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Up-Regulation of α_{1B}-Adrenergic Receptors with Defects in G Protein Coupling: Ligand-Induced Protection from Receptor Instability

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ABSTRACT

The biochemical basis for the unexpected agonist-induced up-regulation of the number of radioligand binding sites for two mutated α_{1B}-adrenergic receptors reported previously was investigated. Up-regulation was independent of the expression vector used and was not prevented by cycloheximide or actinomycin D, eliminating several potential transcriptional mechanisms and new receptor protein synthesis. Antagonists were also able to induce up-regulation, suggesting that ligand occupancy without signal generation was sufficient to induce the increase in binding sites. Accordingly, we hypothesized that up-regulation results from ligand-induced protection from inherent instability of these mutated receptors. Studies with receptors in isolated membranes revealed that the two mutated receptors that exhibited up-regulation in intact cells also exhibited an inherent instability of their ligand binding capacity, and binding of either agonists or antagonists to these receptors could protect against the loss of binding. In contrast, the wild-type receptor and other mutated receptors that did not exhibit up-regulation in intact cells did not exhibit instability or ligand-induced protection in isolated membranes. The occurrence of instability and protection in isolated membranes for only those mutated receptors and ligands that exhibit up-regulation in intact cells provides compelling evidence that the apparent up-regulation of binding sites in intact cells results from ligand-induced protection from an inherent instability of these G protein coupling-defective receptors. Inclusion of protease inhibitors markedly reduced the loss of binding in isolated membranes, implicating membrane-localized proteolysis as the likely mechanism for the instability.

Similar to other G protein-coupled receptors (GPCRs), the α_{1B}-adrenergic receptors (α_{1B}-ARs) are subject to dynamic regulation, with the number of receptors, their subcellular localization, and their function all subject to modification during development, in response to changes in physiological status, in various disease states, and during the course of drug treatment (Cotecchia et al., 1998; Garcia-Sainz et al., 2000; Michelotti et al., 2000). Much of our understanding of these adaptive changes comes from studies of the "desensitization" that occurs in response to repeated or prolonged exposure to agonist drugs that activate the receptors. In addition to activating the receptors and downstream signaling pathways, agonist binding also induces a rapid "uncoupling" of these receptors from further G protein activation, a rapid "sequestration" within the plasma membrane and/or "endocytosis" into intracellular vesicles, and a much slower decrease in the number of receptors detectable in radioligand binding assays, referred to as "down-regulation" (Zhu and Toews, 1995). Down-regulation is the least understood of the components of desensitization, and the mechanisms that regulate the stability and degradation of receptor protein in general also remain poorly characterized for most GPCRs.

We recently investigated the sequestration and endocytosis properties of mutated α_{1B}-ARs that are defective in G protein coupling and activation of the phosphoinositide (PI) hydrolysis pathway (Wang et al., 1997, 2002). Three of these mutated α_{1B}-ARs, Y348A, Δ12, and Δ5, were able to undergo agonist-induced sequestration within the plasma membrane but did not exhibit endocytosis into intracellular vesicles. However, a fourth coupling-defective receptor, Δ[246–261], exhibited both sequestration and endocytosis essentially identical to the wild-type α_{1B}-AR. We also characterized the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic receptor; PI, phosphoinositide; UTR, untranslated region; DMSO, dimethyl sulfoxide; NF-κB, nuclear factor-κB; CMV, cytomegalovirus; E-64, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride; UK-14,304, brimonidine; CHO, Chinese hamster ovary; RSV, Rous sarcoma virus; 5HT, 5-hydroxytryptamine (serotonin).

ability of these receptors to undergo down-regulation after 24-h agonist exposure (Wang et al., 2002). Unexpectedly, the Δ12 and Δ5 receptors exhibited a marked "up-regulation" of receptor binding sites rather than the down-regulation that was observed for the wild-type α_{1B}-AR and the Δ[246–261] receptor; the Y348A receptor exhibited neither up-regulation nor down-regulation.

Up-regulation of receptor binding capacity has been reported recently for several other GPCRs, with a variety of different mechanisms likely to be involved (Cowen et al., 1997; Gether et al., 1997; Jewell-Motz et al., 1997; Leurs et al., 1998). In the current study, we have further characterized the up-regulation of G protein-coupling-defective α_{1B}-ARs and investigated the molecular mechanisms involved. The results clearly point to a ligand-induced "protection" from "inherent instability" that can be detected in isolated membranes as the major mechanism for the apparent up-regulation of the binding capacity of these mutated receptors observed in intact cells. Furthermore, the protective effect of protease inhibitors implicates membrane-localized proteolysis as a major component of this inherent instability.

Materials and Methods

Chemicals and Reagents. Cell culture medium, serum, and trypsin were obtained from Invitrogen (Carlsbad, CA), and G418 was from Calbiochem (La Jolla, CA). Enzymes for molecular biology protocols were from New England BioLabs (Beverly, MA). [^{3}H]Prazosin and the Expre^{35}S^{35}S protein labeling mix were from PerkinElmer Life Sciences (Boston, MA). Epinephrine and other adrenergic receptor ligands were from Sigma-Aldrich (St. Louis, MO), except for UK-14,304 (brimonidine), which was obtained from Allergan (Irvine, CA). Cycloheximide, actinomycin D, protease inhibitor cocktail (P8340), and all other biochemicals were from Sigma-Aldrich.

Plasmid Preparation and Transfections. The preparation of CHO cells expressing the wild-type α_{1B}-AR from the plasmid pRC-CMV (Wang et al., 1997) and the Δ12-mutated α_{1B}-AR from pCMV (Wang et al., 2002) have been described previously. The cDNA for the wild-type receptor included approximately 540 nucleotides of 3′-untranslated sequence (Cotecchia et al., 1988), whereas the cDNA for the Δ12 receptor contained only the coding sequence (Wu et al., 1995). To insert the wild-type α_{1B}-AR cDNA into pRC-RSV, the receptor cDNA was cleaved from pRC-CMV with *Hin*dIII and *Xba*I and ligated into pRC-RSV that had been digested with *Hin*dIII and *Xba*I. To prepare matched plasmids expressing the Δ12 α_{1B}-AR in pRC-CMV and pRC-RSV, the Δ12 cDNA was cleaved from pCMV with *Hin*dIII and ligated into both pRC-CMV and pRC-RSV that had been digested with *Hin*dIII. To prepare matched constructs with and without the 3′-untranslated region (UTR), both the wild-type and Δ12 cDNAs in pRC-CMV were digested with *Bst*EII and *Bsu*36I to excise the region (nucleotides 480–770) encompassing the residues mutated in Δ12. This segment from the wild-type receptor cDNA was then ligated into the plasmid previously containing the Δ12 receptor, which does not include the 3′-untranslated region. Similarly, this segment from the Δ12 receptor cDNA was ligated into the plasmid previously containing the wild-type receptor, which does include the 3′-untranslated region. The correct sequences of all constructs were then confirmed before calcium phosphate-mediated transfection into CHO-K1 cells. Cells expressing these constructs were selected by G418 resistance, and experiments with these cells used "batch-selected" cells rather than individually isolated clones.

Cell Culture. CHO cells transfected with the wild-type or various mutated α_{1B}-ARs were grown in monolayer in Ham's F-12 medium supplemented with 10% fetal bovine serum and 200 μg/ml G418 at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Cells from confluent flasks were trypsinized and plated for experiments in culture dishes at 3,000 to 5,000 cells/cm^2.

Intact Cell Incubations to Induce Up-Regulation. Up-regulation assays were performed as in our previous study (Wang et al., 2002). Cells plated on 100-mm dishes were treated on day 3 with 1 mM ascorbate for control cells or with epinephrine or other ligands in ascorbate to induce up-regulation. After 24-h treatment, cells were washed twice with ice-cold wash buffer (10 mM Tris, pH 7.4, and 140 mM NaCl) and twice with ice-cold hypotonic lysis buffer (1 mM Tris, pH 7.4, and 2 mM EDTA). Cells were then lysed by scraping, and cell membranes were isolated from the lysate by centrifugation for 30 min at 20,000 rpm in an SM-24 rotor in an RC-5B-Plus refrigerated centrifuge (Sorvall, Newton, CT). The membrane pellets were resuspended by homogenization for 10 s with a tissue disruptor (Ultra-Turrax T-25; IKA Works, Inc., Wilmington, NC) in 1 ml of binding buffer (20 mM Tris, pH 7.4, 2 mM $MgCl_2$, and 140 mM NaCl) and assayed for [^{3}H]prazosin binding as described below.

Membrane Isolation and Treatments to Monitor Instability and Protection. Cells plated on 150-mm dishes were washed and lysed by scraping in hypotonic buffer as described above for up-regulation assays, except that the cells were lysed on day 4 without any prior treatment on day 3. The lysate was also homogenized for 10 s with a tissue disruptor before collecting the membranes by centrifugation as described above for up-regulation assays. The resulting membrane pellet was resuspended by homogenization with a tissue disruptor in a volume of binding buffer appropriate for the number of treatments and assays to be conducted (typically 4–5 ml). This membrane preparation was transferred in 1-ml aliquots to polycarbonate centrifuge tubes. Various ligands or vehicle was added to the tubes, which were then capped and incubated for various times on ice or at 22°C (room temperature); for routine experiments, the incubation was for 18 h. In experiments to investigate proteolytic mechanisms, Sigma protease inhibitor cocktail (P8340, containing 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64) was included during these incubations, and control samples contained 1% DMSO, the solvent for the protease inhibitor cocktail. At the end of this incubation, 2 ml of binding buffer was added to each tube and the membranes were isolated by centrifugation as described above. The membrane pellet was washed two additional times by resuspending in 3 ml of binding buffer by vortexing and then isolation of the membranes by centrifugation. The final washed membrane pellet was resuspended in 1 ml of binding buffer by homogenization with a tissue disruptor and assayed for [^{3}H]prazosin binding as described below.

[^{3}H]Prazosin Binding Assays. Aliquots (200 μl) of the membrane suspensions from the two protocols described above were incubated with [^{3}H]prazosin in a final volume of 500 μl of binding buffer for 60 min at 37°C; 100 μM phentolamine was used to define nonspecific binding. The reactions were stopped by filtration over GF/B filters (Whatman, Maidstone, UK) followed by washing three times with wash buffer and quantitation by liquid scintillation counting. Routine assays used a single concentration of [^{3}H]prazosin, typically 3 nM to avoid binding more than 10% of the radioligand. For saturation assays, seven concentrations ranging from approximately 5 to 500 pM were used; for competition binding assays, the [^{3}H]prazosin concentration was approximately 160 pM.

Data Analysis and Presentation. Nonlinear regression analyses of saturation and competition binding data and statistical comparisons were performed with Prism (GraphPad Software Inc., San Diego, CA). Data for up-regulation are presented as -fold of control, whereas data for instability are presented as the percentage of control. Data are the means ± S.E.M. from the indicated number of experiments, with duplicate or triplicate determinations within each experiment.

Results

Characterization of Up-Regulation. The Δ12 receptor contains a three-amino acid deletion, residues 227 to 229, YIV, at the N terminus of the third intracellular loop, whereas the Δ5 receptor has 35 amino acids deleted, residues 227 to 261 (Wu et al., 1995). We focused on the Δ12 receptor for further studies of up-regulation, because it contains a much smaller deletion than Δ5 but exhibits an essentially identical phenotype in terms of G protein coupling, internalization, and up-regulation (Wang et al., 2002). The term up-regulation is used here to denote an increase in the radioligand binding capacity of the receptors, which may or may not be associated with an increase in the number of receptor protein molecules.

The time and agonist concentration dependence of Δ12 up-regulation was investigated. Up-regulation induced by 10 μM epinephrine occurred in a steady and progressive manner, with up-regulation readily detectable at 8 h and continuing beyond 24 h of agonist exposure (Fig. 1A). Up-regulation occurred at quite low concentrations of epinephrine, the EC_{50} value for epinephrine for inducing up-regulation in 24-h assays being 6 ± 1 nM (Fig. 1B), consistent with the high-affinity binding of epinephrine to the Δ12 receptors reported previously (Wang et al., 2002).

Saturation binding assays indicated that up-regulation results from an increase in the number of binding sites (B_{max}) with no change in the affinity for [^{3}H]prazosin (Fig. 2A). For this set of experiments, the average fold increase in B_{max} values was 3.8 ± 0.8 fold (n = 4); the average K_D values for [^{3}H]prazosin were 63 ± 4 pM for control cells and 62 ± 5 pM for the epinephrine-pretreated up-regulated cells. In contrast, exposure of cells expressing the wild-type α_{1B}-AR to epinephrine in the same experiments consistently led to down-regulation, which was also caused by a decrease in B_{max} with no change in affinity; the average decrease in B_{max} was 43 ± 11% (n = 3), and the average K_D values were 30 ± 5 pM for control cells and 29 ± 5 pM for epinephrine-pretreated down-regulated cells (data not shown). The lower affinity for [^{3}H]prazosin of the Δ12 receptor compared with the wild-type receptor also has been reported previously (Wang et al., 2002).

We next conducted experiments to determine whether the up-regulated receptors were in fact Δ12 receptors expressed from the transfected plasmid or whether agonist binding to the Δ12 receptors on the cell surface might somehow lead to expression of the endogenous wild-type α_{1B}-AR gene, which is normally not expressed in CHO cells. The up-regulated receptors from Δ12 cells exhibited the same high binding affinity for epinephrine as the receptors from control Δ12 cells, both of which were 30- to 40-fold higher than the affinity for epinephrine of the wild-type receptor (Fig. 2B). The calculated K_i values were 32 ± 9 nM for the control Δ12 receptors and 26 ± 7 nM for the epinephrine-pretreated and up-regulated Δ12 receptors, whereas the value for the wild-type receptors was 1.1 ± 0.3 μM, all similar to the values reported previously (Wang et al., 2002). Furthermore, even after up-regulation of the receptors, there was no detectable stimulation of PI hydrolysis in the Δ12 cells (data not shown). Together, these results clearly indicate that the up-regulated receptors are the Δ12-mutated receptors and not wild-type receptors expressed from the endogenous gene.

Investigation of NF-κB and CMV Promoter Involvement in Up-Regulation. A previous study reported that agonist-induced up-regulation of wild-type $5HT_{1A}$ serotonin receptors in transfected CHO cells was mediated by activation of NF-κB and increased receptor transcription mediated by an NF-κB enhancer element present in the CMV promoter of the plasmid used in their transfection (Cowen et al., 1997). We examined the possibility that this might be the mechanism for agonist-induced up-regulation of the Δ12 α_{1B}-AR, because it was also transfected into CHO cells with a CMV enhancer-driven plasmid, pCMV (Wang et al., 2002). Although the Δ12 receptor was known to be uncoupled from its classical G protein-mediated PI hydrolysis pathway, it was not unreasonable to suspect that it might nonetheless retain coupling by an alternate mechanism to the NF-κB signaling pathway. One strong line of evidence for NF-κB involvement in the studies with the $5HT_{1A}$ serotonin receptors came from experiments comparing results for the receptor expressed in a CMV promoter-based plasmid, which contains an NF-κB enhancer element, and in an RSV promoter-based plasmid, which does not contain an NF-κB enhancer; up-regulation was observed only with the CMV promoter-containing plasmid (Cowen et al., 1997).

To address the possible role of this NF-κB mechanism in

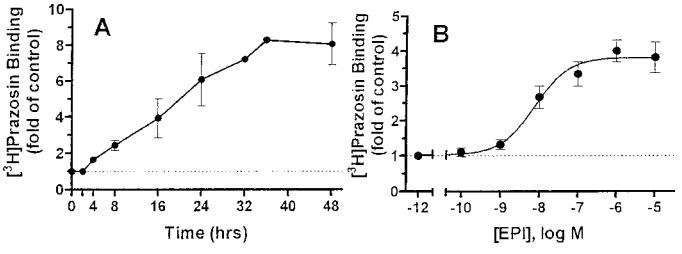

Fig. 1. Time course and concentration dependence for epinephrine-induced up-regulation of Δ12 α_{1B}-AR binding. Cells were incubated for the indicated times with 10 μM epinephrine (A) or for 24 h with the indicated concentrations of epinephrine (EPI, B) before cell lysis and measurement of [^{3}H]prazosin binding to isolated membranes. Data are presented as -fold up-regulation and are the means ± S.E.M. from three to four experiments, each performed in triplicate.

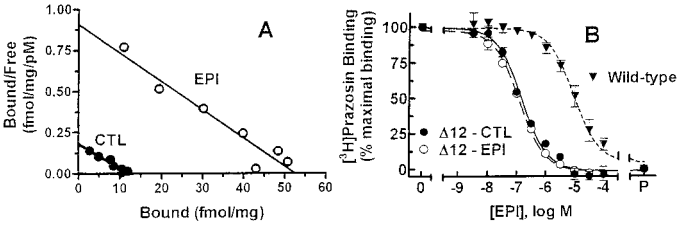

Fig. 2. Binding properties of up-regulated Δ12 α_{1B}-ARs. Cells expressing the Δ12 α_{1B}-AR were incubated for 24 h in the absence (control, CTL, ●) or presence of 10 μM epinephrine (EPI, ○) to induce up-regulation. Cells were lysed and binding of [^{3}H]prazosin to isolated membranes was measured in saturation assays with different concentrations of [^{3}H]prazosin (A) or in competition assays with different concentrations of epinephrine (B); the corresponding epinephrine competition curve for the wild-type α_{1B}-AR (▼) is also shown for comparison. Data for the saturation experiment in A are from a single experiment performed in duplicate and are presented in the form of a Rosenthal plot of specifically bound radioligand versus the ratio of specifically bound radioligand to free radioligand concentration. The average values from all experiments are presented under *Results*. For the competition assays in B, binding in the presence of epinephrine is presented as the percentage of the specific binding in the absence of epinephrine ("0" on the x-axis); "P" represents the nonspecific binding obtained in the presence of 100 μM phentolamine. Data are the means ± S.E.M. from three to four experiments, each performed in triplicate.

Δ12 α_{1B}-AR up-regulation, the wild-type and Δ12 α_{1B}-AR cDNAs were both inserted into the plasmids pRC-CMV and pRC-RSV for side-by-side comparison. Up-regulation would not be expected with pRC-RSV if an NF-κB mechanism were involved. Generating these constructs also allowed us to test the possibility that the occurrence of up-regulation for Δ12 versus down-regulation for the wild-type receptor was an artifact of the different transfection protocols used to generate the Δ12 versus wild-type cells. The wild-type receptor in our previous studies was transfected with pRC-CMV, a single plasmid that encodes G-418 resistance and contains the CMV promoter to drive expression of the inserted α_{1B}-AR cDNA. In contrast, the Δ12 receptor in our previous studies was expressed in pCMV, which contains the CMV promoter but no resistance genes; thus, our original Δ12 cells were generated by cotransfection with the pMAMneo plasmid expressing the neomycin resistance gene (Wang et al., 2002). Furthermore, the wild-type receptor plasmid included approximately 540 base pairs of 3′-untranslated sequence, whereas the Δ12 construct did not contain any 3′-untranslated sequence. Either of these differences in the generation of our wild-type and Δ12 cells could possibly have accounted for the differential up- versus down-regulation observed between these two constructs. As shown in Fig. 3, down-regulation was observed for the wild-type receptor regardless of the vector used for its expression and regardless of the presence or absence of the 3′-untranslated sequence. Similarly, comparable amounts of up-regulation were observed for the Δ12 receptor regardless of the presence or absence of the CMV promoter and its NF-κB enhancer element or the 3′-untranslated sequence. These studies thus provide strong evidence that the NF-κB mechanism postulated for the $5HT_{1A}$ serotonin receptor up-regulation is not the mechanism for Δ12 α_{1B}-AR up-regulation (Cowen et al., 1997). Furthermore, they clearly indicate that up-regulation is an inherent property of the Δ12 receptor and not of the specific vector in which it is expressed or of regulatory sequences in noncoding segments of the cDNA.

Lack of Requirement for New Receptor Synthesis in Up-Regulation. To determine whether transcription and translation were involved in up-regulation by mechanisms other than the NF-κB pathway investigated above, the effects of the mRNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide on Δ12 α_{1B}-AR up-regulation were determined. As shown in Fig. 4, neither actinomycin D nor cycloheximide was able to prevent up-regulation. Although cycloheximide did not prevent up-regulation, it inhibited incorporation of ^{35}S-labeled amino acids (Expre^{35}S^{35}S protein labeling mix) into trichloroacetic acid-precipitable material by 94 ± 2% (n = 3), indicating that it was effective at blocking protein synthesis under these conditions. The fold up-regulation in the absence of inhibitors for this set of experiments was 2.9 ± 0.4 (n = 8). Treatment with cycloheximide in the absence of epinephrine reduced binding to 46 ± 7% (n = 8) of that in control cells treated without cycloheximide. Including epinephrine during the incubation with cycloheximide gave binding that was 113 ± 40% of that in control cells treated without cycloheximide, giving an average up-regulation value of 2.2 ± 0.4-fold. Similarly, treatment with actinomycin D alone reduced binding to 46 ± 18% (n = 3) of control, whereas including epinephrine along with actinomycin D gave binding that was 141 ± 42% of control for an average up-regulation value of 3.5 ± 0.5-fold. In contrast, treatment of cells expressing the wild-type receptor with cycloheximide produced only a small and not statistically significant decrease in binding, giving a binding value 85 ±

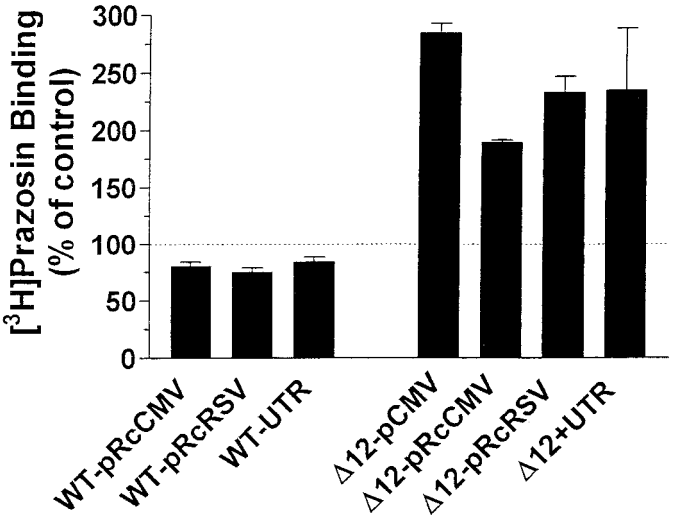

Fig. 3. Up- and down-regulation properties of Δ12 and wild-type α_{1B}-ARs expressed from different vectors. The wild-type (WT) and Δ12 α_{1B}-ARs were expressed in either pRC-CMV or pRC-RSV vectors with the 3′-UTR absent (−UTR) or present (+UTR) in the cDNA, as described under *Materials and Methods*. The wild-type receptor in pRC-CMV with the 3′-untranslated region present is the construct referred to as "wild-type" in all of the other experiments; the Δ12 receptor in pCMV with the 3′-UTR absent is the construct referred to as "Δ12" in all of the other experiments. Cells were incubated for 24 h in the absence or presence of 10 μM epinephrine to induce up- or down-regulation and then lysed. Binding of [^{3}H]prazosin to isolated membranes was then measured. Data are expressed as the percentage of binding to control cells not treated with epinephrine and are the means ± S.E.M. from three experiments, each performed in triplicate. There were no statistically significant differences between the down-regulation values for the wild-type α_{1B}-AR in the different constructs or between the up-regulation values for the Δ12 α_{1B}-AR in the different constructs (analysis of variance followed by Dunnett's post test; $p < 0.05$).

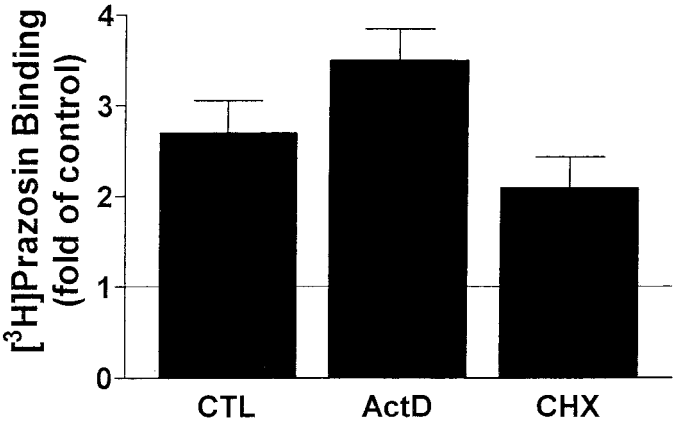

Fig. 4. Effects of actinomycin D and cycloheximide on Δ12 α_{1B}-AR up-regulation. Cells expressing the Δ12 α_{1B}-AR were incubated for 24 h in the absence or presence of 10 μM epinephrine to induce up-regulation, each in the absence (control, CTL) or presence of 1 μg/ml actinomycin D (ActD) or 5 μg/ml cycloheximide (CHX); DMSO, the vehicle for actinomycin D, was included in the control samples for those experiments. Cells were then lysed and [^{3}H]prazosin binding to isolated membranes was measured. Data are expressed as the -fold up-regulation and are the means ± S.E.M. from three experiments for actinomycin D and eight experiments for cycloheximide, each performed in triplicate. The values for up-regulation in the presence of actinomycin D or cycloheximide were not statistically significantly different from that in their absence (analysis of variance followed by Dunnett's post test; $p < 0.05$).

26% (n = 5) of that for cells incubated in the absence of cycloheximide. Thus, the Δ12 receptor loses its binding capacity much faster than the wild-type receptor when new protein synthesis is blocked, and epinephrine can completely prevent this loss of binding. These results suggest that the major component of epinephrine-induced up-regulation is an epinephrine-induced increase in stability of pre-existing Δ12 receptors and not new synthesis of additional receptor protein molecules in the presence of epinephrine.

Up-Regulation by Both α_{1B}-AR Agonists and Antagonists. The ligand specificity for induction of up-regulation was investigated next. As shown in Fig. 5, the nonselective adrenergic receptor agonist norepinephrine induced up-regulation similar to that with epinephrine, as did the α_1-AR-selective agonist phenylephrine. The potency for norepinephrine was similar to that for epinephrine in Fig. 1B, whereas that for phenylephrine was about 10-fold lower, consistent with their known potencies for binding to α_{1B}-ARs. The α_1-AR antagonists phentolamine and WB4101 induced up-regulation that was similar in magnitude to that induced by the agonists and with potencies expected for their binding to α_{1B}-ARs. The antagonist prazosin also induced up-regulation, although to a smaller extent than for the other α_1-AR ligands, perhaps because it is less effective or perhaps because of residual prazosin from the pretreatment (because of its lipophilicity) causing inhibition during the subsequent binding assay. It should be noted that these classical α_1-AR antagonists are all inverse agonists when tested in systems with constitutive activity (Rossier et al., 1999). In contrast to the up-regulation that was observed with all α_1-AR agonists and antagonists, agonists and antagonists selective for other adrenergic receptors caused no up-regulation or induced up-regulation only at relatively high concentrations, concentrations at which they would also bind to α_{1B}-ARs. Thus, no up-regulation was observed with the β-AR antagonist propranolol, and the β-AR agonist isoproterenol and the α_2-AR agonist UK-14,304 induced modest up-regulation, but only at high concentrations where they would be expected to interact with α_{1B}-ARs. Together, these results suggest that ligand binding to the Δ12 receptors is sufficient to mediate their up-regulation and that no agonist-specific conformational change or signal pathway activation is required.

Inherent Instability and Ligand-Induced Protection of Δ12 α_{1B}-ARs. The results mentioned above are all consistent with several recent studies that suggest protection from inherent instability of mutated receptors as a mechanism for up-regulation of other GPCR constructs, including various mutated adrenergic receptors (Gether et al., 1997; Wilson and Limbird, 2000; Alewijnse et al., 2000). Accordingly, the stability of α_{1B}-ARs assessed by retention of [^{3}H]prazosin binding activity after incubation of isolated membranes at 22°C for various times was assessed (Fig. 6). The wild-type receptor retained essentially all of its binding activity over 18-h incubation at 22°C, whereas the Δ12 receptor consistently exhibited a progressive instability under these conditions, typically losing about 50% of its binding activity over 18 h. Including the agonist epinephrine (10 μM) during the 18-h incubation completely protected the Δ12 receptor from this inherent instability, yielding a stability curve essentially identical to that for the wild-type receptor in the absence of ligand. Similar to the term "up-regulation" for the intact cell studies above, the term "instability" in these isolated membrane assays is used to denote a decrease in the radioligand binding capacity of the receptors, which may or may not be associated with a decrease in the number of receptor protein molecules.

Half-maximal protection from instability in isolated membranes was observed with 25 ± 6 nM epinephrine (Fig. 7A), a slightly higher concentration than that for up-regulation in intact cells. Saturation binding assays with [^{3}H]prazosin indicated that the decrease in binding in the membrane instability assays was caused by a decrease in B_{max}, with no change in K_D for the remaining receptors and no evidence for residual lower affinity binding to the "destabilized" receptors (Fig. 7B). The average values were a 39 ± 1% decrease in B_{max} and K_D values of 95 ± 9 pM for receptors in the control membranes maintained on ice and 113 ± 7 pM for the receptors in membranes incubated for 18 h at 22°C (n = 2).

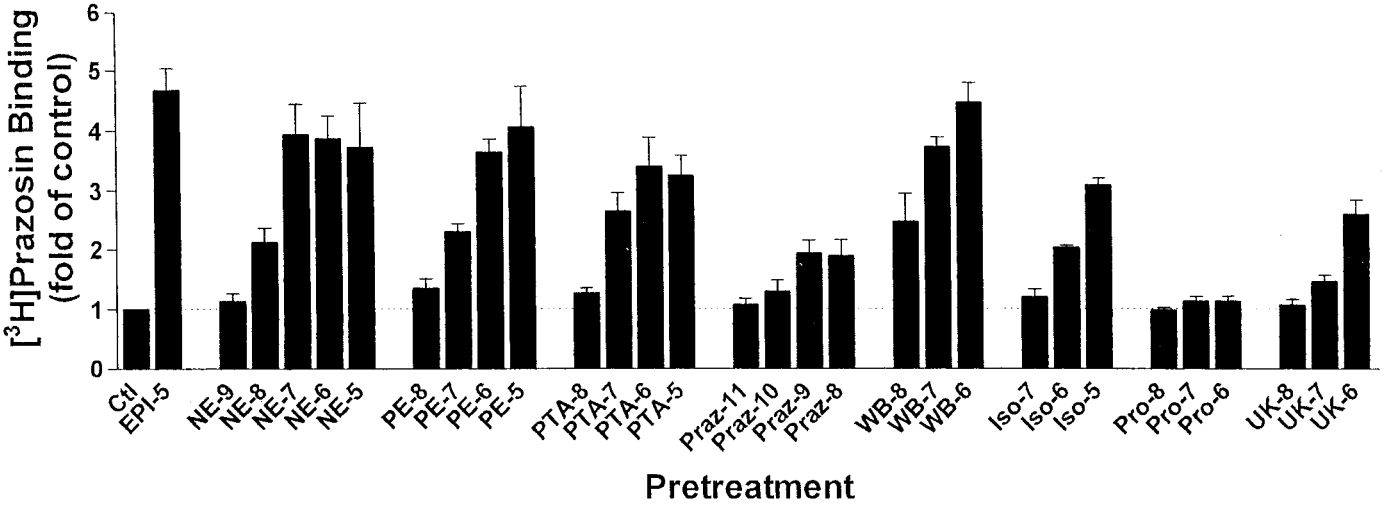

Fig. 5. Ligand specificity and concentration dependence for Δ12 α_{1B}-AR up-regulation. Cells expressing the Δ12 α_{1B}-AR were incubated for 24 h in the absence or presence of 10 μM epinephrine or the indicated concentrations (log M values) of other adrenergic receptor agonists and antagonists. Cells were then lysed and [^{3}H]prazosin binding to isolated membranes was measured. Data are expressed as the -fold up-regulation and are the means ± S.E.M. from three or more experiments, each performed in triplicate. Ctl, ascorbate vehicle control; EPI, 10 μM epinephrine; NE, norepinephrine; PE, phenylephrine; PTA, phentolamine; WB, WB-1401; ISO, isoproterenol; PRO, propranolol; UK, UK-14,304.

In a series of experiments to investigate the ligand and receptor specificity of the instability and protection observed in isolated membranes, the Δ12 receptor exhibited 61 ± 9% loss of binding activity (Fig. 8A). Similar to the intact cell up-regulation, both the agonist epinephrine (10 μM) and the antagonist phentolamine (10 μM) were able to protect the receptor from this instability. In contrast, inclusion of epinephrine or phentolamine had little or no effect on the binding activity of the wild-type receptor under the same conditions. In a subsequent series of experiments further investigating the ligand specificity of protection from instability (Fig. 8B), the Δ12 receptor exhibited a somewhat smaller loss of binding activity, 39 ± 10%. The α_1-AR-selective agonist phenylephrine (10 μM) afforded essentially complete protection, similar to that with epinephrine. Similar to results with phentolamine, the α_1-AR antagonist WB-4101 (1 μM) also afforded essentially complete protection. Consistent with their low potencies for intact cell up-regulation, the non-α_1-AR ligands isoproterenol, propranolol, and UK-14,304 also provided little or no protection in these membrane instability assays.

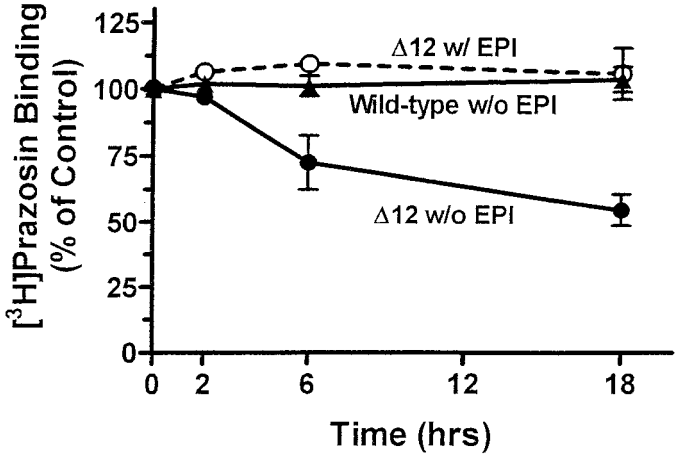

Fig. 6. Time-dependent instability of Δ12 α_{1B}-ARs in isolated membranes and protection from instability by epinephrine. Membranes isolated from cells expressing the wild-type (▲) or Δ12 (●, ○) α_{1B}-ARs were either maintained on ice for 18 h or incubated for the indicated times at room temperature (22°C) in the absence (▲, ●) or presence (○) of 10 μM epinephrine (EPI); all samples were assayed after 18 h, with the 2- and 6-h samples maintained on ice for 16 and 10 h, respectively, before being switched to 22°C for the final 2 or 6 h. Membranes from all treatment conditions were then collected and washed by repeated centrifugation to remove the epinephrine, and [^{3}H]prazosin binding to the membranes was then measured. Data are expressed as the percentage of the binding to membranes maintained on ice for the entire 18 h (plotted as the 100% value at time 0 on the graph) and are the means ± S.E.M. from three to five experiments, each performed in triplicate.

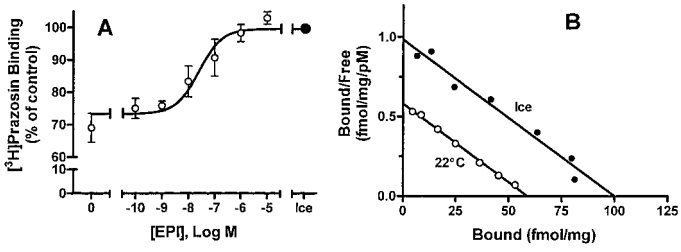

Fig. 7. Further characterization of Δ12 α_{1B}-AR instability and epinephrine-induced protection from instability. For the experiments shown in A, membranes isolated from cells expressing the Δ12 α_{1B}-AR were either maintained on ice (●) or incubated at 22°C (○) for 18 h in the absence (0) or presence of the indicated concentrations of epinephrine (EPI). Membranes were then collected and washed by repeated centrifugation to remove the pretreatment epinephrine, and [^{3}H]prazosin binding to the membranes was measured. Data are expressed as the percentage of the binding to membranes maintained on ice and are the means ± S.E.M. from three experiments, each performed in triplicate. For the experiments shown in B, membranes isolated from cells expressing the Δ12 α_{1B}-AR were either maintained on ice (●) or incubated at 22°C (○) for 18 h. These membranes were washed and then binding of various concentrations of [^{3}H]prazosin to the membranes was measured. Data are presented in the form of a Rosenthal plot as in Fig. 2A and are from a single experiment performed in duplicate; the average values from all experiments are presented under *Results*.

Membrane Instability and Ligand-Induced Protection for Other Mutated α_{1B}-ARs. Other mutated α_{1B}-ARs were tested for their stability/instability and for the ability of both the agonist epinephrine and the antagonist phentolamine to protect from instability (Fig. 9). The Δ5 α_{1B}-AR has 35 amino acids deleted at the N-terminal end of its third intracellular loop, including the three residues deleted in Δ12; similar to the Δ12 receptor, Δ5 is also defective in G protein coupling and in endocytosis and exhibits up-regulation in intact cells assays (Wang et al., 2002). The Δ5 receptor exhibited instability similar to that for Δ12, and this instability was prevented by both epinephrine and phentolamine. The Δ[246–261] α_{1B}-AR has 16 amino acids deleted near the center of the third intracellular loop, the C-terminal 16 residues of those deleted in Δ5; del[246–261] shares the G protein-coupling defect of Δ12 and Δ5, but agonist treatment induces both endocytosis and down-regulation similar to the wild-type receptor, rather than the up-regulation seen with Δ12 and Δ5 (Wang et al., 2002). In the membrane instability assay, del[246–261] exhibited little or no instability and essentially no change in binding after incubation with either epinephrine or phentolamine. The Y348A α_{1B}-AR is a point mutation in the tyrosine of the NPxxY motif in the seventh transmembrane domain; this receptor is defective in both G protein coupling and endocytosis, and it neither up-regulates nor down-regulates with long-term agonist treatment (Wang et al., 1997, 2002). Consistent with its lack of up-regulation, the Y348A receptor did not exhibit instability or ligand-induced changes in binding in the membrane instability assay. Finally, the Tr366 receptor is truncated at residue 366 and lacks nearly all of the C-terminal tail; it exhibits normal G protein coupling but is completely defective in agonist-induced sequestration and endocytosis, and it neither down-regulates nor up-regulates (Wang et al., 2000). The Tr366 receptor did not exhibit instability or ligand-induced changes in binding in the membrane instability assay. Thus, the receptor mutations that lead to instability in isolated membranes and the ligands that induce protection from this instability are the same as those receptor mutations and ligands that result in up-regulation in intact cells.

Effects of Protease Inhibitors on Membrane Instability. In preliminary experiments to explore the molecular basis for the instability of the Δ12 α_{1B}-AR, a variety of agents were tested for their potential ability to either prevent instability of the Δ12 α_{1B}-AR or to induce instability in the wild-type α_{1B}-AR; these included dimethyl sulfoxide and glycerol as "chemical chaperones" (Morello et al., 2000), oxidizing and reducing agents, as well as varying pH, salt, and other ion concentrations during the incubations, none of which were found to be effective (data not shown). The potential involvement of proteolysis in the inherent instability of the Δ12 α_{1B}-AR was assessed, even though studies for several other

GPCRs found no change in the level of receptor protein in Western blots between conditions for instability and those for protection of radioligand binding activity (Gether et al., 1997; Wilson and Limbird, 2000; Chen et al., 2002). Inclusion of a protease inhibitor cocktail (P8340; Sigma-Aldrich) during the instability and protection incubation markedly reduced the loss of binding activity compared with the control cells incubated in the absence of the protease inhibitor cocktail (Fig. 10). The protection of binding activity by the protease inhibitor cocktail was somewhat less than that by the agonist epinephrine alone or by epinephrine together with the protease inhibitors, although statistical comparisons indicated that none of the three protected conditions was significantly different from the control membranes maintained on ice. These data implicate a membrane-associated protease as a major contributor to the instability of the Δ12 α_{1B}-AR.

Discussion

These studies provide compelling evidence that the up-regulation of ligand binding capacity reported previously for two G protein-coupling-defective α_{1B}-ARs, Δ12 and Δ5 (Wang et al., 2002), results from ligand-induced stabilization or protection from an inherent instability of these receptors. First, up-regulation was not blocked by actinomycin D or cycloheximide, indicating that new receptor synthesis is not required and that the apparent up-regulation must use pre-existing receptor protein. Second, antagonists were as effective as agonists in inducing up-regulation, indicating that neither receptor activation nor downstream signaling pathways are involved. Third, the two mutated receptors that exhibit up-regulation in intact cells also exhibit an inherent instability in isolated membrane preparations, whereas the wild-type receptors and mutated receptors that do not exhibit up-regulation are quite stable under these same conditions. Finally, the same panel of drugs that is able to induce up-regulation in intact cells, including both agonists and antagonists with inverse agonist activity, is able to protect these mutated receptors against the instability observed in isolated membrane preparations.

We first tested and eliminated several other potential mechanisms for up-regulation. The possibility that agonist binding to the Δ12 receptors might somehow activate expression of the endogenous wild-type α_{1B}-AR gene that is not normally expressed in CHO cells was eliminated by showing that the up-regulated receptors have the properties of Δ12 receptors and not wild-type receptors, including their higher agonist binding affinity and their inability to stimulate PI hydrolysis. The NF-κB-mediated transcriptional up-regulation mechanism reported for the 5-HT_{1A} receptor expressed in a CMV promoter-driven plasmid (Cowen et al., 1997), similar to the plasmid from which the Δ12 α_{1B}-AR is expressed, was eliminated by showing that up-regulation occurred similarly when the Δ12 receptor was expressed in an RSV promoter-driven plasmid that lacks an NF-κB site. Finally, the ability of up-regulation to occur in the presence of cycloheximide and actinomycin D strongly argued against any other transcriptional or translational mechanism, instead pointing to a change in the binding properties of pre-existing receptors as the mechanism for up-regulation. The greater loss of binding capacity for the Δ12 receptor compared with the wild-type receptor during incubation with cycloheximide further suggested that a decrease in stability of the Δ12 receptor was a likely mechanism for up-regulation.

Our data and conclusions are consistent with several recent studies of up-regulation and/or receptor instability with other mutated adrenergic receptors and other GPCRs. Up-regulation of several mutated α_2-ARs was discovered during studies of receptor sequences involved in agonist-induced

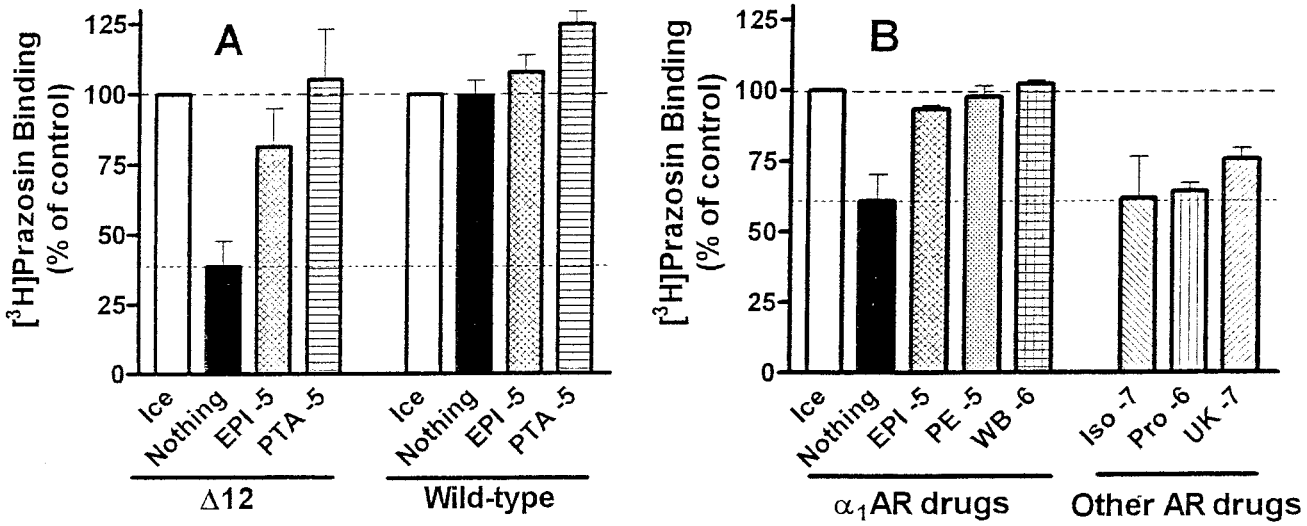

Fig. 8. Ligand specificity for protection from instability of Δ12 α_{1B}-ARs in isolated membranes. For the experiments in A, membranes isolated from cells expressing the wild-type or Δ12 α_{1B}-ARs were either maintained on ice or incubated at room temperature (22°C) for 18 h in the absence (nothing) or presence of 10 μM epinephrine (EPI-5) or 10 μM phentolamine (PTA-5). For the experiments in B, membranes isolated from cells expressing the Δ12 α_{1B}-AR were either maintained on ice or incubated for 18 h at room temperature (22°C) in the absence (nothing) or presence of 10 μM epinephrine (EPI-5), 10 μM phenylephrine (PE-5), 1 μM WB-4101 (WB-6), 100 nM isoproterenol (Iso-7), 1 μM propranolol (Pro-6), or 100 nM UK-14,304 (brimonidine; UK-7). For both sets of experiments, membranes from all treatment conditions were collected and washed by repeated centrifugation to remove the pretreatment drug, and [3H]prazosin binding to the membranes was then measured. Data are expressed as the percentage of the binding to membranes maintained on ice and are the means ± S.E.M. from at least three experiments, each performed in triplicate. The dashed and dotted horizontal lines represent the values for the membranes maintained on ice or incubated at 22°C in the absence of ligand, respectively, for ease of comparison.

down-regulation (Jewell-Motz et al., 1997), similar to our discovery of the up-regulation of the Δ12 and Δ5 α_{1B}-ARs during studies of down-regulation (Wang et al., 2002). Most of the other reports of up-regulation and instability originated from studies of constitutively active mutated receptors, including thyrotropin-releasing hormone receptors (Heinflink et al., 1995), β_2-ARs (Gether et al., 1997; Samama et al., 1997; Rasmussen et al., 1999), α_2-ARs (Betuing et al., 1997), α_{1B}-ARs (Lee et al., 1997), and H_2 histamine receptors (Smit et al., 1996; Alewijnse et al., 1998). These receptors were all found to up-regulate after prolonged incubation with inverse agonists, which are thought to stabilize the "inactive" or "ground state" of constitutively active receptors. These initial studies of up-regulation for constitutively active receptors generally focused on two likely mechanisms: 1) these inverse agonists induced an apparent up-regulation by preventing the constitutive down-regulation that should otherwise occur for these constitutively active receptors, or 2) the activated state of these receptors was somehow less stable than the inactive state, and the inverse agonists prevented this instability by maintaining the receptors in the more stable but inactive ground state. The demonstration that temperature-dependent receptor instability could be observed with detergent-solubilized and purified constitutively active β_2-ARs supported the latter possibility; however, the further observation that agonists as well as antagonists or inverse agonists could protect against this instability suggested that mechanisms unrelated to inverse agonist activity might also be involved (Gether et al., 1997). Subsequent studies showed that agonists as well as antagonists or inverse agonists could induce up-regulation of constitutively active α_{1B}-ARs also (Stevens et al., 2000). This study further separated up-regulation from constitutive activity by showing that not all constitutively active α_{1B}-ARs were up-regulated by inverse agonists and that up-regulation could occur for receptors with little or no constitutive activity. Studies with α_{2A}-ARs, α_{1B}-ARs, and H_2 histamine receptors have all showed that up-regulation of binding sites on intact cells and/or inherent instability in detergent-solubilized preparations can be observed not only for constitutively active receptors but also for mutated receptors defective in G protein coupling and signaling (Alewijnse et al., 2000; Wilson and Limbird, 2000; Wilson et al., 2001).

The relationship between the G protein-coupling status of a receptor and the occurrence of instability and/or up-regulation is intriguing. Nearly all of the examples of these phenomena, as cited above, are for receptors with altered G protein coupling; interestingly, either the enhanced G protein coupling of the constitutively active mutants, or absent or defective G protein coupling, as in the current study, can lead to instability and up-regulation. In contrast, wild-type receptors with their "normal" level of G protein coupling are generally stable and do not exhibit ligand-induced up-regulation, except perhaps when expressed under conditions in which they exhibit constitutive activity (Alewijnse et al., 1998; Stevens et al., 2000). Similarly, we did not observe up-regulation for several mutated α_{1B}-ARs with normal G protein coupling but with marked defects in other aspects of receptor responses, including sequestration, endocytosis, and down-regulation, indicating that neither instability nor up-regulation is a feature of mutated α_{1B}-ARs in general. To our knowledge, the only examples of up-regulation for α_{1B}-ARs with normal G protein coupling are from a study with green fluorescent protein-tagged constructs, in which ligand-induced up-regulation was reported for both the wild-type α_{1B}-AR and one mutated receptor with eight carboxyl-termi-

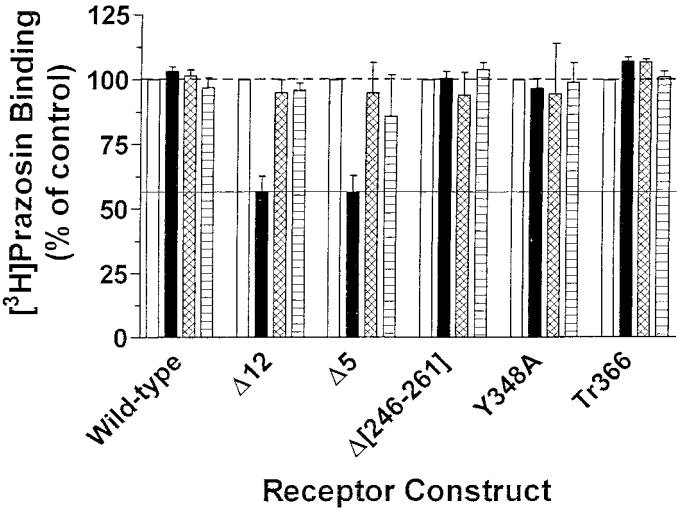

Fig. 9. Stability or ligand-induced protection from instability for various mutated α_{1B}-ARs. Membranes isolated from cells expressing the wild-type, Δ12, or other mutated α_{1B}-ARs were either maintained on ice (□) or incubated for 18 h at room temperature (22°C) in the absence (■) or presence of 10 μM epinephrine (▨) or 10 μM phentolamine (▤). Membranes from all treatment conditions were then collected and washed by repeated centrifugation to remove the epinephrine or phentolamine, and [3H]prazosin binding to the membranes was then measured. Data are expressed as the percentage of the binding to membranes maintained on ice and are the means ± S.E.M. from three or more experiments, each performed in triplicate.

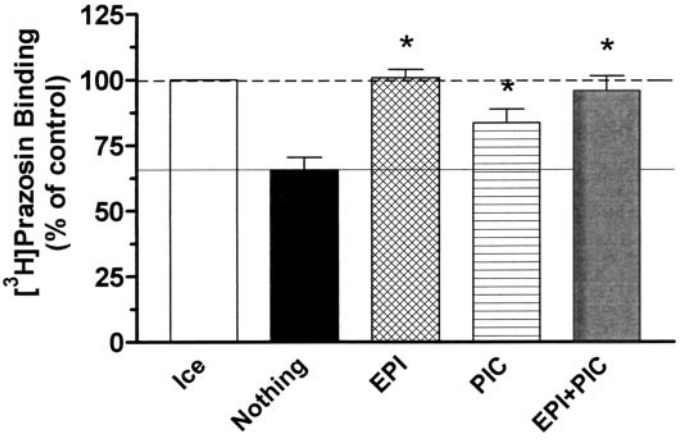

Fig. 10. Effects of protease inhibitors on instability of the Δ12 mutated α_{1B}-AR. Membranes isolated from cells expressing the Δ12 mutated α_{1B}-ARs were either maintained on ice (ice) or incubated for 18 h at room temperature (22°C) in the absence (nothing) or presence of 10 μM epinephrine (EPI), a protease inhibitor cocktail (PIC) or 10 μM epinephrine plus protease inhibitor cocktail (EPI + PIC). All incubations included 1% DMSO, the vehicle for the protease inhibitor cocktail. Membranes from all treatment conditions were then collected and washed by repeated centrifugation to remove the pretreatment agents, and [3H]prazosin binding to the membranes was then measured. The DMSO vehicle had little or no effect on the binding to membranes maintained on ice or incubated in the absence or presence of epinephrine in several experiments in which these three conditions were compared in the absence and presence of DMSO (data not shown). Data are expressed as the percentage of the binding to membranes maintained on ice and are the means ± S.E.M. from five experiments, each performed in triplicate. The asterisks indicate that the values for the three protected samples are not significantly different from the ice control ($p > 0.05$) using Bonferroni's multiple comparisons test.

nal tail serines mutated to alanines but with normal G protein coupling (Stevens et al., 2000). In contrast, neither our wild-type α_{1B}-AR nor our Tr366 α_{1B}-AR, which exhibits normal G protein coupling but lacks almost all of its C-terminal tail, exhibited either up-regulation in intact cells (Wang et al., 2000) or instability in isolated membranes in the current studies. It is also clear that not all α_{1B}-ARs with mutations in G protein coupling are unstable or subject to up-regulation. We did not observe either up-regulation or instability for our Y348A or Δ[246–261] α_{1B}-ARs, both of which lack G protein coupling (Wang et al., 2002). Another recent study with mutations of Phe303 of the α_{1B}-AR found that two of the substitutions that were defective in G protein coupling exhibited relatively little instability, whereas two substitutions that conferred constitutive activity exhibited a marked instability as well as ligand-induced protection (Chen et al., 2002). Thus, there seems to be no obligate linkage between G protein coupling and instability or up-regulation, even though most examples of these phenomena are for receptors with altered G protein coupling.

An intriguing possibility is that up-regulation of the Δ12 and Δ5 α_{1B}-ARs is a result of a critical role of the N terminus of the third intracellular loop of the α_{1B}-AR in maintaining receptor structural stability, perhaps independent of the critical role of this portion of the receptor in mediating G protein coupling (Cotecchia et al., 1990; Wu et al., 1995). This idea arises from a study of α_{2A}-AR up- and down-regulation, in which a series of constructs with alterations in the N-terminal portion of the third intracellular loop were all found to exhibit up-regulation rather than down-regulation, in spite of the fact that some of these receptors were defective in coupling to G_i or G_s, whereas others exhibited normal coupling (Jewell-Motz et al., 1997). The failure of our Δ[246–261] α_{1B}-AR mutation near the center of the third intracellular loop to confer up-regulation in spite of its uncoupling from G protein activation provides evidence for this model. On the other hand, instability and/or up-regulation are clearly not limited to receptors with mutations in this specific domain, because these phenomena have been observed for both constitutively active and for uncoupled receptors with mutations in various other regions of multiple GPCRs (Lee et al., 1997; Rasmussen et al., 1999; Alewijnse et al., 2000; Stevens et al., 2000; Wilson and Limbird, 2000; Chen et al., 2002).

The instability for the α_{1B}-AR in our studies is defined as a loss of high-affinity [^{3}H]prazosin binding, and the precise chemical nature of this instability remains to be determined. This is also the case for the other GPCRs that have been shown to exhibit this instability. Our data showing the ability of protease inhibitors to protect against the loss of binding for the Δ12 α_{1B}-AR clearly indicate that proteolysis is involved, suggesting that a different mechanism may mediate the instability of G protein coupling-defective α_{1B}-ARs than for the instability observed in previous studies with other mutated GPCRs. For several other GPCRs, the loss of ligand binding activity in instability assays has been shown to occur without proteolysis of the receptor protein, suggesting that a conformational change or some more extensive unfolding of receptor structure may mediate the instability without actual degradation of receptor protein (Gether et al., 1997; Rasmussen et al., 1999; Wilson and Limbird, 2000; Chen et al., 2002). However, it should be noted that protease inhibitors were routinely included in all incubations in those studies, and thus any protease-mediated instability mechanisms would have been missed. In contrast, receptor up-regulation in intact cells did seem to be accompanied by a corresponding increase in receptor protein assessed by Western blotting in one study with a mutated α_{2A}-AR (Jewell-Motz et al., 1997) and assessed by fluorescence in one study with wild-type and mutated green fluorescent protein-tagged α_{1B}-ARs (Stevens et al., 2000). The nature of the specific protease(s) involved in the instability of the Δ12 α_{1B}-AR, and whether the receptor itself or another associated protein is the relevant substrate, both remain to be determined. Because of the lack of α_{1B}-AR antibodies suitable for Western blotting (Vicentic et al., 2002), we have not yet been able to demonstrate definitively whether changes in α_{1B}-AR protein occur during either up-regulation or instability. Alternate mechanisms that could alter radioligand binding sites without changes in receptor protein content are also being investigated, including changes in covalent modifications, in membrane compartmentation, and/or in receptor protein folding such as those mediated by so-called "pharmacological chaperones" for some other GPCRs (Morello et al., 2000). Further studies and probably new experimental tools will be required to fully delineate the molecular basis for both instability and protection in isolated membranes and for up-regulation in intact cells.

In summary, our studies provide strong evidence that the protection from inherent instability for the Δ12 and Δ5 α_{1B}-ARs seen in isolated membrane preparations is the mechanism for their ligand-induced up-regulation seen in intact cells, because both the panel of mutated receptors that exhibit instability and the panel of ligands that exhibit protection are exactly the same as those that exhibit up-regulation in assays with intact cells. Our data further implicate a membrane-associated proteolytic mechanism as a major contributor to the instability. Detailed identification of the chemical and biophysical mechanisms involved in the instability and ligand-induced protection for these and other related mutated receptors may reveal fundamental insights into the structural features that normally maintain receptor stability and perhaps also into destabilizing mechanisms that may contribute to the agonist-induced down-regulation that occurs for these receptors in various physiological, pathological, or therapeutic settings.

Acknowledgments

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