

The μ -Opioid Receptor Down-Regulates Differently from the δ -Opioid Receptor: Requirement of a High Affinity Receptor/G Protein Complex Formation

SUMITA CHAKRABARTI,¹ WANLING YANG, PING-YEE LAW, and HORACE H. LOH

Department of Pharmacology, University of Minnesota, Medical School, Minneapolis, Minnesota 55455

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SUMMARY

Chronic opioid treatment of Neuro_{2A} cells stably expressing either δ -opioid receptor (DOR) or μ -opioid receptor (MOR) resulted in agonist-dependent receptor down-regulation. Although there is high homology in the DOR and MOR amino acid sequences, there is an apparent difference in the regulation of the cellular levels of these two receptors. The ability of 24-hr [D-Pen², D-Pen⁵]enkephalin (DPDPE) treatment to internalize and down-regulate DORs expressed in Neuro_{2A} remained intact after pertussis toxin (PTX) pretreatment, which uncouples the receptor from G proteins. In contrast, the ability of [D-Ala², N-MePhe⁴, Gly-ol⁵]enkephalin (DAMGO) to internalize and down-regulate MORs in Neuro_{2A} cells was completely abolished by PTX pretreatment. The requirement of functional MOR but not DOR in agonist-induced receptor down-regulation was further demonstrated by site-directed mutagenesis of the receptors. When Asp114 in transmembrane 2 of MOR was converted to alanine, the ability was abolished of DAMGO or morphine to inhibit forskolin-stimulated [³H]cAMP production in

Neuro_{2A} cells stably expressing this mutant receptor. There was a parallel decrease in agonist affinity and elimination of the agonist-induced receptor down-regulation. On the other hand, although the equivalent mutation of Asp95 to alanine in DOR likewise resulted in the inability of DPDPE to inhibit [³H]cAMP production, the ability of DPDPE to down-regulate this mutant receptor after 24-hr treatment was unaffected. This difference in MOR and DOR down-regulation could be caused by the differences in the ability of these two receptors to form high affinity complexes with G proteins. DOR retained the ability to form high affinity complexes even after PTX pretreatment or after mutation of Asp95 in transmembrane 2. In contrast, MOR existed only in the low affinity, uncoupled state after PTX pretreatment or after conversion of Asp114 to alanine. Therefore, in Neuro_{2A} cells, agonist-induced opioid receptor down-regulation seems to depend directly on the formation of the high affinity receptor complexes and not on the activation of the receptors and subsequent transduction of the signals.

Opioid receptors belong to the superfamily of G protein-coupled receptors. They mediate the effects of opioid alkaloids and peptides toward many important physiological functions, including stress, nociception, mood, reward, locomotor activities, respiratory functions, neurotransmitter release, and immune responses (1). Opioid receptors are classified into three different types, μ , δ , and κ , on the basis of extensive pharmacological, physiological, and behavioral studies (2–4). All three types of opioid receptors have been cloned recently (5–10). They are highly homologous (11) and well characterized when expressed transiently in COS-7, a green monkey kidney cell line (12), or after stable transfection in Chinese hamster ovary cells (13) or murine neuroblas-

toma Neuro_{2A} cells (14). These cellular models expressing cloned opioid receptors are extremely important for the study of ligand/receptor interactions and molecular mechanisms underlying signal transduction mechanisms.

It has been reported that chronic agonist stimulation of different cell lines expressing MORs and/or DORs desensitized effector mechanisms and decreased membrane receptor density (15, 16). Decrease in receptor number on chronic agonist treatment was initially reported in neuroblastoma X glioma NG108–15 hybrid cells expressing DORs (17, 18). Later, several lines of evidence indicated receptor down-regulation for both MORs and DORs in human neuroblastoma SHSY5Y cells on chronic treatment with selective agonists (19–21). Recently, after the cloning of all three opioid receptors, chronic agonist-induced receptor down-regulation has been reported in different cell lines expressing cloned DORs or MORs (13, 14).

Despite being studied for a long time, the molecular mech-

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¹ Current affiliation: Department of Biochemistry, State University of New York, Brooklyn, NY 11203-2098.

ABBREVIATIONS: MOR, μ -opioid receptor; DOR, δ -opioid receptor; PTX, pertussis toxin; DAMGO, [D-Ala², N-MePhe⁴, Gly-ol⁵]enkephalin; DPDPE, [D-Pen², D-Pen⁵]enkephalin; DADLE, [D-Ala², D-Leu⁵]enkephalin; Gpp(NH)p, guanosine-5'-(β , γ -imido)triphosphate; HA, hemagglutinin; TM2, transmembrane 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

anisms of receptor regulation by opioid receptor subtypes remain unknown. Previous studies have shown that chronic exposure of NG108–15 cells to [³H]DADLE resulted in the translocation of DORs from the membrane surface to lysosomal compartments, where they were subsequently degraded (22). Inhibition of receptor synthesis does not seem to affect this receptor down-regulation. Further studies have suggested that this agonist-induced DOR internalization does not involve receptor activation of G protein because PTX treatment did not affect either receptor down-regulation (23) or formation of high affinity receptor/G protein complexes in NG108–15 cells (24). Although MOR in Neuro_{2A} cells seems to internalize by a pathway² similar to that of DORs in NG108–15 cells, it is not clear how either receptor type is regulated. Therefore, in the current study, we investigated the role of G protein/receptor interactions in agonist-induced down-regulation of MOR and DOR expressed in Neuro_{2A} cells. By uncoupling receptor activation of G proteins through PTX pretreatment or mutation of an aspartic acid residue in TM2 that has been reported to be critical to agonist affinity and activity (25, 26), it could be demonstrated that MOR down-regulation requires the formation of high affinity complexes.

Experimental Procedures

Construction of mutant receptors. MORs and DORs were mutated by oligonucleotide-directed mutagenesis using the pAlter-1 mutagenesis system and the protocol supplied by Promega (Madison, WI). For DORD95A (Asp95 converted to alanine), oligonucleotides were synthesized to introduce both the mutation and a restriction enzyme *Pvu*II site between Ala94 and Ala96. For MORD114A, the restriction enzyme *Aha*II site located at the amino acid being mutated was removed by the oligonucleotides. The presence of these mutations could then be confirmed by restriction enzyme analysis. The mutant receptor cDNAs were then subcloned into pcDNA3 plasmid at the *Eco*RI/*Xba*I sites for heterologous expression in Neuro_{2A} cells.

Cell culture and transfection. Murine neuroblastoma Neuro_{2A} cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin in a humidified atmosphere of 90% air/10% CO₂. These cells have no expression of endogenous opioid receptors. Constructs of wild-type MOR, as well as MORD114A or MORD114N, wild-type DOR, and DORD95A cDNAs were transfected into Neuro_{2A} cells. All transfections were performed by using calcium phosphate precipitation method as previously described by Chen and Okayama (27). Isolation of colonies and maintenance of clones were carried out in the presence of geneticin (G418) as previously described (14). Colonies were screened for opioid receptor binding as determined by whole-cell binding assays using 2 nM [³H]diprenorphine in the presence and absence of 10 μM naloxone (15).

Radioligand binding assay. Saturable and competition binding assays were performed using cell membranes (150–200 μg) in 25 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂ and [³H]diprenorphine (specific activity, 39 Ci/mmol) in the presence and absence of naloxone (10 μM) unless otherwise mentioned. [³H]Diprenorphine (2 nM) was used for competition, and 0.2–12 nM was used for saturation binding assay. Binding assays were carried out at 25° for 90 min as previously described (28) and terminated by rapid filtration through GF/B filter papers. Filters were washed three times with 5 ml of ice-cold 25 mM HEPES buffer to remove unbound radioactivity, and bound radioactivity was determined by scintillation counting in a

Beckman 5000 scintillation counter (Beckman Instruments, Palo Alto, CA). Calculation of IC₅₀ values and maximal inhibition of total binding was performed using computer curve fitting of dose-response curves with the program SigmaPlot (Jandel Scientific, San Rafael, CA).

Opioid agonist regulation of intracellular [³H]cAMP level. Measurement of [³H]cAMP level was determined as previously described (14). Briefly, cells were plated onto 17-mm plates, prelabeled with [³H]adenine (2.5 mCi/ml) for 1 hr, and treated with 10 μM forskolin and 0.5 mM isobutyrylmethyl-xanthine in Krebs-Ringer-HEPES buffer at 37° for 15 min in the presence and absence of opioid drugs. The [³H]cAMP generated was then separated from other [³H]nucleotides with a double-column chromatographic procedure as described by White and Karr (29). Again, calculation of IC₅₀ values and maximal inhibition by agonists was performed using computer curve fitting of dose-response curves with SigmaPlot.

Materials. [³H]Diprenorphine (39 Ci/mmol), [³H]adenine (18 Ci/mmol), and [α-³²P]ATP (30 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). [α-³²P]NAD⁺ (30 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium and geneticin were purchased from GIBCO (Grand Island, NY). Opioid peptides DPDPE and DAMGO were obtained from Peninsula Laboratories (Belmont, CA), and opioid alkaloids like morphine, diprenorphine, and naloxone were obtained from the National Institute on Drug Abuse (Bethesda, MD). PTX was purchased from List Biologicals (Campbell, CA). All other chemicals were from Sigma Chemical (St. Louis, MO).

Results

Previous studies with NG108–15 indicated that agonists could down-regulate DORs even after PTX pretreatment (23). These data suggested that DOR down-regulation did not require the activation of G_i/G_o. In the current study, we addressed whether these previous observations were also applicable to MORs or were limited to DORs in NG108–15 cells. Neuro_{2A} cell lines stably expressing either MOR or DORs were used in the current study. Among multiple clones of Neuro_{2A} cells stably expressing MOR cDNA, one clone, MORIA3, was used that expressed 2.06 ± 0.17 pmol of receptor/mg of protein, as characterized and reported previously (14). A clone of Neuro_{2A} cells stably expressing DOR, DORD9, was established and selected as described above. This clone expressed 3.4 ± 0.5 pmol/mg of protein DOR with high affinity for [³H]diprenorphine ($K_d = 0.87 \pm 0.2$ nM).² In both MORIA3 and DORD9, respective receptor agonists inhibited the forskolin-stimulated intracellular [³H]cAMP production in a dose-dependent manner. The agonist-induced inhibition of forskolin-stimulated intracellular cAMP level could be blocked by 4-hr pretreatment with PTX (100 ng/ml) in these cells as it did for DORs in NG108–15 cells (23).

When DORD9 cells were pretreated with 10 μM DPDPE for 24 hr, as expected, a decrease in [³H]diprenorphine binding was observed (Fig. 1A). The amount of receptor binding remaining after DPDPE treatment was determined to be 20 ± 4% of control. It could be demonstrated through the use of Western analysis of the total cellular receptor proteins and immunofluorescence studies using receptor-specific antibodies that this decrease in [³H]diprenorphine binding was indeed due to down-regulation of the receptors.³ When Neuro_{2A} DORD9 was pretreated with 100 ng/ml PTX for 4 hr followed by exposure of the cells to DPDPE for 24 hr, DORs remained

² S. Chakrabarti, U. Arvidsson, M. Riedl, R. Elde, P.-Y. Law, and H. H. Loh, manuscript in preparation.

³ J. L. Ko, U. Arvidsson, R. Elde, P.-Y. Law, and H. H. Loh, unpublished observations.

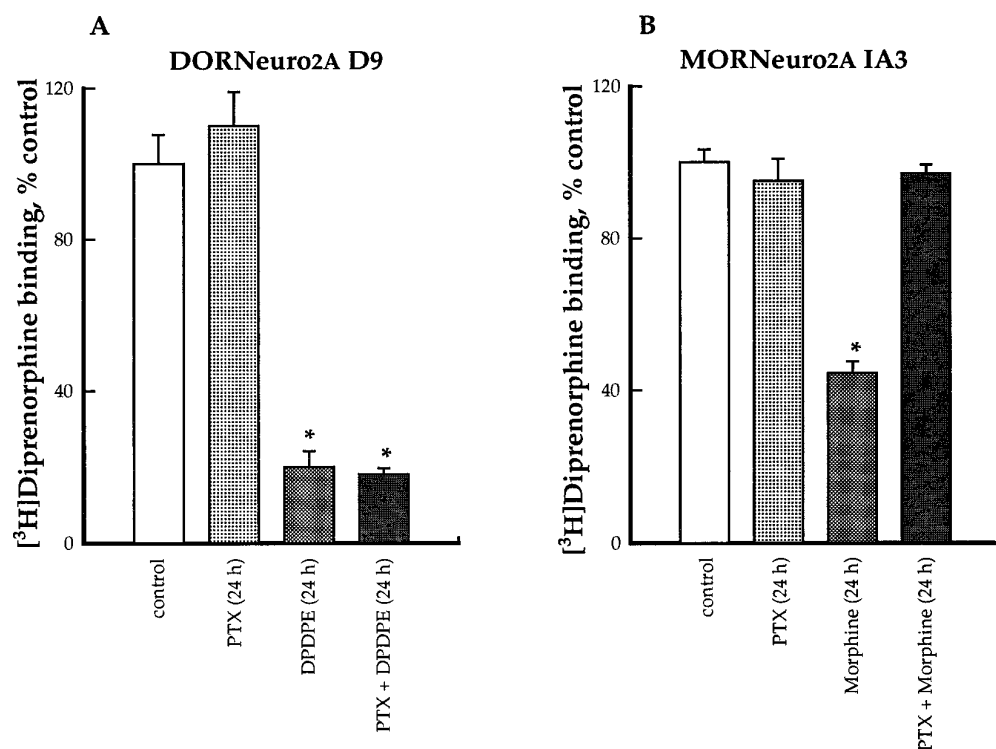


Fig. 1. Effect of PTX pretreatments on chronic agonist-induced receptor down-regulation of MORs and DORs expressed in Neuro_{2A} cells. PTX (100 ng/ml) pretreatment was applied for 4 hr followed by DPDPE (10 μ M) or morphine (50 μ M) treatments for 24 hr at 37°. After chronic pretreatments of drugs, MOR and DOR binding was determined using 2 nM [³H]diprenorphine in whole-cell binding assays. Selective agonists such as 10 μ M DAMGO or 5 μ M DPDPE was used for determining MOR and DOR binding respectively. All variables were compared from their respective controls. *, Significantly ($p < 0.005$) different from control. Data show average \pm standard error from triplicate determinations and are representative of three similar individual experimental results. Receptor densities in Neuro_{2A} cell lines expressing DORD9 or MORIA3 were 3.4 ± 0.5 and 2.06 ± 0.17 pmol/mg of protein, respectively.

down-regulated by the agonist, similar to previous observations with NG108–15 cells (23). PTX itself did not change the amount of receptors at the cell surface, and the amount of [³H]diprenorphine binding remaining after PTX and chronic DPDPE treatment was determined to be $18 \pm 2\%$, which was not significantly different from that of DPDPE alone (Fig. 1A).

However, when MORIA3 cells were pretreated with PTX and agonist, a different picture emerged. As demonstrated earlier (14), pretreatment of MORIA3 with 50 μ M morphine resulted in down-regulation of the MORs (Fig. 1B). The amount of receptors remaining after 24 hr of morphine treatment was determined to be $45 \pm 3\%$ of control. Similar results were obtained when MORIA3 cells were pretreated with 500 nM DAMGO for 24 hr (14). In contrast to the results obtained with DORs, 4 hr of pretreatment of MORIA3 with 100 ng/ml PTX followed by 50 μ M morphine exposure for 24 hr resulted in a complete blockade of agonist-induced receptor down-regulation (Fig. 1B). Again, PTX pretreatment did not significantly alter the level of MORs in MORIA3. The amount of [³H]diprenorphine binding after chronic PTX and morphine pretreatment was $97 \pm 2\%$ of control. Similarly, the amount of [³H]diprenorphine binding did not significantly change from control when PTX and DAMGO was pretreated for 24 hr (data not shown). Hence, when PTX treatment completely blocked signal transduction through agonist-activated MORs, receptor down-regulation after chronic agonist treatment was not observed.

In addition to the results obtained with radioligand binding studies, receptor internalization studies were conducted with HA-epitope-tagged MOR (MORTAG) and DOR (DORTAG) cells, stably transfected in Neuro_{2A} cells (receptor densities = 1.87 and 2.6 pmol/mg of protein, respectively). Receptor redistribution after agonist treatment was monitored with anti-HA-lissamine rhodamine immunostaining

and confocal microscopy. Similar to the results obtained with binding studies, immunocytochemical staining studies also showed that MORTAG, which was mostly localized on the cell membrane under control condition without agonist, internalizes intracellularly after 1 hr of DAMGO (500 nM) treatment (Fig. 2, A and C). However, when these cells were pretreated with PTX (100 ng/ml) for 4 hr, further treatment of DAMGO (500 nM) could not internalize MORTAG (Fig. 2D). On the other hand, as also demonstrated in Fig. 2 (E and G), the δ -opioid selective agonist DPDPE stimulated internalization of DORTAG. However, unlike MORTAG, PTX pretreatment could not prevent DPDPE-induced internalization of DORTAG (Fig. 2H). Again, PTX treatment itself did not internalize MORTAG or DORTAG. Therefore, it is clear that PTX treatment, which initiates uncoupling of receptors from G_i/G_o, prevents agonist-stimulated internalization and down-regulation of MORs but not DORs.

It is possible that the divergent observations seen with Neuro_{2A} MORIA3 and DORD9 were due to the phenotypic differences between these two clones. Thus, we decided to coexpress MORs and DORs in the same cells. DORs subcloned into the expression vector pREP4 were used to transfect MORIA3; pREP4 vector was chosen because it provides another antibiotic resistance gene, hygromycin, and its expression is episomal. Theoretically, introduction of DORs by this plasmid system would not interfere with the chromosomal expression of the MOR in Neuro_{2A} MORIA3. Using both hygromycin and geneticin selection, we were able to isolate several clones that expressed both DOR and MOR. One clone of Neuro_{2A} cells expressing both receptors, MOR-DOR17, expressed 2.37 ± 0.09 pmol/mg of protein MORs and 2.84 ± 0.11 pmol/mg of protein DORs with high affinities (1.1 and 1 nM, respectively) for [³H]diprenorphine and was chosen for receptor down-regulation studies. Whole-cell binding assay was carried out using [³H]diprenorphine binding in the

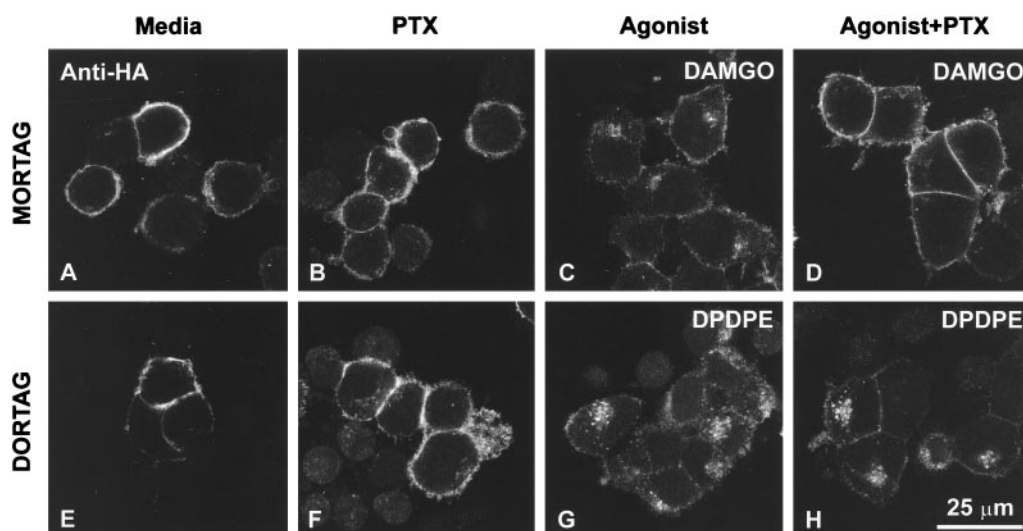


Fig. 2. Agonist-induced receptor internalization is inhibited in MORs but not in DORs pretreated with PTX. Neuro_{2A} cells were used stably expressing MORTAG or DORTAG with densities of 1.87 ± 0.2 and 2.6 ± 0.2 pmol/mg of protein, respectively. Receptors were immunostained with anti-HA-rhodamine at 0°, and after the excess staining was washed off, receptor trafficking was chased under the absence or presence of agonist treatments at 37°. Some cells were pretreated with PTX (100 ng/ml, 4 hr). The μ -opioid agonist DAMGO (500 nM) and δ -opioid agonist DPDPE (100 nM) were treated for 1 hr to MORTAG and DORTAG cells, respectively, with and without PTX pretreatment. Internalization of MORTAG (C) and DORTAG (G) was observed after treatment with DAMGO or DPDPE, respectively. Receptor internalization was not observed in media without drugs (A and E). PTX pretreatment prevented DAMGO-induced internalization of MORTAG (D) but not DPDPE-induced internalization of DORTAG (H) and by itself had no effect on either receptors (B and F). Data are representative of one of three experiments with similar results.

presence of 10 μ M DAMGO or 5 μ M DPDPE to determine MOR binding and DOR binding, respectively. When the clone MORDOR17 was treated with 50 μ M morphine for 24 hr, decrease in [³H]diprenorphine binding ($55 \pm 7\%$ of control), indicative of MOR down-regulation, was observed (Fig. 3A). Again, similar to the observation with MORIA3, pretreatment of PTX could block morphine-induced MOR down-regulation. When MORDOR17 was treated with 100 ng/ml PTX for 4 hr followed by 50 μ M morphine treatment for 24 hr, the

amount of [³H]diprenorphine binding was $110 \pm 12\%$ of control (Fig. 3A). In a similar manner, although $44 \pm 10\%$ μ -opioid binding remained after DAMGO pretreatment, $102 \pm 3.7\%$ control binding was found to remain after chronic treatment of PTX and DAMGO (data not shown). On the contrary, pretreatment with PTX for 4 hr was not able to change DOR down-regulation when MORDOR17 cells were pretreated with PTX followed by chronic exposure of DPDPE (10 μ M for 24 hr), resulting in remaining [³H]diprenorphine

MORDORNeuro2A 17

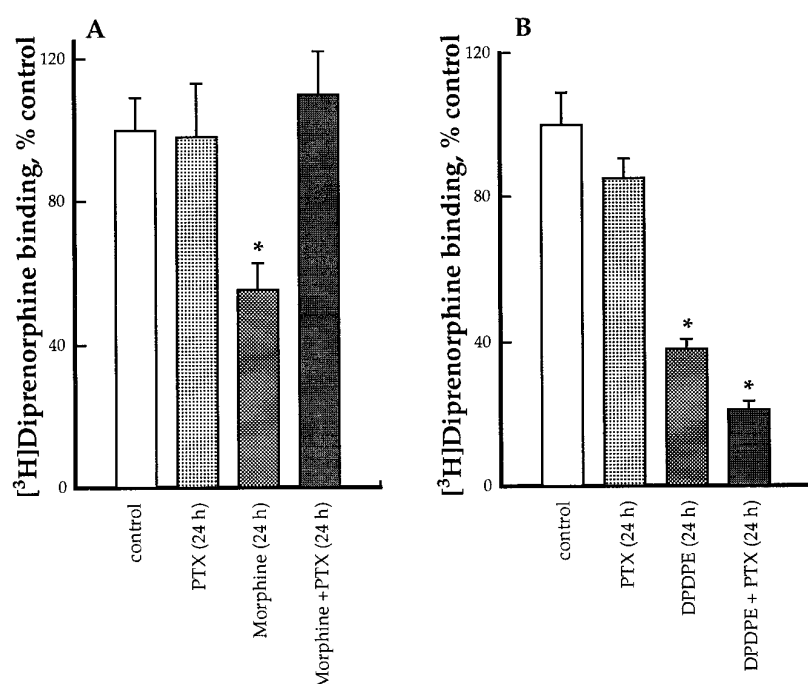


Fig. 3. Selective down-regulation of DORs with DPDPE (10 μ M) but not MORs with morphine (50 μ M) in PTX-pretreated Neuro_{2A} cells expressing both MOR and DOR. MORDOR17 Neuro_{2A} cells was used expressing MOR and DOR at densities of 2.37 ± 0.09 and 2.84 ± 0.11 pmol/mg of protein, respectively. PTX and DPDPE or morphine pretreatment was applied in a similar manner as described in the legend to Fig. 1. MOR and DOR bindings were determined in whole-cell binding assays after drug pretreatments. Nonspecific binding was determined by the inclusion of either 10 μ M DAMGO or 5 μ M DPDPE in the binding buffer. Details of the experiment are given in the text. Bars, [³H]diprenorphine binding (percent of control) expressed as average \pm standard error of triplicate determinations and representative of three similar individual experimental results. *, Significantly ($p < 0.01$) different from control.

binding of $21 \pm 2\%$ of control, which was not significantly different from that of cells treated with DPDPE alone ($38 \pm 3\%$ of control) (Fig. 3B). PTX treatment alone had no significant effect on MOR or DOR binding. Furthermore, DPDPE treatment induced no apparent change in MOR binding. The ability of PTX to block the agonist-induced down-regulation of MOR but not DORs was also observed with other Neuro_{2A} clones expressing both MOR and DORs and in clones of Chinese hamster ovary cells expressing either receptors (13, 30) (data not shown). Therefore, it is likely that the blockade of MOR down-regulation by PTX is not due to phenotypic differences among clones or cell lines.

Although both Neuro_{2A} cell lines expressing MORs or DORs were similarly pretreated with PTX before chronic stimulation with selective agonists, possibly the divergence in results is due to incomplete ADP-ribosylation of G_i/G_o coupled to DORs. To test this possibility, PTX-induced ADP-ribosylation of membranes from untreated and PTX-pretreated DORD9 cells were performed as previously described (31). Results showed PTX-induced [α -³²P]NAD⁺ incorporation into G_i/G_o proteins of control membranes prepared from DORD9 cells (Fig. 4, lane 2). However, no such PTX-induced [α -³²P]NAD⁺ incorporation into G_i/G_o was observed in membranes pretreated with PTX, indicating complete ADP-ribosylation with the toxin during pretreatment of DORD9 cells (Fig. 4, lane 4).

To investigate the possibility that the differences in the abilities of μ - and δ -opioid agonists to induce receptor down-regulation after PTX treatment was due to the ability of DORs to retain high affinity state, mutagenesis of the receptors was performed. We chose to mutate the conserved aspartic acid in the TM2 domain because earlier reports have suggested that the conversion of this amino acid to either alanine or asparagine results in the inhibition of opioid receptor activities (25, 26). Thus, through site-directed mutagenesis, we converted Asp114 in MORs and Asp95 in DORs to alanine. Subsequently, stable cell lines expressing these mutants, MORD114A and DORD95A, were established. Two clones were selected for current studies. Neuro_{2A} MORD114A#D1 had a B_{\max} value of 2.1 ± 0.1 pmol/mg of protein and a K_d value of 0.52 ± 0.04 nM for [³H]diprenor-

phine. Neuro_{2A} DORD95A#29 had a B_{\max} value of 0.59 ± 0.1 pmol/mg of protein and a K_d value of 0.31 ± 0.1 nM for [³H]diprenorphine. Similar to earlier reports in other G protein-coupled receptors (32, 33), mutation of this particular aspartic acid resulted in a reduction of agonist affinities with minimal change in antagonists affinities (25, 26). As summarized in Table 1, there was no apparent change in antagonist affinities, whereas the affinities for the agonists DAMGO and morphine decreased 272- and 63-fold, respectively, in MORD114A#D1. In DORD95A#29, the decrease in agonist affinities was not as marked as that in MORD114A#D1 (Table 2). The affinities of DPDPE and DADLE were decreased 1.7- and 3-fold, respectively (Table 2). Nevertheless, in agreement with an earlier report (26), agonist interaction with these two mutant opioid receptors was unable to regulate adenylyl cyclase activities. Therefore, although in Neuro_{2A} cells expressing wild-type MORs or DORs, DAMGO and DPDPE ($1 \mu\text{M}$) inhibited forskolin-stimulated intracellular [³H]cAMP production by 51% and 70%, respectively, $10 \mu\text{M}$ concentrations of the same agonists could not significantly inhibit cAMP production in cells expressing the aspartic acid mutants. Significant inhibition of forskolin-stimulated cAMP levels was not observed with agonists concentrations 100-fold higher than their K_d values. Asparagine substitution of this aspartate in MOR mutants, MORD114N, also had similar results like that of MORD114A. Thus, MORD114A/MORD114N and DORD95A mutations indeed have functionally uncoupled opioid receptors from the adenylyl cyclase (Fig. 5).

When Neuro_{2A} MORD114A#D1 cells were pretreated with various concentrations of morphine for 24 hr, the amount of mutant receptors remaining on the cell surface was similar to that of cells without pretreatment. As shown in Fig. 6A, exposure of the MORD114A#D1 cells to 5, 10, or $50 \mu\text{M}$ morphine for 24 hr did not significantly change [³H]diprenorphine binding. The concentrations of morphine used were much higher than its K_d value for the mutant receptor (Table 1), and the same concentrations of morphine elicited significant down-regulation of the wild-type MORs in MORIA3 cells (data not shown). Therefore, loss of agonist-induced down-regulation in PTX-pretreated MORs after chronic agonist stimulation was not a special case because the same phenomenon was also observed in cells expressing mutant MORD114A receptors. Similarly, receptor down-regulation was not observed with chronic stimulation with high concentrations ($50 \mu\text{M}$) of morphine to Neuro_{2A} cells expressing

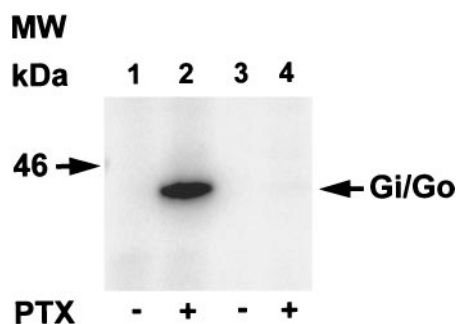


Fig. 4. ADP-ribosylation of G_i/G_o in the presence of PTX. ADP-ribosylation of G_i/G_o proteins were carried out from control membranes (lanes 1 and 2) and PTX pretreated (100 ng/ml, 4 hr) membranes (lanes 3 and 4) of DORD9 cells in the absence or presence of PTX, respectively. Subsequent to PTX-induced ADP-ribosylation, membranes were washed well and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with prestained molecular mass protein markers. [α -³²P]NAD⁺ was incorporated into G_i/G_o proteins of control membranes (lane 2) but not of membranes prepared from PTX-pretreated cells (lane 4).

TABLE 1

K_i values of opioid ligands for wild-type MOR and mutant MORD114A receptors expressed in Neuro_{2A} cells

K_i values (average \pm standard error from three experiments) were calculated from competition binding studies using membranes of Neuro_{2A} cells expressing wild-type or mutant opioid receptors, 2 nM [³H]diprenorphine, and different concentrations of opioid ligands (1–1000 nM) as described in the text.

	K_i	
	MOR	MOR D114A
	nM	
Morphine	3.7 ± 0.2	234 ± 47
DAMGO	2.6 ± 0.15	707 ± 98
Etorphine	0.31 ± 0.03	120 ± 35
Naloxone	6.0 ± 2	9.5 ± 0.8
Diprenorphine	1.9 ± 0.31	0.9 ± 0.1

TABLE 2

K_i values of opioid ligands for wild-type DOR and mutant DORD95A receptors expressed in Neuro_{2A} cells

K_i values (average ± standard error from three experiments) were calculated from competition binding studies using membranes of Neuro_{2A} cells expressing wild-type or mutant opioid receptors, 2 nM [³H]diprenorphine, and different concentrations of opioid ligands (1–1000 nM) as described in the text.

	K _i	
	DOR	DORD95A
	nM	
DPDPE	2.8 ± 0.3	4.8 ± 0.7
DADLE	1.9 ± 0.2	5.95 ± 0.4
Naloxone	20 ± 2.6	14 ± 4.2
Naltrindole	0.4 ± 0.07	0.16 ± 0.01

another mutant MORD114N (99 ± 2.3% of control [³H]diprenorphine binding). On the other hand, when DORD95A#29 cells were treated with DPDPE, receptor down-regulation was observed. As shown in Fig. 6B, concentrations of 1, 5, or 10 μM DPDPE, which was high above their K_d value to DORD95A (Table 2) in these cells, for 24 hr resulted in an average of 38 ± 2% decrease in [³H]diprenorphine binding. Similar to the results with PTX, although the δ-opioid agonists could not activate the mutant receptor, they remained potent for the induction of receptor down-regulation.

There is an apparent correlation between the ability of receptors to form the ternary high affinity complexes with the heterotrimeric G proteins and the ability of agonists to induce receptor down-regulation. Formation of ternary high affinity complexes could be demonstrated simply by the ability of guanine nucleotide to decrease agonists affinities or shift the agonist competition curves to the right (34, 35). Opioid receptor coupling and subsequent activation of the heterotrimeric G proteins require the presence of monovalent cation Na⁺ (36). Therefore, under the coupling conditions (i.e., in the presence of Na⁺ and guanine nucleotide GTP), opioid receptors exist in multiple affinity states (37, 38). As shown in Fig. 7, PTX treatment, which reduces the ability of opioid agonist DPDPE to induce receptor/G protein coupling, reduced the affinity of DPDPE for DOR. Similarly, addition of Na⁺ and Gpp(NH)p resulted in a further right shift of the competition concentration-dependent curve (Fig. 7). Interestingly, Na⁺ and Gpp(NH)p could still decrease DPDPE affinity in DORD9 cells after PTX pretreatment. As summarized in Table 3, Na⁺ and Gpp(NH)p decreased DPDPE affinity by 7-fold in DORD9 cells pretreated with PTX, whereas the same agents decreased the affinity by 13-fold in control cells without pretreatment. In contrast, Na⁺ and Gpp(NH)p decreased morphine affinity in MORIA3 cells by 68-fold but only 4.5-fold in same cells pretreated with PTX (Table 3). Furthermore, although PTX pretreatment decreased DPDPE affinity by ~5-fold in DORD9 cells, it decreased morphine affinity by ~17-fold in MORIA3 cells. These data suggest that after PTX treatment, a higher percentage of the DORs existed in the high affinity complexes with G proteins compared with the MORs after similar PTX treatment.

It has been found that MORD114A mutation converted MORs into a state incapable of forming high affinity complexes with G protein. As summarized in Table 3, morphine affinity for the mutant MORD114A receptors in the presence of Mg²⁺ was similar to that of the wild-type MOR in the

presence of Na⁺ and Gpp(NH)p. The addition of Na⁺ and Gpp(NH)p to the competition binding assays did not alter morphine affinity for MORD114A. Similarly, PTX pretreatment of MORD114A could decrease morphine affinity by only 2-fold. These data confirmed that MORD114A receptors in Neuro_{2A} cells were unable to associate with G proteins. In contrast, mutation of Asp95 in DOR to alanine did not drastically reduce the DPDPE affinity. There was a 2-fold decrease in DPDPE affinity compared with the wild-type DOR (Table 2). Interestingly, Na⁺ and Gpp(NH)p reduced DPDPE affinity only 2-fold in DORD95A mutant. The DPDPE affinity for the mutant receptor in the presence of these two agents was even higher than that observed with wild-type receptors. This is in agreement with other G protein-coupled receptors studies, indicating that this conserved aspartic acid in the TM2 domain is involved in the Na⁺ cation effects on receptor functions (32, 33). The lack of Na⁺ and Gpp(NH)p effect on the DPDPE affinity for DORD95A could be demonstrated further by the inability of these two agents to alter the affinity of this agonist in cells pretreated with PTX. These data suggested that although DPDPE could not activate DORD95A, the ability of this mutant receptor to form high affinity complexes with G proteins remained.

Discussion

The current study clearly demonstrates the role of G protein coupling in agonist-induced MOR down-regulation. Uncoupling of MORs from G proteins by PTX pretreatment not only inactivated receptors but also prevented them from down-regulation after chronic agonist treatment. Furthermore, Asp114 mutant MORs, which could not form an agonist/receptor/G protein ternary complex, failed to down-regulate. In contrast to the results obtained with MORs, results from the current study showed that DORs could be down-regulated even though they were functionally uncoupled by PTX pretreatment, as previously suggested by observations in NG108–15 cells (23). In addition, Asp95 mutant DORs, which did not activate receptor/G protein/ligand ternary complex, were able to mediate DPDPE-induced DOR down-regulation.

These data collectively indicate that unlike MORs, DORs may not require an active G_i/G_o interaction for receptor down-regulation. This is the first demonstration of differences in functional regulation among opioid receptor subtypes. This is also somewhat surprising because both receptors belong to the opioid family of receptors bearing high homology (64%) to each other (7) and are colocalized in some brain areas with similar affinities to endogenous opioids such as enkephalins and endorphins (12).

Previous studies in NG108–15 cells reported that opioid receptors exist in multiple affinity states (38, 39). In the high affinity state, receptors should be able to form a receptor/G protein/ligand complex and turn on effector signals when the ligand is an agonist. The intrinsic activity of the ligand was also reported to reflect the percentage of high affinity receptors for β₂-adrenergic receptors (39). However, when DORs in NG108–15 cells were pretreated with PTX, under which conditions they could not regulate adenylyl cyclase activity, ~40% of receptors remained in the high affinity state (24). This interesting phenomenon was also observed in the current study when part of the population of cloned DORs ex-

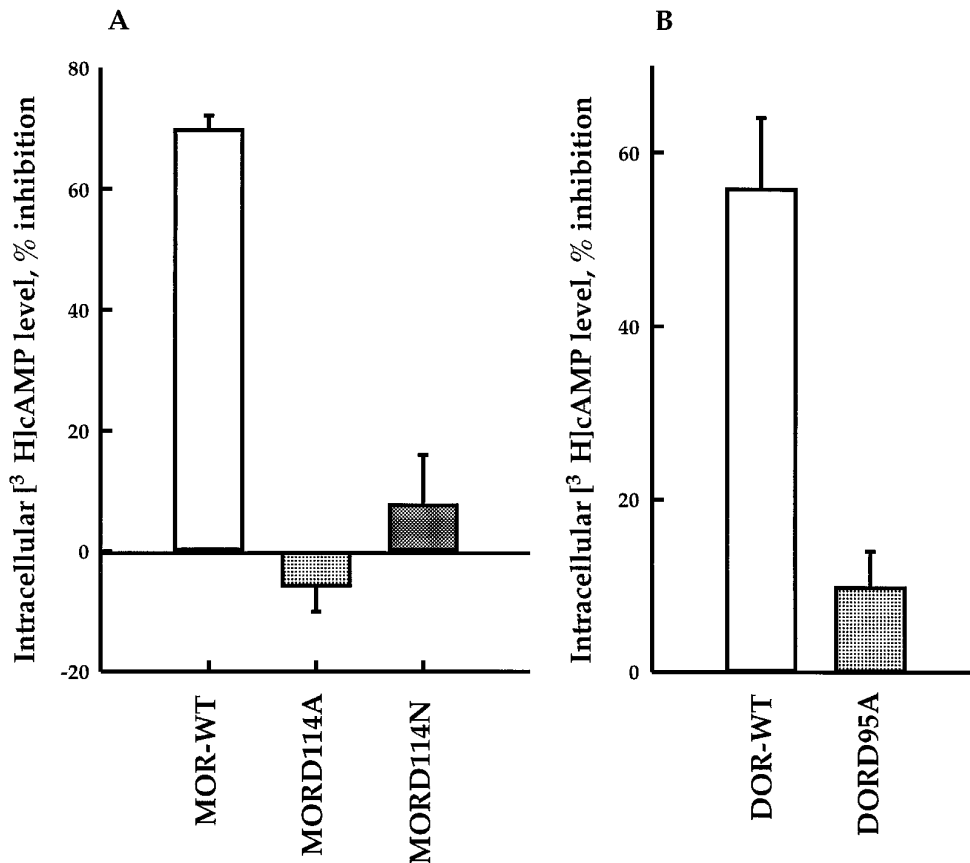


Fig. 5. Agonist-induced regulation of intracellular [3 H]cAMP level in wild-type and mutant opioid receptors. Comparative views of selective agonist-induced regulation of forskolin-stimulated intracellular cAMP levels in wild-type and TM2 Asp mutants of MOR and DOR, respectively (details are given in the text). Briefly, cAMP levels of cells plated onto 17-mm plates were labeled with [3 H]adenine and measured after treatment with forskolin ($10 \mu\text{M}$) in the absence and presence of μ - and δ -selective opioid agonists at 37° for 15 min. Unlike that of wild-type receptors, significant opioid agonist-induced inhibition was not found in mutant MORD114A, MORD114N, and DORD95A receptors. Bars, average \pm standard error of triplicate determinations.

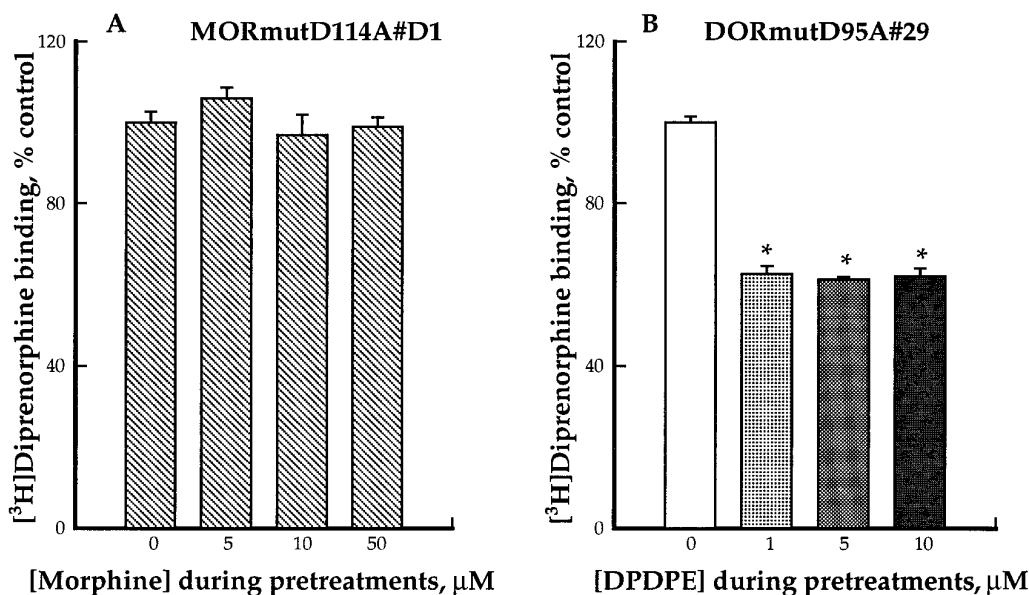


Fig. 6. Comparison of receptor down-regulation with selective agonists using mutant opioid receptors MORD114A and DORD95A. No significant differences in receptor densities were observed in MORD114A Neuro_{2A} cells after 10–50 μM morphine pretreatment for 24 hr from that of control cells without pretreatments (A). In contrast, a significant decrease of receptor density was observed in DORD95A Neuro_{2A} cells after 24 hr of 1–10 μM DPDPE pretreatment (B). Each bar, [3 H]diprenorphine binding (percent of control) expressed as average \pm standard error of triplicate determinations. Each experiment was repeated twice. *, Significantly ($p < 0.0005$) different from control. Receptor densities in selected clones expressing MORD114AD1 and D95A#29 were 2.1 ± 0.1 and 0.59 ± 0.1 pmol/mg of protein, respectively.

pressed in Neuro_{2A} cells were in an $\text{Na}^+/\text{Gpp}(\text{NH})\text{p}$ -sensitive high affinity state even after PTX pretreatment.

Competition binding studies, which showed the PTX pretreated wild-type or Asp95 mutant DORs to retain some high affinity binding sensitive to Na^+ and $\text{Gpp}(\text{NH})\text{p}$, suggest a physical association between DORs and G proteins. Thus, previous studies in NG108–15 cells and current results suggest that PTX pretreatment does not totally uncouple DORs from G proteins. A reasonable explanation could be a tight association between the DOR and G proteins, as previously

suggested (36). However, this is not the case for MORs. Previous studies have observed that PTX pretreatment functionally uncoupled opioid receptors from their effector mechanisms (31), and in the current study, we find not only functional uncoupling but also a profound decrease of affinity of morphine after PTX pretreatment. Thus, PTX pretreatment not only functionally but physically uncouples MORs from G proteins. This drastic decrease of high affinity sites after PTX treatment, which also affected chronic agonist-induced MOR down-regulation, suggests a high affinity bind-

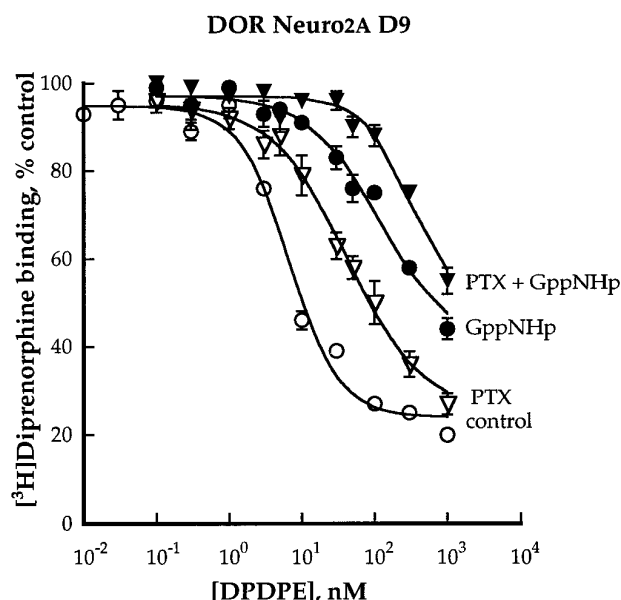


Fig. 7. Competition binding studies using [^3H]diprenorphine and different concentrations of DPDPE in Neuro_{2A} cells expressing DORs. Binding assays were carried out with membranes prepared from Neuro_{2A} cells expressing DORs with and without PTX (100 ng/ml, 4 hr) pretreatment in the presence of MgCl_2 (10 mM) or Gpp(NH)p (10 μM) and NaCl (100 mM) as described in the text. Data points, average \pm standard error of triplicate determinations that are representative of two identical experiments. The different K_i values are described in Table 3.

TABLE 3

Relative affinities of selective agonists for wild-type MORs, DORs, mutant MORD114A, and mutant DORD95A

	Control		PTX pretreated	
	Mg^{2+}	Na^+ Gpp(NH)p	Mg^{2+}	Na^+ Gpp(NH)p
	nM			
MOR morphine	3.7 ± 0.2	253 ± 73	65 ± 5	290 ± 48
MORD114A morphine	234 ± 47	157 ± 26	461 ± 122	601 ± 120
DOR DPDPE	2.8 ± 0.3	37 ± 15	13.5 ± 2.1	93 ± 39
D95A DPDPE	4.8 ± 0.7	10 ± 1.9	9.1 ± 0.6	14 ± 1.5

K_i values of selective opioid agonists (average \pm standard error of three experiments) from control with no pretreatment and PTX-pretreated Neuro_{2A} cells expressing MOR, DOR, MOR D114A, and DOR D95A. K_i values were calculated from competition binding studies using membranes from different clones expressing wild-type or mutant receptors, 2 nM [^3H]diprenorphine with or without selective agonists in the presence of either 10 mM MgCl_2 or 10 μM Gpp(NH)p, and 100 mM NaCl as described in the text. Morphine was chosen as a μ -selective opioid agonist for MOR and DPDPE and a δ -opioid selective agonist for DOR.

ing conformation or G protein interaction necessary for the opioid receptor down-regulation. The fact that DORs could exist in high affinity state after PTX treatment or Asp95 mutation and could be down-regulated supports the hypothesis of requirement of physical interaction between the receptor and G protein for down-regulation. This hypothesis has been demonstrated previously with β_2 -adrenergic receptors. Mutation and deletion analysis revealed regions involved in G protein coupling of this receptor, without which it fails to undergo rapid internalization on agonist stimulation (40). Detailed examination of these deletion mutant β_2 -adrenergic receptors suggested that a secondary structure of the receptor was also important in addition to its primary sequence for the interaction between the receptor and G_s and, hence, its internalization (40).

In conclusion, data from the current study suggest that a tight physical association of G proteins and opioid receptor is critical for agonist-induced receptor down-regulation. Even after PTX treatment, DORs retained high affinity binding complexes, permitting internalization and down-regulation after chronic agonist treatments. Furthermore, the ability of agonist to down-regulate mutant DORD95A also suggested that activation of G proteins by DORs might not be required for receptor down-regulation. The importance of high affinity G protein/receptor complexes for opioid receptor down-regulation was demonstrated in MOR down-regulation. Receptor down-regulation was not observed with PTX pretreatment, which severely decreased the affinity of MORs for morphine or DAMGO, indicating that a decrease of physical or functional association accounted for the lack of chronic agonist-stimulated receptor down-regulation.

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Send reprint requests to: Dr. S. Chakrabarti, Department of Biochemistry, Box 8, State University of New York, Health Sciences Center at Brooklyn, 450 Clarkson Ave., Brooklyn, NY, 11203-2098. E-mail: schakrabarti@netmail.uscbklyn.edu
