0026-895X/00/040687-08$3.00/0

MOLECULAR PHARMACOLOGY, **57:**687–694 (2000).

Identification of Distinct Carboxyl-Terminal Domains Mediating Internalization and Down-Regulation of the Hamster α_{1B}-Adrenergic Receptor

JIEFA WANG, LEI WANG, JIALIN ZHENG, JODI L. ANDERSON, and MYRON L. TOEWS

Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska

Received September 7, 1999; accepted December 20, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

The roles of the carboxyl-terminal tail of the α_{1B}-adrenergic receptor in its expression, function, and regulation were investigated by site-directed mutagenesis. The receptor construct truncated after residue 363 seemed not to be properly expressed. In contrast, the receptor truncated after residue 366 and all of the longer receptor constructs were properly expressed and exhibited agonist and antagonist binding and activation of phosphoinositide hydrolysis similar to the wild-type receptor. Agonist-induced sequestration of receptors within the plasma membrane, endocytosis into intracellular vesicles, and eventual down-regulation were all absent in the receptor truncated after residue 366. A series of sequential truncations and a deletion mutation identified a critical role for residues 403 to 425, which include the previously identified sites for G protein-coupled receptor kinase phosphorylation, in agonist-induced internalization of the receptor. Similar studies identified a critical role for residues 367 to 380 in agonist-induced down-regulation. Individual point mutations converting either cysteine 367 or serine 369 to alanine selectively eliminated down-regulation, thus identifying two specific amino acid residues required for down-regulation. Importantly, several of the mutated receptors that failed to show rapid agonist-induced internalization nonetheless exhibited normal agonist-induced down-regulation. In addition to identifying specific regions and individual residues of the α_{1B}-adrenergic receptor involved in internalization and down-regulation, these studies provide mutated receptors that internalize but do not down-regulate, that down-regulate without internalization, and that are defective in both internalization and down-regulation, all of which should be useful tools for further studies of the specific cellular compartments and molecular mechanisms involved in receptor internalization and down-regulation.

Agonist binding to G protein-coupled receptors (GPCRs) leads not only to their activation and the generation of intracellular responses but also to adaptive changes in the receptors that limit their subsequent responsiveness. These receptor-specific changes include a rapid uncoupling of receptors from activation of their cognate G proteins, mediating functional desensitization; a rapid redistribution of receptors into relatively inaccessible compartments in the plasma membrane or inside the cell, variously referred to as sequestration, endocytosis, or internalization; and a slower loss of radioligand binding sites with prolonged agonist exposure, termed down-regulation (Perkins et al., 1991). Considerable progress has been made in recent years in identifying the receptor modifications involved in these changes for the prototypical β_2-adrenergic receptor (β_2AR) and various other receptors (Ferguson et al., 1996; Bohm et al., 1997; Krupnick and Benovic, 1998), although many of the details of the molecular modifications and protein-protein interactions that are involved in bringing about these changes remain to be determined.

Recent studies have focused on the role of the carboxyl-terminal tail of GPCRs in mediating these adaptive changes. For many GPCRs, the carboxyl-terminal tail seems to be required for functional desensitization and/or for internalization, as well as for the receptor phosphorylation that is thought to play an important role in mediating both desensitization and internalization. In addition to the extensive studies with β_2ARs cited above, carboxyl-terminal tail involvement has been documented for other G_s-coupled receptors such as H_2 histamine receptors (Fukushima et al., 1997), for G_i-coupled receptors such as SSTR3 (Roth et al., 1997), and for G_q-coupled receptors such as $P2Y_2$ nucleotide receptors (Garrad et al., 1998), to cite only a few examples. The receptor sequences and molecular mechanisms involved in GPCR internalization have received considerable recent attention. However, much less is known about the role of the carboxyl-terminal tail or other receptor domains in the receptor down-regulation that occurs with long-term agonist treatment, which remains the most poorly understood of the

This work was supported in part by National Institutes of Health Research Grant GM34500 to M.L.T.

ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic receptor; GRK, G protein-coupled receptor kinase; PI, phosphoinositide.

An erratum has been published:
http://molpharm.aspetjournals.org/content/58/1/246.full.pdf

adaptive changes that occur after agonist activation of GPCRs. However, a few studies have implicated specific carboxyl-terminal tail residues in down-regulation of GPCRs as well, including G_s-coupled β_2ARs (Valiquette et al., 1990), G_i-coupled δ-opioid receptors (Cvejic et al., 1996), and G_q-coupled m3 muscarinic acetylcholine receptors (Yang et al., 1993).

Similar to other GPCRs, α_{1B}ARs also undergo agonist-induced desensitization, internalization, and down-regulation (Hoffman, 1987; Toews et al., 1991; Cotecchia et al., 1995; Bird et al., 1997), although there are differences in the extent to which these processes occur among the various systems that have been studied. In cultured cell systems, functional desensitization of α_{1B}ARs can be induced by agonists or by protein kinase C-activating phorbol esters, and desensitization is accompanied by receptor phosphorylation (Leeb-Lundberg et al., 1985, 1987). Internalization of α_{1B}ARs occurs, although the extent to which sequestration within the plasma membrane versus endocytosis into intracellular vesicles contribute to internalization and the effects of protein kinase C activators on these changes appear to be cell type-specific (Leeb-Lundberg et al., 1987; Toews, 1987; Cowlen and Toews, 1988; Zhu et al., 1996). We have obtained a range of results in our studies of long-term regulation of α_{1B}AR expression, with down-regulation, no down-regulation, or even up-regulation occurring in different cell types or in different clones of α_{1B}AR-transfected cells (Toews, 1987; Zhu et al., 1996; Bird et al., 1997).

The functional domains of the α_{1B}AR mediating desensitization and internalization are beginning to be elucidated. A previous study showed that truncation of most of the carboxyl-terminal tail of the α_{1B}AR prevented its phosphorylation and desensitization and slowed its internalization after agonist exposure (Lattion et al., 1994). A subsequent study identified multiple specific carboxyl-terminal tail serine residues involved in phosphorylation and functional desensitization of the α_{1B}AR in response to both agonists and protein kinase C activators (Diviani et al., 1997). However, the specific carboxyl-terminal tail sequences involved in α_{1B}AR internalization and down-regulation have not been determined. In these studies, we have generated α_{1B}ARs with truncations, deletions, and individual point mutations within the carboxyl-terminal tail to identify the α_{1B}AR sequences involved in agonist-induced internalization and down-regulation. Our results indicate that both processes require sequences within the carboxyl-terminal tail of the receptor, but that the regions involved in these two processes are distinct. Our results further demonstrate that down-regulation can occur even for receptors that fail to exhibit rapid receptor internalization.

Experimental Procedures

Materials. Cell culture medium, serum, trypsin, G418, and LipofectAMINE reagent were obtained from Life Technologies (Grand Island, NY). The Muta-Gene In Vitro Mutagenesis Kit was obtained from Bio-Rad (Richmond, CA); other enzymes were purchased from New England Biolabs (Beverly, MA). [^{3}H]Prazosin was obtained from DuPont-New England Nuclear (Boston, MA) and [^{3}H]inositol was purchased from Amersham (Arlington Heights, IL). Epinephrine, phentolamine, sucrose, and other biochemicals were obtained from Sigma (St. Louis, MO).

Site-Directed Mutagenesis. The cDNA encoding the α_{1B}AR was cleaved from the plasmid pRC/CMV at the *Hin*dIII/*Xba*I sites and subcloned to phage M13 mp18 also digested with *Hin*dIII/*Xba*I, and mutations to the coding sequence were then generated by oligonucleotide-directed mutagenesis using the Bio-Rad Muta-Gene M13 Kit, all as in our previous study (Wang et al., 1997).

The mutagenic primers for the truncation mutants were as follows, where the underlined nucleotides represent the stop codon that was introduced: ATGCGTATCCTTTAGTGCCAGTGCCGT for the receptor truncated after Leu363 (Tr363); CTTGGGTGCCAGTAGCGTAGTGGCCGT for the receptor truncated after Gln366 (Tr366); CGTCGTCTGGGCTAGGCGTGCGCTTAC for the receptor truncated after Gly380 (Tr380); CAGTCGCGGAAGTAGGACTCCCTGGAC for the receptor truncated after Lys402 (Tr402); GCGTCGCCCAGCTAGCCGGGCTACCTG for the receptor truncated after Ser425 (Tr425); AAATCCGGGGCTTAGCTGCTCAGTCTG for the receptor truncated after Ala449 (Tr449); and CTCTTGGGAGAGTAGCCGGAGAGCCCG for the receptor truncated after Pro477 (Tr477). For Tr363 and Tr366, the TAG stop codon was substituted for the following amino acid normally present; for the other truncations, the TAG stop codon was inserted ahead of the following amino acid normally present. The mutagenic primers for the deletion mutants were as follows (the carat represents the site at which the deletion was introduced): ATCCTTGGGTGCCAG^GCGTGCGCTTACACC for Del[367–380], deleting residues 367 through 380, and TCGCAGTCGCGGAAG^CCGGGCTACCTGGGT for Del[403–425], deleting residues 403 through 425. The mutagenic primers for the two point mutations were as follows, where the underlined nucleotides represent the altered amino acid: CTTGGGTGCCAGGCTCGTAGTGGCCGT for the receptor with Ala substituted for Cys367 (C367A); and TGCCAGTGCCGTGCAGGCCGTCGCCGC for the receptor with Ala substituted for Ser369 (S369A).

After confirmation of the mutations by DNA sequencing, the mutated α_{1B}ARs were cut from M13 mp18 using *Hin*dIII/*Xba*I and subcloned into the expression vector pRC/CMV, followed by DNA sequencing to reconfirm the mutation. The wild-type and mutated α_{1B}AR plasmids were then stably transfected into CHO-K1 Chinese hamster ovary cells using LipofectAMINE, and clones resistant to 400 μg/ml G418 were isolated and screened for α_{1B}AR expression, as in our previous study (Wang et al., 1997).

Cell Culture. Cells were maintained in monolayer culture in Ham's F12 medium supplemented with 10% fetal bovine serum and 200 μg/ml G418 at 37° in a humidified incubator with a 5% CO_2 atmosphere. Cells from confluent flasks were trypsinized and plated in culture dishes at 3000 to 5000 cells/cm^2. Cells were typically used for experiments on the fourth day of culture.

Radioligand Binding Assays. For membrane preparation, cells grown on 150-mm dishes were rinsed twice with 10 ml of ice-cold wash buffer (10 mM Tris, pH 7.4, 140 mM NaCl) and then twice with 10 ml of ice-cold lysis buffer (1 mM Tris, pH 7.4, 2 mM EDTA) and allowed to swell for 10 min on ice. Cells were then lysed by scraping from the dish with a rubber policeman. The lysate was centrifuged for 30 min at 20,000 rpm in an SM24 rotor in a Sorvall RC5B refrigerated centrifuge. The membrane pellet was resuspended in binding buffer (20 mM Tris, pH 7.4, 2 mM $MgCl_2$, 140 mM NaCl) with a Tissumizer (Tekmar, Cincinnati, OH), and this membrane suspension was used in radioligand binding assays. Membranes were incubated with [^{3}H]prazosin in binding buffer for 60 min at 37° in a shaking water bath. The reactions were stopped by filtration over Whatman GF/B glass fiber filters on a Brandel (Gaithersburg, MD) cell harvester and washing three times with 4 ml of wash buffer. Liquid scintillation counting was used to quantify radioactivity associated with the filters. For saturation assays, six to seven different concentrations of [^{3}H]prazosin were used. For competition binding assays, [^{3}H]prazosin was used at approximately 300 pM and the concentrations of competing ligands were varied. In all cases, nonspecific binding was defined as that occurring in the presence of 10 μM phentolamine.

Phosphoinositide (PI) Hydrolysis Assays. Cells grown on 35-mm dishes were labeled for 18 to 24 h with 2 μCi [^{3}H]inositol in

1 ml of inositol-free high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After labeling, cells were rinsed once with Ham's-HEPES (Ham's F12 medium, 20 mM HEPES, pH 7.4) and then stimulated for 20 min with various concentrations of epinephrine in Ham's-HEPES containing 10 mM LiCl. Labeled compounds were then extracted from the cells with methanol, and chloroform and water were added, as described (Nakahata et al., 1986). Inositol phosphates in the resulting aqueous phase were separated on Dowex 1-X8 (formate form) columns. Total inositol phosphates were eluted with 8 ml of 1 M ammonium formate and 0.1 M formic acid. Radioactivity in a 3-ml portion of the eluate (a) and a 0.375-ml portion of the organic phase containing the inositol phospholipids (b) were determined by liquid scintillation counting. The percentage of conversion of inositol phospholipids to inositol phosphates was then calculated by the formula $a / (a + b) \times 100\%$.

Cell Surface Accessibility of Receptors by Assays of Radioligand Binding on Ice. Cells in growth medium on 35-mm dishes were exposed to 10 μM epinephrine plus 1 mM ascorbate for 30 min at 37° to induce redistribution. Control cells were exposed only to the 1 mM ascorbate vehicle. Cells were rinsed twice with 2 ml of Ham's-HEPES and then incubated on ice for 4 h with 1.8 nM [^{3}H]prazosin in Ham's-HEPES. Cells were then rinsed twice with 2 ml of Ham's-HEPES containing 10 μM phentolamine to remove unbound radioligand and dissolved in 1 ml of 0.2 N NaOH. Radioactivity associated with the dissolved cells was assessed by liquid scintillation counting. Nonspecific binding was defined as that occurring in the presence of 10 μM phentolamine.

Receptor Redistribution by Sucrose Density Gradient Centrifugation Assays. Cells grown on 100-mm dishes were given fresh growth medium on the day before the experiment. Cells were exposed to 10 μM epinephrine or vehicle for 30 min at 37° to induce internalization. Cells were rinsed twice with 10 ml of ice-cold wash buffer and then twice with ice-cold lysis buffer and allowed to swell for 10 min on ice. Cells were then lysed by scraping from the dishes in 0.8 ml of lysis buffer with a rubber policeman. This lysate was layered on top of a discontinuous sucrose density gradient consisting of 1.7 ml of 15% sucrose, 5.0 ml of 30% sucrose, and 2.5 ml of 60% sucrose. Samples were centrifuged at 28,000 rpm for 60 min at 4° in an SW41 rotor in a Beckman L8-70 refrigerated ultracentrifuge. Fractions of 0.8 ml each were then collected from the top of the tubes. Binding of [^{3}H]prazosin (1.4 nM) to the membranes in each fraction was then determined essentially as described above.

Down-Regulation Assays. For down-regulation assays, cells grown on 100-mm dishes were incubated in the absence or presence of 10 μM epinephrine for 24 h at 37°. Cells were then rinsed and lysed as for sucrose gradient assays. Centrifugation and resuspension of the membranes and binding of [^{3}H]prazosin (3.7 nM) to the isolated membranes were then conducted as described in *Radioligand Binding Assays*, above. In most cases, binding assays were conducted on the day that the membranes were isolated; however, for some experiments, the membrane pellets were stored at −80°C for a few days before resuspension and assay.

Data Analysis. Nonlinear regression analyses of saturation and competition binding assay and dose-response curve data were performed with GraphPad PRISM (GraphPad Software, Inc., San Diego, CA). Values for all parameters for all mutations are the averages of a minimum of three values determined in duplicate or triplicate, including assays with at least two different clones and performed on at least two different days. Data are presented as the mean ± S.E. (n = x, y), where x indicates the total number of determinations and y represents the number of different clones tested. Statistical comparisons of data for all of the mutated receptors to those for the wild-type receptor were by one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism (GraphPad Software), with statistically significant difference from the wild-type receptor taken at $P < .05$.

Results

Site-directed mutagenesis was used to generate hamster α_{1B}ARs with truncations, deletions, and individual point mutations in their carboxyl-terminal tails (Fig. 1). The characteristics of each mutated receptor were determined after stable transfection into Chinese hamster ovary cells. Receptor expression levels and antagonist binding affinities were

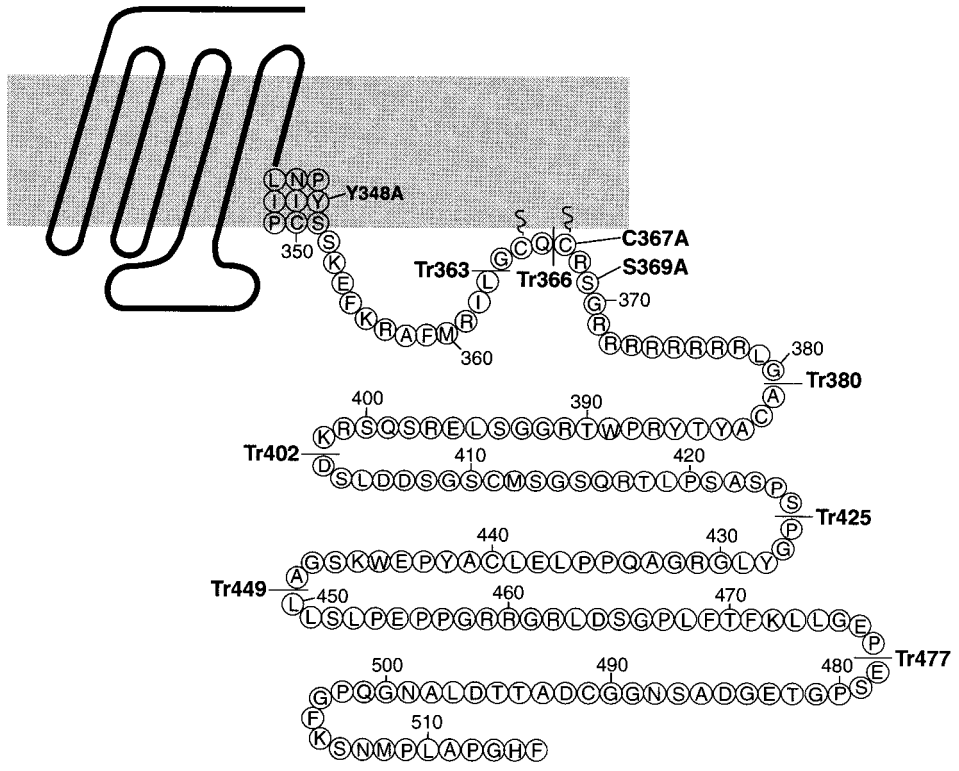

Fig. 1. Schematic diagram of amino acid residues and mutations in the seventh transmembrane domain and carboxyl-terminal tail of the α_{1B}AR, using the single letter amino acid code.

determined by saturation binding assays with the antagonist radioligand [^{3}H]prazosin. Agonist binding properties were determined in competition binding assays with [^{3}H]prazosin, and agonist efficacies and potencies for activation of PI hydrolysis were determined in assays of [^{3}H]inositol phosphates formation. Agonist-induced receptor internalization was assessed after 30-min exposure to 10 μM epinephrine using two different assays, one measuring the percentage of receptor binding sites that become inaccessible for [^{3}H]prazosin binding on ice as a measure of cell surface accessibility and the other measuring the percentage of the receptors that shift from the plasma membrane fraction to the light vesicle fraction on sucrose density gradients as a measure of endocytosis into intracellular vesicles. Both assays were used based on our previous studies with the α_{1B}AR, which showed that agonist exposure could cause these receptors to lose their cell surface accessibility but remain plasma membrane-associated, presumably by becoming sequestered in a specialized compartment within the plasma membrane but not endocytosed into intracellular vesicles (Cowlen and Toews, 1988; Wang et al., 1997). Down-regulation was assessed by measuring [^{3}H]prazosin binding to membrane preparations from cells pretreated for 24 h in the absence or presence of 10 μM epinephrine. The results from these assays are summarized below for each of the sets of mutations that were generated.

Initial Truncation Mutations. Previous studies found that truncation of the hamster α_{1B}AR after Arg368 (Tr368 mutant) (thus deleting most of the carboxyl-terminal tail) generated a receptor with binding and functional properties similar to those of the wild-type receptor but with marked defects in agonist-induced receptor phosphorylation and desensitization and a slowed rate of internalization (Lattion et al., 1994). In our initial mutagenesis, we generated two shorter α_{1B}AR constructs to determine whether the more membrane-proximal portion of the carboxyl-terminal tail was important for receptor expression, binding, or function. The Tr366 receptor, truncated after Gln366, was expressed at levels similar to the wild-type receptor and exhibited binding and functional properties similar to the wild-type receptor (Table 1) but was completely defective in agonist-induced sequestration, endocytosis, and down-regulation (Fig. 2), as described in more detail below. Transfection of cells with cDNA coding for the Tr363 receptor, truncated after Leu363 and only three amino acids shorter than the Tr366 receptor, did not yield any receptor-positive clones as assessed by [^{3}H]prazosin binding. Stimulation of PI hydrolysis by epinephrine also was not observed with any of the Tr363 clones tested. These results suggest that the sequence from Gly364 through Gln366 is critical for proper receptor expression and/or ligand binding. Preliminary reverse transcriptase polymerase chain reaction experiments indicated that the

TABLE 1

Binding and functional properties of wild-type and mutated α_{1B}AR constructs

K_d and B_{max} values for the radiolabeled antagonist [^{3}H]prazosin were determined in saturation binding assays. IC_{50} values for the agonist epinephrine were determined in competition binding assays with [^{3}H]prazosin as the radioligand, and K_i values were calculated from the single-site IC_{50} values based on the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Potencies (EC_{50} values) and efficacies (fold stimulation values) for PI hydrolysis were determined in assays of [^{3}H]inositol phosphates formation. Values are the mean ± S.E.; n values in parentheses indicate the total number of determinations and the number of different clones tested. The asterisk indicates that the value for the mutated receptor was significantly different from the corresponding value for the wild-type receptor, $P < .05$.

Receptor Construct	Expression Level Range[a]	Saturation Assays	Competition Assays[b]	PI Hydrolysis Assays	
		[^{3}H]Prazosin K_d	Epinephrine K_i	Epinephrine EC_{50}	Fold Stimulation[c]
	pmol/mg	*pM*	*μM*	*nM*	
Wild-type[d]	0.5–4.9	43 ± 1 (n = 7,5)	2.0 ± 0.2 (n = 7,5)	43 ± 6 (n = 15,4)	4.4 ± 0.6
Tr477	0.8–9.0	62 ± 5 (n = 4,3)	4.6 ± 0.4 (n = 4,3)	102 ± 18 (n = 3,2)	7.0 ± 0.6
Tr449	0.8–10.2	61 ± 4 (n = 4,2)	3.8 ± 0.2 (n = 4,2)	116 ± 18 (n = 4,2)	6.4 ± 2.9
Tr425	0.1–7.5	50 ± 4 (n = 4,3)	3.6 ± 0.5 (n = 5,4)	80 ± 12 (n = 3,2)	2.5 ± 0.4
Tr402	2.2–7.8	68 ± 5 (n = 4,3)	2.5 ± 0.3 (n = 5,4)	146 ± 49 (n = 4,2)	9.9 ± 2.3
Tr380	0.3–6.9	42 ± 2 (n = 3,3)	2.4 ± 0.1 (n = 3,3)	158 ± 11 (n = 4,2)	2.4 ± 0.3
Tr366	0.5–0.6	37 ± 3 (n = 5,4)	2.0 ± 0.2 (n = 6,4)	21 ± 3 (n = 8,3)	6.0 ± 2.3
Del[403–425]	0.8–13.4	71 ± 8 (n = 8,4)	8.4 ± 1.2* (n = 4,4)	36 ± 7 (n = 4,2)	4.7 ± 1.1
Del[367–380]	0.5–7.4	47 ± 1 (n = 5,3)	2.2 ± 0.1 (n = 5,5)	130 ± 20 (n = 3,3)	5.9 ± 1.4
S369A	0.8–10.2	80 ± 5 (n = 5,2)	8.3 ± 0.6* (n = 4,2)	113 ± 22 (n = 4,3)	3.1 ± 0.6
C367A	0.7–8.2	45 ± 3 (n = 4,3)	2.8 ± 0.2 (n = 5,4)	118 ± 7 (n = 4,3)	4.4 ± 1.0

[a] Values are the range of receptor expression levels in picomoles per milligram of membrane protein from both saturation and down-regulation assays for which membrane protein concentrations were determined.

[b] Competition data were fitted to both one- and two-site models; for all of the receptor constructs, the one-site fit was preferred. Data were also fitted to a variable slope model; the Hill coefficient for the wild-type receptor was 0.76 ± 0.05 and the range for the mutated receptors was from 0.73 to 0.92, none of which was significantly different from the wild-type receptor.

[c] The n values for the fold stimulation column are identical to those for the EC_{50} determinations in the previous column.

[d] Values for the wild-type receptor include data from experiments reported previously (Wang et al., 1997) and from additional experiments together with the new mutated receptors reported here.

Tr363 cDNA was expressed similarly to wild-type cDNA, suggesting that the defect in expression of the Tr363 receptor is post-transcriptional.

Sequential Truncations. The results with the Tr366 receptor clearly implicated the carboxyl-terminal tail of the α_{1B}AR in its sequestration, endocytosis, and down-regulation. To delineate in more detail the regions of the carboxyl-terminal tail involved in each of these processes, we first generated a series of receptors with sequential truncations of the carboxyl-terminal tail (Fig. 1): Tr477, truncated after Pro477; Tr449, truncated after Ala449; Tr425, truncated after Ser425; Tr402, truncated after Lys402; and Tr380, truncated after Gly380. All of these receptor constructs were expressed at high levels similar to those for the wild-type receptor (Table 1); no consistent differences in regulatory properties were observed between the higher and lower expressing clones. In saturation binding assays with isolated membrane preparations, K_D values for [^{3}H]prazosin ranged from 37 to 68 pM, similar to the K_D value of 43 pM for the wild-type receptor (Table 1). Similarly, in the competition binding assays (Table 1), all of the truncated receptors exhibited single-site K_i values for epinephrine between 2.0 and 4.6 μM, similar to the value of 2.0 μM for the wild-type receptor.

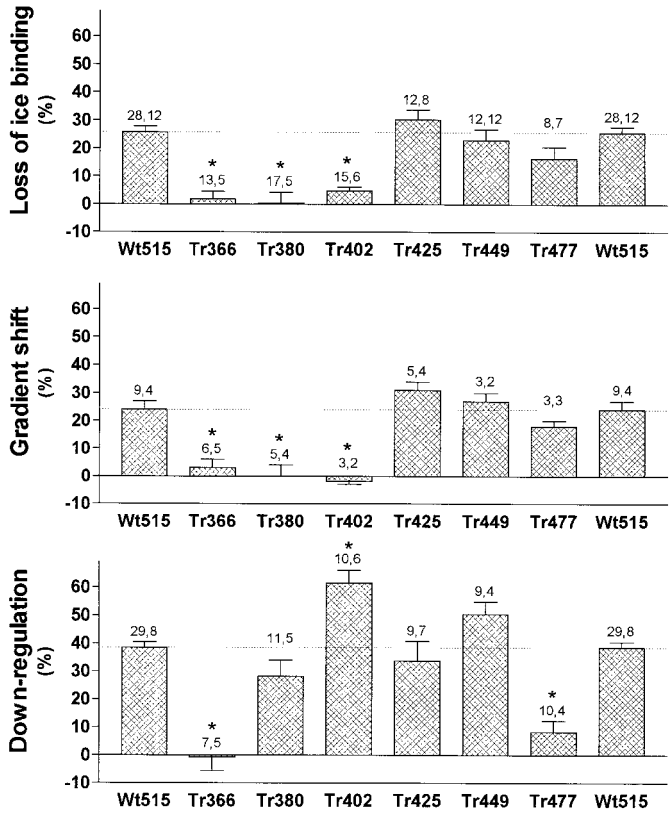

Fig. 2. Internalization and down-regulation properties of wild-type and carboxyl-terminal tail-truncated α_{1B}ARs. Agonist-induced decreases in the surface accessibility of α_{1B}ARs were monitored as the loss of [^{3}H]prazosin binding to intact cells on ice after incubation in the absence or presence of 10 μM epinephrine for 30 min *(loss of ice binding*, top). Endocytosis into what are presumed to be intracellular vesicles was monitored as the shift of receptors from the plasma membrane fraction to the light vesicle fraction on sucrose density gradients after incubation in the absence or presence of 10 μM epinephrine for 30 min (*gradient shift*, middle). Down-regulation was monitored as the decrease in radioligand binding to membranes prepared from cells incubated in the absence or presence of 10 μM epinephrine for 24 h (bottom). For the ice-binding and down-regulation assays, binding to epinephrine-pretreated samples is expressed as the percentage decrease compared to binding to the control samples incubated in the absence of epinephrine. For the gradient shifts, the fraction of receptors that shift to the light vesicle fraction is expressed as the percentage of the receptors present in the plasma membrane fraction from the control cells. In each panel, the values for the wild-type receptor (Wt515) are presented on the far left and far right and as the horizontal dashed line for ease of comparison. All values are the mean ± S.E.; the numbers above each bar represent the total number of determinations and the number of different clones tested, respectively. The asterisk indicates that the value for the mutated receptor is significantly different from the corresponding value for the wild-type receptor, $P < .05$.

All of the truncated receptors were functional in assays of epinephrine-stimulated PI hydrolysis, with fold-stimulation values ranging from 2.5- to 9.9-fold, compared with 4.4-fold stimulation for the wild-type receptor (Table 1). There was no clear indication of constitutive activity for any of the mutated receptors, although detailed studies of the correlation of basal or stimulated PI hydrolysis values for specific clones of each mutation with the corresponding levels of receptor expression in those clones were not undertaken. The potencies of epinephrine for stimulation of PI hydrolysis were somewhat variable among the mutated receptors, with EC_{50} values ranging from 21 pM for Tr366 to 158 pM for Tr380 compared with the value of 43 pM for the wild-type receptor. The differences were not statistically significant, and there was no obvious correlation between the EC_{50} values and the extent of truncation of the carboxyl-terminal tail. The higher potencies of epinephrine in functional assays of PI hydrolysis than in the competition binding assays most likely result from the presence of spare receptors or receptor reserve caused by the high levels of receptor expression in these transfected cells. However, the different experimental conditions for these two assays may also contribute to the observed differences.

In assays of agonist-induced receptor internalization (Fig. 2, top and middle), Tr477, Tr449, and Tr425 all exhibited values similar to those for the wild-type receptor in both assays, with approximately 20 to 30% of the original cell surface receptors becoming inaccessible for [^{3}H]prazosin binding on ice and a similar percentage of the original plasma membrane receptors shifting to the light vesicle fraction on sucrose gradients. Both assays for internalization gave somewhat lower values than wild-type for Tr477 and somewhat higher values than wild-type for Tr425. In contrast, the shorter receptors Tr402 and Tr380 were almost completely defective in internalization in both assays, similar to the results with the Tr366 receptor. These results suggest that residues 403 to 425 of the α_{1B}AR are required for its agonist-induced internalization.

In assays of down-regulation for the truncated receptors (Fig. 2, bottom), the Tr477 receptor, the longest of the truncated receptors, was markedly defective; Tr477 down-regulated by only 8%, compared with 39% down-regulation for the wild-type receptor. Surprisingly, the shorter receptors Tr449, Tr425, Tr402, and Tr380 all down-regulated well, with Tr449 and Tr402 down-regulating to a somewhat greater extent than the wild-type receptor and Tr425 and Tr380 down-regulating somewhat less than the wild-type receptor. The observation of nearly normal down-regulation with Tr380 and the complete lack of down-regulation with Tr366 suggest that residues 367 to 380 of the α_{1B}AR are important for its agonist-induced down-regulation. Sequences in the more distal part of the carboxyl-terminal tail may also be involved in

down-regulation based on the different properties of the Tr477 receptor compared with the wild-type and Tr449 receptors.

Deletion Mutations. Results from the sequential truncation mutations suggested that residues 403 to 425 and 367 to 380 contain sequences critical for internalization and down-regulation, respectively. However, it was also possible that these specific sequences are not required, but rather that a certain length of the carboxyl-terminal tail is required, independent of the specific amino acid sequence. To test the roles of the 403-to-425 and 367-to-380 regions in the context of a more normal carboxyl-terminal tail, we generated deletion mutations eliminating these sequences from an otherwise full-length receptor. Both the Del[403–425] and Del[367–380] receptors were expressed at levels comparable to the wild-type receptor (Table 1). The K_D values for [^{3}H]prazosin binding were similar to those for the wild-type receptor and the various truncated receptors (Table 1). Del[403–425] exhibited approximately 4-fold lower affinity than the wild-type receptor for binding of the agonist epinephrine, whereas epinephrine binding for Del[367–380] was the same as for wild-type (Table 1). The epinephrine potencies and fold stimulation values for PI hydrolysis were not significantly different from those for the wild-type and truncated receptors (Table 1). Thus neither of these regions is critical for agonist or antagonist binding or for receptor activation.

In agreement with the results from the sequential truncations, Del[403–425] was completely defective in both assays for internalization but down-regulated at least as well as the wild-type receptor (Fig. 3). These results confirm an important role for residues 403 to 425 in mediating rapid receptor internalization. In addition, they suggest that neither these specific residues nor rapid receptor internalization are required for the receptor down-regulation that occurs during long-term agonist exposure. Also in agreement with results from the sequential truncations, Del[367–380] was markedly defective in down-regulation (Fig. 3), exhibiting only 4.5% down-regulation, 12% of the down-regulation observed with the wild-type receptor. Del[367–380] was clearly able to undergo agonist-induced internalization assessed by both assays; the extent of internalization was somewhat lower than that for wild-type in both assays, although the differences were not statistically significant (Fig. 3).

Point Mutations. To further investigate individual residues within the 367-to-380 domain that are involved in down-regulation, we mutated Cys367 and Ser369 to Ala (C367A and S369A, respectively; Fig. 1). The C367A receptor exhibited binding affinities for [^{3}H]prazosin and for epinephrine that were similar to those for the wild-type receptor, whereas S369A exhibited somewhat lower affinities for both ligands (Table 1). Both C367A and S369A stimulated PI hydrolysis with similar potencies and fold-stimulation values as for the wild-type and other mutated receptors (Table 1). Strikingly, both of these individual amino acid mutations generated receptors that were completely defective in down-regulation with little or no defect in internalization (Fig. 3).

Discussion

These studies identify distinct domains of the α_{1B}AR carboxyl-terminal tail involved in agonist-induced internalization and down-regulation. Together with previous studies investigating receptor phosphorylation and functional desensitization (Lattion et al., 1994; Diviani et al., 1997), our results indicate that the carboxyl-terminal tail is critical for all of the well-characterized adaptive changes in the α_{1B}AR that occur after agonist exposure. In contrast, none of the carboxyl-terminal tail mutations caused marked alterations in receptor expression, binding or function, except for Tr363, the shortest construct, which prevented functional receptor expression.

The initial focus of our studies was to identify regions of the carboxyl-terminal tail mediating receptor internalization, including both sequestration within the plasma membrane and endocytosis into intracellular vesicles. None of the carboxyl-terminal tail mutations generated in this study had differential effects on sequestration versus endocytosis like those observed with the Y348A α_{1B}AR in our previous study (Wang et al., 1997); all were either similar to wild-type for both responses or were essentially completely defective in both responses. Our results clearly demonstrate that residues 403 to 425 of the α_{1B}AR are required for normal agonist-induced receptor internalization measured by either assay. Both the ability of Tr425 but not Tr402 to internalize normally and the selective loss of internalization in Del[403–425] support this conclusion. This region of the receptor contains the serine residues at positions 404, 408, and 410 that have recently been identified as the sites required for GPCR kinase (GRK)-mediated phosphorylation and functional desensitization of the α_{1B}AR (Diviani et al., 1997). For the β_2AR, GRK-mediated phosphorylation has been shown to promote binding of β-arrestin to the receptor, which prevents functional coupling of the receptor with its G-protein, thus causing desensitization (Ferguson et al., 1996; Krupnick and Benovic, 1998). More recently, β-arrestin bound to phosphorylated receptors has been shown to also serve as an adaptor protein linking the desensitized receptors to the clathrin-mediated endocytosis machinery (Krupnick and Benovic, 1998). Specific carboxyl-terminal tail serine and threonine residues or regions containing such residues have been shown to be required for internalization of several other GPCRs, with m3 muscarinic acetylcholine receptors (Yang et al., 1995), AT_{1a} angiotensin receptors (Thomas, 1999), and bradykinin receptors (Pizard et al., 1999) as examples for G_q-coupled receptors. Based on these studies, it seems likely that the presence of the GRK phosphorylation sites in the 403-to-425 region accounts for the requirement of this region for agonist-induced internalization.

Although the mechanisms involved in GPCR internalization are beginning to be understood, much less is known about the molecular mechanisms or specific receptor sequences involved in GPCR down-regulation. Although we have observed little or no down-regulation (Toews, 1987) and even up-regulation of α_{1B}ARs (Zhu et al., 1996; Bird et al., 1997) in some of our previous studies, 30 to 40% down-regulation is the most typical result for transfected α_{1B}ARs (Bird et al., 1997), and this extent of down-regulation was consistently observed for the wild-type receptor in the current studies.

The ability of Tr380 but not Tr366 to undergo normal down-regulation as well as the relatively selective loss of down-regulation in Del[367–380] clearly implicate residues 367 to 380 of the α_{1B}AR in its agonist-induced down-regulation. This region contains at least three features of potential

interest: Cys367, a potential site of receptor palmitoylation; Ser369, a potential site of receptor phosphorylation; and a string of consecutive Arg residues (eight in the α_{1B}AR) that is found in the α_{1B}AR and the α_{1D}AR but not in the α_{1A}AR or many other GPCRs. The studies presented here indicate that both the Cys367 and Ser369 residues are critical for down-regulation, because mutation of either residue to Ala essentially completely eliminated down-regulation.

Palmitoylation of Cys367 is a reasonable candidate for the role of this region in down-regulation, because there is evidence for palmitoylation playing a role in down-regulation of α_{2A}ARs (Eason et al., 1994). Whether the α_{1B}AR is palmitoylated, whether Cys367 or Cys365 (or both) is the site of palmitoylation, and the significance of palmitoylation for down-regulation or other aspects of α_{1B}AR function are all areas for further study. Phosphorylation of Ser369 is also a reasonable mechanism for involvement in down-regulation, because this serine is close to basic amino acids on both its amino- and carboxyl-terminal sides and should be a good phosphorylation site for protein kinase C (Newton, 1995). Interestingly, a recent study of down-regulation of the glucose-dependent insulinotropic peptide receptor also indicated that both the cysteine residue at the potential palmitoylation site and a nearby serine residue were involved in its down-regulation (Tseng and Zhang, 1998). Finally, a recent study suggests the possibility that the arginine string of the α_{1B}AR could also be involved in down-regulation. This study showed that the arginine string was required for association of the α_{1B}AR with a protein termed gC1q-R (the receptor for the globular heads of the C1q protein) and that over-expression of gC1q-R down-regulated expression of the α_{1B}AR (Xu et al., 1999). Thus it is possible that Cys367, Ser369, and the arginine string could all play important roles in agonist-induced down-regulation.

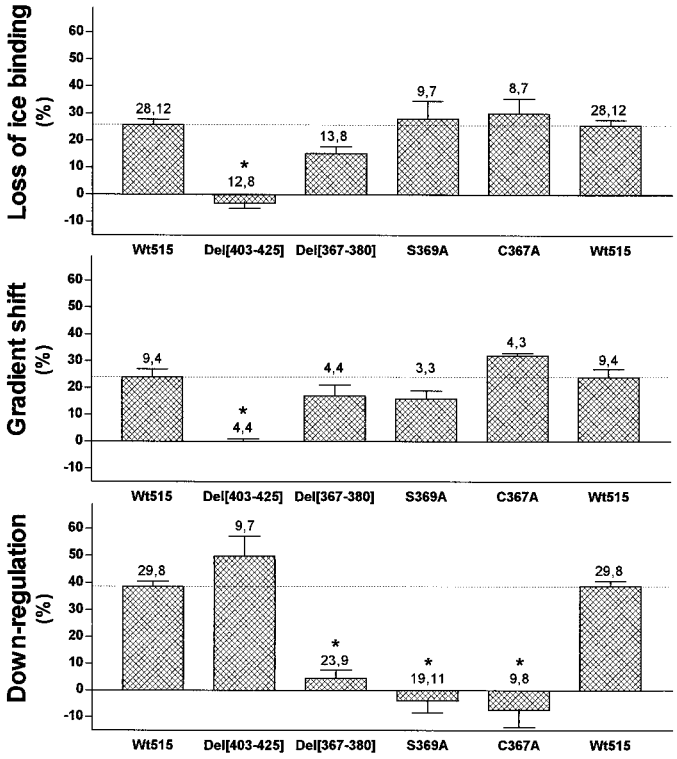

Fig. 3. Internalization and down-regulation properties of wild-type and deletion- and substitution-mutated α_{1B}ARs. Loss of ice binding, gradient shift, and down-regulation were all determined as described in the legend to Fig. 2. In each panel, the values for the wild-type receptor (Wt515) are presented on the far left and far right and as the horizontal dashed line for ease of comparison. All values are the mean ± S.E.; the numbers above each bar represent the total number of determinations and the number of different clones tested, respectively. The asterisk indicates that the value for the mutated receptor is significantly different from the corresponding value for the wild-type receptor, $P < .05$.

Our data suggest that the distal portion of the α_{1B}AR carboxyl-terminal tail may also be important for down-regulation, based on the properties of the Tr477 and Tr449 receptors. The marked decrease in down-regulation with truncation at residue 477 and the recovery of down-regulation with further truncation to residue 449 can be explained if a down-regulation-inhibiting domain is present between residues 450 to 477 and if the inhibitory activity of this domain is normally blocked by a counter-regulatory domain present between residues 478 to 515. Truncation at residue 477 would then unmask the inhibitory domain and decrease down-regulation, whereas further truncation to residue 449 would remove the inhibitory domain and restore down-regulation. There is precedent for this idea, because previous studies have reported both positive and negative signals for down-regulation in the carboxyl-terminal tails of βARs (Hertel et al., 1990) and H_2 histamine receptors (Smit et al., 1996).

The ability of receptors that are defective in internalization to nonetheless down-regulate normally, as seen for Tr402, Tr380 and Del[403–425], was an unexpected outcome of our studies. A study with β_2ARs also described mutations that allowed normal down-regulation without receptor internalization (Hausdorff et al., 1991). Most current models assume that down-regulation results from proteolytic degradation of receptors after their endocytosis and delivery to lysosomes (Perkins et al., 1991; Ferguson et al., 1996; Bohm et al., 1997). If this model is correct, then internalization should obviously be required for down-regulation to occur. One possible explanation for the ability of our internalization-defective receptors to down-regulate normally is that these mutated receptors do in fact internalize, but they do so at a rate that is too slow to detect in our assays. In this regard, it should be emphasized that internalization is measured after short-term (30-min) exposure to agonist, whereas down-regulation is measured after much longer (24-h) agonist treatment. Thus an undetectably low rate of internalization occurring over a 24-h period could perhaps provide sufficient internalized receptors to allow the normal extent of down-regulation. If this were the case, we would anticipate that down-regulation might occur more slowly for the internalization-defective receptors, but we have not observed this in preliminary experiments. The other possibility is that down-regulation does in fact occur by a mechanism that is independent of receptor internalization. This would require the development of new hypotheses for down-regulation, and our mutated receptors would be useful for exploring various alternate mechanisms.

The failure of the Tr363 construct to generate receptors that were competent for either radioligand binding or signal generation, coupled with the normal expression, binding, and function of the Tr366 receptor, suggests that the 364-to-366 region of the receptor is critical for normal receptor expression. The presence and/or palmitoylation of Cys365 may be

important for normal α_{1B}AR expression, because truncation of both β_2ARs (Dixon et al., 1987) and α_2ARs (Kennedy and Limbird, 1994) on the membrane-proximal side of their palmitoylation sites prevented receptor expression. Truncation after Leu363 could also interfere with the function of the "dihydrophobic" Ile-Leu sequence at residues 362 and 363, corresponding to a motif required for proper cell surface delivery of V_2 vasopressin receptors (Schulein et al., 1998). Together these studies implicate the sequences surrounding and including the potential palmitoylation sites as being critical for regulation of receptor expression, both for proper expression of newly synthesized receptors and for the decreased expression that occurs during down-regulation.

In summary, we have generated and characterized receptors that internalize and down-regulate normally (Tr449, Tr425), identifying regions of the receptor not critical for either process; receptors that internalize normally but are defective in down-regulation (Del[367–380], C367A, S369A), identifying residues selectively involved in down-regulation; receptors that are defective in internalization but down-regulate normally (Tr402, Tr380, Del[403–425]), identifying a region critical for internalization and indicating that internalization may not be required for down-regulation; and a receptor defective in both internalization and down-regulation (Tr366). These receptor constructs should prove useful for elucidating in greater detail the molecular mechanisms regulating both the expression and the localization of α_{1B}ARs, with the results of likely relevance to other GPCRs as well.

References

Bird KS, Anderson JL and Toews ML (1997) Modulation of α_{1B}-adrenoceptor expression by agonist and protein kinase inhibitors. *Eur J Pharmacol* **340:**267–275.

Bohm SK, Grady EF and Bunnett NW (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J* **322:**1–18.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Cotecchia S, Lattion AL, Diviani D and Cavalli A (1995) Signalling and regulation of the α_{1B}-adrenergic receptor. *Biochem Soc Trans* **23:**121–125.

Cowlen MS and Toews ML (1988) Evidence for α_1-adrenergic receptor internalization in DDT_1 MF-2 cells following exposure to agonists plus protein kinase C activators. *Mol Pharmacol* **34:**340–346.

Cvejic S, Trapaidze N, Cyr C and Devi LA (1996) Thr^{353}, located within the COOH-terminal tail of the delta opiate receptor, is involved in receptor down-regulation. *J Biol Chem* **271:**4073–4076.

Diviani D, Lattion AL and Cotecchia S (1997) Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the α_{1B}-adrenergic receptor. *J Biol Chem* **272:**28712–28719.

Dixon RA, Sigal IS, Candelore MR, Register RB, Scattergood W, Rands E and Strader CD (1987) Structural features required for ligand binding to the β-adrenergic receptor. *EMBO J* **6:**3269–3275.

Eason MG, Jacinto MT, Theiss CT and Liggett SB (1994) The palmitoylated cysteine of the cytoplasmic tail of α_{2A}-adrenergic receptors confers subtype-specific agonist-promoted downregulation. *Proc Natl Acad Sci USA* **91:**11178–11182.

Ferguson SS, Barak LS, Zhang J and Caron MG (1996) G-protein-coupled receptor regulation: Role of G-protein-coupled receptor kinases and arrestins. *Can J Physiol Pharmacol* **74:**1095–1110.

Fukushima Y, Asano T, Takata K, Funaki M, Ogihara T, Anai M, Tsukuda K, Saitoh T, Katagiri H, Aihara M, Matsuhashi N, Oka Y, Yazaki Y and Sugano K (1997) Role of the C terminus in histamine H2 receptor signaling, desensitization, and agonist-induced internalization. *J Biol Chem* **272:**19464–19470.

Garrad RC, Otero MA, Erb L, Theiss PM, Clarke LL, Gonzalez FA, Turner JT and Weisman GA (1998) Structural basis of agonist-induced desensitization and sequestration of the $P2Y_2$ nucleotide receptor. Consequences of truncation of the C terminus. *J Biol Chem* **273:**29437–29444.

Hausdorff WP, Campbell PT, Ostrowski J, Yu SS, Caron MG and Lefkowitz RJ (1991) A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc Natl Acad Sci USA* **88:**2979–2983.

Hertel C, Nunnally MH, Wong SK, Murphy EA, Ross EM and Perkins JP (1990) A truncation mutation in the avian β-adrenergic receptor causes agonist-induced internalization and GTP-sensitive agonist binding characteristic of mammalian receptors. *J Biol Chem* **265:**17988–17994.

Hoffman BB (1987) Regulation of alpha-1 adrenergic receptors, in *The Alpha-1 Adrenergic Receptors* (Ruffolo RR Jr ed) pp 457–474, Humana Press, Clifton, NJ.

Kennedy ME and Limbird LE (1994) Palmitoylation of the α_{2A}-adrenergic receptor. Analysis of the sequence requirements for and the dynamic properties of α_{2A}-adrenergic receptor palmitoylation. *J Biol Chem* **269:**31915–31922.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.

Lattion AL, Diviani D and Cotecchia S (1994) Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the α_{1B}-adrenergic receptor. *J Biol Chem* **269:**22887–22893.

Leeb-Lundberg LM, Cotecchia S, DeBlasi A, Caron MG and Lefkowitz RJ (1987) Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT_1 MF-2 smooth muscle cells. *J Biol Chem* **262:**3098–3105.

Leeb-Lundberg LM, Cotecchia S, Lomasney JW, DeBernardis JF, Lefkowitz RJ and Caron MG (1985) Phorbol esters promote α_1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc Natl Acad Sci USA* **82:**5651–5655.

Nakahata N, Martin MW, Hughes AR, Hepler JR and Harden TK (1986) H_1-histamine receptors on human astrocytoma cells. *Mol Pharmacol* **29:**188–195.

Newton AC (1995) Protein kinase C: Structure, function, and regulation. *J Biol Chem* **270:**28495–28498.

Perkins JP, Hausdorff WP and Lefkowitz RJ (1991) Mechanisms of ligand-induced desensitization of β-adrenergic receptors, in *The Beta-Adrenergic Receptors* (Perkins JP ed) pp 73–124, Humana Press, Clifton, NJ.

Pizard A, Blaukat A, Muller-Esterl W, Alhenc-Gelas F and Rajerison RM (1999) Bradykinin-induced internalization of the human B_2 receptor requires phosphorylation of three serine and two threonine residues at its carboxyl tail. *J Biol Chem* **274:**12738–12747.

Roth A, Kreienkamp HJ, Meyerhof W and Richter D (1997) Phosphorylation of four amino acid residues in the carboxyl terminus of the rat somatostatin receptor subtype 3 is crucial for its desensitization and internalization. *J Biol Chem* **272:**23769–23774.

Schulein R, Hermosilla R, Oksche A, Dehe M, Wiesner B, Krause G and Rosenthal W (1998) A dileucine sequence and an upstream glutamate residue in the intracellular carboxyl terminus of the vasopressin V_2 receptor are essential for cell surface transport in COS.M6 cells. *Mol Pharmacol* **54:**525–535.

Smit MJ, Timmerman H, Blauw J, Beukers MW, Roovers E, Jacobs EH, Hoffmann M and Leurs R (1996) The C terminal tail of the histamine H_2 receptor contains positive and negative signals important for signal transduction and receptor down-regulation. *J Neurochem* **67:**1791–1800.

Thomas WG (1999) Regulation of angiotensin II type 1 (AT_1) receptor function. *Regul Pept* **79:**9–23.

Toews ML (1987) Comparison of agonist-induced changes in β- and α_1-adrenergic receptors of DDT_1 MF-2 cells. *Mol Pharmacol* **31:**58–68.

Toews ML, Shreve PE and Bylund DB (1991) Regulation of adrenergic receptors, in *Signaling Mechanisms in Secretory and Immune Cells* (Martinez JR, Edwards BS and Seagrave JC eds) pp 1–17, San Francisco Press, Inc., San Francisco, CA.

Tseng CC and Zhang XY (1998) The cysteine of the cytoplasmic tail of glucose-dependent insulinotropic peptide receptor mediates its chronic desensitization and down-regulation. *Mol Cell Endocrinol* **139:**179–186.

Valiquette M, Bonin H, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M (1990) Involvement of tyrosine residues located in the carboxyl tail of the human β_2-adrenergic receptor in agonist-induced down-regulation of the receptor. *Proc Natl Acad Sci USA* **87:**5089–5093.

Wang J, Zheng J, Anderson JL and Toews ML (1997) A mutation in the hamster α_{1B}-adrenergic receptor that differentiates two steps in the pathway of receptor internalization. *Mol Pharmacol* **52:**306–313.

Xu Z, Hirasawa A, Shinoura H and Tsujimoto G (1999) Interaction of the α_{1B}-adrenergic receptor with gC1q-R, a multifunctional protein. *J Biol Chem* **274:**21149–21154.

Yang J, Logsdon CD, Johansen TE and Williams JA (1993) Human m3 muscarinic acetylcholine receptor carboxyl-terminal threonine residues are required for agonist-induced receptor down-regulation. *Mol Pharmacol* **44:**1158–1164.

Yang J, Williams JA, Yule DI and Logsdon CD (1995) Mutation of carboxyl-terminal threonine residues in human m3 muscarinic acetylcholine receptor modulates the extent of sequestration and desensitization. *Mol Pharmacol* **48:**477–485.

Zhu SJ, Cerutis DR, Anderson JL and Toews ML (1996) Regulation of hamster α_{1B}-adrenoceptors expressed in Chinese hamster ovary cells. *Eur J Pharmacol* **299:**205–212.

Send reprint requests to: Myron L. Toews, Ph.D., Department of Pharmacology, University of Nebraska Medical Center, 986260 Nebraska Medical Center, Omaha, NE 68198-6260. E-mail: mtoews@unmc.edu

0026-895X/00/070246-01$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:246, 2000 /1/844356

Erratum

In the article by Wang et al. [Wang J, Wang L, Zheng J, Anderson JL and Toews ML (2000) Identification of distinct carboxyl-terminal domains mediating internalization and down-regulation of the hamster α_{1B}-adrenergic receptor. *Mol Pharmacol* **57:**687–694] a correction in the text, which is of potential scientific importance, was not made before printing.

Under *Results* in the *Initial Truncation Mutations* section on p. 690, text that read "The Tr366 receptor, truncated after Gln366, was expressed at levels similar to the wild-type receptor and exhibited binding and functional properties similar to the wild-type receptor (Table 1) but was completely defective. . . ", should read "The Tr366 receptor, truncated after Gln366, was expressed at lower levels than the wild-type receptor but exhibited binding and functional properties similar to the wild-type receptor (Table 1); however, it was completely defective. . . . ". We regret any inconvenience caused by this error.