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Effects of Chronic Morphine Exposure on Opioid Inhibition of Adenylyl Cyclase in 7315c Cell Membranes: A Useful Model for the Study of Tolerance at μ Opioid Receptors

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SUMMARY

The effects of prolonged morphine exposure on the μ opioid receptor in 7315c pituitary tumor cell membranes have been examined. Since a low concentration of naloxone reversed the inhibition of forskolin-stimulated adenylyl cyclase induced by the μ-selective agonist, Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO), and by high concentrations of [D-Pen^2-D-Pen^5]enkephalin (DPDPE), we suggest that these cells contain a homogeneous population of μ opioid receptors coupled to adenylyl cyclase via a guanyl nucleotide-binding protein. Studies measuring the ability of [D-Ala^2-D-Leu^5]enkephalin (DADLE), an opioid agonist, to inhibit adenylyl cyclase in cells that had been exposed to 100 μM morphine for varying periods of time, indicated that the agonist no longer inhibited enzyme activity after 5 hr of morphine exposure. Measurements of ^{3}H-antagonist binding in membranes from cells exposed to morphine demonstrated a decreased receptor density after 24 hr of 100 μM morphine exposure with no change in the antagonist affinity. Computer analysis indicated a 20% decrease in the number of μ receptors labeled after 24 hr of morphine exposure and a 60% decrease after 72 hr of exposure. Computer analysis of agonist competition against ^{3}H-antagonist binding confirmed the existence of one binding site with an affinity intermediate between the high and low apparent affinity states observed in membranes from untreated cells. Addition of 10 μM GTPγS did not affect the agonist affinity or receptor density in membranes from morphine-treated cells, suggesting that the receptors were uncoupled from G proteins, as observed in 7315c cell membranes that have been treated with pertussis toxin. Thus chronic morphine treatment induced a rapid loss of opioid μ receptor-mediated inhibition of adenylyl cyclase (desensitization), and a more slowly developing reduction in receptor number. The desensitization was accompanied by a loss of guanyl nucleotide regulation of agonist affinity. These findings are comparable to results reported for the δ opioid receptor and the β-adrenergic receptor upon prolonged agonist exposure.

Previous studies have indicated that prolonged opioid agonist exposure of the δ opioid receptor in the NG108-15 cell produced at least three different cellular adaptative processes which all involve the coupled state of the receptor. The first step involved a rapid desensitization of the δ opioid receptor. The second step, observed 24 hr later, involved receptor down-regulation (1, 2). After withdrawal of the chronically administered opioid agonist, an increase above baseline in adenylyl cyclase activity was reported (3). We now present the first report examining the effects of chronic morphine exposure in the 7315c cell, a pituitary tumor cell containing a homogeneous population of μ opioid receptors (4, 5). In these cells, opioids inhibit adenylyl cyclase through a mechanism in which a guanyl nucleotide-binding protein is implicated (6, 7). In this paper, we have also confirmed that opioid inhibition of adenylyl cyclase is mediated via μ receptors. Since morphine and related analgesics and anesthetic agents act primarily through μ opioid receptors (8), our results may be relevant to the mechanisms underlying opiate drug tolerance in intact animals.

In other receptor systems in which receptors are coupled to adenylyl cyclase by G proteins, chronic agonist exposure has resulted in an initial loss of agonist-induced enzyme inhibition or activation (desensitization), and a later reduction in the number of receptors (down-regulation) measurable by radioligand binding techniques (1, 2, 9). In order to detect time-dependent changes induced by agonist treatment of 7315c cells, we have measured opioid inhibition of adenylyl cyclase, and

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The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the *NIH Guide to the Care and Use of Laboratory Animals*, National Institutes of Health Publication No. 85.23, Revised 1985.

ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DADLE, [D-Ala^2-D-Leu^5]enkephalin; DAGO, Tyr-D-Ala^2-Gly-N(Me)Phe-Gly-ol; DIP, diprenorphine; DMEM, Dulbecco's modified essential medium; DPDPE, [D-Pen^5-D-Pen^5]enkephalin; EDTA, ethylenediaminetetraacetate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ICI174,864, allyl$_2$-Tyr-(α-aminoisobutyric acid)$_2$-Phe-Leu-OH; K_{app}, apparent dissociation constant (macroscopic dissociation constant); K_i, equilibrium dissociation constant for competing ligand; U50,488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(pyrrolidinyl)-cyclohexyl]benzeneacetamide methansulfonate hydrate.

agonist and antagonist binding in membranes of cells exposed to morphine for varying periods of time. Our results confirm that both desensitization and receptor-down-regulation occur following chronic activation of 7315c cell μ opioid receptors.

It is well known that GTP and other guanyl nucleotides reduce agonist affinities at opioid and other G protein-coupled receptors (10–14). In NG108-15 cells, it has also been shown that GTP is essential for opioid inhibition of adenylyl cyclase activity (15). Treatment of NG108-15 cells with pertussis toxin, a process which is thought to uncouple receptors from G proteins which can act as pertussis toxin substrates (16, 17), also reduces agonist affinity and concomitantly renders the receptors insensitive to regulation by guanyl nucleotides (5, 18, 19). Thus, guanyl nucleotide effects on agonist affinity probably provide a measurable indication of functional coupling between agonist-occupied receptors and associated G proteins. We have recently shown that GTP and GTPγS are capable of inducing a very low agonist affinity state of the μ-opioid receptor in 7315c cells which may be associated with receptor-mediated activation of a G protein (5). We have therefore evaluated the ability of guanyl nucleotides to regulate agonist binding affinities in membranes from 7315c cells in which opioid inhibition of adenylyl cyclase is markedly reduced as a result of prior agonist exposure. Our results suggest that G protein coupling of μ opioid receptors may be reduced following sustained agonist exposure. We believe this is the first demonstration of agonist-induced desensitization and receptor down-regulation at μ opioid receptors.

Materials and Methods

Chemicals. Chemicals and reagents were obtained from the following sources: [^{3}H]DIP (46.5 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); [^{3}H]cAMP (31.2 Ci/mmol) from New England Nuclear Corp. (Boston, MA); bovine serum albumin (fraction V), Boehringer Mannheim Biochemicals (Indianapolis, IN), DAGO, ICI174,864, DPDPE, and DADLE from Cambridge Research Biochemicals Ltd. (Atlantic Beach, NY); Antifoam A, ATP, cAMP, GTP, and HEPES from Sigma Chemical Co. (St. Louis, MO); unlabeled DIP from Research Technology Branch, National Institute of Drug Abuse (Rockville, MD); GTPγS from Boehringer Mannheim Biochemicals; DMEM and glutamine from Biofluids (Rockville, MD); fetal calf serum from KC Biochemicals (Lenexa, KS); Vitrogen 100 from Collagen Corp. (Palo Alto, CA); morphine sulfate from Merck (Rahway, NJ), and unlabeled naloxone from Endo Labs (Garden City, NY). Bovine adrenal "binding protein" was generously donated by Dr. T. E. Cote.

Chronic treatment of 7315c cells. The 7315c tumor was maintained by serial implantation in the peritoneal cavity of female Buffalo rats (40–60 g; National Cancer Institute, Frederick MD). Cells were prepared as previously described (5, 6). Briefly, tumors were removed and finely minced with a razor blade in 10 ml of DMEM supplemented with 0.25% bovine serum albumin. The cell suspension was filtered through gauze to remove large tissue fragments and red blood cells and centrifuged twice at 200 × *g* for 5 min. The cells were then incubated for the desired time period in 75-cm^2 collagen-coated tissue culture flasks at a concentration of 2 × 10^6 cells/ml of DMEM containing 2 nM glutamine and 10% fetal calf serum. For studies involving treated cells, the medium was supplemented with 100 μM morphine. For each flask of treated cells, a control flask was incubated for the same time interval without morphine. Aliquots of cell suspensions were examined under the microscope and the viability of the 7315c cells during the incubation period was monitored using the trypan blue exclusion method. The number of cells was counted using a hemocytometer. Preliminary studies demonstrated the necessity for the 7315c cells to adhere to a growth surface for cell viability in culture. When the cells were placed on collagen-coated 75-cm^2 tissue culture flasks, it was possible to measure opioid radioligand binding and cyclase activity during periods as long as 18 days in culture. Cell counts and trypan blue exclusion measurements indicated that about 60% of the cells in the initial suspension attached to the plate within 1 hr, and these cells remained viable without increasing in number for at least 72 hr in the presence or absence of morphine.

Preparation of 7315c membranes. Control and treated cells were centrifuged three times at 200 × *g* for 5 min. To ensure that residual morphine did not remain in the preparation after chronic treatment, we examined the retention of [^{3}H]morphine through the regular washing procedure. Cells were incubated with [^{3}H]morphine at 100 μM for 48 hr and washed as usual. All of the [^{3}H]morphine was removed from the 7315c cells by three washes. Prior to washing, a 250-μl aliquot of cell suspension contained 28,900 ± 400 dpm (mean ± SE of triplicate determinations). After three washes, an aliquot contained 3 ± 5 dpm. Cell pellets were resuspended in Krebs HEPES (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH adjusted to 7.4) and disrupted by homogenization using a Teflon-glass homogenizer (four passes at speed setting 70 on the T-line laboratory stirrer; Thomas Scientific, Philadelphia, PA).

Determination of adenylyl cyclase activity. Cyclase activity was assayed in the presence of 10 μM forskolin to increase basal activity of the enzyme, thus improving quantitation of opiate-mediated inhibition of adenylyl cyclase. Approximately 100 μg of protein/ml of control or treated 7315c membranes were added to the assay buffer (80 mM Tris-HCl, 10mM theophylline, 1 mM $MgSO_4$, 8 mM EDTA, 0.01 mM GTP, 0.25 mM ATP). After a 10-min incubation at 30°, the reaction was terminated by boiling the assay tubes for 1 min. The amount of cAMP was determined by a protein binding assay (20).

Opioid binding assays. Receptor binding was carried out by incubating the membrane suspension in a 1-ml final reaction volume of modified Krebs buffer at 37°. Radioligand binding assays were performed as described previously (21). Competition of 1 nM [^{3}H]DIP by DIP or DAGO was used to characterize binding to μ opioid receptors in 7315c cell membranes. Competition studies employed 18 unlabeled antagonist concentrations or 28 unlabeled agonist concentrations between 0.5 nM and 10 μM. In experiments examining the effects of GTPγS, the nonhydrolyzable GTP analogue, 10 μM was chosen as the concentration maximally effective in inhibiting agonist binding at high and low concentrations (4). Protein concentrations were determined using a modification of the Lowry procedure (22).

Analysis of binding data. Competition data were analyzed and plotted by the computer program LIGAND (23). This program utilizes a nonlinear least squares curve-fitting algorithm and assumes the simultaneous contribution of one or more independent binding sites. Details of analysis have been reported previously (4, 5). Briefly, the model best fitting the experimental data was selected on the basis of *F* test comparisons of the residual variances, and a runs test examining the sequences of positive and negative differences between the actual data points and the estimated binding curve. If the estimates for a two-site model did not result in a significant improvement in fit over a one-site fit, the simpler model is reported. In order to compare the binding parameters of DAGO in the presence of GTPγS to those in its absence, computer-generated fits for GTPγS curves were compared to fits in which the affinities were constrained to values obtained in the absence of GTPγS. To be accepted as an optimal fit, the parameters modeled in the presence of the nucleotide had to produce a significant improvement, as determined by the *F* test ($p < 0.05$), over those where parameters were constrained to be equal to those modeled in the absence of added nucleotide. To compare the binding parameters of unlabeled DIP obtained in untreated cell membranes to those obtained in treated 7315c membranes, computer-generated fits for curves in treated membranes were compared to fits in which the affinities were constrained to values obtained in unexposed membranes. Nonspecific binding was modeled as an independent parameter in the computer analysis. For graphical representation of data, the modeled nonspecific

binding estimate has been subtracted from the total amount bound and is reported in the figure legend. The estimated reliability of a reported value is indicated as a standard error of the parameter estimate calculated by the LIGAND program from the pooled data from three or more independent experiments, each of which contained triplicate samples at each concentration of unlabeled competing ligand.

Analysis of adenylyl cyclase data. All studies involving determination of adenylyl cyclase activity represent the average of three or more independent experiments each of which contained triplicate samples of each unlabeled ligand concentration.

Results

Characterization of μ-mediated inhibition of adenylyl cyclase activity in 7315c membranes. In order to confirm earlier reports demonstrating μ-mediated inhibition of adenylyl cyclase activity in the 7315c cells (6), dose response curves were constructed using DAGO, the μ-selective agonist. DAGO produced a dose-related depression of adenylyl cyclase activity (Fig. 1A). The concentration of DAGO producing 50% of the maximum inhibitory effect of DAGO was approximately 100 nM. At a concentration of 10 μM, DAGO produced a maximum inhibition of adenylyl cyclase activity of about 80%.

To confirm that DAGO was acting through a μ opioid receptor to mediate inhibition of adenylyl cyclase activity, the ability of naloxone, a partially μ-selective antagonist, to antagonize DAGO-mediated effects was examined. DAGO concentration response curves were constructed in the absence and presence of 10 nM naloxone (Fig. 1A). Dose response curves performed in the presence of 10 nM naloxone indicated that this concentration was sufficient to completely reverse the DAGO-mediated inhibition of cyclase activity. Conversely, a 300 nM concentration of ICI174,864, the δ-selective antagonist, produced only a slight antagonism of the DAGO-mediated depression of cyclase activity (data not shown). The possibility that δ receptors, in addition to μ, inhibit adenylyl cyclase activity in the 7315c cell was also evaluated by comparing the effects of the δ-selective agonist, DPDPE, with those of DAGO (Fig. 1B). DPDPE concentration response curves were constructed in the absence and presence of the antagonist naloxone. DPDPE was not as potent as DAGO, the μ-selective agonist, in reducing cyclase activity. Little inhibition was observed at concentrations below 1 μM; at 10 μM DPDPE, 50–70% inhibition was observed. Naloxone (10 nM) significantly antagonized the inhibitory effect of 10 μM DPDPE (Fig. 1B), but antagonism by 300 nM ICI174,864 was very weak (data not shown).

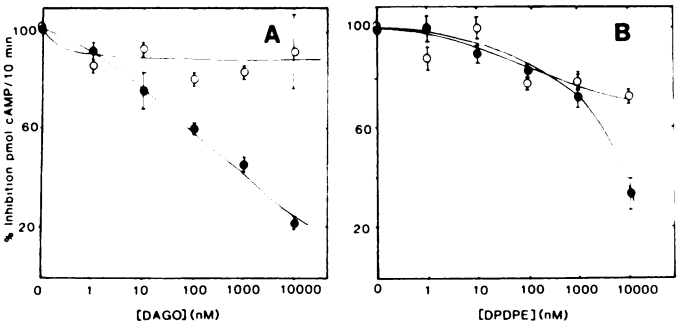

Fig. 1. Naloxone effects on DAGO (A)- and DPDPE (B)-mediated inhibition of cyclase activity in 7315c membranes. Membranes were prepared and adenylyl cyclase activity was assayed in the absence and presence of 10 nM naloxone. Naloxone antagonized the inhibitory action of DAGO, and of 1 and 10 μM concentrations of the δ-selective agonist DPDPE. The figures are mean values from two replicate experiments. The *error bars* represent standard error estimates from six determinations at each concentration of antagonist. ●, DAGO (A) or DPDPE (B) alone; ○, DAGO (A) or DPDPE (B) in the presence of 10 nM naloxone.

Adenylyl cyclase assays performed in the presence of increasing concentrations of the opioid antagonist naloxone indicated that concentrations of this drug between 10 nM and 1 μM inhibited cyclase activity by about 20% in the 7315c cell membranes. Because of this, we were unable to determine K_e values for reversal of agonist-mediated inhibition in this preparation. In contrast to the inhibitory effects observed with naloxone, enzyme activity was unaffected by another opioid antagonist, diprenorphine (10 nM to 1 μM). Unfortunately, DIP does not discriminate well between μ and δ receptors (5) and, therefore, could not be used to determine the receptors mediating adenylyl cyclase inhibition in 7315c cells. It is clear that naloxone readily antagonizes the actions of submicromolar concentrations of DAGO and micromolar concentrations of DPDPE, a result consistent with their action through μ opioid receptors. The limited effectiveness of ICI174,864 in antagonizing the actions of DAGO or DPDPE also supports the suggestion that the action of both these peptides was mediated through the μ and not the δ opioid receptor. To eliminate the possibility that inhibition of adenylyl cyclase activity was mediated through κ opioid receptors, the ability of the κ-selective agonists U50,488H and ethylketocyclazocine to inhibit cyclase activity was evaluated (Table 1). In both cases, concentrations up to 100 nM were unable to significantly depress the enzyme activity.

Adenylyl cyclase activity in membranes from control and morphine-treated 7315c cells. In order to examine the adaptative processes induced by tolerance in 7315c cell membranes, cells were treated for varying periods of time with 100 μM morphine and the ability of the opioid agonist DADLE to inhibit cyclase activity was examined. In untreated cells maintained in culture for varying time periods, DADLE significantly depressed adenylyl cyclase activity in a dose-dependent manner (Fig. 2). The agonist concentration producing 50% inhibition of cyclase activity was approximately 10–100 nM. At a concentration of 10 μM, DADLE produced a maximum inhibition of adenylyl cyclase activity of approximately 80% at all incubation times. In other experiments (data not shown), DAGO-mediated inhibition of adenylyl cyclase has been observed after maintenance of 7315c cells for periods as long as 18 days in culture. Although the cells remained viable at 37°, cell counts and

TABLE 1

Effect of κ-selective agonists on adenylyl cyclase activity in 7315c cell membranes

Cell membranes were assayed in the presence of increasing concentrations of opioid agonists. For details of the adenylyl cyclase assay, see Materials and Methods. The number of independent replicate experiments is indicated in the table (*N*). Independent experiments contained triplicate determinations at each agonist concentration. The tabulated values are the estimates of the percentage inhibition (± standard error) produced by the agonist from a combined analysis of the results from *N* independent experiments.

Agonist	*N*	[Agonist]	% Inhibition of adenylyl cyclase activity
		nM	
U50,488H	3	1	2 ± 5
		10	1 ± 2
		100	8 ± 12
Ethylketocyclazocine	2	1	6 ± 8
		10	2 ± 10
		100	7 ± 5

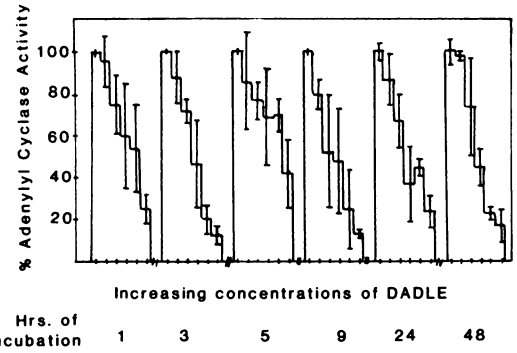

Fig. 2. DADLE inhibits adenylyl cyclase activity in untreated 7315c cell membranes after incubation of dispersed primary cultures of intact 7315c cells at 37° for the indicated times. After the specified incubation period, membranes were prepared and adenylyl cyclase activity was assayed in the absence and presence concentrations of DADLE ranging from 1 nM to 10 μM. At each incubation time, the *leftmost bar* indicates the control adenylyl cyclase activity in the absence of added drug, set at 100%. Each step then indicates the effect of 10-fold increases in DADLE concentrations ranging from 1 nM to 10 μM. Mean control adenylyl cyclase values in the absence of added DADLE did not vary as incubation time was increased (1 hr, 15 pmol of cAMP/mg of protein/10 min; 3 hr, 18.5 pmol of cAMP/mg of protein/10 min; 5 hr 17 pmol of cAMP/mg of protein/10 min; 9 hr, 15 pmol of cAMP/mg of protein/10 min; 24 hr, 16.5 pmol of cAMP/mg of protein/10 min; 48 hr, 21 pmol of cAMP/mg of protein/10 min). The histograms report mean values from four replicate experiments. The *error bars* represent the standard error estimate.

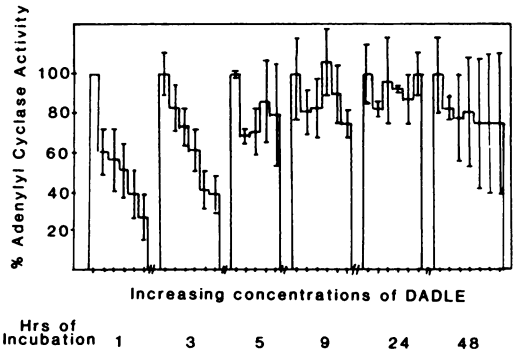

Fig. 3. DADLE-mediated inhibition of adenylyl cyclase in treated 7315c cell membranes. The 7315c cells were exposed to 100 μM morphine at 37° for the indicated times. Membranes were prepared and adenylyl cyclase activity was assayed in the absence and presence of concentrations of DADLE ranging from 1 nM to 10 μM as described in the legend to Fig. 2. Mean control adenylyl cyclase values in the absence of added DADLE did not vary as incubation time was increased (1 hr, 18 pmol of cAMP/mg of protein/10 min; 3 hr, 21 pmol of cAMP/mg of protein/10 min; 5 hr, 17.5 pmol of cAMP/mg of protein/10 min; 9 hr, 16.8 pmol of cAMP/mg protein/10 min; 24 hr, 20.4 pmol cAMP/mg protein/10 min; 48 hr, 16 pmol cAMP/mg of protein/10 min). The histograms report mean values from four replicate experiments. The *error bars* represent the standard error estimate.

protein determinations indicated that cells were not dividing or increasing in tissue mass during incubation. Protein concentrations in membrane preparations remained similar throughout incubations for at least 72 hr (1 hr, 250 ± 50 and 72 hr, 261 ± 17 μg/250 μl of membrane suspension). In cells that were treated with 100 μM morphine for up to 72 hr, membrane protein concentrations also remained stable and did not differ significantly from control values (1 hr, 201 ± 31 and 72 hr, 296 ± 36 μg/250 μl of membrane suspension). After 1- and 3-hr time periods in 100 μM morphine, DADLE decreased adenylyl cyclase activity in a dose-dependent fashion, although the maximum inhibition of adenylyl cyclase activity was reduced from 80% in untreated cells to 60–70% at 3 hr (Fig. 3). The DADLE IC_{50} value obtained under these conditions was very similar to that obtained in untreated cells. After approximately 5 hr of 100 μM morphine treatment, DADLE inhibition of adenylyl cyclase activity was much reduced. By 9 hr, the adenylyl cyclase activity in the presence of 10 μM DADLE was comparable to the activity measured in the absence of peptide (Fig. 3).

Binding studies in control and treated 7315c cell membranes. In order to detect time-dependent changes in receptor density in chronically exposed 7315c cells, ^{3}H-antagonist competition studies were performed on membranes from cells that had been exposed to 100 μM morphine for varying periods of time. When [^{3}H]DIP binding was competed by unlabeled DIP in control cell membranes, computer analysis resulted in a one-site model with an antagonist K_i of 0.77 ± 0.01 nM and receptor density of 44 ± 2 fmol/mg of protein (Table 2).

Competition studies in membranes from cells that had been treated with 100 μM morphine for 5 hr yielded similar results (Fig. 4A). Monophasic DIP competition curves with very similar DIP K_i values were also obtained after 24 and 72 hr of morphine treatment. However, the number of receptors was slightly reduced after 24 hr, and had decreased to 32% of the numbers found in membranes from untreated cells after 72 hr of morphine treatment (Fig. 4, B and C, Table 2).

TABLE 2

Diprenorphine binding in membranes from control and treated 7315c cells

The 7315c cells were incubated at 37° in the absence or presence of 100 μM morphine for the indicated time. The number of independent replicate experiments under each condition is indicated in the table (*N*). Parameters for untreated 7315c cells were obtained from cells incubated for 5, 24, and 72 hr in the absence of 100 μM morphine. Results were analyzed by a nonlinear curve-fitting algorithm (23). The tabulated values are the estimates of K_i and B_{max} (± standard error of the parameter estimates) for DIP from a combined analysis from *N* experiments. The concentration of [^{3}H]DIP was 1 nM.

Hours of 100 μM morphine treatment	*N*	K_i	B_{max}
		nM	*fmol/mg protein*
untreated	9	0.77 ± 0.01	44 ± 1.8
5	3	0.75 ± 0.3	43 ± 7
24	3	0.73 ± 0.1	35 ± 1.5
72	3	0.84 ± 0.26	14 ± 1.3

Guanine nucleotide regulation of agonist binding in membranes from morphine-treated 7315c cells. To detect changes in the agonist-receptor-G protein interactions during the development of tolerance, the ability of GTPγS, the non-hydrolyzable GTP analogue, to regulate agonist binding in 7315c cell membranes was examined. The possibility that changes in agonist/receptor coupling may occur during prolonged agonist exposure was investigated by measuring the agonist K_{app} in membranes from cells that had been treated with morphine for varying amounts of time in the absence and presence of GTPγS. The agonist K_{app} was determined by competition against ^{3}H-antagonist binding.

Initially, competition studies in the presence and absence of GTPγS were performed in untreated cell membranes (Fig. 5A). Competition against [^{3}H]DIP binding by DAGO in the absence of added nucleotide resulted in a complex displacement curve. Computer analysis indicated two components of agonist bind-

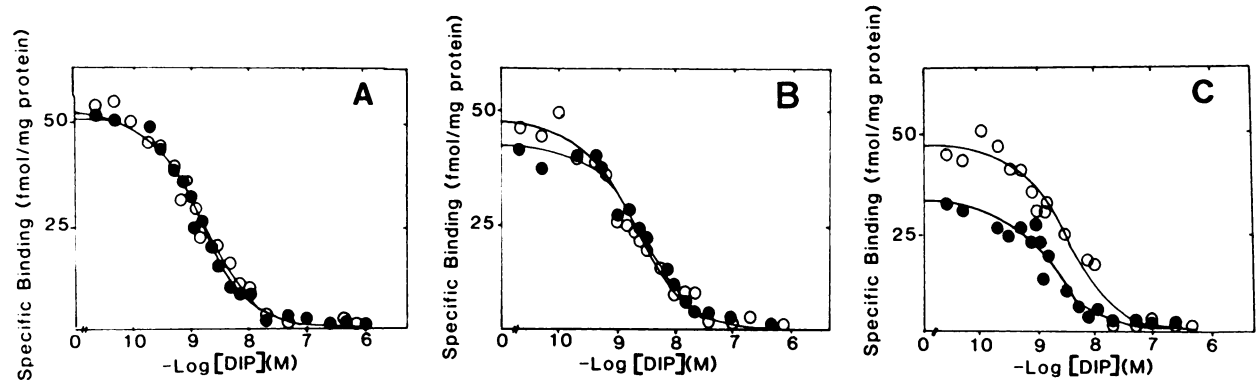

Fig. 4. Competition for [^{3}H]DIP binding by unlabeled DIP in control (○) and treated (●) 7315c cell membranes exposed for 5 hr (A), 24 hr (B), and 72 hr (C) to 100 μM morphine. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for one binding site under each condition. Nonspecific binding was a modeled parameter equal to 19% of total binding in the untreated cells, 21% in cells treated for 5 hr, 24% in cells treated for 24 hr, and 18% in cells treated for 72 hr with 100 μM morphine. Essentially equivalent nonspecific binding was observed when other unlabeled ligands were used to estimate nonspecific binding. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided under Materials and Methods. Data are from a single experiment which was repeated with similar results.

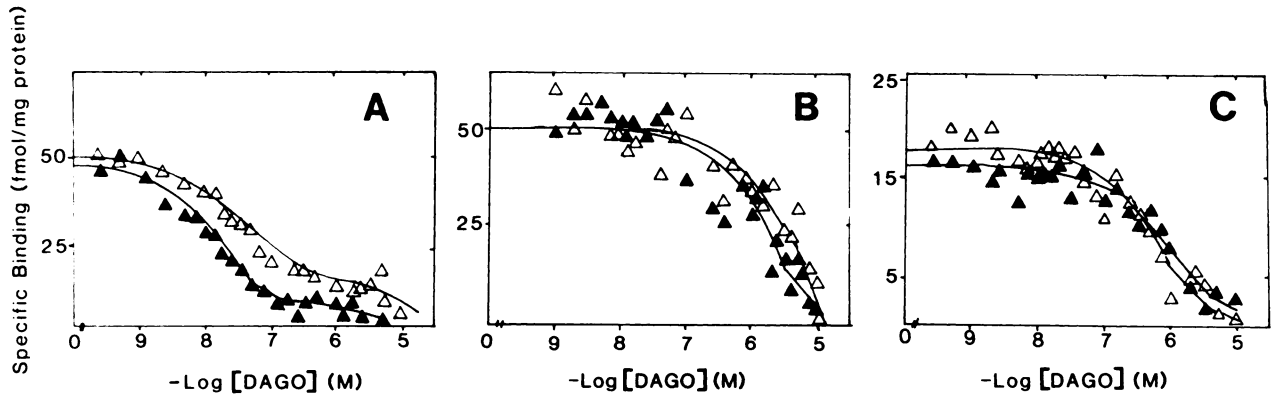

Fig. 5. Competition for [^{3}H]DIP binding by unlabeled DAGO in control (A) and treated 7315c cell membranes exposed for 5 hr (B) and 72 hr (C) to 100 μM morphine. Studies were performed in the absence (▲) and presence (△) of 10 μM GTPγS. The *lines* are traced from the best fits generated by the LIGAND program. The optimal fits produced from data in control membranes (A) were for two binding sites in both the presence and absence of 10 μM GTPγS. The best fit produced from 5 (B)- and 72 (C)-hr treated 7315c cell membranes resulted in one binding site in both the absence and presence of 10 μM GTPγS. In control studies, membranes from cells incubated for 5 or 72 hr in the absence of morphine yielded biphasic competition curves as expected. After these periods in culture in the absence of morphine, GTPγS was still able to reduce agonist affinity (data not shown). Nonspecific binding was a modeled parameter equal to 19% in the absence and 22% in the presence of GTPγS in untreated membranes, 24% in the absence, and 17% in the presence of GTPγS in treated membranes. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of binding are provided under Materials and Methods. Data are from a single experiment which was repeated with similar results.

ing with high and low affinities. The high affinity site had a K_{app} of 1–10 nM and the low affinity site a K_{app} of 100–500 nM. The addition of 10 μM GTPγS caused a rightward shift in both the high and low affinity sites (Table 3) and an increase in the proportion of binding sites in the low affinity state, with a concomitant increase in the total number of measured binding sites (from 47 ± 17 fmol/mg of protein to 93 ± 21 fmol/mg of protein). These observations are similar to those obtained when competition studies against [^{3}H]naloxone binding by DAGO in the absence and presence of GTPγS were performed (5).

The competition of [^{3}H]DIP binding by DAGO in membranes from cells that had been treated with morphine for 5 hr resulted in a monophasic curve (Fig. 5B). Analysis suggested that a one-site model fit the experimental data significantly better than a two-site model (Table 3). The K_{app} for DAGO was now 404 ± 200 nM, whereas the receptor density (44 ± 9 fmol/mg of protein) was comparable to that estimated when [^{3}H]DIP was subjected to competition by DAGO or DIP in untreated membranes (Tables 2 and 3). The addition of 10 μM GTPγS produced no significant change in the agonist K_{app} or B_{max} in membranes from cells subjected to 5 hr of morphine treatment ($p > 0.05$).

[^{3}H]DIP binding competed by DAGO in membranes from cells that had been treated with morphine for 72 hr also yielded a monophasic competition curve (Fig. 5C). Computer analysis confirmed the existence of a single agonist affinity state with a K_{app} for DAGO of 134 ± 44 nM (Table 3). These studies also confirmed that the number of [^{3}H]DIP-binding sites present after 72 hr of morphine treatment was reduced significantly ($p < 0.01$) relative to control values. There was still no regulation of agonist affinity by GTPγS after 72 hr of incubation with morphine (Table 3). Thus, both high affinity and very low affinity states of the receptor detectable in the absence and presence of guanyl nucleotides, respectively, were no longer apparent after exposure to morphine for a period as brief as 5 hr. Reduction in agonist apparent affinity and guanyl nucleotide regulation of agonist affinity occurred before there was a significant reduction in the number of binding sites.

TABLE 3

Opioid binding by membranes from treated 7315c cells in the presence and absence of GTPγS

Cell membranes were incubated for 20 min at 37° in modified Krebs buffer in the absence or presence of 10 μM GTPγS, the nonhydrolzable GTP analogue. The number of independent replicate experiments under each condition is indicated in the table (*N*). Results were analyzed by a nonlinear curve-fitting algorithm (23). The tabulated values are the estimates of K_D and B_{max} (± standard error of the parameter estimate) for DAGO from a combined analysis of the results from *N* independent experiments. Competition studies against [^{3}H]NAL in untreated cells were obtained from a previous paper (5). Similar results were obtained using [^{3}H]DIP ($N = 1$). The concentration of [^{3}H]DIP was 1 nM.

Hours of 100 μM morphine treatment	Radioligand	*N*	[GTPγS]	DAGO	
				K_{app}	B_{max}
			μM	nM	fmol/mg protein
0	NAL	3	none	7 ± 2	28 ± 3
				465 ± 220	16 ± 20
		3	10	36 ± 15	41 ± 4
				3600 ± 1200	60 ± 13
0	DIP	1	none	10 ± 2	27 ± 5
				400 ± 20	20 ± 12
		1	10	40 ± 5	35 ± 13
				4000 ± 1000	58 ± 8
5	DIP	3	none	404 ± 200	44 ± 9
		3	10	238 ± 100	41 ± 4
72	DIP	3	none	134 ± 44	11 ± 1
		3	10	110 ± 33	9 ± 1

TABLE 4

Functional and/or binding changes after 100 μM morphine treatment of 7315c cells

Hours of treatment	Change
5	loss in the ability of the opioid agonist to inhibit adenylyl cyclase activity (desensitization) decrease in the agonist affinity loss of GTPγS regulation of opioid agonist binding
24	desensitization small decrease (20%) in receptor density (down-regulation) (agonist affinity and GTPγS regulation not measured)
72	desensitization approximately 60% down-regulation decrease in agonist affinity loss of GTPγS regulation of opioid agonist binding

Discussion

We report the occurrence of two time-dependent processes: desensitization, characterized as the loss in the ability of the opioid agonist to inhibit adenylyl cyclase activity, and down-regulation, defined as the decrease in the number of binding sites, upon chronic morphine treatment of the μ receptor in the 7315c cell. In correlation with these findings our binding data show that, after desensitization has occurred, there is a decrease in the opioid agonist affinity, and agonist binding is no longer under GTP control. There was no change in the receptor density after 5 hr of morphine treatment when the agonist response was significantly attenuated. However, after 72 hr of 100 μM morphine exposure, there was a significant decrease in the receptor density. Again, no GTP effect on agonist binding was observed. The adaptative processes described for the μ opioid receptor are very similar to those observed for the δ opioid receptor in the NG108-15 cell (1, 2, 24) and the β-adrenergic receptor (25, 26) upon chronic agonist treatment.

Observations described herein are not complicated by the presence of other opioid receptor types, since ligand binding studies suggest that 7315c cells contain a homogeneous population of μ opioid receptors (4, 5). We reconfirmed initial observations that opioid inhibition of adenylyl cyclase was mediated through the μ receptor (6). DAGO, the μ-selective agonist, decreased adenylyl cyclase activity in a dose-dependent fashion, whereas DPDPE, the δ-selective agonist, and EKC and U50,488H, both κ agonists, were unable to inhibit cyclase activity at low concentrations. Naloxone (10 nM) antagonized both DAGO- and DPDPE-mediated suppression of cyclase. These results are consistent with the view that opioid inhibition of adenylyl cyclase in 7315c cell membranes results from activation of μ opioid receptors.

Although the dispersed 7315c cells did not divide in culture, the cells survived for at least 18 days. Opioid inhibition of adenylyl cyclase was observed in membranes from cells cultured for that period of time. The membrane protein content of each incubate did not change during 72 hr of incubation. Thus changes in receptor number reported here presumably reflect changes in receptor synthesis, internalization, or degradation and are not related to or influenced by cell growth or replication.

Morphine was chosen as the appropriate drug for chronic treatment. Morphine, a relatively μ-selective agonist (8), has been used widely to study the development of opioid tolerance and dependence. We chose conditions analogous to the studies of Law *et al.* (1, 24) describing δ opioid receptor desensitization and down-regulation for the NG108-15 cells upon chronic agonist exposure. Maximal inhibition of prostaglandin E_1-stimulated adenylyl cyclase activity in both control and morphine-treated NG108-15 cells (1, 3) was observed with 100 μM morphine. This concentration should give almost complete occupation of all functional receptors, including the low agonist affinity states of the μ receptor (4), throughout the chronic exposure period. We have shown that, despite the high concentration, morphine can be completely removed from cell membranes prior to assay of enzyme or receptor properties. The opioid agonist DADLE was chosen to examine opioid inhibition of adenylyl cyclase activity in cells that had been treated with 100 μM morphine for varying time periods. This peptide has

been found to act as a full agonist at the μ opioid receptor in the neurons of the rat locus coeruleus (27) and in peripheral μ opiate receptors (28), and was chosen to correlate with earlier studies examining agonist-mediated inhibition of cyclase activity in NG108-15 cells (4).

We observed loss of DADLE inhibition of adenylyl cyclase after 5 hr of 100 μM morphine treatment, and after 9 hr of exposure, concentrations of DADLE as high as 10 μM failed to inhibit cyclase activity. Forskolin-stimulated enzyme activity remained at levels comparable to those observed in untreated cells even after incubation with morphine for 48 hours. The onset of desensitization in the 7315c cells appeared to occur later than reported for the δ opioid receptor in the NG108-15 cells after 10 nM etorphine exposure (1) or the β-adrenoceptor in S49 lymphoma cells after 10 μM isoproterenol exposure (29). In these systems significant desensitization was apparent after 3 hr of agonist exposure, at a time when the agonist effect was only slightly reduced in the 7315c cells.

The mechanisms underlying loss of agonist potency or effect have been investigated in many receptor systems (1, 9, 29–32), and several biochemical events appear to be associated with this phenomenon. Measurement of the number of opioid-binding sites detectable in 7315c cell membranes with the labeled antagonist [^{3}H]DIP after exposure of cells to morphine for 5 hr, a time when desensitization was clearly apparent, shows that the number of receptors was not changed. However, their ability to interact with agonist, and the regulation of agonist affinity, may have been impaired. Binding experiments were performed on membranes from cells exposed to 100 μM morphine for 5 hr (onset of desensitization) in the presence and absence of 10 μM GTPγS. Since the binding affinity of opioid antagonists at μ receptors in 7315c cells was unaffected by guanyl nucleotides (5), we used [^{3}H]DIP to label μ sites and examined the pattern of competition by DAGO. Employing an agonist concentration range spanning 5 orders of magnitude, we were able to identify only one agonist affinity state in membranes from 7315c cells treated for 5 hr. Computer analysis yielded a preferred one-site fit with a K_{app} for DAGO of approximately 200 nM. This is in contrast to the identification of at least two agonist affinity states of the μ receptor in membranes prepared from untreated 7315c cells or from guinea pig cortical membranes (5). The DAGO affinity for μ receptors in membrane exposed to 100 μM morphine for 5 hr was 30-fold lower than the high affinity state identified in untreated membranes. The existence of reduced agonist affinity states of G protein-coupled receptors following short-term agonist exposure has been reported for the δ opioid receptor (2) and the β-adrenergic receptor (25, 26). The state of the 7315c cell μ receptor, with a K_{app} of 200–400 nM after 5 hr of morphine exposure, is apparently comparable to that identified in pertussis toxin-treated 7315c cell membranes with a K_{app} of 255 ± 100 nM (5). These results are consistent with previous work (19, 31) in which investigators reported a similarity in opioid agonist binding characteristics at δ receptors in NG108-15 cells that had been treated with pertussis toxin and those that had been chronically treated with the opioid agonist, DADLE.

The addition of GTPγS, the nonhydrolyzable GTP analogue, to membranes from morphine-treated cells did not significantly change the agonist K_{app} or B_{max} value. The loss in guanyl nucleotide regulation of agonist binding is thought to be associated with the onset of the desensitization process in several receptor systems (1, 24–26) and is reminiscent of the agonist insensitivity to GTP regulation observed in pertussis toxin-treated 7315c membranes. The results suggest that an impaired interaction of the μ opioid receptor with the guanyl nucleotide-binding protein might be responsible for the desensitization observed in the 7315c cells upon chronic morphine treatment.

In addition to desensitization, a second cellular adaptative process, down-regulation, was observed in 7315c cell membranes following continuous opioid agonist exposure. Competition studies employing the opioid antagonist unlabeled DIP against [^{3}H]DIP demonstrated a gradual decline in the total number of μ opioid receptors as the duration of 100 μM morphine treatment increased. The mechanism of down-regulation is not known, although investigators have suggested an internalization of the ligand-receptor complex into sequestered regions of the membrane that are inaccessible to hydrophilic ligands (33, 34). Unlike the fairly rapid onset of desensitization, down-regulation occurred only after approximately 24 hr exposure of 7315c cells to morphine.

After 72 hr of 100 μM morphine treatment, there was a 60–70% reduction in the number of labeled μ opioid-binding sites. Thus, it is possible that receptor degradation followed internalization. It is possible that desensitized receptors uncoupled from G proteins are more readily internalized and degraded than G protein-coupled receptors. It may be relevant that a reduction in μ receptor density was also observed following sustained pertussis toxin treatment in 7315c cells (5). Earlier studies have reported a 50–70% decrease in the number of δ opioid receptors following pertussis toxin treatment in the NG108-15 cells (5, 19).

Using binding and functional studies, we have demonstrated the occurrence of two different cellular adaptation processes which occur upon chronic morphine exposure of the μ opioid receptor in the 7315c cell (Table 4). These processes are similar to those described for other receptor systems coupled to guanyl nucleotide-binding proteins after chronic agonist exposure. Specific mechanisms involved in μ opioid receptor desensitization and down-regulation remain to be elucidated. Evidence presented here suggests that desensitization of the μ opioid receptor in the 7315c cells is associated with the uncoupling of the ligand receptor complex from the guanyl nucleotide-binding protein. Desensitization is followed by a loss of opioid receptors by an as yet undefined mechanism.

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