0026-895X/97/050896-07$3.00/0

MOLECULAR PHARMACOLOGY, **52:**896–902 (1997).

Down-regulation of μ-Opioid Receptor by Full but Not Partial Agonists Is Independent of G Protein Coupling

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Received March 28, 1997; Accepted July 22, 1997

SUMMARY

In C_6 glial cells stably expressing rat μ-opioid receptor, opioid agonist activation is negatively coupled to adenylyl cyclase through pertussis toxin-sensitive G proteins. In membranes, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) increases guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate (GTP[γ-^{35}S]) binding by 367% with an EC_{50} value of 28 nM. Prolonged exposure to agonists induced desensitization of the receptor as estimated by a reduction in the maximal stimulation of GTP[γ-^{35}S] binding by DAMGO and rightward shifts in the dose-response curves. In cells treated with 10 μM concentrations of etorphine, DAMGO, β-endorphin, morphine, and butorphanol, DAMGO-stimulated GTP[γ-^{35}S] binding was 58%, 149%, 205%, 286%, and 325%, respectively. Guanine nucleotide regulation of agonist binding was correspondingly lower in membranes from tolerant cells. Furthermore, chronic opioid treatment increased forskolin-stimulated adenylyl cyclase activity, and potency of DAMGO to inhibit cAMP accumulation was lower in morphine- and DAMGO-tolerant cells (EC_{50} = 55 and 170 nM versus 18 nM for control). Chronic treatment with agonists reduced [^{3}H]DAMGO binding in membranes with the rank order of etorphine > DAMGO = β-endorphin > morphine > butorphanol, and the affinity of DAMGO in alkaloid- but not peptide-treated membranes was significantly lower in comparison with control. Pertussis toxin treatment of the cells before agonist treatment did not prevent the down-regulation by full agonists; DAMGO and etorphine exhibited ~80% internalization, whereas the ability of partial agonists was greatly impaired. In addition to establishing this cell line as a good model for further studies on the mechanisms of opioid tolerance, these results indicate important differences in the inactivation pathways of receptor triggered by full and partial agonists.

Opioid receptors are activated by endogenous opioid peptides and alkaloids, which cause a multitude of important physiological functions. Recent cloning of μ-, δ-, and κ-opioid receptors showed that these proteins contain seven transmembrane domains and belong to the family of GPCRs (1). The μ-opioid receptor is the molecular target for potent analgesics such as morphine and fentanyl, which are indispensable in the management of pain despite their abuse potential (2). The biochemical mechanisms of tolerance have been studied in many systems, including cell lines containing δ-opioid receptors such as N4TG1 (3) and NG108–15 (4) cells. Although studies conducted in the central nervous system often led to inconsistent results due to the heterogeneity of the system, experiments carried out in a single brain region, such as locus ceruleus, demonstrated the physiological relevance of the cellular model originally proposed by Sharma *et al.* (5) in NG108–15 cells based on the alterations in the opioid/AC system. Subsequent studies using 7315c (6) and SH-SY5Y (7, 8) cells examined altered properties of μ-opioid receptor/effector components during tolerance; however, the exact mechanisms involved in this process are largely unknown. To study the molecular mechanisms of μ-opioid receptor selectively, we transfected C_6 glial cells that express many other receptors, but not opioid receptors (9), with the rat μ receptor cDNA. Transfected μ receptor in these cells is coupled to AC through PTX-sensitive G proteins (10). We characterized opioid agonist efficacies (11) and showed that this cell line exhibits sodium regulation of receptor in much the same fashion as SH-SY5Y cells (12).

The major goal of the current study was to investigate the molecular changes involved in the development of tolerance by different agonists of varying efficacies. In the C_6 cell line stably expressing high levels of μ receptor (~8 pmol/mg), tolerance to peptides and alkaloids was induced, and alterations were examined at every step of the signal transduction pathway (i.e., ligand/receptor interactions, G protein and effector functions). The diminished receptor activation of G protein, as measured by agonist stimulation of GTP[γ^{35}S] binding (11), reflected the desensitization that could be com-

This work was supported by the United States Public Health Service Grant DA04087.

ABBREVIATIONS: GPCR, G protein-coupled receptor; DAMGO, [D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]-enkephalin; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; PTX, pertussis toxin; AC, adenylyl cyclase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pared with a reduction in the guanyl nucleotide regulation of the ligand/receptor interactions. Down-regulation of the receptor was induced by all ligands, including partial agonists, but the extent was dependent on the efficacy of the agonist used for inducing tolerance. We also show that down-regulation by full agonists proceeds almost completely in the absence of a functional G protein, whereas partial agonists exhibit only a marginal effect. Finally, changes observed in the AC system establish the validity of this cell line as a model for further studies on opioid tolerance.

Experimental Procedures

Materials. [^{3}H]DAMGO (60 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). GTP[γ-^{35}S] (1300 Ci/mmol) was from Dupont-New England Nuclear (Boston, MA). [^{3}H]Naloxone (57.5 Ci/mmol) was from New England Nuclear Research Products (Boston, MA). [^{3}H]Naltrexone (9.2 Ci/mmol) was provided by the National Institute on Drug Abuse (Bethesda, MD). Biochemicals, including Dulbecco's modified Eagle's medium, were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum and geneticin were from GIBCO (Grand Island, NY). PTX was purchased from List Biochemicals (Campbell, CA). Unlabeled opioids were obtained through the Narcotic Drug and Opiate Peptide Basic Research Center at the University of Michigan.

Cell culture and treatments. C_6 glial cells were transfected with the rat μ receptor cDNA and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and geneticin (1 mg/ml) essentially as described previously (11).

Tolerance to drugs and peptides was induced by adding 10 μl of each agonist (10 mM) to a culture flask (10 ml) followed by incubation at 37° for 24 hr in a typical experiment. In experiments to establish the time dependence of tolerance, the incubation was stopped at various periods of time as described below. PTX was added (20 ng/ml) for 24 hr to inactivate the inhibitory G proteins. In experiments designed to study the receptor down-regulation, cells were grown for an additional 12 hr in the presence of 10 μM concentrations of agonists.

At the end of each incubation, cells were washed four times with phosphate-buffered saline and lifted off the flask surface by incubation with Versene buffer (5 mM HEPES, 5 mM KCl,137 mM NaCl, 1 mM EGTA, 5.6 mM glucose, pH 7.4) for 5 min and spun (5 min at 200 $\times$ *g*) and the pellet was resuspended in physiological buffer A (128 mM NaCl, 2.4 mM KCl, 2.0 mM $NaHCO_3$, 3.0 mM $MgSO_4$, 10 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 10 mM glucose, pH 7.4, at 37°), incubated at 37° for 10 min, and pelleted. This cell pellet was used either to prepare membranes or to conduct adenylate cyclase assays in whole cells.

Membrane preparation. The washed cell pellet was lysed in hypotonic phosphate buffer (0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, pH 7.4), homogenized in a Dounce tissue dispenser, and centrifuged at 20,000 $\times$ *g* for 20 min. The pellet was resuspended in 50 mM Tris·HCl, pH 7.4, and aliquots were frozen at −80°. These membranes were used for ligand and [GTPγ^{35}S] binding.

Ligand binding. Saturation binding of DAMGO was carried out in 50 mM Tris·HCl, pH 7.4. The assay mixture contained various concentrations of [^{3}H]DAMGO (0.5–16 nM) and 10–15 μg of membrane protein in a final volume of 500 μl. [^{3}H]Naloxone binding was carried out in the presence of 100 mM NaCl. Nonspecific binding was determined with the addition of 10 μM DAMGO/naltrexone. After incubation to achieve equilibrium at 25°, samples were quickly filtered and subjected to liquid scintillation counting. Details particular to each experiment are given in the figure legends.

GTP[γ^{35}S] binding. The assay mixture contained (in a final volume of 100 μl) 50 mM Tris·HCl, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 50 μM GDP, and 50 pM GTP[γ^{35}S]. Agonist stimulation of basal GTP[γ^{35}S] binding (11) was estimated by the addition of 10 μl of DAMGO (final concentrations, 1 nM to 10 μM). Nonspecific binding was measured in the presence of 10 μM cold GTPγS. Incubation was started by the addition of 40 μl of membranes (5–15 μg of protein). After incubation at 25° for 30 min, binding was terminated by the addition of 2 ml of ice-cold wash buffer (50 mM Tris·HCl, 5 mM $MgCl_2 \cdot 6H_2O$, 100 mM NaCl), and the contents were filtered through GF/C filters.

Adenylyl cyclase assay. Cells were collected and resuspended in physiological buffer A containing 8 mM theophylline. Cells (15–25 μg of protein/50 μl) were added to tubes containing 10 μM forskolin and various concentrations of DAMGO in a final volume of 100 μl and incubated at 37° for 15 min. Enzyme activity was stopped by the addition of 50 μl of 0.15 M HCl and heating the samples at 70° for 2 min. These samples were then frozen at −80° until further use. After thawing and neutralization of the samples with Tris base, the content of cAMP was determined with a radioligand binding assay kit (Diagnostic Products, Los Angeles, CA).

Protein determination. Protein concentration was estimated according to Lowry *et al.* (13) using bovine serum albumin as standard in samples solubilized in 1 N NaOH for 60 min at 37°.

Data analysis. The data from ligand binding experiments were analyzed using Prism (GraphPAD, San Diego, CA), and the data from saturation experiments were fit to a single binding site. Dose-response curves for AC assays and GTP[γ^{35}S] binding studies were obtained by fitting the data to a sigmoidal curve using the same program. Radioligand displacement curves were fit using a one-site binding curve with variable slope.

Results

To study the mechanism of tolerance, cells were treated with 10 μM DAMGO for various time points, and agonist-stimulated GTP[γ^{35}S] binding was measured in membranes prepared from control and tolerant cells. As presented in Fig. 1, the magnitude of DAMGO-stimulated GTP[γ^{35}S] binding

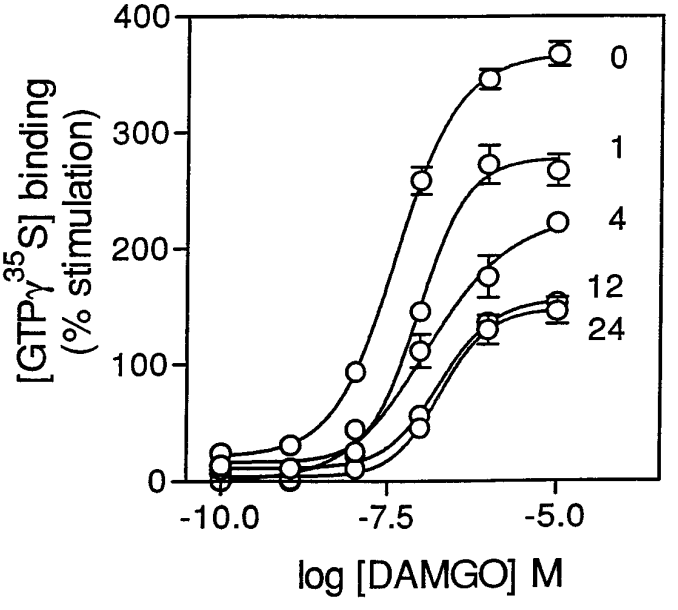

Fig. 1. GTP[γ^{35}S] binding in membranes. $C_6\mu$ cells were exposed to 10 μM DAMGO for various periods of time [1–24 hr (*right of each curve*)] and washed with phosphate-buffered saline, and crude membranes were prepared as described in the text. DAMGO stimulation of GTP[γ^{35}S] binding was determined with 50 pM GTP[γ^{35}S] in the presence of 0.1 nm to 10 μM DAMGO, and data are expressed as binding relative to that obtained in the absence of agonist. Results are from a representative experiment that was repeated twice.

decreased with the time of DAMGO preincubation. DAMGO stimulated GTP[γ^{35}S] binding by 360–390% over basal levels (measured in the absence of any agonist) with an EC_{50} value of 28 ± 6 nM. The number of GTP[γ^{35}S] binding sites stimulated by 10 μM DAMGO was 7.93 ± 0.73 pmol/mg of protein. One-hour treatment of cells with 10 μM DAMGO reduced the maximal stimulation to 280% and decreased the potency to 96 nM. Treatment of the cells with same concentration of DAMGO for prolonged periods of time further reduced the maximal levels with a concurrent drop in the potency of the agonist, thus demonstrating the uncoupling of the receptor from the G protein. Importantly, the basal values of GTP[γ^{35}S] binding measured in the absence of DAMGO in all samples were similar, showing that the cells were washed thoroughly and free of the ligand (cpm values from a typical experiment are 90 for control, 109 for 2 hr, 115 for 12 hr, and 109 for 24 hr). These results are in line with the reduced GTPase activity in NG108–15 cells under the conditions of tolerance (14).

The ability of different agonists to uncouple the receptor from G protein was examined by inducing tolerance to 10 μM concentrations of various agonists and measuring DAMGO stimulation of GTP[γ^{35}S] binding in membranes. As presented in Table 1, maximal stimulation was reduced in tolerant samples, in accordance with the efficacies of the agonists (11). Although etorphine pretreatment reduced the agonist stimulation of GTP[γ^{35}S] binding to 58%, DAMGO pretreatment decreased it from 390% in control to 149%; butorphanol, a partial agonist, showed a minimal reduction to 325%. In addition, the potency of DAMGO to stimulate GTP[γ^{35}S] binding was decreased.

In addition to the uncoupling and desensitization of the receptor, biochemical changes in the effector system are known to occur in response to persistent stimulation (7). DAMGO, morphine, and butorphanol inhibit forskolin-stimulated cAMP levels in these cells with EC_{50} values of 18, 30, and 60 nM and maximal inhibitions of 80%, 60%, and 40% (data not shown). When tolerance was induced in these cells, with 10 μM concentrations of DAMGO and morphine, potency of DAMGO to inhibit the forskolin-stimulated cAMP levels was reduced with no attenuation of the maximal inhibition. The dose-response curves generated from these experiments yielded EC_{50} values of 18 ± 7.0, 163 ± 37, and 59 ± 5.0 nM for control and DAMGO- and morphine-treated samples, respectively (Fig. 2). In SH-SY5Y cells, a 4-fold shift was produced

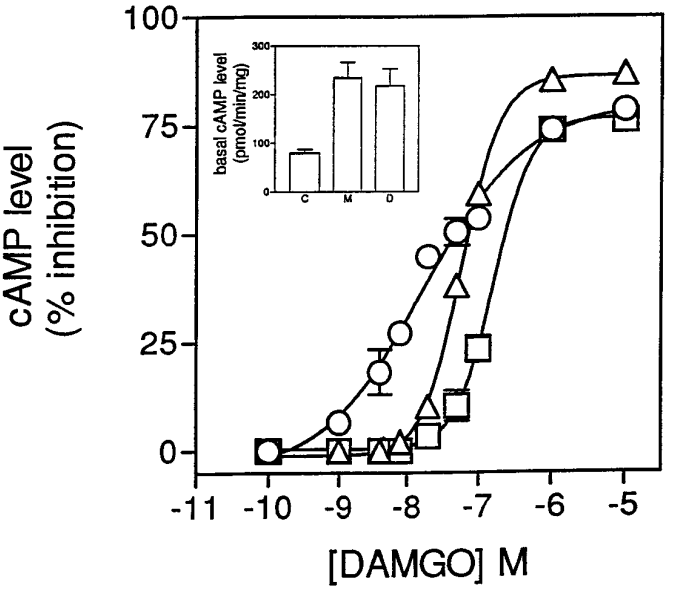

Fig. 2. Opioid inhibition of forskolin-stimulated AC activity in intact cells. After pretreatment with 10 μM DAMGO (□) or morphine (△) for 24 hr, $C_6\mu$ cells were collected and AC activity was assayed in control (○) as well as agonist-treated samples in the presence of DAMGO (1 nM to 10 μM) and 10 μM forskolin at 37° for 15 min. The accumulated cAMP levels were measured by radioimmunoassay as described in the text. *Inset*, forskolin-stimulated cAMP levels in control (*C*) and DAMGO- (*D*) or morphine- (*M*) treated cells in the absence of opioid agonist. Values are mean ± standard error.

by prolonged morphine exposure (15). In addition, as depicted in Fig. 2 (*inset*), there was a compensatory increase in the basal cAMP levels in the agonist-pretreated samples. The cAMP levels increased by 2.5-fold in DAMGO-treated cells [from 75 ± 4.5 (control) to 187 ± 12 pmol/min/mg] and 2.7-fold in morphine-treated cells (202 ± 6.0 pmol/min/mg).

Binding properties of the receptor in the state of tolerance were determined to examine whether the observed alterations in the signal transduction pathway were due to any changes in the ligand/receptor interactions. [^{3}H]DAMGO binding was studied in membranes prepared from cells treated with 10 μM concentrations of various agonists for 24 hr (Table 2). The number of total binding sites (B_{max}) decreased with the agonist treatment; a full agonist like etorphine reduced the B_{max} value by >95%, but butorphanol, a partial agonist, decreased it by 50%. Both the peptides tested (β-endorphin and DAMGO) diminished the binding by 80% in

TABLE 1

DAMGO stimulation of [GTPγ^{35}S] binding in membranes from agonist-pretreated cells

Confluent cells in medium were incubated with 10 μM of each agonist for 24 hr; then, cells were washed with PBS and collected, and membranes were prepared as described in Experimental Procedures. [GTPγ^{35}S] binding to membranes was measured in the presence of DAMGO (1 nM to 10 μM) at 25° for 30 min. Data are expressed as percentage stimulation over that obtained in the absence of added DAMGO.

Treatment	EC_{50}	Maximal stimulation
	nM	*% over control*
Control	28 ± 6	367 ± 21
DAMGO	209 ± 14	149 ± 10
Morphine	103 ± 10	286 ± 16
Butorphanol	58 ± 18	325 ± 9
Etorphine	>600	58 ± 7
β-Endorphin	240 ± 12	205 ± 9

Values are mean ± standard error from four or five experiments.

TABLE 2

Parameters of [^{3}H]DAMGO binding in membranes

Equilibrium binding of [^{3}H]DAMGO was carried out in membranes prepared from control and agonist-treated cells (10 μM for 24 hr) as described in Experimental Procedures.

Treatment	[^{3}H]DAMGO binding		
	K_d	B_{max}	Reduction in B_{max}
	nM	*fmol/mg*	*%*
Control	0.48 ± 0.11	8219 ± 582	
DAMGO	0.39 ± 0.046	1465 ± 280	82
Morphine	1.29 ± 0.24	3373 ± 423	59
Butorphanol	2.17 ± 0.57	4140 ± 434	50
Etorphine	3.63 ± 0.51	244 ± 59	97
β-Endorphin	0.41 ± 0.06	1212 ± 60	85

Values are mean ± standard error from three or four experiments.

accordance with their capacities to function as full agonists. All the alkaloids, but not the peptides, used for inducing tolerance reduced the affinity of the receptor for [^{3}H]DAMGO. In preliminary experiments using [^{3}H]morphine and [^{3}H]naloxone for binding, we obtained similar results. [^{3}H]Morphine binding in control and DAMGO- and morphine-treated samples exhibited K_d values of 2.94, 4.02, and 8.11 nM, whereas [^{3}H]naloxone binding gave dissociation constants of 1.13, 1.32, and 3.1 nM (mean; two experiments). A partial agonist like morphine binds to as many sites as a full agonist in this system; [^{3}H]morphine binding yields a B_{max} value of 8.5 ± 0.6 pmol/mg of protein. In these studies, binding in C6μ cells increased from ~4 to 10 pmol/mg of protein with the number of passages. This increase in receptor number may be due to the selection of receptor-expressing cells during culture in the presence of geneticin. Although same results were obtained in cultures expressing either 4 or 8–10 pmol/mg of receptor, all the experiments were conducted in cells from passages expressing consistently 8–10 pmol/mg of receptor.

To evaluate the sensitivity of agonist binding to guanine nucleotides in these membranes, [^{3}H]DAMGO binding was studied in the presence of 10 μM GTPγS (Fig. 3). GTPγS regulation of [^{3}H]DAMGO binding was maximal in control membranes; it inhibited 88% of the specific binding. Tolerance to agonists markedly reduced the modulation of receptor by GTPγS as revealed by the increased [^{3}H]DAMGO binding in presence of this agent in comparison with control, as reported previously (16). The inhibition of agonist binding in membranes prepared from etorphine-, DAMGO-, morphine-, and butorphanol-treated cells was 39%, 59%, 73%, and 87%, respectively. It is remarkable that even in membranes tolerant to full agonists, there remains a considerable extent of binding that is modulated by guanine nucleotides.

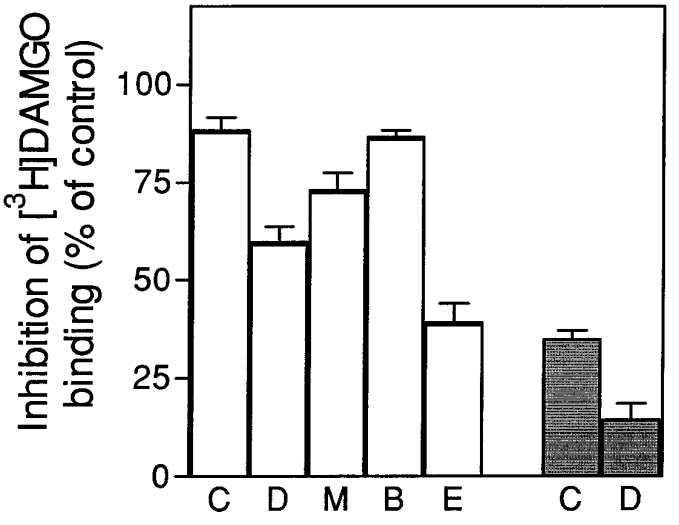

Fig. 3. Effect of GTPγS on agonist binding in membranes. Confluent C$_6\mu$ cells were treated with 10 μM concentrations of various ligands for 24 hr, and membranes were prepared as described in the text. [^{3}H]DAMGO binding (1.2 nM) was studied in control (*C*) and DAMGO- (*D*), morphine- (*M*), butorphanol- (*B*), and etorphine- (*E*) treated samples in 50 mM Tris·HCl, pH 7.4, buffer with 100 mM NaCl. Reduction of binding in the presence of 10 μM GTPγS is expressed as percentage inhibition for each sample. *Filled bars*, inhibition of [^{3}H]DAMGO binding obtained in the absence of sodium in control (*C*) and DAMGO-treated (*D*) membranes. Values are mean ± standard error from three independent experiments.

The same experiment was carried out in the absence of sodium (Fig. 3); inhibition of [^{3}H]DAMGO binding by GTPγS was only 34 ± 3.0% in control membranes and was further reduced in DAMGO-treated membranes, as expected (14 ± 4.1%). However, the diminished GTPγS effect in DAMGO-treated membranes in the presence of sodium constituted 67% of that observed in control, whereas it was 41% in the absence of sodium. The smaller guanyl nucleotide effect in the absence of sodium suggests that the conformation of the receptor is different and corresponds with the inefficient signal transfer by this state (12).

It was interesting that DAMGO desensitized and down-regulated the receptor without causing any apparent changes in the affinity of the protein for the ligands. To further examine whether this high affinity binding depended on the interaction of the receptor with G protein, cells were first incubated with 10 μM DAMGO for 6 hr; then, PTX (20 ng/ml) was added to the medium. Results from DAMGO displacement of [^{3}H]naltrexone binding are shown in Fig. 4. DAMGO preincubation protected the high affinity binding of agonist; EC_{50} values obtained in control and treated membranes were 10.5 ± 1.1 and 8.9 ± 1.2 nM. At the same time, preincubation with naltrexone did not prevent the conversion of high affinity receptors to a low affinity state by PTX treatment (Fig. 4).

The role of G protein in the agonist-induced down-regulation of the receptor was examined next because changes in G protein levels were reported under the conditions of tolerance (17). Cells were grown in presence of 20 ng/ml PTX for 24 hr before incubation with 10 μM DAMGO for additional 12 hr. PTX treatment completely abolished the agonist stimulation

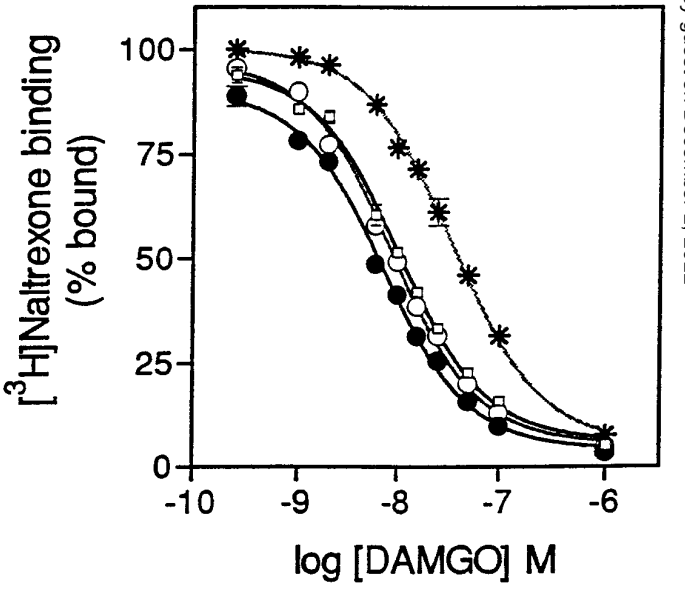

Fig. 4. Effect of PTX treatment on the high affinity binding in membranes prepared from DAMGO-tolerant cells. DAMGO was added to confluent cells at a final concentration of 10 μM followed by PTX (20 ng/ml) after 6 hr, and the incubation was continued for 24 hr. Cells were washed with phosphate-buffered saline and collected, and membranes were prepared as described in the text. Displacement of [^{3}H]naltrexone (0.5 nM) binding by DAMGO was determined in control membrane samples (□), DAMGO-treated membrane samples (●), naltrexone-treated followed by PTX-treated membrane samples (*), and DAMGO-treated followed by PTX-treated membrane samples (○). Data are from a representative experiment that was repeated three times.

of GTP[γ^{35}S] binding (Fig. 5, *inset*), yet the binding as measured by [^{3}H]naloxone was lower in DAMGO-treated membranes (Fig. 5). In an attempt to understand the nature of this phenomenon, a variety of agonists were used for pretreatment after G protein inactivation and [^{3}H]naloxone binding was estimated (Table 3). Under these experimental conditions, DAMGO caused receptor loss of ~48% compared with 58% in control membranes containing functional G protein. This corresponded to 80% (48% versus 58%) of internalization observed in cells containing active G protein. The ability of partial agonists to induce down-regulation, however, was greatly impaired in PTX-treated cells. [^{3}H]Naloxone binding decreased by only 13% and 4% in morphine- and butorphanol-treated membranes, respectively. This reduction in B_{max} value constituted 27% and 9% of the down-regulation induced by DAMGO (13% and 4% versus 48%) (Table 3). In sharp contrast, in PTX-naive cells, morphine and butorphanol displayed 72% and 61% of the effect shown by DAMGO (59% and 50% reduction in B_{max} value by morphine and butorphanol compared with 82% by DAMGO) (Table 2). To rule out the possibility of the presence of residual active G protein, cells were treated with higher concentrations of PTX (200 ng/ml) in the subsequent experiment. DAMGO induced ~45% down-regulation under these conditions, conclusively demonstrating that full agonists can indeed induce down-regulation in a fashion independent of G protein coupling.

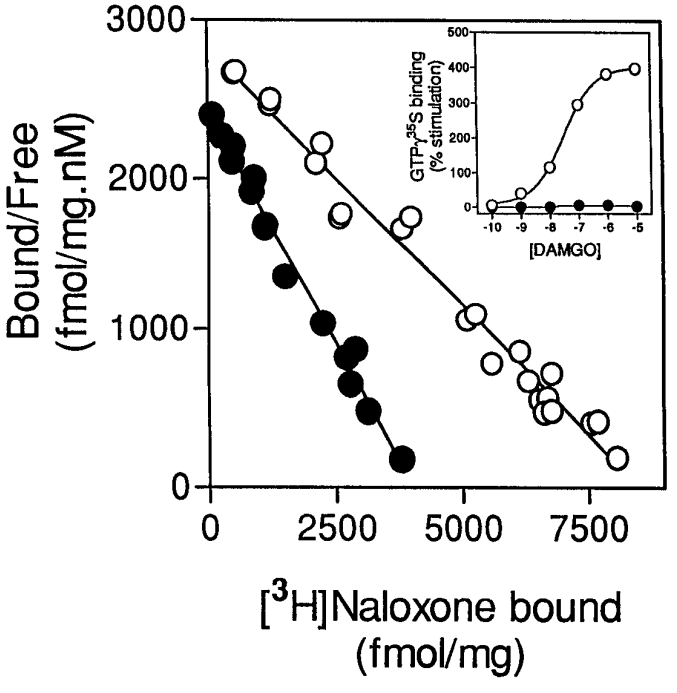

Fig. 5. Antagonist binding in membranes prepared from G protein-inactivated DAMGO-tolerant cells. Confluent cells were treated with PTX (100 ng/ml) for 24 hr, and the incubation was continued with 10 μM DAMGO for another 12 hr. At the end of incubation, cells were washed and collected, and membranes were prepared. [^{3}H]Naloxone binding was studied in samples from control (PTX treatment alone) (○) and from PTX-treated followed by DAMGO-treated samples (●) as described in the text. *Inset*, DAMGO-stimulated GTP[γ^{35}S] binding in the same samples.

TABLE 3

Parameters of [^{3}H]Naloxone binding in membranes from PTX-treated, agonist-tolerant cells

[^{3}H]Naloxone binding was measured in membranes prepared from cells treated with PTX (20 ng/ml for 24 hr) followed by 10 μM agonist for an additional 12 hr. Cells treated with only PTX in the absence of any agonist constituted control for this experiment. Under these conditions, DAMGO induced down-regulation of 58% of the [^{3}H]naloxone binding sites in cells containing functional inhibitory G protein (*b*; Table 2). Values are mean ± standard error from three experiments. Mean and range values are given for experiments repeated in duplicate.

Treatment (10 μM)	[^{3}H]Naloxone binding			
	K_d	B_{max}	Reduction in B_{max} (a)	Change[a]
	nM	*fmol/mg*	*%*	*%*
Control	1.73 ± 0.15	8617 (365)		
DAMGO	1.72 ± 0.17	4458 (68)	48	83
Morphine	2.21 ± 0.21	7480 (190)	13	22
Butorphanol	4.93 (2.28)	8244 (248)	4	7
Etorphine	5.46 (2.36)	4623 (361)	46	79

[a] Percentage of the reduction in B_{max} in PTX-treated cells (a) divided by the reduction in B_{max} in control cells (Table 2).

Discussion

In this report, we exposed cells to high concentrations of peptides and alkaloids of various efficacies to examine opioid tolerance in C6μ cells stably expressing μ receptor and attempt to understand the molecular basis underlying agonism.

Chronic treatment of the transfected cells with agonists leads to an impaired activation of the G protein; stimulation of GTP[γ^{35}S] binding by DAMGO was higher in membranes treated with partial agonists compared with full agonists, indicating the importance of efficacy of an agonist in the desensitization process (4, 7, 14, 18). Importantly, the degree of desensitization could be correlated directly to the extent of down-regulation induced by each agonist. Thus, DAMGO and butorphanol pretreatments reduced G protein activation by ~60% and ~12% of control, and the B_{max} value was reduced by 82% and 50%, respectively. Although efficacy of the ligands was related to desensitization in NG108–15 cells (4) and SH-SY5Y (8, 14, 19) cells, partial agonists failed to down-regulate the receptor in those studies. We, however, found that down-regulation of the receptor by all the ligands studied and rank order of efficacy was the same for inducing internalization and desensitization of the receptor. Moreover, affinity of the receptor in membranes tolerant to drugs, and not peptides, was significantly lower. The exact reasons for this difference are not clear, but in consideration of the hydrophobic nature of the alkaloids, the possibility of residual ligand from the pretreatments giving rise to the observed marginal differences in the affinity cannot be ruled out completely, although the low basal GTP[γ^{35}S] binding measured in membranes indicates complete washout of the drugs. Regulation of the receptor binding properties by chronic opiate treatment has been controversial (20 and references therein) in general, and it could be due to the nature and dose of the agonist used. Interestingly, time dependence of desensitization displayed rightward shifts in dose-response curves concomitant with reduction in the maximal stimulation of GTP[γ^{35}S] binding by DAMGO. Although the decrease in the stimulation can be partly accounted for by the disappearance of receptor sites from the plasma membrane, the rightward shifts may be due to the "inefficient" coupling or uncoupling

of the receptor from the transducer. Guanine nucleotide regulation of agonist binding was attenuated consequent to pretreatment with DAMGO in either the presence (59% versus 88% in control) or absence (14% versus 34%) of sodium, indicating a partial coupling of the remaining receptor sites on the membrane. Thus, guanine nucleotide sensitivity retained in the membranes was inversely related to the efficacy of the drug used for inducing tolerance in the following order: DAMGO > morphine > butorphanol. Reductions in μ-opioid activation of G proteins in specific brain regions have also been reported recently, using GTP[γ^{35}S] autoradiography (21).

Measurements of opioid inhibition of AC corroborate the findings obtained at the G protein level. Potency of DAMGO decreased in cells made tolerant to DAMGO and morphine, in this order, whereas the maximal inhibition remained unaltered. These results are in accordance with the development of tolerance and dependence, namely, the requirement for a higher dose to maintain the same physiological response (5, 22). The curves for DAMGO inhibition of forskolin-stimulated cAMP levels were steeper in tolerant cells. Although the basis for this change is not clear, it could be due to the increase in basal AC activity; the $G_{\alpha i}$ subunit released as a result of receptor activation may have to accumulate before any inhibition is observed because the basal cAMP levels were increased >2.5-fold on agonist pretreatment. The rightward shifts observed in dose-response curves for the inhibition of cAMP levels by DAMGO in cells made tolerant to DAMGO and morphine (9.2- and 3.3-fold) were comparable to 7.5- and 3.6-fold decreases in potencies, respectively, obtained in agonist-stimulated GTP[γ^{35}S] binding assay in membranes. However, in consideration of the extent of down-regulation (82%) and extensive uncoupling of the remaining 18% sites (7.5-fold reduction in potency and 2.5-fold reduction in maximal G protein activation), it is interesting to explore how the system adapted to exhibit the same maximal response at the effector level. It could be due to greater signal amplification at the effector level; alterations in the levels of G proteins may provide increased sensitivity (17). There was no stimulation of cAMP levels by opioids in the tolerant cells as in earlier studies in which opioid receptors were shown to couple to G_s and stimulate AC directly (23). However, AC supersensitization was detected when activated directly by forskolin, in a stimulatory receptor-independent manner. Increased affinity of the enzyme for modulators, including α_i/α_o, is a possibility because cycloheximide was shown to not affect the development of cAMP overshoot (24). On the other hand, an increase in the mRNA of AC type VIII, as demonstrated in the amygdala and locus ceruleus of chronic morphine-treated rats (25), may explain the enhanced forskolin interactions directly with the AC isoform that serves as the effector for the opioid system and the increased basal cAMP levels in tolerant cells.

Antagonist binding in membranes from PTX-pretreated opioid-tolerant cells illustrates several important points. Down-regulation observed in the absence of functional G protein is also dependent on the efficacies of the agonists. This implies that agonists can induce conformational changes in the receptor independent of the interactions with the G protein. Different ligands may induce different conformational changes (26) or amounts of activated receptor (27), which in turn would determine the extent of signal transmission to the effector. This isomerization of the receptor is accommodated in the extended version of ternary complex model (28). Whether these conformational changes can be equated to those that are induced in a natural environment must be pursued because G proteins are needed to stabilize the active conformation of the receptor. The data from the protection experiments also support this contention. When cells were incubated with DAMGO, but not naltrexone, before the addition of PTX, the high affinity binding was retained, providing direct evidence for the induction of conformational changes by agonists. Although DAMGO produced considerable down-regulation (82%) in PTX-treated cells, a G protein-dependent component cannot be ruled out. Partial agonists presumably need stabilization of the conformation by G protein more than do full agonists and consequently caused very little down-regulation in PTX-treated cells. Rapid regulation of opioid receptors has also been shown to depend on the agonist used (29, 30).

The intracellular domains of the receptor, exposed by ligand binding for G protein activation, may also serve as cellular trafficking signals because they contain serine/threonine residues that are phosphorylated by a variety of kinases. These in turn are bound by β-arrestins, which leads to internalization of the receptor (31). The observed differences in internalization can be explained if full and partial agonists cause different extents of phosphorylation (27). In this context, it is important to note that cAMP-dependent protein kinase (32), protein kinase C (33), and β-adrenergic receptor kinase, a GPCR kinase (34), have been implicated in opioid receptor tolerance. However, the role of phosphorylation kinases for down-regulation in the absence of signal transduction (i.e., as a result of inactivation of functional G protein) remains to be seen. On the other hand, in Chinese hamster ovary cells transfected with δ-opioid receptor, the COOH-terminal tail was shown to be necessary for down-regulation but not for functional coupling, suggesting that entirely different domains could be involved in triggering of the down-regulation of receptor (35). Nevertheless, the differences observed between full and partial agonists in our experiments suggest the involvement of distinct inactivation pathways; understanding these processes should help in the design of novel therapeutic agents with less abuse potential.

Agonist-induced down-regulation of GPCRs (36, 37) in the absence of functional coupling indicates important aspects of complexity in biological functions. The prototypic low-density lipoprotein receptor serves the function of transport of a ligand (low-density lipoprotein) into the cytoplasm. The ligand/receptor complex is internalized and delivered into the lysosomal compartment, in which the ligand dissociates from the receptor and becomes available for metabolism while the receptor is recycled back into the membrane. In the case of GPCRs, the original mode of itinerary for the agonist/receptor complex seems to be retained because full agonists (all endogenous opioid peptides are full agonists) induced almost complete down-regulation in cells devoid of functional inhibitory G proteins. The additional function of signal transfer is accomplished by the introduction of a transducer in the membrane domain of the receptor; the same mechanism is useful in protecting the cell from excessive signal.

In summary, we examined systematically alterations in the properties and signal transduction of μ receptor stably transfected into the C_6 glial cells under the state of tolerance

to various agonists and demonstrated that this transfected cell system is a good model for further studies. Experiments are in progress to elucidate the biochemical mechanisms involved in the development of tolerance to different drugs, especially at the receptor/G protein interface, using membranes that are free of active G protein.

Acknowledgments

We thank Dr. Huda Akil for providing the $C_6\mu$ cell line and Dr. Ann E. Remmers for critical review of the manuscript.

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