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Thr^{353}, Located within the COOH-terminal Tail of the δ Opiate Receptor, Is Involved in Receptor Down-regulation*

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Svetlana Cvejic, Nino Trapaidze, Curt Cyr‡, and Lakshmi A. Devi§

From the Department of Pharmacology, New York University Medical Center, New York, New York 10016

Prolonged exposure to abused drugs such as opiates causes decreased response to the drug; this reduced sensitivity is thought to be due to the loss of receptors, or down-regulation. The molecular mechanism of the opiate receptor down-regulation is not known. In order to address this, we generated a number of mutants of the δ opiate receptor COOH-terminal tail. When expressed in the Chinese hamster ovary cells, both the wild type and the receptor with a deletion of 37 COOH-terminal residues bind diprenorphine with comparable affinities and show similar decreases in cAMP levels in response to D-Ala^2, D-Leu^5, enkephalin (DADLE). However, the truncated receptor does not show down-regulation from the cell surface upon prolonged exposure (2–48 h) to DADLE. In contrast, both the wild type receptor and the receptor with the deletion of only 15 COOH-terminal residues show substantial down-regulation upon long term DADLE treatment. These results suggest that the region located between 15 and 37 residues from the COOH terminus is involved in the receptor down-regulation. In order to identify residues that play a key role in down-regulation, point mutations of residues within this region were examined for their ability to modulate receptor down-regulation. The receptor with a mutation of Thr^{353} to Ala does not down-regulate, whereas the receptor with a mutation of Ser^{344} to Gly down-regulates with a time course similar to that of the wild type receptor. Taken together, these results suggest that the COOH-terminal tail is not essential for functional coupling but is necessary for down-regulation and that Thr^{353} is critical for the agonist-mediated down-regulation of the δ opiate receptor.

It has been well established that chronic exposure to opiates such as morphine causes a decrease in the sensitivity to opiates (1). This reduced sensitivity is thought to be due to the loss of receptors from the cell's surface. This phenomenon, termed "down-regulation," has been implicated in the opiate tolerance/dependence that is seen in narcotic addicts. The molecular basis of the down-regulation phenomenon is not known.

Cell lines that express high levels of opiate receptors have been used for studies with agonist-mediated receptor function such as down-regulation (2–4). A neuroblastoma × glioma hybrid cell line (NG108-15) has been widely used to characterize the δ subtype of the opiate receptor (2–4). Upon exposure to agonist the receptor number on the NG108-15 cells rapidly decreases (as detected with radioligand binding assays). Treatment for up to 24 h leads to a decrease of about 50–80% of the cell surface receptors (2, 3). This receptor down-regulation is thought to be due to internalization of the ligand-receptor complex followed by degradation of the receptor (4).

The primary structure of the opiate receptors as deduced from the cDNA has revealed that all three subtypes of the opiate receptors are members of the G-protein-coupled receptor family (5–7). Many structural features that are conserved in other G-protein-coupled receptors are found in the opioid receptors; these include the consensus *N*-linked glycosylation sites near the amino terminus, a palmitoylation site in the COOH-terminal tail, and sites for phosphorylation in the first and third intracellular domain and in the COOH-terminal tail (5–7). Extensive studies have been carried out to examine the role of COOH-terminal tail in modulating receptor-mediated events in the case of adrenergic receptors (8). In contrast, relatively few studies have examined the role of COOH-terminal tail in modulating such events by other neuropeptide receptors. We have therefore examined the role of COOH-terminal tail in agonist-mediated down-regulation of the opiate receptor using mutations of the COOH-terminal tail. We find that a portion of the COOH-terminal tail is necessary for the agonist-mediated down-regulation and that Thr^{353} plays an important role in the down-regulation of the δ opiate receptor.

EXPERIMENTAL PROCEDURES

Generation of Mutants and Cell Lines Expressing Full-length or Truncated δ Opiate Receptor—Flag-epitope (ADDDDKYD) tagged δ opiate receptor was subcloned into the pCDNA3 expression vector. Two deletion mutants, ΔC15 and ΔC37 were generated using polymerase chain reaction to amplify regions of flag-tagged δ opiate receptor from Thr^{211} to Val^{357} (for ΔC15) or from Thr^{211} to Thr^{335} (for ΔC37). The amino acid numbering is adopted from the numbering of mouse δ opiate receptor (6). The schematic drawing of the primary structure of full-length receptor and the positions of insertions for generating the flag epitope-tagged receptor are given in Fig. 1. Polymerase chain reaction fragments were restriction-digested with unique restriction enzymes and subcloned into the corresponding restriction sites of pCDNA3-δOR that was digested with the same restriction enzymes. The point mutations were generated by oligonucleotide-directed mutagenesis using an Altered Sites II *in vitro* mutagenesis kit from Promega (Madison, WI) according to the manufacturer's directions. Nucleotide sequence was confirmed by double-stranded DNA sequencing (9). The resulting COOH-terminal truncations and point mutations are shown in the *lower panel* of Fig. 1.

Approximately 3×10^5 Chinese hamster ovary (CHO)[1] cells were transfected with 5 μg of Qiagen-purified plasmid DNA using Lipofectin reagent (Life Technologies Inc.). Colonies with stable expression were selected in medium containing 500 μg/ml of Geneticin (Life Technologies Inc.). 24–48 colonies were tested for receptor expression by binding

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‡ A recipient of the NIDA postdoctoral training grant DA-07254. Current Address: Dept. of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195.

§ To whom correspondence should be addressed: Dept. of Pharmacology, New York University Medical Center, MSB 411, 550 First Ave., New York, NY 10016. Tel.: 212-263-7119; Fax: 212-263-7133; E-mail: Lakshmi.Devi@med.nyu.edu.

[1] The abbreviations used are: CHO, Chinese hamster ovary; DADLE, [d]Ala^2, [d]Leu^5, enkephalin.

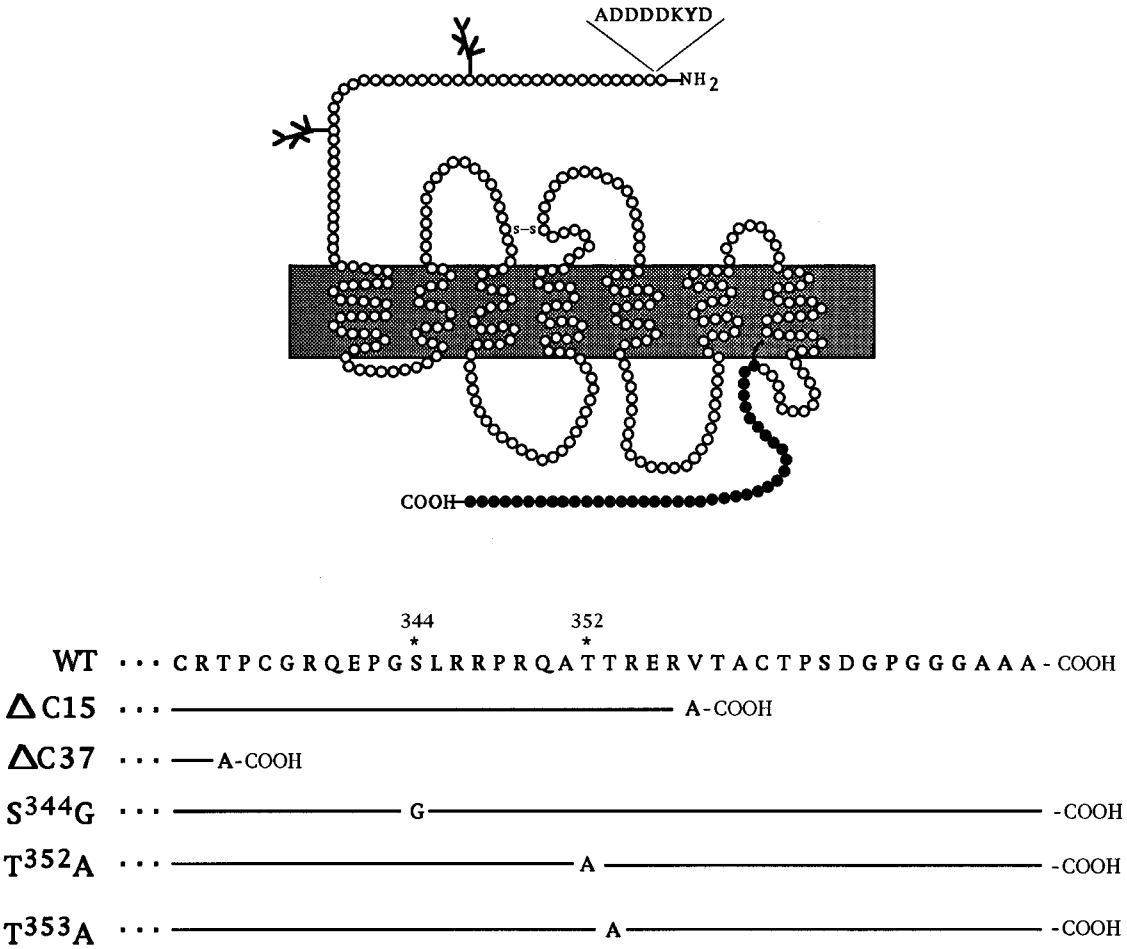

FIG. 1. **Schematic representation of the structure of the full-length mouse δ opiate receptor, WT.** The putative glycosylation sites are shown as *branched chains*, and the putative palmitoylation site is shown as a *beaded line* near the COOH-terminal tail. The COOH-terminal residues 333–372 are shown by *filled circles*. The flag epitope-tagged receptor (F-WT) contains additional amino acid sequence shown in *capital letters* near the NH_2 terminus. The *lower panel* shows the COOH-terminal tail residues 333–372 of the wild type receptor in single-letter amino acid code. The *asterisks* point to the putative protein kinase C phosphorylation sites, and the *numbers* indicate the amino acid positions; the numbering is according to Evans *et al.* (6). The amino acid sequence of the mutants identical to the wild type is represented by a *line*, and the changes are as indicated.

assay using [^{3}H]diprenorphine (10). Specific binding is defined as the difference between the radioactivity bound to the cells in the presence and absence of 10 μM diprenorphine. Expression of the receptor was also confirmed by Western blotting of the membranes from the stably expressing cells using a flag tag-specific antibody, M1 (IBI/Kodak).

Binding Assay—Approximately 10^6 cells were incubated with [^{3}H]diprenorphine for 20 min in 0.5 ml of Krebs-Ringer-HEPES buffer, pH 7.4, at 37 °C, without or with the unlabeled diprenorphine. The cells were collected on Whatman GF-B filters and washed extensively with 50 mM Tris-Cl, pH 7.4. The radioactivity on the filters was determined after an overnight incubation of filters in Biosafe scintillation fluid (Beckman). K_d and B_{max} values were determined by Scatchard analysis using the Ligand program.

Functional Coupling—Functional coupling of the wild type and mutant receptors was determined by assaying for changes in levels of intracellular cAMP after the stimulation of cells with various concentrations of DADLE. For this, 1–2 × 10^5 cells/well were plated onto a 24-well plate. The next day the cells were pretreated for 1 h with 10 μM forskolin followed by increasing doses of DADLE (shown in Fig. 2) for 20 min. Treatment was terminated with 5% trichloroacetic acid, and the level of cAMP was determined by radioimmunoassay following neutralization of the cell extract with 2.5 M potassium carbonate.

cAMP Assay—For the cAMP radioimmunoassay, 10–50 μl of neutralized cell extract, a dilution of cAMP antiserum (Biomedical Technology Inc.) that gives approximately 30% binding of ^{125}I-cAMP, and approximately 5,000 cpm of ^{125}I-cAMP were incubated in 50 mM Tris-Cl buffer, pH 7.5, in a final volume of 300 μl. Following overnight incubation at 4 °C, the radioimmunoassay was terminated by the addition of 50 μl of calf serum and 1 ml of 17.5% polyethylene glycol-8000 in 50 mM sodium phosphate buffer, pH 7.5. The antigen-antibody complex was

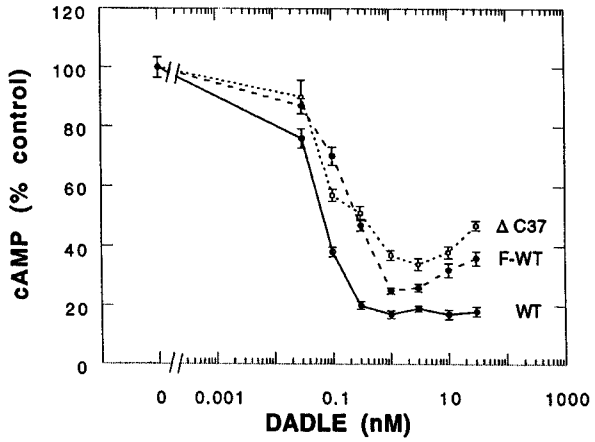

FIG. 2. **Functional coupling of the wild type (WT, ○), flag-tagged wild type (F-WT, ●), or mutant lacking the COOH-terminal 37 amino acids (ΔC37, □) receptors.** The functional coupling was examined by changes in cAMP levels as a measure of the inhibition of adenylate cyclase. Cells were treated with various doses of DADLE, and the cAMP was determined by radioimmunoassay as described under "Experimental Procedures." The cAMP in control cells that was not treated with DADLE is taken as 100%. The data represent the average ± S.E. of triplicate values from three separate determinations. The data for cells expressing 1–2 × 10^5 receptors/cell are presented; similar dose-response curves were observed with additional clonal cultures expressing different numbers of receptors.

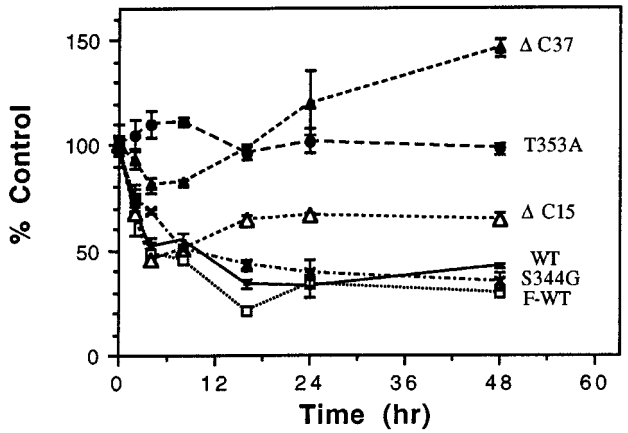

FIG. 3. **Time-dependent decrease in [^{3}H]diprenorphine binding during chronic DADLE treatment.** Cells expressing the WT (○), F-WT (□), ΔC15 (△), ΔC37 (▲), S344G (×), or T353A (●) were treated with 100 nM DADLE for various periods of time. After extensive washing with buffer, [^{3}H]diprenorphine binding to the cells was measured as described under "Experimental Procedures." [^{3}H]diprenorphine binding to untreated cells (treated 1–3 min with 100 nM DADLE prior to extensive washing) is taken as "control" (100%). The data represent the average ± S.E. of triplicate determinations. The data for cells expressing 1–2 × 10^5 receptors/cell is presented. For each construct, a similar time course of down-regulation was observed with at least two additional clonal cultures expressing different numbers of receptors.

collected by centrifugation of the precipitate following a 15-min incubation at 4 °C. The radioactivity in the precipitate was determined using a γ-counter.

Down-regulation of the Receptor—For down-regulation studies, 1–2 × 10^6 cells were treated with 100 nM DADLE for various periods of time shown in Fig. 3. Following treatment, the cells were extensively washed with Krebs-Ringer-HEPES buffer. The binding assay was carried out on whole cells as described above. K_d and B_{max} values were determined by Scatchard analysis using the Ligand program.

RESULTS AND DISCUSSION

We chose CHO cells as a suitable host cell line to express high levels of delta opiate receptor, since CHO cells have been useful for stable expression of a number of neuropeptide receptors (10–14). We stably transformed the CHO cells with full-length or mutated δ opiate receptor; a schematic of the primary structure of full-length and mutated δ opiate receptor is presented in Fig. 1. We selected 24–48 clones expressing varying numbers of receptors and analyzed two or three clones in each case. The cell lines expressing wild type or mutated δ opiate receptor exhibited high affinity for diprenorphine (Table I). The affinity is similar to the reported affinity of NG108-15 for diprenorphine (2). It should be pointed out that although the receptor number varied 100-fold between cell lines, there was no substantial difference in the affinity for diprenorphine between the various clones. The modest 2–3-fold variation in affinity is in agreement with the 2–3-fold variation in the K_d reported for CHO cells expressing varying levels of the full-length receptor (10).

DADLE, an opiate receptor agonist, was used to determine the inhibition of adenylate cyclase, as a measure of functional coupling, in these cell lines. Dose-response curves of the inhibition of cAMP accumulation by 20-min DADLE treatment show that the cAMP levels decrease with increasing amounts of DADLE *i.e.* the percentage of inhibition of cAMP increases with increasing amounts of DADLE (Fig. 2). The IC_{50} value for the DADLE-induced inhibition of cAMP accumulation by cells expressing the full-length receptor is identical to the IC_{50} value reported for CHO cells expressing full-length receptor (10) and about 10-fold lower than the IC_{50} value reported for NG108-15 cells (2). Interestingly, the dose-response curve for the DADLE-induced inhibition of cAMP accumulation in receptor lacking

TABLE I

Expression of the wild type and mutant δ opiate receptor in Chinese hamster ovary cells

K_d and B_{max} values of diprenorphine binding were determined on whole cells, using at least eight concentrations of diprenorphine, and the data were analyzed using the Ligand program. Standard error of the mean is included for each value of B_{max}.

Cell line	K_d	B_{max}	Receptors/cell
	nM	*fmol/10^6 cells*	
WT			
Clone A	0.7	80 ± 66	50,000
Clone B	0.3	368 ± 44	195,000
Flag-tagged WT			
Clone C	2.7	4307 ± 473	2,500,000
Clone D	1.0	1300 ± 117	760,000
Clone E	1.1	289 ± 34	165,000
ΔC15			
Clone F	1.3	498 ± 24	300,000
Clone G	1.5	223 ± 15	130,000
Clone H	2.5	120 ± 29	70,000
ΔC37			
Clone I	2.9	204 ± 37	120,000
Clone J	0.9	81 ± 14	50,000
S344G			
Clone K	0.6	231 ± 64	135,000
Clone L	0.1	127 ± 11	70,000
T353A			
Clone M	1.0	424 ± 55	250,000
Clone N	0.4	30 ± 7	20,000

the COOH-terminal 37 amino acids is comparable with the dose-response curves for the wild type receptors (Fig. 2). The fact that the removal of the COOH-terminal 37 amino acids does not affect the efficiency of functional coupling suggests that the COOH-terminal tail does not play a role in signal transduction by the δ opiate receptor. This is in contrast to other G-protein coupled receptors where the COOH-terminal tail is thought to play an important role in functional coupling to G-proteins (15). Studies with deletion and other mutational analyses in β_2 adrenergic and other G-protein-coupled receptors have shown that in these receptors the COOH-terminal tail is an integral part of signal transduction (16, 17).

To examine the role of the COOH-terminal tail in receptor down-regulation, cells expressing the wild type or the mutant receptors were treated with 100 nM DADLE for various time periods, and the amount of receptors was determined by the binding of [^{3}H]diprenorphine to intact cells. Cells expressing the full-length receptors (untagged or flag-epitope-tagged) exhibit a time-dependent decrease in diprenorphine binding with a maximal decrease of about 60–70% by about 16 h (Fig. 3). Comparable results were obtained with two other transfected cultures expressing different numbers of receptors (data not shown). The cells expressing ΔC15 receptor (lacking the COOH-terminal 15 amino acids) exhibit a time-dependent decrease in diprenorphine binding similar to the pattern of down-regulation seen with the wild-type receptor, although the maximal decrease is only about 40% even after 48 h of treatment (Fig. 3). In contrast, the cells expressing ΔC37 receptor (lacking the COOH-terminal 37 amino acids) show no decrease in diprenorphine binding even upon 48 h of treatment with DADLE. The cell surface diprenorphine binding actually increases after 48 h as compared with the control (untreated) cells (Fig. 3). The finding that the ΔC15 receptor shows down-regulation and ΔC37 receptor does not suggests that the region located between 15 and 37 residues from the COOH terminus plays an important role in δ opiate receptor down-regulation.

In order to identify the residues involved in this down-regulation process, we generated receptors with mutations of three phosphorylatable residues within the region between 15 and 37 amino acids from the COOH terminus, namely, Ser^{344}, Thr^{352},

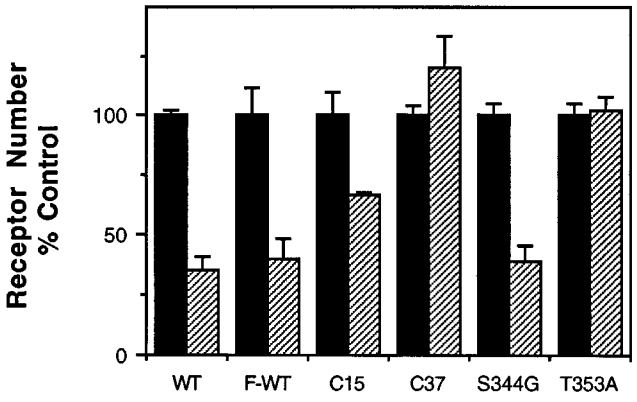

FIG. 4. **Down-regulation of the δ opiate receptor in cell lines expressing wild-type and mutant receptors.** The cell lines were treated with 100 nM DADLE for 1–5 min (*filled bars*) or for 24 h (*striped bars*), and the diprenorphine binding was carried out as described under "Experimental Procedures." K_d and B_{max} values of diprenorphine binding were determined by whole cell binding studies, using at least five concentrations of diprenorphine, and the data were analyzed using the Ligand program. The receptor number in cells treated for 1–3 min with DADLE is taken as "control" (100%). Standard error of the mean is included for each value of the B_{max}.

and Thr^{353}. The cells expressing the $Thr^{353} \rightarrow Ala$ receptor do not exhibit a decrease in diprenorphine binding even after 48 h of treatment (Figs. 3 and 4). In contrast, the cells expressing $Ser^{344} \rightarrow Gly$ receptor exhibit a time course of decrease in diprenorphine binding identical to the time course seen for the wild type receptor (Figs. 3 and 4). This change in diprenorphine binding in cells treated with DADLE represents a change in the receptor number and not a change in affinity as examined by Scatchard analyses (Fig. 4). It should be pointed out that we have been unable to isolate cell lines expressing significant levels of the $Thr^{352} \rightarrow Ala$ receptors among 96 clones tested. Taken together, these results suggest that Thr^{353} is critical for the agonist-induced down-regulation of the δ opiate receptor.

There is growing evidence that in many of the G-protein-coupled receptors the COOH-terminal tail plays a role in receptor down-regulation. Studies involving the prototypical β_2 adrenergic receptor have demonstrated the importance of this receptor's carboxyl terminus in mediating desensitization through both second messenger-dependent and independent kinases, which phosphorylate multiple serines and threonines in this region (8). The COOH-terminal tail of the opiate receptor contains putative phosphorylation sites (6, 7); phosphorylation could play a role in opiate receptor down-regulation. Studies in cell lines and in *Xenopus laevis* oocytes have implicated the involvement of phosphorylation in opiate receptor function (18–21).

Phosphorylation by protein kinase C has been implicated in the down-regulation of other G-protein-coupled receptors (22–24). It is possible that protein kinase C plays a role in δ opiate receptor desensitization and/or down-regulation since the COOH-terminal tail of the δ opiate receptor contains residues that fit the consensus for phosphorylation by protein kinase C. Studies examining the desensitization of δ opiate receptors have shown that a β-ARK-related kinase, and not protein kinase C, plays an important role in the receptor desensitization (19). In contrast, studies with the down-regulation of the endogenous opiate receptors in NG108-15 cells have shown that modulators of protein kinase C affect receptor down-regulation, suggesting an involvement of protein kinase C in this process (25). In this study we find that mutation of a protein kinase C consensus site (Ser^{344}) does not affect down-regulation, suggesting that phosphorylation by protein kinase C at this site may not be involved in δ opiate receptor down-regulation. This is further supported by studies with modulators of protein kinase C on receptor down-regulation. Pretreatment of the cells expressing wild type receptor with 30 nM phorbol ester for 3 h (to activate protein kinase C) or with 1 μM phorbol ester for 24 h (to deplete protein kinase C) does not affect the DADLE-mediated down-regulation (data not shown). Furthermore, treatment of the cells for 24 h with 0.5 μM calphostin C has no effect on down-regulation (not shown). Taken together, these results suggest that protein kinase C does not play a major role in the down-regulation of the δ opiate receptor expressed in CHO cells.

The possibility that the Thr^{353} may play a role other than being phosphorylated cannot be ruled out. It is possible that the Thr^{353} identified in this study specifically recognizes some cellular factor(s) involved in the process of receptor down-regulation. Such a possibility is supported by the studies with the avian β_2 adrenergic receptor that does not exhibit agonist-mediated down-regulation (26, 27). Deletion of a certain portion of the COOH-terminal tail of this receptor results in down-regulation (26), and addition of this COOH-terminal portion to the mammalian β_2 adrenergic receptor results in a dramatic decrease in the down-regulation of the latter receptor (27). These results suggest that COOH-terminal domains could interact with certain cellular protein(s) that mediate receptor down-regulation.

In summary, our study has shown that removal of the COOH-terminal tail does not affect functional coupling but completely abolishes down-regulation of the δ opiate receptor. In addition, these studies demonstrate that Thr^{353} is essential for δ opiate receptor down-regulation.

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