic acid (inhibitor of protein phosphatase 2A family of phosphatases). Pretreatment with okadaic acid inhibited the recovery of H2r sites (Fig. 3A) and abolished the resensitization of cAMP response to amthamine (Fig. 3B), suggesting that the recovery of H2r active sites and the response resensitization depends on H2r dephosphorylation.

Role of Arrestins in H2r Regulation. Nonvisual arrestins play a key role not only in GPCR uncoupling but also in receptor internalization caused by their ability to function

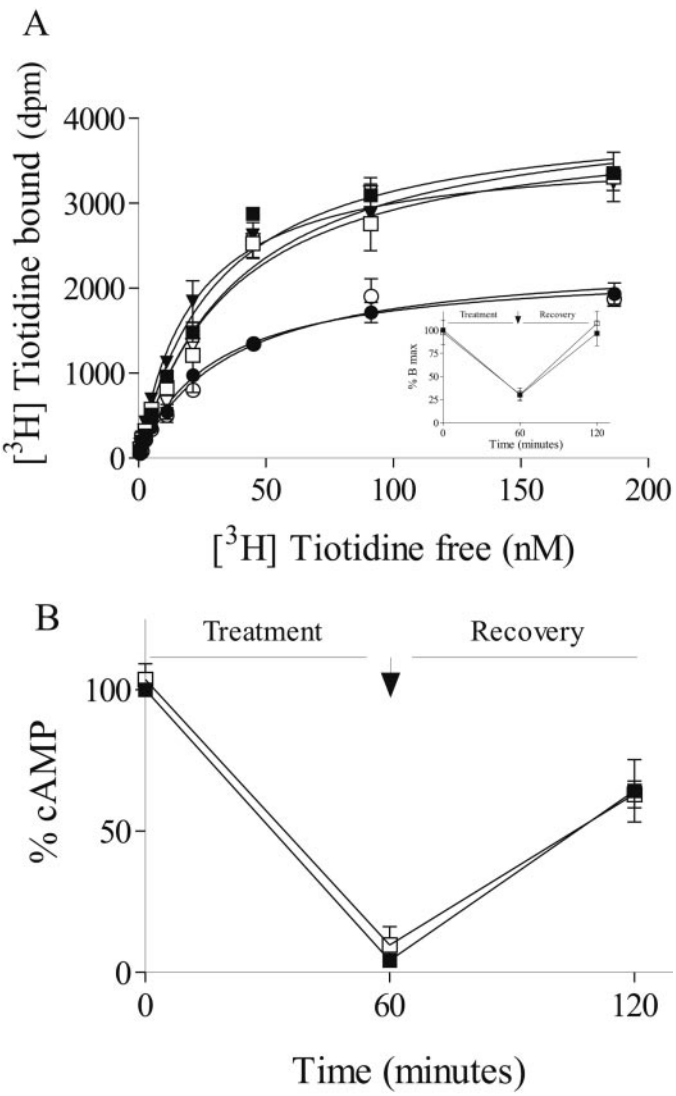

Fig. 2. H2r desensitization, internalization, and recovery in the presence of cycloheximide. A, internalization and recovery of H2r membrane sites. [^{3}H]Tiotidine saturation assays were performed in H2r-transfected COS7 cells: untreated (■), treated with 10 μM amthamine for 60 min (●), or treated with 10 μM amthamine for 60 min, washed, and further incubated for 60 min in fresh medium (▼). Open symbols correspond to cells pretreated with 50 μM cycloheximide. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Inset, data represent the percentage B_{max} value fitted by nonlinear regression of [^{3}H]tiotidine saturation assay, calculated as the means ± S.E.M. (n = 3). B, resensitization of the H2r. H2r-transfected COS7 cells were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 60 min in fresh medium. Assays were carried out in the absence (■) or presence of cycloheximide (□). Data represent the percentage of cAMP measured after stimulation with 10 μM amthamine in the presence of 1 mM IBMX and calculated as the means ± S.E.M. (n = 3). A and B, 100% corresponds to untreated cells in the absence of cycloheximide.

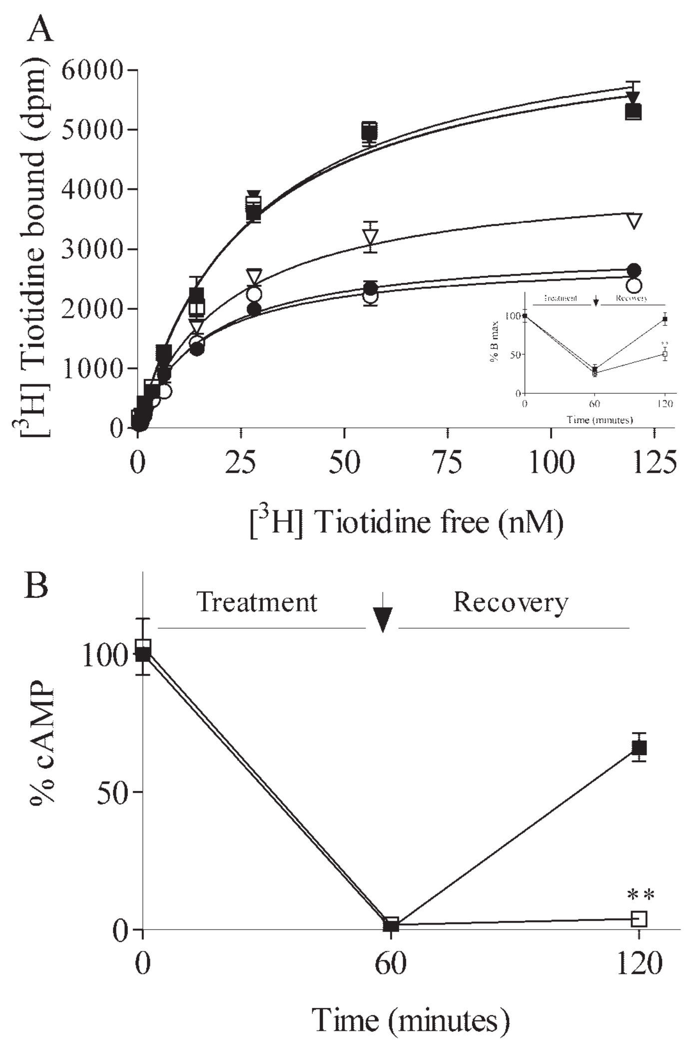

Fig. 3. H2r desensitization, internalization, and recovery in the presence of okadaic acid. A, internalization and recovery of H2r membrane sites. [^{3}H]Tiotidine saturation assays were carried out in H2r-transfected COS7 cells: untreated (■), treated with 10 μM amthamine for 60 min (●), or treated with 10 μM amthamine for 60 min, washed (↓), and further incubated for 60 min in fresh medium (▼). Open symbols represent treatments in the presence of 0.5 μM okadaic acid. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Inset, data represent the B_{max} value fitted by nonlinear regression of [^{3}H]tiotidine saturation assays, calculated as the means ± S.E.M. (n = 3). B, resensitization of the H2r. H2r-transfected COS7 cells were treated with 10 μM amthamine for 60 min, washed (↓), and incubated for 60 min in fresh medium. Assay was carried out in the absence (■) or in the presence of okadaic acid (□). Data represent the percentage of cAMP measured after stimulation with 10 μM amthamine in the presence of 1 mM IBMX, calculated as the means ± S.E.M. (n = 3). **, p < 0.001 versus resensitization in the absence of okadaic acid. A and B, 100% corresponds to untreated cells in the absence of okadaic acid.

as adapter proteins binding to both phosphorylated receptors and clathrin, thus allowing endocytosis. To assess the potential involvement of β-arrestins in H2r desensitization, COS7 cells were transiently cotransfected with H2r and arrestin 2, arrestin 3, or β-arrestin (319–418), a dominant-negative mutant lacking the receptor binding domain that inhibits receptor internalization by binding constitutively to clathrin and AP-2 (Krupnick et al., 1997) (Fig. 4A). As shown in Fig. 4B, only arrestin 3 overexpression led to a decrease in both H2r basal sites and amthamine-induced response. The reduction in amthamine-induced cAMP response when arrestin 3 is overexpressed may result from increased H2r desensitization and/or internalization. However, because cAMP response diminished up to a similar extent as the number of receptors, it is likely that receptor internalization may account for the reduction in amthamine response.

To further understand the role of arrestins in receptor internalization and recycling, H2r sites and cAMP response were assessed in COS7 transfected cells exposed to 10 μM amthamine at different time points and after agonist removal. We found that arrestin 3 overexpression reduced H2r membrane sites not only in untreated cells (as observed previously) but also in amthamine-treated cells. β-Arrestin (319–418) consistently abolished amthamine-induced internalization, supporting the idea that arrestin is involved in H2r endocytosis. The overexpression of arrestin 2 failed to modify H2r sites (Fig. 4C).

Desensitization kinetic assays showed a faster desensitization only in arrestin 3-cotransfected cells (Fig. 4D). Although β-arrestin (319–418) dampened receptor internalization, it did not prevent receptor desensitization. Recycling and resensitization experiments showed that after 60 min of agonist removal, there were no differences in the amount of H2r membrane sites among the studied groups (Fig. 4E), but when cAMP response was assessed, β-arrestin (319–418) prevented H2r resensitization (Fig. 4F). These results show that H2r has to be first internalized to be resensitized, with β-arrestins playing a crucial role in both processes.

To confirm the specificity of arrestin 3 in the internalization and resensitization processes, we carried out the experiments cotransfecting the receptor with RNAi specifically designed to knock down the expression of arrestin 2 or arrestin 3. RNAi targeting either arrestin 2 or arrestin 3 specifically reduced protein levels by ~80% (Fig. 5A). As shown in Fig. 5, B and C, only cotransfection with RNAi against arrestin 3 had a significant effect on receptor internalization and resensitization.

Role of Dynamin in H2r Internalization and Resensitization. Dynamins are proteins that assemble into rings at the neck of invaginated coated pits, and their GTPase

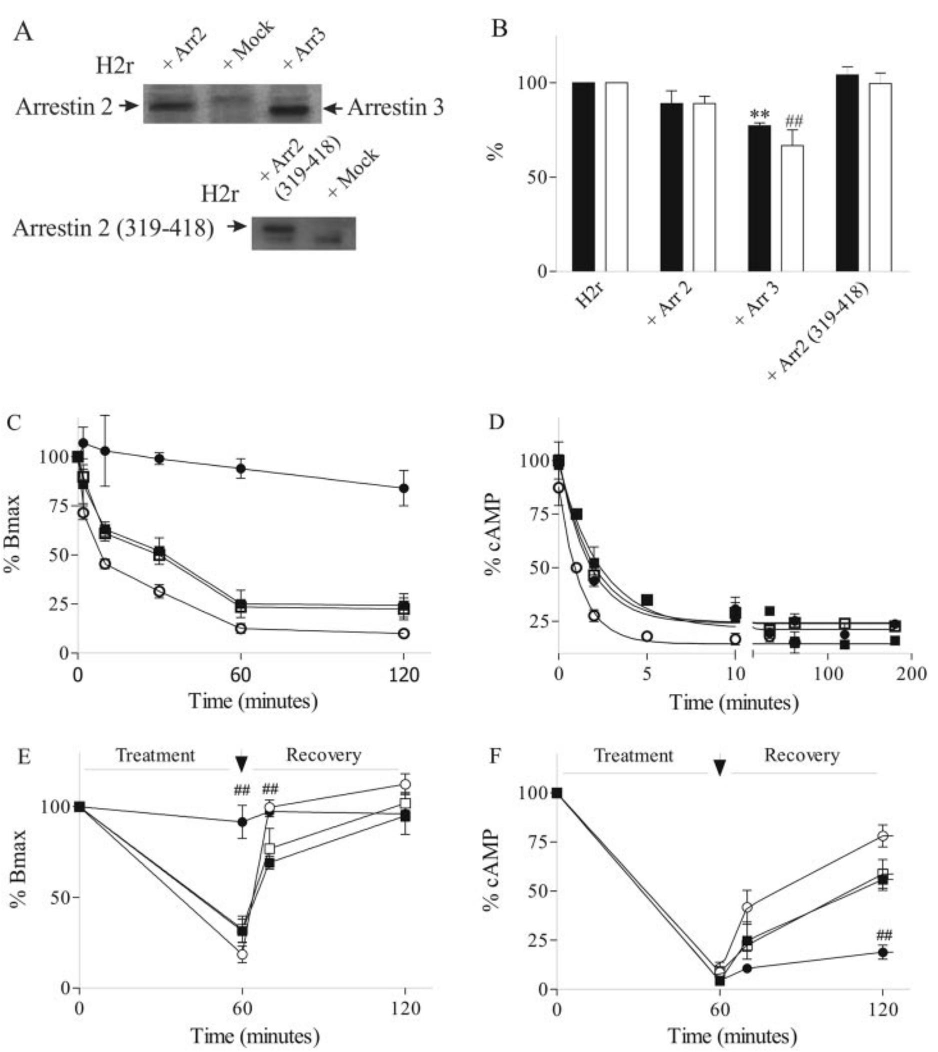

Fig. 4. Arrestin involvement on H2r desensitization, internalization, and resensitization. A, arrestin overexpression in COS7-transfected cells. COS7 cells were transiently cotransfected with H2r and arrestin 2, arrestin 3, arrestin (319–418), or empty vector (mock). Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and probed with an anti-β-arrestins (top) or anti-C-term β-arrestin 1 (bottom) antibodies. Representative Western blots are shown. B, H2r basal levels and cAMP response. COS7 cells were transiently cotransfected with H2r and Arr2, Arr3, or Arr(319–418). B_{max} value from [^{3}H]tiotidine saturation assays (■) and cAMP response to 10 μM amthamine (□) were determined. ##, **, $p < 0.001$ versus control cells. C, H2r internalization time course. COS7 cells were treated for the indicated periods of time with 10 μM amthamine. B_{max} value was determined by nonlinear regression fit from [^{3}H]tiotidine saturation assays. D, H2r desensitization time course. COS7 cells were treated for the indicated periods of time with 10 μM amthamine. cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined as detailed under *Materials and Methods*. E, recovery of H2r membrane sites. COS7 cells were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 10 or 60 min with fresh medium. B_{max} value was determined by nonlinear regression fit from [^{3}H]tiotidine saturation assays. F, Resensitization of the H2r. COS7 cells were treated for 60 min with 10 μM amthamine, washed (↓), and incubated for 10 or 60 min in fresh medium. cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined as detailed under *Materials and Methods*. C to F, COS7 cells cotransfected with H2r and mock (■), Arr3 (○), Arr2 (□), or βArr(319–418) (●). Data are the means ± S.E.M. (n = 3); 100% corresponds to untreated cells for each transfection condition. ##, $p < 0.001$ versus H2r-transfected COS7 cells.

activity is required for the scission of the vesicles from the plasma membrane, thus regulating receptor endocytosis. To further investigate H2r internalization, COS7 cells were cotransfected with H2r and a dominant-negative mutant for dynamin (dynaminK44A). This mutant is defective in its GTP binding site, effectively blocking dynamin-mediated endocytosis at a stage after the initiation of the coat assembly and preceding the sequestration into deeply invaginated coated pits (van der Bliek et al., 1993). DynaminK44A expression in COS7 cells abolished amthamine-induced H2r internalization (Fig. 6B) without modifying H2r basal number of sites or amthamine cAMP maximal response (Fig. 6A). We noticed again that when the process of internalization was abolished, in this case by cotransfection with dynamin-K44A, the recovery of H2r active sites was also inhibited (Fig. 6C), suggesting that agonist-induced internalization and resensitization are dynamin-dependent.

Clathrin Involvement in H2r Internalization and Resensitization. To shed light on the role of clathrin in H2r internalization and resensitization, we investigated amthamine-induced H2r internalization in the presence of two dominant-negative mutants of Eps15 protein, DIII and EH29, which specifically disrupt clathrin-coated pit-mediated endocytosis (Benmerah et al., 1999). The cotransfection with an irrelevant mutant (DIIIΔ2) was used as a negative control. The expression of the dominant-negative constructs modified neither the number of H2r sites nor amthamine-

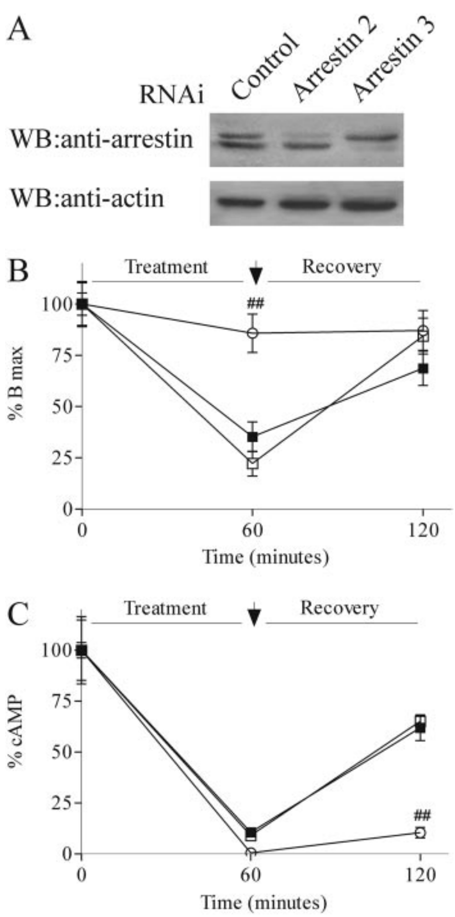

Fig. 5. Effect of arrestin knockdown on H2r internalization and resensitization. A, specific arrestin knock down in COS7 RNAi-transfected cells. Cells were cotransfected with H2r and Stealth RNAi against arrestin 2 or arrestin 3 and harvested 72 h later. Blots were incubated with a mouse monoclonal antibody anti-arrestin 2 that cross-reacts with arrestin 3. Blots were stripped and reprobed for actin for loading control. B, internalization and recovery of H2r membrane sites. COS7 cells cotransfected with H2r and RNAi-negative control (■), RNAi against arrestin 3 (○) or arrestin 2 (□) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 60 min with fresh medium. B_{max} value was determined by nonlinear regression fit from [^{3}H]tiotidine saturation assays. C, desensitization and resensitization of the H2r. COS7 cells cotransfected with H2r and RNAi-negative control (■), RNAi against arrestin 3 (○), or arrestin 2 (□) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 60 min in fresh medium. cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined as detailed under *Materials and Methods*. B and C, data are the means ± S.E.M. (n = 3). 100% corresponds to untreated cells for each transfection condition. ##, $p < 0.001$ versus control COS7 cells.

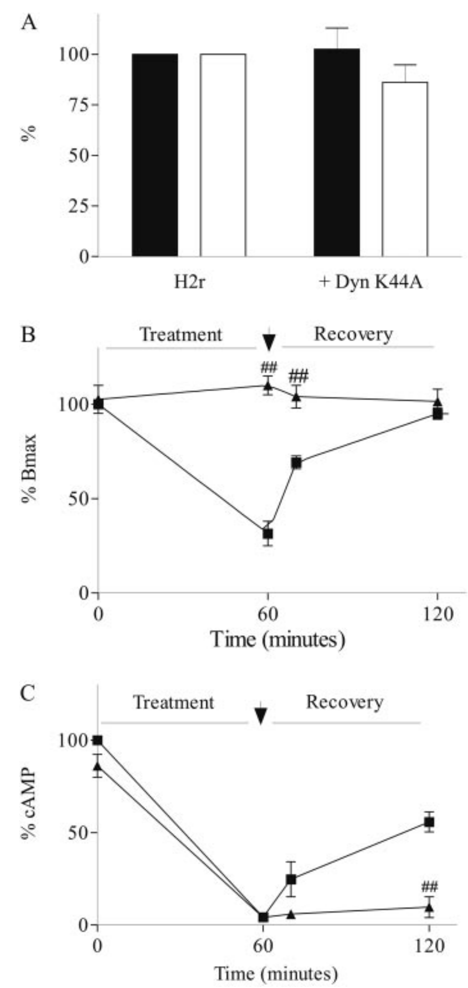

Fig. 6. Dynamin involvement on H2r desensitization, internalization, and resensitization. A, basal H2r internalization and cAMP response. COS7 cells were transiently cotransfected with H2r and DynK44A, and B_{max} values from [^{3}H]tiotidine saturation assay (■) and cAMP response to 10 μM amthamine (□) with respect to control cells were assessed. B, internalization and recovery of H2r membrane sites. COS7 cells cotransfected with H2r and mock (■) or DynK44A (▲) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 10 or 60 min in fresh medium, and B_{max} value was determined by nonlinear regression fit from [^{3}H]tiotidine saturation assays. C, resensitization of the H2r. COS7 cells cotransfected with H2r and mock (■) or DynK44A (▲) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 10 or 60 min in fresh medium. cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined as detailed under *Materials and Methods*. A to C, data represent the means ± S.E.M. (n = 3); 100% correspond to untreated control cells. ##, $p < 0.001$ versus H2r-transfected COS7 cells.

induced cAMP response under nonstimulated conditions (Fig. 7A).

DIII and EH29 abolished receptor internalization after amthamine treatment for 60 min (Fig. 7B). Consistent with previous results, the conditions that impeded receptor internalization also inhibited receptor resensitization (Fig. 7C). These findings support that receptor resensitization is dependent on clathrin-mediated internalization.

H2r Internalization and Resensitization in U937 Cells. In COS7 cells, the expression of H2r and that of the constructs was induced by transfection. Because this experimental procedure may eventually modify the stoichiometry among the components of the signaling pathway and ultimately have an impact on the cellular response, we evaluated the role of arrestin and dynamin in H2r internalization in U937 cells. In this leukemic cell line, we reported previously moderate expression of H2r and its coupling to the Gs pathway, its desensitization mechanism, and its participation in cell maturation (Lemos Legnazzi et al., 2000; Fernández et al., 2002). U937 cells were stably transfected with β-arrestin (319–418) or dynaminK44A. The expression of these constructs in the resulting clones was confirmed by reverse transcription-polymerase chain reaction. As shown in Fig. 8, A and B, clones expressing β-arrestin (319–418) or dynaminK44A did not significantly differ from naive cells regarding the number of H2r basal sites or amthamine-induced cAMP response.

When H2r internalization and recovery was evaluated in U937 cells, the number of H2r sites diminished approximately by 50% after stimulation with amthamine for 60 min. However, the expression of β-arrestin (319–418) or dynaminK44A in U937-derived clones reduced H2r endocytosis (Fig. 9A).

To evaluate the role of arrestin and dynamin in the resensitization of H2r in U937 cells, U937-arr (319–418) and U937-dynK44A, cells were exposed to 10 μM amthamine for 60 min, extensively washed, and then assayed at different time points for amthamine-induced cAMP production recovery. The expression of either β-arrestin (319–418) or dynaminK44A led to a significant reduction in H2r resensitization compared with U937-naive cells (Fig. 9B). These results indicate that in the presence of dominant-negative mutants, which dampened H2r internalization, the ability of the desensitized receptors to resensitize was significantly reduced, as observed in COS7 transfected cells.

We next addressed whether H2r resensitization depended on de novo protein synthesis by pretreating U937 cells with cycloheximide. As shown in Fig. 10, H2r resensitization in cycloheximide U937-treated cells was not significantly different from untreated cells. However, in U937-derived clones,

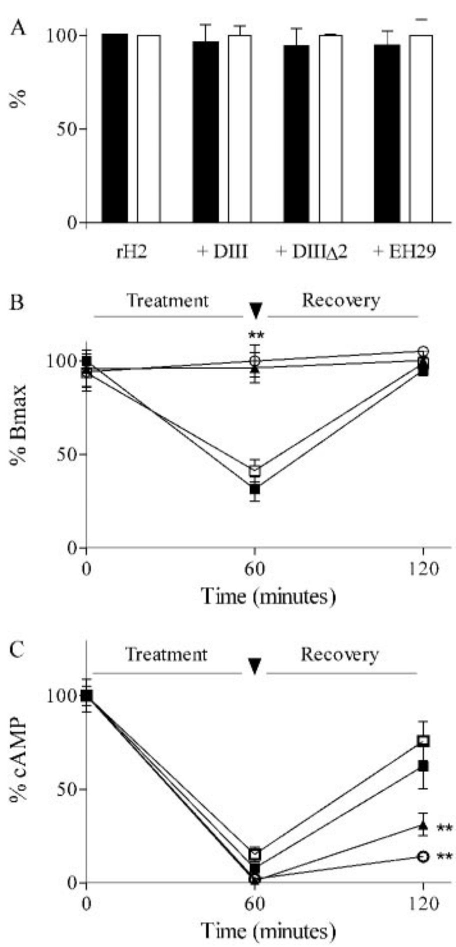

Fig. 7. Clathrin involvement on H2r desensitization, internalization, and resensitization. A, H2r basal internalization and cAMP response. COS7 cells were transiently cotransfected with H2r and DIII, DIIIΔ2, or EH29, and B_{max} values from [^{3}H]tiotidine saturation assay (■) and cAMP response to 10 μM amthamine (□) were determined. B, internalization and recovery of H2r membrane sites. COS7 cells cotransfected with H2r and mock (■), DIII (▲), DIIIΔ2 (□), or EH29 (○) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 60 min in fresh medium, and B_{max} was determined by nonlinear regression fit from [^{3}H]tiotidine saturation assays. C, resensitization of the H2r. COS7 cells cotransfected with H2r and mock (■), DIII (▲), DIIIΔ2 (□), or EH29 (○) were exposed to 10 μM amthamine, washed (↓), and further incubated for 60 min in fresh medium. cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined. A to C, data were calculated as the means ± S.E.M. (n = 3); 100% correspond to untreated control cells. **, $p < 0.001$ versus H2r-transfected COS7 cells.

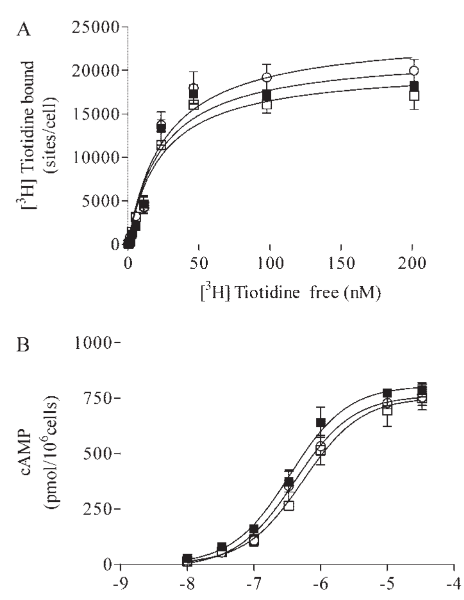

Fig. 8. Characterization of U937 clones obtained by stable transfection with arrestin (319–418) or dynaminK44A. A, H2r membrane sites. Saturation assays for [^{3}H]tiotidine were carried out in U937 (■), U937-Arr(319–418) (□), and U937-DynK44A (○) cells. B, H2r cAMP response. U937 (■), U937-βArr(319–418) (□), and U937-DynK44A (○) cells were exposed for 9 min to increasing concentrations of amthamine at 37°C in the presence of 1 mM IBMX. cAMP levels were determined as detailed under *Materials and Methods*. A and B, data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

H2r resensitization differed between cycloheximide-treated and untreated cells, supporting the idea that H2r de novo synthesis may serve as an alternative mechanism to achieve resensitization when receptor recycling is inhibited.

Discussion

Virtually all GPCRs undergo ligand-induced internalization, a process originally considered as a mechanism tending to remove desensitized receptors from the cell surface. However, it is now well accepted that receptor endocytosis serves a variety of purposes, including receptor down-regulation, desensitization, recycling, and relocalization of the cell signaling. The major findings of the present study are that H2r internalization is necessary for the recovery of H2r active sites in the membrane and that arrestin 3, dynamin, and clathrin are involved in both processes.

We have reported previously that H2r exposure to amthamine induces a rapid and homologous desensitization (Lemos Legnazzi et al., 2000; Shayo et al., 2001). In the present study, we found that H2r internalized after agonist exposure and that the loss of cAMP response was observed earlier than that of H2r membrane sites (Fig. 1A).

In COS7-transfected cells, only arrestin 3 overexpression significantly increased both basal and amthamine-induced internalization and desensitization (Fig. 4, A–D). The decrease in H2r response suggests augmented receptor internalization rather than diminished H2r coupling to heterotrimeric G proteins because a similar reduction in H2r membrane sites was observed. The internalization of H2r after exposure to amthamine was completely abolished in the presence of dominant-negative mutants for arrestin or dynamin or RNAi against arrestin 3 (Figs. 4C, 5B, and 6B). The fact that these mutants did not reduce the basal level of H2r internalization (Figs. 4B and 6A) suggests that H2r may not exhibit a high level of constitutive internalization in the absence of agonists. Overall, these results indicate that arrestin 3 and dynamin play a relevant role in H2r internalization. Similar results were reported for the β2-AR in HEK 293 cells (Zhang et al., 1997).

It has been suggested that cells expressing low endogenous levels of GRKs and arrestins, such as COS cells (Fig. 4A), are not a suitable model to study dominant-negative proteins or RNAi because they may not exhibit a high level of receptor internalization. Therefore, cells expressing higher levels of these proteins, such as HEK 293 or Chinese hamster ovary cells, are preferred to study β2-AR internalization. However, we observed sequestration of more than 60% of surface receptors and a complete inhibition of H2r internalization in the presence of dominant-negative mutants for arrestin, dynamin, or a specific RNAi against arrestin 3, supporting the idea that COS7 cells represent an appropriate model to study H2r.

Dynamin, a 100-kDa GTPase, originally isolated as a nucleotide-dependent microtubule binding protein, has been identified as a major component and marker of the clathrin-mediated endocytic pathway (van der Bliek and Meyerowitz, 1991; Shpetner and Vallee, 1992). In the same way, β-arrestins have been shown to interact with clathrin and the AP-2 complex (Laporte et al., 1999). Furthermore, β-arrestin (318–419) was reported to localize in clathrin-coated pits in the absence of agonist stimulus and to effectively block endogenous clathrin-binding sites. Therefore, the participation of both arrestin and dynamin in the regulation of H2r sequestration suggests a role for clathrin-coated pits in this process. A wide spectrum of molecular mechanisms underlying GPCR internalization has been reported, including arrestin-dependent, dynamin/clathrin-independent, arrestin-independent, dynamin/clathrin-dependent, and dynamin-dependent, clathrin-independent mechanisms (Prossnitz, 2004). Furthermore, some GPCRs undergo internalization via caveolae (Haasemann et al., 1998;

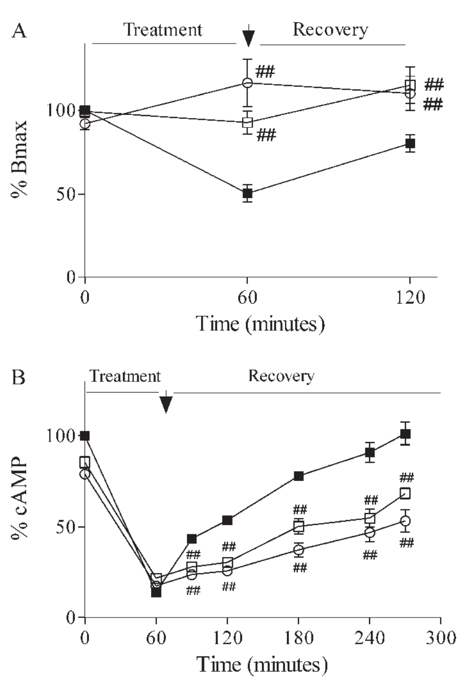

Fig. 9. H2r desensitization, internalization, and resensitization in U937 cells and derived clones. A, internalization and recovery of H2r membrane sites. U937 (■), U937-Arr(319–418) (□), and U937-DynK44A (○) cells were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 60 min in fresh medium, B_{max} value was determined by nonlinear regression fit from saturation [^{3}H]tiotidine assays. B, desensitization and resensitization of the H2r. U937 (■), U937-βArr(319–418) (□), and U937-DynK44A (○) cells were treated for 60 min with 10 μM amthamine and washed (↓). The maximal cAMP response to 10 μM amthamine in the presence of 1 mM IBMX was determined at different time points. A and B, data were calculated as the means ± S.E.M. (n = 3); ##, $p < 0.001$ versus U937 cells; 100% corresponds to untreated U937 cells.

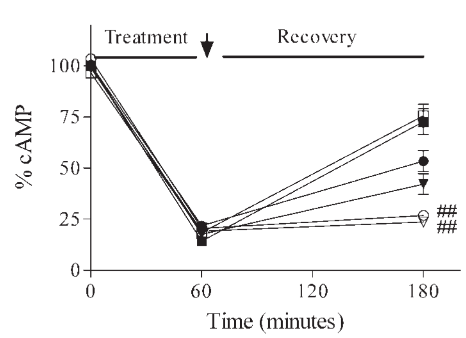

Fig. 10. H2r resensitization in U937 cells and derived clones in the presence of cycloheximide. U937 (■), U937-Arr(319–418) (●), and U937-DynK44A (▼) were treated for 60 min with 10 μM amthamine, washed (↓), and further incubated for 120 min in fresh medium. cAMP maximal response to 10 μM amthamine in the presence of 1 mM IBMX was determined as detailed under *Materials and Methods*. Open symbols correspond to the same treatment in the presence of 50 μM cycloheximide. Data were calculated as the means ± S.E.M. (n = 3). ##, $p < 0.05$ versus similar assay in the absence of cycloheximide; 100% correspond to untreated U937 cells in the absence of cycloheximide.

Ginés et al., 2001; Mueller et al., 2002). The mechanisms underlying this process are largely unknown, but phosphorylation by protein kinases other than GRKs may mediate the targeting for receptor internalization (Rapacciuolo et al., 2003). Therefore, in the present study, we next addressed the role of clathrin in H2r endocytosis. For this purpose, dominant-negative mutants for the Eps15 protein, a constituent of plasma membrane clathrin-coated pits that is ubiquitously and constitutively associated with AP-2, were used. This construct has been shown to inhibit clathrin-dependent endocytosis by disrupting the assembly of the clathrin-coated pits (Benmerah et al., 1999). Coexpression of H2r with dominant-negative Eps15 mutants, DIII or EH29, dampened H2r internalization after amthamine treatment. Although these findings do not provide direct evidence for H2r-clathrin association, they suggest clathrin involvement in H2r internalization.

On the other hand, the internalization of H1r is mediated by a clathrin-independent mechanism. It is interesting that the authors provide evidence that H1r internalization occurs through a mechanism involving lipid rafts or caveolae. In addition, β1-AR phosphorylation by protein kinase A also leads to receptor internalization via caveolae (Rapacciuolo et al., 2003).

The trafficking of GPCRs is critical for the regulation of temporal and spatial aspects of the receptor response. Thus, it serves as a mechanism aiming to modulate the expression of receptors on the cell surface to ensure that extracellular stimuli are transduced into intracellular signals with the appropriate magnitude, duration, and specificity.

The present results show that stimulus removal led to a rapid recovery of H2r sites, whereas H2r response resensitized slower. This finding suggests that H2r membrane localization is not sufficient to induce cAMP response, because a fraction of membrane receptors may be inactivated. This is consistent with the observation that after 60 min of amthamine exposure, 25% of the receptors remaining in the cell surface were unable to evoke a response.

To investigate the role of receptor sequestration in H2r signaling, we studied receptor fate after internalization. Overall, results show that H2r sequestration serves to the resensitization of receptors in the cell surface. This was supported by the observation that when receptor sequestration was inhibited, as in the presence of β-arrestin (319–418), small interfering RNA for arrestin 3, dynaminK44A, and DIII or EH29 constructs, the system was unable to evoke cAMP response even 1 h after stimulus removal.

In the resensitization of H2r receptor, dephosphorylation by protein phosphatase 2A family of phosphatases seems to be a crucial step, because pretreatment with okadaic acid reduced the recovery of both H2r membrane sites and cAMP response. A previous work shows that endocytosis is critical for β2-AR resensitization (Zhang et al., 1997). The authors propose that receptors are dephosphorylated and resensitized in early endosomes by a mechanism involving a conformational change in the receptor brought about by acidification in the endosomal compartment, which enhances dephosphorylation of GRK phosphorylated sites. This intracellular dephosphorylation is consistent with our findings showing that not only a phosphatase inhibitor but also the blockade of H2r internalization dampened H2r resensitization. In accordance, we have described previously that H2r desensitization and phosphorylation are mediated by GRK2 in COS7 cells (Shayo et al., 2001).

Based on the characteristics of the interaction between the receptor and β-arrestins, GPCRs are divided into two major classes. Class A receptors, which includes receptors such as the β2-AR among others, transiently bind arrestin after activation and are rapidly recycled back from the endosomes to the cell surface (Oakley et al., 2000). Class B receptors, including V2 vasopressin receptor, seem to retain stable complexes with arrestins on endosomal vesicles, are poorly recycled back to the plasma membrane, and eventually are targeted for lysosomal degradation. Class A and B GPCRs also differ in their binding preference for arrestins. Indeed, whereas class A receptors bind to arrestin 3 with high affinity, class B receptors show no preference for any of the two β-arrestins.

Although our results do not provide direct evidence of an association between H2r and arrestin 3, they indicate that arrestin 3 is preferentially involved in H2r internalization and its rapid recycling to the cell surface. Therefore, based on these observations, H2r would belong to class A GPCRs.

It is interesting that when dephosphorylation was inhibited by okadaic acid, a significant reduction in the number of receptors returning to the cell surface was observed. It seems likely that dephosphorylation blockade may change H2r fate from recycling to endosomal degradation. In this regard, it has been reported previously that the density of GPCR phosphorylation sites may be involved in regulating the stability of the interaction between the receptor and arrestin (Oakley et al., 2001).

Because H2r is endogenously expressed in U937 cell line, and receptor desensitization is critically involved in leukemic cell differentiation, we investigated H2r internalization and the role of the accessory proteins in this cell line. The findings obtained in U937-derived clones stably transfected with dominant-negative constructs for arrestin or dynamin were similar to those observed in COS7 cells and further support the physiological relevance of the mechanisms described.

It is worth noting that cycloheximide treatment reduced H2r resensitization only in U937-derived clones, in which H2r internalization was disrupted. These results suggest that de novo H2r synthesis plays an alternative role in receptor resensitization only when internalization is abolished.

H2 ligands are among the most widely prescribed and over-the-counter-sold drugs in the world. Because of their widespread use to treat non–life-threatening disorders such as gastric ulcers, they are generally used as long-term therapy rather than being restricted for short-term manifestations. Therefore, the assessment of the potential adverse or undesired effects is highly important.

Considering the clinical widespread use of H2r ligands, their involvement in leukemic cell maturation and their cardioprotective effects in patients with chronic heart failure, the characterization of their mechanism(s) of desensitization and recycling becomes crucial to further understand the long-term effects of these ligands.

Acknowledgments

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