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Agonist-Induced Functional Desensitization of the μ-Opioid Receptor Is Mediated by Loss of Membrane Receptors Rather than Uncoupling from G Protein

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SUMMARY

The effects of acute exposure of the opioid peptide [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) on the μ-opioid receptor were examined in Chinese hamster ovary (CHO) K-1 and baby hamster kidney stable transfectants. In the CHO cell line, acute 1-hr treatment with DAMGO decreased the density of receptors without affecting the affinity or proportion of agonist-detected sites and attenuated the ability of the agonist to inhibit forskolin-stimulated cAMP accumulation. In contrast, similar 1-hr treatment of baby hamster kidney cells did not affect receptor density or agonist ability to inhibit cAMP accumulation, but longer duration of agonist exposure resulted in a reduction in membrane receptor, identical to the CHO cells. These results suggested that for the μ-opioid receptor, alteration in receptor density was the major determinant for the observed agonist-induced desensitization. Consistent with this notion, the ratio of the DAMGO concentration yielding half-maximal occupation of the μ receptor to that yielding half-maximal functional response was <1. This suggests the necessity for a high μ receptor occupancy rate for maximal functional response, so that any loss of cell surface opioid-binding sites was a critical determinant in reducing the maximal response. This hypothesis was further supported by the observation that irreversible inactivation of fixed proportions of opioid-binding sites with β-chlornaltrexamine demonstrated that there were few spare receptors, which is in contrast to what has been reported for other G protein-coupled receptors, including the δ-opioid receptor. Taken together, these data suggest that the opioid agonist DAMGO has a high affinity for the μ receptor but must occupy >70% of the available receptors to generate the maximal second messenger-linked response.

The cellular effects of hormones and neurotransmitters are regulated at several levels, most notably at the level of the receptor. Even in the continued presence of hormone or neurotransmitters, the cellular response can be attenuated by modulation of the agonist affinity, G protein-coupling efficiency, or density of receptors. This phenomenon of desensitization is observed with almost every receptor and can be separated into two categories depending on agonist specificity. For the β-adrenergic receptor and rhodopsin, agonist-specific homologous desensitization occurs rapidly, within minutes, through uncoupling of receptor from G proteins (1, 2), whereas heterologous desensitization occurs much more slowly (1, 2).

In contrast to what has been considered prototypic for G protein-coupled receptors based on the above examples, it has become evident that the mechanisms of desensitization may vary among individual receptors. We are particularly interested in the opioid receptor as one of the primary mediators of analgesia and for its potential role in drug addiction. Better understanding of the mechanism of addiction and dependence that is linked to receptor desensitization (3, 4) may provide useful information for the design of therapeutic agents without undesirable side effects. Previous studies have shown that chronic opioid agonist treatment of the endogenous δ-opioid receptor in NG108-15 cells or the cloned κ receptor expressed in COS-7 cells produces at least two separate cellular adaptive processes. In both receptor subtypes, chronic exposure to opioid agonist resulted in reduced affinity of receptor for agonist, with receptor uncoupling from G protein resulting in diminished ability to inhibit cAMP accumulation (5–7). The second adaptation occurred with more prolonged exposure to agonist and involved down-regulation of receptor (8, 9).

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ABBREVIATIONS: CHO, Chinese hamster ovary; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; BHK, baby hamster kidney; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GRK; G protein-coupled receptor kinase; β-CNA, β-chlornaltrexamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline.

In the current study, we examined the mechanism of desensitization of the cloned μ-opioid receptor and found striking differences from that described for the prototypic G protein-coupled receptors and even for the other opioid receptors. We also report interesting differences in the μ-opioid receptor expressed in CHO and BHK cells in terms of functional efficacy and desensitization after agonist activation. Thus, the μ receptor expressed in CHO cells demonstrated more rapid desensitization and receptor down-regulation concurrently, whereas the μ receptor expressed in BHK cells was more resistant to acute desensitization and did not show any decrease in receptor-binding sites until more prolonged agonist exposure occurred. Our results suggest that for the μ-opioid receptor, down-regulation of receptor is the predominant mechanism mediating the loss of agonist-dependent, forskolin-stimulated cAMP accumulation after agonist exposure.

Materials and Methods

Expression in mammalian cells. The full-length cDNA for μ-opioid receptor (OP-48) was cloned into the expression vector pRc/CMV (InVitrogen, San Diego). For stable expression, cell line CHO K-1 (no. CCL61; American Type Culture Collection, Rockville, MD) or BHK-21tk$^-$ (no. CRL1621; American Type Culture Collection) was grown to 50% confluency in a 100-mm dish and subsequently transfected with pCMV-OP-48 using Lipofectamine reagent (GIBCO BRL, Baltimore, MD) or *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (Boehringer-Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's recommendations. Stable transfectants were selected in α-minimal essential medium or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 mg/ml geneticin (Life Technologies, Burlington, Ontario, Canada), and clones with the appropriate expression level were screened by radioligand binding assay and selected.

Membrane preparation. Cells were grown until apparent confluency and then washed twice with 10 ml of ice-cold PBS, harvested, and centrifuged at 100 × *g* for 10 min. Cells were then lysed in hypotonic buffer [5 mM Tris·HCl, pH 7.8, 0.5 mM $MgCl_2$, 0.1 mM EGTA containing protease inhibitor cocktail (10 μg/ml benzamidine, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor)] with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) with two 30-sec bursts at the 5.5 setting. The lysate was then centrifuged at 100 × *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was collected and centrifuged at 30,000 × *g* for 30 min, washed once with the hypotonic binding buffer, and then centrifuged again at high speed. The resulting pellet was resuspended in buffer containing 50 mM Tris·HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitor cocktail and used immediately for radioligand-binding studies. Protein content was determined according to the method of Bradford (10).

Radioligand binding. For saturation experiments, cell membranes (20–30 μg of protein/binding assay tube) were incubated with increasing concentrations of [^{3}H]naloxone (specific activity, 50 Ci/mmol; DuPont-New England Nuclear, Boston, MA) in a total volume of 0.5 ml. Specific binding was determined by calculating the difference in binding of the radiolabeled ligand in the absence and presence of the μ-selective antagonist naltrexone (10 μM). Competition experiments were performed using [^{3}H]naloxone at approximately its K_d value and other drugs at concentrations ranging from 10^{-12} to 10^{-4} M. Competition experiments showing a shift in the inhibition curve of the agonist DAMGO were performed in the presence and absence of GTP (200 μM), guanosine-5′-(β,γ-imido)triphosphate (100 μM), or GTPγS (50–800 μM). After a 2-hr incubation at room temperature, bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Montreal, Quebec, Canada) onto 0.5% polyethylenimine-presoaked GF/C Whatman filters. Filters were washed with 10 ml of ice-cold 50 mM Tris·HCl buffer, pH 7.4, and placed in glass vials with 5 ml of scintillation fluid (cytoscint; ICN, Costa Mesa, CA) and counted for tritium. All experiments were done in duplicate, and each experiment was repeated at least twice.

Treatment of stably transfected cells with pertussis toxin. Pertussis toxin (1–2 μg/ml) was added to the medium over a monolayer of stably transfected cells expressing the μ-opioid receptor and incubated for 24 hr before harvesting.

[^{32}P]ADP-ribosylation of transfected cell membrane components by pertussis toxin. The [^{32}P]ADP-ribosylation experiments were performed essentially as described previously (11). In brief, pertussis toxin (20 μg/ml) was preactivated as described and added to a tube containing 100 μg of membrane protein in 10 mM Tris·HCl, pH 7.4, 25 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 0.1 mM GTP, 10 μM NAD^+, 2 μl of protease inhibitor cocktail (as above), and 50 μCi of [^{32}P]NAD^+ in a final volume of 50 μl and then incubated at 37° for 60 min. Control samples were treated in the same manner in the absence of pertussis toxin. Reactions were stopped by the addition of SDS-PAGE sample buffer (50 mM Tris·HCl, pH 6.5, 10% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.003% bromphenol blue) and analyzed by 12% SDS-PAGE. Gels were dried and exposed to Kodak XAR-5 film.

Adenylyl cyclase assay. Adenylyl cyclase assays were conducted essentially as described previously (12). The assay mix contained 20 μl of membrane suspension (15–20 μg of protein), 12 μM ATP, 0.1 mM cAMP, 53 μM GTP, 2.7 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 unit of myokinase, and 0.13 μCi of [^{32}P]ATP in a final volume of 50 μl. Enzyme activities were determined in triplicate assay tubes containing decreasing concentrations (10^{-3} to 10^{-12} M) of DAMGO with 10 μM forskolin. Adenylyl cyclase activity was also measured in the presence of H_2O (basal), forskolin alone, or 10 mM sodium fluoride and incubated at 27° for 20 min. Reactions were stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (25,000 cpm). cAMP was isolated by sequential column chromatography using Dowex cation-exchange resin and aluminum oxide. Adenylyl cyclase assay was performed on membranes that had been grown in the presence or absence of pertussis toxin. Data were analyzed by nonlinear least-squares regression.

β-CNA treatment. β-CNA was dissolved in acidified anhydrous ethanol and was added to the cultured cells. The same amount of ethanol as in the final concentration of CNA solution was added to control cells as well. The final concentration of ethanol was ≤0.5%. After a 1-hr incubation at 37°, cells were washed with 100 ml of warm PBS solution, and the membrane preparation procedure was followed for the radioligand binding or adenylyl cyclase assays.

Data analysis. The binding data from saturation and competition experiments were fit by nonlinear least-squares regression using the data analysis program LIGAND (13). The data from multiple experiments were averaged and expressed as mean ± standard error. The results were considered significantly different when the probability of randomly obtaining a mean difference was <0.05 using the paired Student's *t* test.

Results

Binding studies of the μ-opioid receptor expressed in cell lines. When cells were transfected with pCMV plasmid containing the μ-opioid receptor cDNA, 75 clones were obtained after selection with geneticin. The clones were subsequently screened for expression using radioligand binding. For comparative purposes, both CHO K-1 and BHK cell lines were stably transfected (CHO-μ and BHK-μ, respectively). The binding of antagonist [^{3}H]naloxone to the μ receptor in both cell lines was saturable and of high affinity (Table 1). Analysis of saturation experiments showed that [^{3}H]nalox-

TABLE 1

Binding parameters of opioid ligands for the stably transfected μ-opioid receptor

Ligands	CHO K-1 cells				BHK cells			
	K_H	K_L	R_H	R_L	K_H	K_L	R_H	R_L
	nM		%		*nM*		%	
Agonist								
Morphine					0.9 ± 0.3	61 ± 20	77 ± 3	23 ± 3
DAMGO	3.4 ± 0.3	305 ± 14	76 ± 2	24 ± 2	2.2 ± 0.8	880 ± 385	82 ± 10	18 ± 14
DADLE					8 ± 6	78 ± 3	71 ± 25	29 ± 25
U 50,488		808 ± 101	0	100	946			
DPDPE		934 ± 20	0	100	1192			
Antagonists								
Naltrexone					0.3 ± 0.1			
Naloxonazine					15			
Naltrindole					34			
Cyprodime					42 ± 22			

K_H and K_L, K_i values of the high (H) and low (L) affinity states; R_H and R_L, proportion of receptor (R) in high and low affinity states; DADLE, [D-*Ala*^{2},D-Leu^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; U 50,488, (+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide.

one bound to the μ receptor with a K_d value of 1.0 ± 0.14 nM and a B_{max} value of 6.86 ± 0.15 pmol/mg protein in CHO-μ (four experiments) and a K_d value of 2.8 nM and a B_{max} value of 11–15 pmol/mg protein in BHK-μ (two experiments), respectively. No specific radioligand binding was detectable in untransfected cells.

The μ agonist DAMGO detected two affinity states of the μ-opioid receptor in both cell lines (Table 1); 80% of the total receptor population was in a high affinity state with a dissociation constant of 2–3 nM, and 20% of the receptor population was in a low affinity state with a dissociation constant of 300–900 nM (Table 1; seven experiments). A number of opioid ligands displaced [^{3}H]naloxone binding to this receptor in the appropriate rank order, consistent with μ-opioid receptor pharmacology (14). The receptor displayed higher affinities for μ-selective agonists and antagonists and lower affinities for the κ-selective and the δ-selective ligands (Table 1).

Guanine nucleotide regulation of agonist binding to the μ-opioid receptor. To detect coupling of μ receptors to endogenous G proteins, competition of [^{3}H]naloxone with DAMGO was performed in the absence or presence of guanine nucleotide and analogs. Only in the presence of high concentrations of the stable guanine nucleotide analog GTPγS (800 μM) was the competition curve for DAMGO shifted to the right completely (Fig. 1), suggesting that under these conditions, the receptor population only existed in a low affinity state for agonist (15).

To determine the nature of the endogenous G proteins coupled to μ receptors, cells were treated with pertussis toxin for 24 hr. Using the membrane from CHO-μ pretreated with 2 μg/ml pertussis toxin, [^{3}H]naloxone competition for DAMGO was shifted to the right completely (Fig. 2A), suggesting that the receptor was coupled to pertussis toxin-sensitive G proteins in the CHO cell line. Untreated CHO-μ cells showed that DAMGO inhibited forskolin-stimulated cAMP accumulation. In contrast, in pertussis toxin-pretreated CHO-μ cells, agonist-mediated inhibition of forskolin-stimulated cAMP accumulation was abolished (Fig. 2B). This suggests that μ receptors were coupled to adenylyl cyclase through pertussis toxin-sensitive G proteins (G_i/G_o). Both the loss of agonist-detected high affinity sites and the abolishment of agonist-mediated inhibition of adenylyl cyclase by pertussis toxin were also documented in BHK cells (data not shown).

To visualize the presence of pertussis toxin-sensitive G proteins in cells, toxin-mediated [^{32}P]ADP-ribosylation was performed in BHK-μ cells (Fig. 2C). A [^{32}P]ADP-ribosylated protein with a molecular mass of ~41 kDa was detected (*lane 1*). Radiolabeling of this protein was specifically inhibited by pertussis toxin preincubation (*lane 2*), indicating that BHK-μ

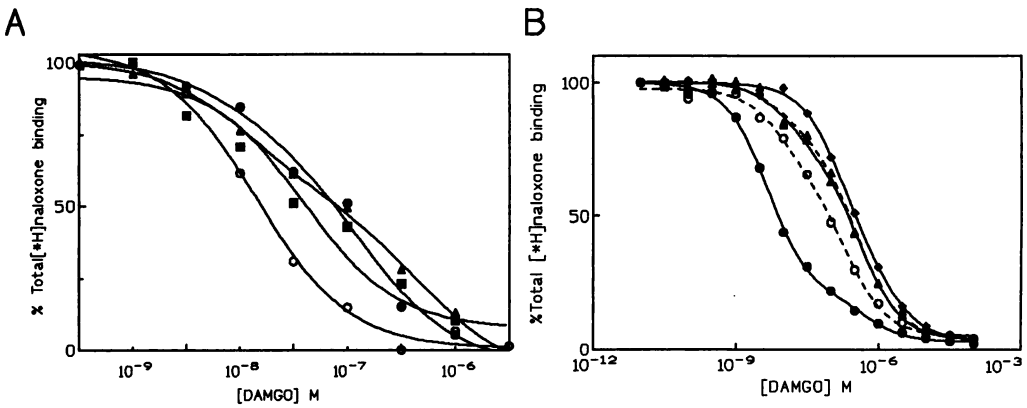

Fig. 1. Guanine nucleotide regulation of agonist binding. Membranes from stably transfected cells expressing μ receptor were incubated with 2 nM [^{3}H]naloxone and increasing concentrations of DAMGO in the absence or presence of guanine nucleotide analogues. A, Membranes from BHK-μ cells: control (○) and presence of 200 μM GTP (▲), 50 μM GTPγS (■), or 100 μM guanosine-5′-(β,γ-imido)triphosphate (●). B, Membranes from CHO-μ cells in the absence of GTPγS (●) and presence of different concentrations of GTPγS: 100 (○), 200 (▲), 400 (△), and 800 (◆) μM.

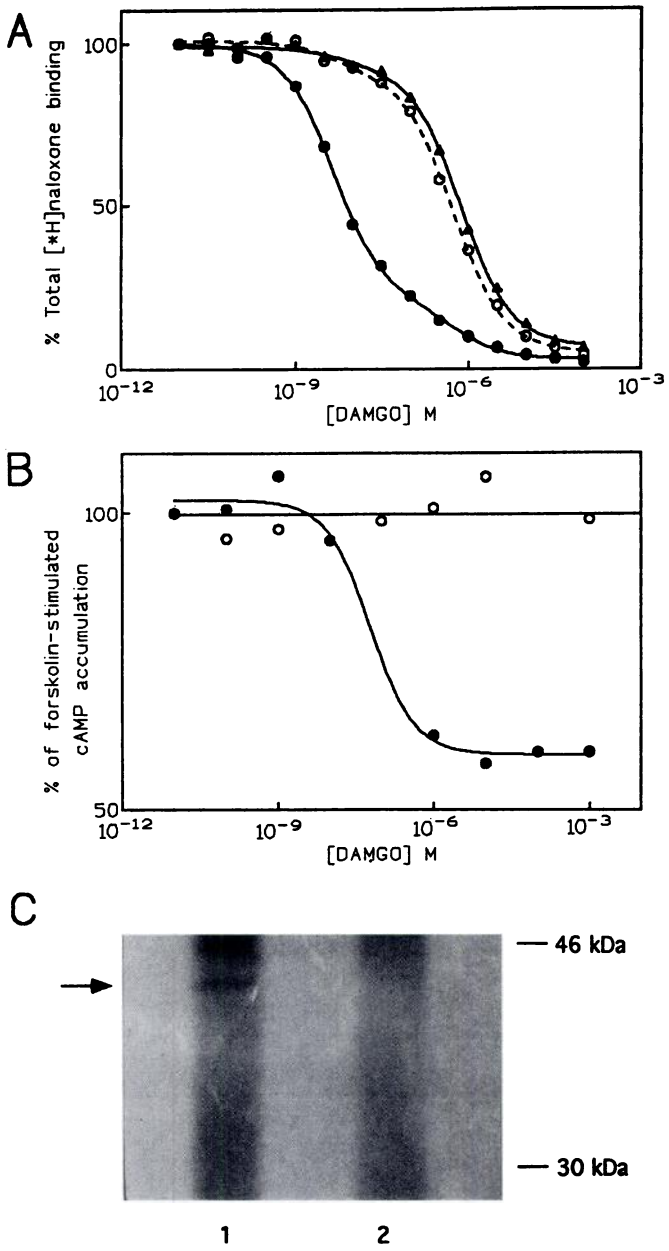

Fig. 2. Agonist competition of [^{3}H]naloxone binding in the presence of pertussis toxin. A, Membranes from CHO-μ cells expressing μ receptor were incubated with [^{3}H]naloxone with increasing concentrations of DAMGO in the absence of pertussis toxin (●) or after treatment with 1 μg/ml (○) or 1.5 μg/ml (▲) pertussis toxin. B, Agonist inhibition of forskolin-stimulated adenylyl cyclase activity after pertussis toxin treatment (2 μg/ml). Membranes from CHO-μ cells were prepared without (●) or with (○) pertussis toxin treatment and incubated with increasing concentration of DAMGO in the presence of 10 μM forskolin. C, SDS-PAGE analysis of pertussis toxin-mediated [^{32}P]ADP-ribosylated BHK cell membrane without (*lane 1*) or with (*lane 2*) pertussis toxin treatment (2 μg/ml). Data are representative of two independent experiments. Details of the experimental procedures are given in Materials and Methods.

cells contained endogenous pertussis toxin-sensitive G proteins.

Agonist-mediated desensitization. To determine the nature of the agonist-induced regulation of the μ receptor, we exposed the cells to 1 μM DAMGO for 1 hr. In untreated cells, DAMGO inhibited the forskolin-stimulated cAMP accumulation in both cell lines (Fig. 3A). In CHO-μ cells pretreated with 1 μM DAMGO for 1 hr, agonist-dependent inhibition of forskolin-stimulated cAMP accumulation was decreased to 60% of the maximum inhibition occurring in untreated cells. This inhibition by DAMGO was dose dependent (Fig. 3B). In control cells, the DAMGO concentration producing 50% maximum inhibition of adenylyl cyclase activity was 43.5 ± 0.16 nM (seven experiments), and maximum inhibition was 45%. Pretreatment of the CHO-μ cells with DAMGO for 1 hr decreased agonist-dependent inhibition of forskolin-stimulated cAMP accumulation to 28% with an IC_{50} of 954 ± 0.31 nM (Fig. 3B). In contrast, exposure of BHK-μ cells to DAMGO for 1 hr did not affect the capacity of agonist-dependent inhibition of adenylyl cyclase activity. To test whether BHK-μ cells could desensitize, we extended the pretreatment with DAMGO for a longer period of 12 hr. At this time point, agonist-dependent inhibition of forskolin-stimulated cAMP accumulation was only minimally evident, identical to that in CHO-μ cells (Fig. 3A).

To investigate possible changes in the receptor/G protein interaction during the desensitization process, we examined the changes in the agonist-detected high and low affinity states of the receptor. [^{3}H]Naloxone competition by DAMGO in membranes from CHO-μ cells pretreated with 1 μM DAMGO for 1 hr revealed that the total number of receptors was decreased by 28% compared with control (Fig. 4A). The proportion of receptors in the agonist-detected high affinity state of receptor and in the low affinity state in untreated membranes remained unchanged from pretreatment values at 73 ± 3% and 27 ± 3%, respectively. To determine whether this apparent reduction in receptor density was the result of receptor retention of free remaining DAMGO or of down-regulation of receptors, stringent washes with PBS were performed on the pretreated CHO-μ membrane subjected to saturation binding assay. Specific [^{3}H]naloxone binding to μ receptors from CHO-μ cells treated with DAMGO for 1 hr was decreased by 30% without alteration in affinity for antagonist (Fig. 4B). Scatchard analysis of the saturation experiments demonstrated that [^{3}H]naloxone had a K_d value of 1.0 ± 0.1 nM (three experiments), which is identical to the K_d value obtained in the untreated membrane (0.98 ± 0.02 nM), but the density of receptors was decreased from 7.11 ± 0.11 to 4.82 ± 0.18 pmol/mg membrane protein (three experiments) (Fig. 4B, *inset*). We conclude that the reduction in receptor density of CHO-μ cells was the result of down-regulation after DAMGO treatment. [^{3}H]Naloxone competition with DAMGO in BHK-μ cells pretreated with DAMGO for 1 hr showed no reduction in receptor density or in the proportion of receptors in high and low affinity states (Fig. 4C).

Because changes in CHO-μ-opioid receptor function depended on the receptor density on the cell surface, we examined whether desensitization without uncoupling of the receptor from the G protein was the result of overexpression of receptors in the cell. For this purpose, we pretreated cells containing different expression levels of receptors with DAMGO to examine the changes in adenylyl cyclase activity, receptor density, and affinity of receptor to ligand. In Table 2, CHO cells with three different expression levels of receptors showed the same degree of receptor activation and affinity. Furthermore, cells showed identical responses of desensitization and receptor down-regulation without alteration of

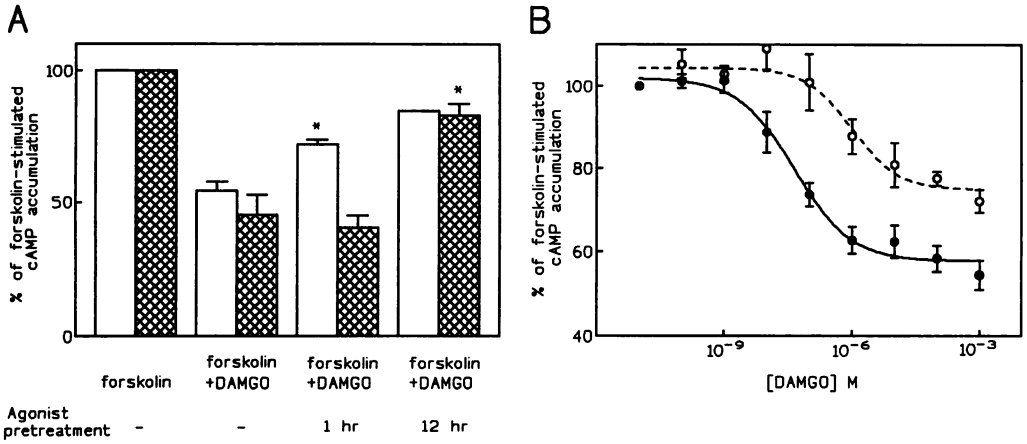

Fig. 3. Effect of agonist pretreatment on forskolin-stimulated adenylyl cyclase activity in cells expressing the μ receptor. A, Both CHO-μ (*open bars*) and BHK-μ (*cross-hatched bars*) cells were preincubated with 1 μM DAMGO for either 1 or 12 hr, and then cells were washed with buffer and stimulated with 10 μM forskolin alone or 10 μM forskolin with 1 mM DAMGO. Data shown are mean ± standard error of three independent experiments. *, $p < 0.05$, significant difference from control using the two-tailed Student's *t* test. B, Membranes from CHO-μ cells were incubated in the absence of DAMGO (●) and after exposure to 1 μM DAMGO for 1 hr (○); the ability of increasing concentrations of DAMGO from 10^{-11} to 10^{-3} M to inhibit the forskolin-stimulated cAMP accumulation was tested. Data shown are the mean ± standard error of seven independent experiments.

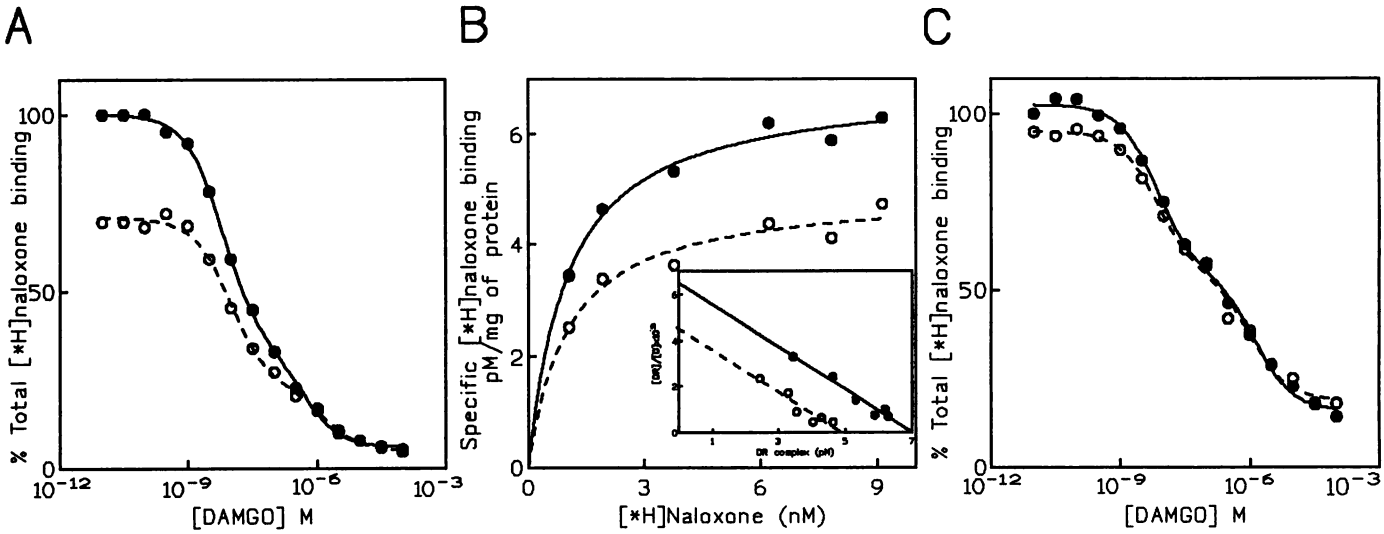

Fig. 4. Effect of agonist pretreatment on μ-opioid receptor binding. Cells expressing the μ receptor were studied in the absence of DAMGO (●) or after treatment with 1 μM DAMGO for 1 hr (○); then, membranes were subjected to binding studies. A and C, Membranes from CHO-μ (A) and BHK-μ (C) cells were incubated with 2 nM [^{3}H]naloxone and increasing concentrations of DAMGO. B, Membranes from CHO-μ cells were incubated with increasing concentration of [^{3}H]naloxone, and specific binding was plotted as a function [^{3}H]naloxone concentration. *Inset*, Scatchard analysis of the saturation data. Experiments were conducted in triplicate; representative data are given.

agonist affinity or proportion of receptor high/low state, suggesting that receptor desensitization resulting from receptor down-regulation from the surface did not relate to the expression-level differences of receptors.

Irreversible inactivation of the μ-opioid receptor by β-CNA. To further investigate the apparent mechanism of desensitization in CHO-μ cells within 1 hr of DAMGO treatment, we irreversibly inactivated the μ receptors using the alkylating agent β-CNA and examined the changes in adenylyl cyclase activity. The aziridinium ion of CNA is believed to covalently bind the opioid-binding site and thereby inactivate receptors (16). The irreversible inactivation of the opioid receptors by CNA is time as well as concentration dependent, with the maximum inactivation occurring within 30 min. We tested the reduction of μ-opioid receptor density with various concentrations of CNA after incubation for 1 hr. After pretreatment with 0.8 nM CNA, 18% of the high affinity state of the μ receptor was inactivated, but DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation remained identical to that in untreated membranes (three experiments) (Fig. 5A). However, a reduction in the number of high affinity state receptors by 28% with 1 nM CNA decreased the agonist-dependent inhibition of cAMP accumulation to 61% of the maximum inhibition. The inactivation of greater numbers of μ receptors using higher concentrations of CNA resulted in progressively greater impairment of agonist inhibition of forskolin-stimulated cAMP accumulation.

Interestingly, 1 nM CNA pretreatment decreased the receptor density and agonist-dependent forskolin-stimulated cAMP accumulation to a degree approximately comparable to that documented after 1-hr DAMGO pretreatment. The number of receptors was decreased by 28% compared with un-

TABLE 2
Effects of DAMGO pretreatment on binding and adenylyl cyclase at different expression levels of μ-opioid receptor in CHO cells

		Untreated	Pretreated with DAMGO
Clone 1	B_{max}	6.86 ± 0.15 pmol/mg protein	4.82 ± 0.18 pmol/mg protein
	K_d	1.0 ± 0.1 nM	0.98 ± 0.02 nM
	EC_{50}	43.5 ± 0.16 nM	954 ± 0.31 nM
Clone 2	B_{max}	3.77 ± 0.86 pmol/mg protein	2.6 ± 0.2 pmol/mg protein
	K_d	0.92 ± 0.17 nM	0.94 ± 0.3 nM
	EC_{50}	8.54 ± 0.16 nM	215 ± 0.13 nM
Clone 3	B_{max}	0.57 ± 0.74 pmol/mg protein	0.45 pmol/mg protein
	K_d	2.41 ± 0.5 nM	2 nM
	EC_{50}	17.7 ± 7.6 nM	886.3 ± 6.46 nM

treated membranes, but the proportion of receptor in the high affinity state and the affinity for the receptor were maintained unchanged at 72% and 2 nM, respectively (Fig. 5B). When DAMGO-dependent inhibition of forskolin-stimulated cAMP accumulation was tested using 1 nM CNA-treated membrane, the degree of inhibition was attenuated to 60% of maximum, and the EC_{50} of DAMGO effect was shifted toward the right (Fig. 5C). Collectively, we conclude that the decrease in the μ-opioid receptor number and the attenuation of μ agonist-dependent inhibition of cAMP accumulation are causally linked processes.

Discussion

Analysis of the μ-opioid receptor-expressing stable transfectants that we established was consistent with recent pharmacological characterizations of transiently expressed cloned μ receptors (17–20) and established opioid-ligand binding profiles in brain tissues (14, 21).

In both transfected cell lines, 76% of the total receptor population was in high affinity for agonist and 24% of the receptor was in the low affinity state. This suggested that a large proportion of the receptors was coupled to endogenous G proteins, confirmed by the guanine nucleotide regulation of the agonist-detected high affinity state of receptor. Although high concentrations of guanine nucleotide analogs reduced agonist affinity for the receptor (Fig. 1), the high affinity state of receptors remained detectable despite very high guanine nucleotide analog concentrations in both cell lines. This may indicate that μ-opioid receptor/G protein coupling is unusually tight. Interestingly, there have been suggestions regarding the variability of receptor affinity for G protein that a substantial difference may exist between inhibitory and stimulatory G protein systems. The α_2-adrenergic receptor and D_2 dopamine receptor that interact with inhibitory-type G protein may be purified by affinity chromatography as stable complexes with functional G proteins (22, 23), suggestive of a high affinity between these receptors and G protein.

The abolishment of high affinity binding of agonist as a result of pertussis toxin treatment also suggested that the agonist-detected high affinity sites were the result of receptor coupling to G protein. Pertussis toxin treatment results in the irreversible detachment of G proteins from receptors (24). The treated cells expressing the μ receptor revealed loss of the high affinity state, with receptors completely shifted to the low affinity state for agonist. Furthermore, these cells displayed a complete loss of DAMGO-induced inhibition of forskolin-stimulated adenylyl cyclase activity.

These results demonstrate that the μ receptors were functionally coupled to pertussis toxin-sensitive endogenous G proteins in both cell lines and that the high affinity state of receptors demonstrated was the functionally active form of receptor.

In the current study, we report that receptor down-regulation was the mechanism responsible for the desensitization of μ-opioid receptor function, resulting in loss of the opioid agonist-induced inhibition adenylyl cyclase activity. The observed desensitization and receptor down-regulation are not due to overexpression of receptors because we obtained identical results in CHO cells with 10–20-fold lower expression levels (Table 2). The results of this study contrast with some of the earlier observations made with chronic treatment of endogenous δ-opioid receptors in NG108-15 cells (3) and μ-opioid receptors in 7315c rat pituitary tumor cells (5). In 7315c cells, the initial attenuation of cAMP accumulation occurred after a 5-hr treatment with morphine, and the effect was accompanied by the uncoupling of receptor from G proteins, as indicated by the loss of sensitivity of agonist binding to guanine nucleotides and by the shift of high affinity ligand binding to low affinity. Only after morphine exposure for 24 hr was a reduction in receptor numbers demonstrated (5). Similar results were described in NG108-15 cells for δ-opioid receptors, as desensitization preceded receptor down-regulation (3). Also, with the dopamine D_1 receptor expressed in Sf9 cells, it was shown that uncoupling of G proteins from receptors was the main mechanism of rapid desensitization of adenylyl cyclase activity, which was a separate process dissociable from the slowly occurring receptor internalization (25). Unlike these examples (3–5), the desensitization of the μ-opioid receptor in CHO cells and BHK cells was not accompanied by the uncoupling of receptor from G protein, indicated by the lack of change in agonist affinity for the receptor after agonist exposure. Also, the proportion of receptors in agonist-detected high and low affinity states remained the same as in control. It may suggest that μ-opioid receptors have a high affinity to G proteins even in the absence of agonist, so that rapid rearrangement of receptors to reassociate with endogenous G proteins may occur immediately after receptor down-regulation.

We demonstrated that agonist concentrations for the half-maximal equilibrium occupation of the μ receptor sites (K_i) in the high affinity state and for that to produce half-maximal inhibitory effect on cAMP accumulation (EC_{50}) were different. For example, in control CHO-μ cells, the K_d for DAMGO was 3 nM at the high affinity site, but the EC_{50} for adenylyl cyclase inhibition was 43 nM. This seems to indicate that although DAMGO binds to the μ receptors avidly, higher concentrations occupying a greater number of receptors may be necessary to generate the maximum effector response. It

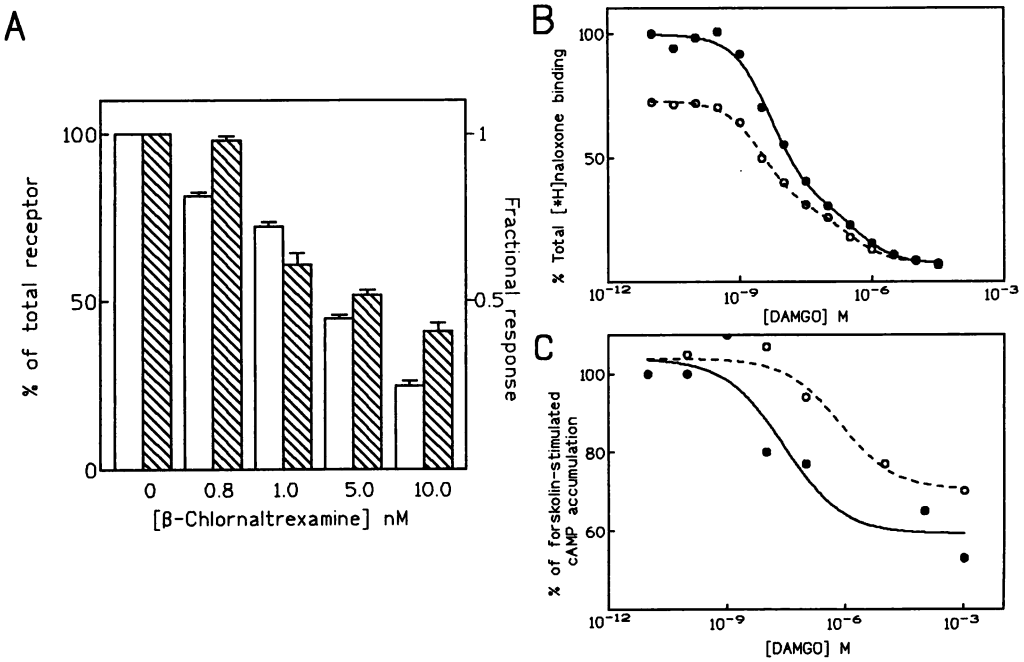

Fig. 5. Effect of CNA pretreatment on receptor binding and on forskolin-stimulated cAMP accumulation. A, CHO-μ membranes were preincubated with varying concentrations of CNA for 1 hr at 37° and subjected to both competition binding and adenylyl cyclase assay. *Open bars*, number of total receptors was calculated by normalizing the density of total receptors before CNA treatment as 100%. *Striped bars*, relative inhibition of adenylyl cyclase activity at different CNA concentrations was calculated based on the maximal inhibition in control cells (three experiments). B, [^{3}H]Naloxone competition binding with DAMGO using membranes pretreated with 1 nM CNA (○) or untreated membranes (●). C, Membranes from CHO-μ cells were preincubated in the presence (○) or absence (●) of 1 nM β-CNA for 1 hr, and the ability of increasing concentrations of DAMGO to inhibit the forskolin-stimulated cAMP accumulation was tested.

has been shown for the endogenous δ-opioid receptor in NG108-15 cells that the ratios of the K_D to IC_{50} were >1 for all δ-selective agonists (26). Subsequently, loss of 90% of the receptors in this cell still maintained the maximum agonist-dependent inhibitory response, suggesting significant receptor reserve (16). However, in our study, the ratio of the K_i to IC_{50} for DAMGO was significantly <1. This may suggest that the maximal opioid peptide effect on μ receptor-linked adenylyl cyclase activity may require equilibrium occupation of a much greater complement of receptors. This may also suggest a reduced functional efficacy of the receptor/effector unit, so that a greater percentage occupancy of receptors is necessary to generate a functional response. Unlike the usual amplification of receptor function via postreceptor mechanisms, these results indicate a dampening of the response cascade and potentially represent a unique regulatory feature linked to μ-opioid receptors.

We tested the role of receptor density in the regulation of μ receptor function in CHO-μ cells using various concentrations of CNA to inactivate receptors systematically, down to 25% of the initial density. Unlike what has been described for the δ-opioid receptor in NG 108–15 cells, a 30% loss of μ receptors caused a significant reduction in the maximal inhibition of adenylyl cyclase activity (Fig. 5A). Interestingly, a 20% loss of receptor density retained the maximal response, but a 10% further reduction of receptors caused a 40% loss of maximal response. This result together with the ratio of K_d to IC_{50} values indicates that the maximal functional activation of μ receptors occurs when ~80% of receptors are occupied and the relationship between receptor density and function is not a simple linear one. Thus, the percentage of receptors that may be considered "spare" is considerably lower than that reported for other receptors, even including other opioid receptors.

There are other examples of μ-opioid receptor-coupled systems in which causally linked receptor down-regulation and desensitization have been observed. For example, after 24-hr treatment with DAMGO, endogenous μ-opioid receptors in differentiated SH-SY5Y human neuroblastoma cells showed concurrent desensitization of the inhibition of adenylyl cyclase activity and receptor down-regulation (27). Furthermore, *in vivo* studies in rat showed that chronic infusion of etorphine increased the IC_{50} for inhibition of adenylyl cyclase activity and was accompanied by a decrease in μ-opioid receptor numbers with no effect on agonist affinity (28). This suggests that even *in vivo*, μ-opioid receptor desensitization is accompanied predominantly by receptor down-regulation rather than any alteration of receptor affinity.

Then, we investigated what features of CHO cells enable desensitization and down-regulation of μ-opioid receptor to occur more rapidly than BHK cells. The inactivation of G protein-coupled receptors requires the sequential action of at least two proteins, GRKs (29, 30) and arrestin-like proteins (31–33), as indicated by several independent lines of evidence. For the β_2-adrenergic receptor, substitution or deletion of serine/threonine residues in the carboxyl tail that are recognition sites of GRKs slows or diminishes agonist-specific desensitization (34). Furthermore, when cells are permeabilized and treated with heparin to block the action of GRK but not other kinases, agonist-specific desensitization is significantly reduced or eliminated (35), suggesting that phosphorylation of receptor by GRKs is an important step for agonist-specific desensitization. The GRKs have tissue-specific distribution (30, 36–38), which may account for the

differential desensitization rates in CHO and BHK and may relate to differences in expression in the ovary and kidney origins of these cell lines. Another possibility leading to cell-type differences in desensitization might be the presence of arrestin-like proteins. Although kinase-mediated receptor phosphorylation reduces G protein-coupled receptor function, it is not sufficient to terminate receptor activation. However, receptors phosphorylated by GRK may bind to arrestin-like cytosolic proteins and then subsequently internalize (29–31). Overexpression of β-arrestin in human embryonic cells rescued the internalization of mutant β_2-adrenergic receptors that were resistant to internalization (33). Even in wild-type β_2-adrenergic receptors expressed in CHO cells, overexpression of GRK2 or β-arrestin has been shown to increase the agonist-promoted desensitization rate (39). Furthermore, internalization of wild-type β_2-adrenergic receptor is inhibited by overexpression of mutant β-arrestin (33). Thus, it may be argued that the balance of GRK and arrestin-like protein expression is an important limiting factor for agonist-induced desensitization in heterologous cell systems. Because the lack of early desensitization in BHK-μ cells was accompanied by a lack of receptor internalization, one possibility may be that reduced efficacy of arrestin-like cytosolic proteins in BHK cells is responsible, at least in part, for the observed difference from the CHO cells.

In summary, based on the correlation between the reduction in receptor density and loss of agonist efficacy, we propose that the μ-opioid receptor requires a greater proportion of receptor occupancy to generate the maximal functional response. Thereby, we report that a reduction in the number of μ-opioid receptors in the high affinity state, resulting from a reduction in total membrane receptors, seems to be the predominant mechanism causing functional desensitization of the μ-opioid receptor. Although the cellular mechanisms underlying the observed decrease in μ receptor number, such as sequestration or internalization, are yet to be elucidated, these results indicate that μ-opioid receptor-transfected cells can serve as a model for investigation of some of the unique features of μ-opioid receptors, which may represent the basis for molecular and cellular mechanisms underlying opioid drug tolerance.

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