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Receptor Mechanisms of Opioid Tolerance in SH-SY5Y Human Neural Cells

BRUCE D. CARTER and FEDOR MEDZIHRADSKY

Departments of Biological Chemistry (B.D.C., F.M.) and Pharmacology (F.M.), The University of Michigan Medical School, Ann Arbor, Michigan 48109

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SUMMARY

In differentiated SH-SY5Y human neuroblastoma cells, various opioids exhibited a wide range of potencies (K_i) in acutely inhibiting adenylate cyclase to different extents (I_{max}). After exposure of the cells to opioids for 24 hr, the initially reduced cAMP content of the cells recovered toward pre-exposure levels. Withdrawal of agonist from, or addition of antagonist to, the tolerant cells rapidly increased the cAMP content to 1.5 times the basal value. Long term treatment of the cells with agonists of high acute potency, such as Tyr-D-Ala-Gly-(Me)Phe-Gly-ol and levorphanol, decreased the B_{max} of the antagonist [^{3}H]naltrexone by 80–95%, increased the K_s for GTPase stimulation 10–14-fold, and increased the K_i for adenylate cyclase inhibition 2–3-fold. On the other hand, these parameters were only marginally affected by agonists of lower acute potency, such as profadol and morphiceptin, regardless of their I_{max} in inhibiting adenylate cyclase. The reduction in the level of receptor binding was experimentally not dissociable from effector desensitization. Tyr-D-Ala-Gly-(Me)Phe-Gly-ol retained the characteristics of a potent agonist in inducing tolerance even under conditions of submaximal signal, produced by lower concentrations of the peptide or by pretreatment with pertussis toxin. Alkylation of receptors by β-chlornaltrexamine, although it reduced [^{3}H]naltrexone binding by 50%, did not significantly alter the rank order of opioid agonists based on their ability to acutely inhibit adenylate cyclase. These results show that in opioid-tolerant SH-SY5Y cells the concurrently occurring down-regulation of receptor and shifts in the concentration dependence of effector response correlate with the potency of a given opioid in producing its acute effect but not with the maximum extent of that effect.

The molecular mechanisms underlying opioid tolerance, desensitization of opioid receptor-effector coupling, and partial agonism have yet to be ascertained. A cellular model for opioid tolerance and dependence based on the regulation of cellular cAMP in NG108–15 neuroblastoma × glioma hybrid cells has been proposed; chronic exposure to morphine was accompanied by time-dependent recovery of the cAMP concentration acutely reduced by the agonist. Upon removal of morphine or addition of opioid antagonist, the cellular cAMP content rebounded to levels above basal, in agreement with a state of dependence (1). The physiological relevance of this cellular model was supported by the observed increase in cAMP levels during morphine withdrawal in rat brain (2). Subsequent investigations have suggested the involvement of two processes in the development of opioid tolerance, i.e., the uncoupling of receptor from G protein and then a down-regulation of receptors. Long term exposure to opioid of NG108–15 cells (3) and 7315c rat tumor cells (4) decreased high affinity opioid receptor binding, representing the G protein-coupled form of the receptor (5). Similar results have been obtained in brain membranes; high affinity μ receptor binding in guinea pig brain was decreased after long term exposure to morphine (6), and the number of μ and δ receptors in rat brain was reduced after chronic etorphine treatment (7). In brain membranes of morphine-treated rats (8) and in Tyr-D-Ala-Gly-Phe-D-Leu-treated NG108–15 cells (9), uncoupling of receptor from G protein was demonstrated by the desensitization of opioid-stimulated GTPase. In differentiated SH-SY5Y human neuroblastoma cells, which preferentially express μ-opioid receptors, the desensitization of adenylate cyclase inhibition after chronic morphine treatment was described (10, 11), but no change in ligand binding or sensitivity to guanine nucleotide was observed (11). The acute effects of opioids on both adenylate cyclase and GTPase in these cells have recently been quantified (12, 13).

The studies cited above have, in part, described altered properties of receptor-effector components during opioid tolerance. However, the mechanisms by which agonists induce these changes remain unknown. Signal generation and effector activation depend on the formation of an active receptor conformation induced with distinct efficiencies by full and partial agonists (14, 15). Although several models have been forwarded

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; β-CNA, β-chlornaltrexamine; DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen-OH; ICI 174864, (allyl)$_2$-Tyr-Aib-Aib-Phe-Leu-OH; PGE_1, prostaglandin E_1; PL017, Tyr-Pro-(NMe)Phe-D-Pro-NH_2.

to explain receptor activation by agonists (see Discussion), many unexplained phenomena remain (e.g., see Ref. 16). In particular, no study has yet compared the mechanisms by which full and partial agonists affect the cascade of signal transduction from receptor to adenylate cyclase via G protein. After characterizing the coupling between components of opioid signal transduction in SH-SY5Y neural cells (13), we have now in these cells investigated effector desensitization and receptor down-regulation by full and partial opioid agonists. On the basis of the results, the suitability of proposed concepts of agonism to predict the ability of an opioid to induce tolerance in SH-SY5Y cells is discussed. Preliminary results of this work have been presented elsewhere (17).

Experimental Procedures

Materials. The μ-selective opioid agonists levorphanol and morphine (18), the partial agonists nalbuphine and profadol (19), the nonselective and δ-selective antagonists naloxone and ICI 174864, respectively (18), and the nonselective irreversible antagonist β-CNA (20) were obtained through the Narcotic Drug and Opiate Peptide Basic Research Center at the University of Michigan. The μ-selective morphiceptin analog PL017 (21) was purchased from Peninsula Laboratories (Belmont, CA). [^{3}H]Naltrexone (22 Ci/mmol) was provided by the National Institute on Drug Abuse, and the cAMP assay kits and [γ-^{32}P]GTP (30 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL) or Diagnostic Products Corp. (Los Angeles, CA). Other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). SH-SY5Y human neuroblastoma cells were a gift from Dr. June Biedler, Memorial Sloan-Kettering Cancer Center (New York, NY).

Cell culture. Human neuroblastoma SH-SY5Y cells were grown under 10% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Retinoic acid (10 μM) was added to the medium 1 day after passage and was maintained during growth to differentiate the cells (10). In all experimental protocols with intact cells, the viability of the cells was ascertained by measuring the cellular K^+/Na^+ ratio. Under all experimental conditions a ratio of 3 or greater, indicative of intact cells with an unperturbed plasma membrane, was obtained (22).

For induction of tolerance, SH-SY5Y cells that had been differentiated with retinoic acid for 6–7 days were exposed for 24 hr to various opioids present in the culture medium at a concentration of 10 μM, except where otherwise specified. Pertussis toxin treatment was carried out by addition of 100 ng/ml to the media for 18–24 hr.

Protein determination. Protein concentration was measured according to the method of Lowry *et al.* (23), with bovine serum albumin as standard. The samples were initially solubilized with 1 N NaOH at 37° for 1 hr, preceded in some instances by exposure to 2% sodium dodecyl sulfate.

Membrane isolation. Surface-growing cells were washed three times in isotonic buffer medium (0.9% NaCl, 0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, pH 7.4) and lifted off the surface by incubation with modified Puck's solution for 10 min (24). The suspension was centrifuged for 4 min at 200 × *g*, and the pellet was resuspended in 100 volumes of hypotonic buffer (0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, 0.2 mM $MgSO_4$, 1 mM dithiothreitol, pH 7.4). The suspension was centrifuged for 15 min at 20,000 × *g* at 4°, and the pellet was dispersed with a Dounce homogenizer in the hypotonic buffer or in 50 mM Tris·HCl, pH 7.4. For the GTPase assay the membranes were stored at −80°.

Receptor alkylation. Surface-growing cells in culture flasks were rinsed three times with the isotonic buffer solution and then incubated for 45 min with 1 nM β-CNA in Dulbecco's modified Eagle's medium in the absence of fetal calf serum. The cells were then rinsed and harvested for assessment of the adenylate cyclase activity or preparation of membranes as described above.

Ligand binding. This procedure was carried out in isolated membranes as described previously (18, 25). Briefly, the assay medium contained 400 μl of membranes (40–140 μg of protein) suspended in 50 mM Tris·HCl, pH 7.4, 100 μl of H_2O containing unlabeled naltrexone, and 25 μl of [^{3}H]naltrexone (0.05–5 nM). Specific binding of [^{3}H]naltrexone was determined with 2 μM naltrexone. After a 15-min preincubation, the assay was initiated by the addition of radiolabeled opioid; the samples were incubated for 30 min at 25° to achieve binding equilibrium, quickly filtered, and subjected to liquid scintillation counting.

Assays of adenylate cyclase. After the exposure to opioids or culture medium alone, cells were washed three times in the isotonic buffer described above and lifted off the surface by incubation with modified Puck's solution (24). The cell suspension was then centrifuged for 4 min at 200 × *g*, and the pellet was resuspended (approximately 4 × 10^6 cells/ml) 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, 8 mM theophylline, pH 7.4 at 37°). After 10 min at 37°, acute inhibition of adenylate cyclase activity was initiated by the addition of 50 μl of a solution of PGE_1 (final concentration, 10 μM) and opioid to 450 μl of cell suspension. The assay was terminated after 15 min by the addition of 250 μl of 0.15 M HCl, followed by heating for 4 min at 75°. These samples were stored at −20° until further treatment. After thawing and neutralization with Tris base, the cellular cAMP content was determined using a radioligand binding assay kit.

To assess the time dependence of cAMP levels, cells grown in 35-mm polystyrene plates were exposed for various times to 10 μM opioid added to the culture medium, and then the medium was removed and replaced with physiological buffer A containing the original concentration of opioid and 10 μM PGE_1. After incubation for 10 min, the assay was terminated by aspiration of the buffer and addition of 1 ml of 0.05 M HCl. The cells were scraped off the plates with a 2-ml H_2O rinse and stored at −20°. After thawing, the samples were centrifuged at 20,000 × *g* for 15 min and neutralized with Tris base, and the concentration of cAMP in the supernatant was determined as described above. The remaining membrane pellets were dissolved in 2% sodium dodecyl sulfate for the determination of protein.

Assay of GTPase. With minor modifications, the method described previously was used (13, 26). Briefly, the assay medium consisted of 30 μl of membranes suspended in hypotonic buffer (5–20 μg of protein/tube), 40 μl of buffer A supplemented with 1 mM 5′-adenylylimidodiphosphate, an ATP-regenerating system (20 mM creatine phosphate, 3 units/tube creatine kinase), 20 μl of GTP solution to give a final concentration of 2 μM (composed of 1.5 μM unlabeled GTP and 0.5 μM [γ-^{32}P]GTP), and 10 μl of H_2O or DAMGO solution. In separate tubes, the opioid-insensitive, high-K_m GTPase activity was measured in the presence of 50 μM GTP. The assay was initiated by the addition of membrane suspension to the prewarmed buffer medium. After incubation for 10 min at 37°, the liberated ^{32}P was separated from the nucleotide by charcoal treatment and subjected to liquid scintillation counting as described.

Data analysis. The binding data from saturation experiments were fit to a one-site model by nonlinear regression analysis, using the program SYSTAT, as described previously (13). The best fit was determined using the *F* ratio test to compare the weighted residual sum of squares, with $p > 0.05$. All data reported are based on regressions for which the distribution of residuals was random and normal.

Nonlinear regression analysis of data for inhibition of adenylate cyclase and stimulation of GTPase was computed with the program GraphPad (ISI Software, Philadelphia, PA). The data from multiple experiments were fit simultaneously to an equation (depicted on a logarithmic scale) that describes many dose-response functions: $Y = A + (B - A)/[1 + (10^X/10^C)^D]$, where Y is the percent inhibition (or stimulation), X is the logarithm of the ligand concentration, A is the minimum (zero) and B the maximum of the curve (I_{max} or S_{max}), C is the IC_{50} (K_i or K_e), and D is the slope factor.

Results

Acute inhibition of adenylate cyclase by opioids. Various opioids attenuated PGE_1-stimulated cAMP formation in intact SH-SY5Y cells with different potencies and maximal effects (Fig. 1; Table 1). Full agonists such as DAMGO and levorphanol had low K_i values and high I_{max} values, whereas partial agonists such as profadol produced less inhibition and required higher concentrations to elicit the same extent of effector response. Interestingly, some opioids induced either strong inhibition with very high K_i values (e.g., morphiceptin) or weak inhibition with low K_i values (e.g., nalbuphine). The acute effects of all the agonists were blocked by the antagonist naloxone, and the dose-response curve for DAMGO was unaffected by the presence of 1 μM ICI 174864.

Time course of cellular cAMP content during long term exposure to opioids. In the continued presence of maximally effective concentrations of a given opioid, the initially attenuated level of cellular cAMP recovered toward the basal value (Fig. 2). Maximum desensitization occurred within 24 hr and, although all the opioids examined produced some level of tolerance, only DAMGO caused complete recovery of the cAMP content. Withdrawal of DAMGO after its long term presence in the medium rapidly elevated the cAMP content to 50% above basal. The subsequent return to basal level was also swift, being nearly complete within 2 hr. Although long term treatment with morphine or the partial agonist profadol resulted in a relatively small recovery of cellular cAMP, the removal of these opioids caused an overshoot of the cyclic nucleotide similar to that obtained with the washout of DAMGO (Fig. 2).

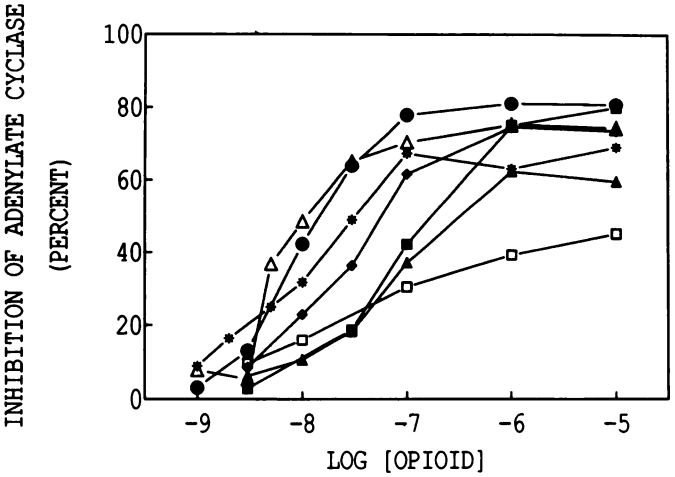

Fig. 1. Inhibition of adenylate cyclase by opioids in SH-SY5Y cells. Intact cells were incubated with DAMGO (●), levorphanol (△), morphiceptin (■), morphine (◆), nalbuphine (□), PL017 (*), or profadol (▲) for 15 min at 37°. Subsequently, the cellular concentration of cAMP was determined and the results are expressed as inhibition of adenylate cyclase, relative to untreated cells. Shown are mean values obtained in two to five experiments, each carried out in duplicate. The corresponding inhibition parameters are listed in Table 1.

TABLE 1

Acute inhibition of adenylate cyclase by opioids

The experiments were carried out as described in the legend to Fig. 1. The listed parameters were determined by nonlinear regression analysis, as described in Experimental Procedures. Shown are mean ± standard error values and *n*, the number of observations (each carried out in duplicate) obtained in the number of experiments given in parentheses.

Opioid	K_i	I_{max}	*n*
	nM	%	
DAMGO	9.7 ± 0.4	81 ± 0.8	32 (5)
Levorphanol	6.7 ± 0.8	74 ± 2.6	24 (4)
Morphiceptin	91.0 ± 2.0	81 ± 0.6	13 (3)
Morphine	28.0 ± 3.8	75 ± 2.5	17 (3)
Nalbuphine	34.0 ± 6.0	47 ± 1.3	12 (2)
PL017	10.8 ± 2.0	70 ± 2.9	20 (3)
Profadol	65.0 ± 14.0	63 ± 3.0	17 (3)

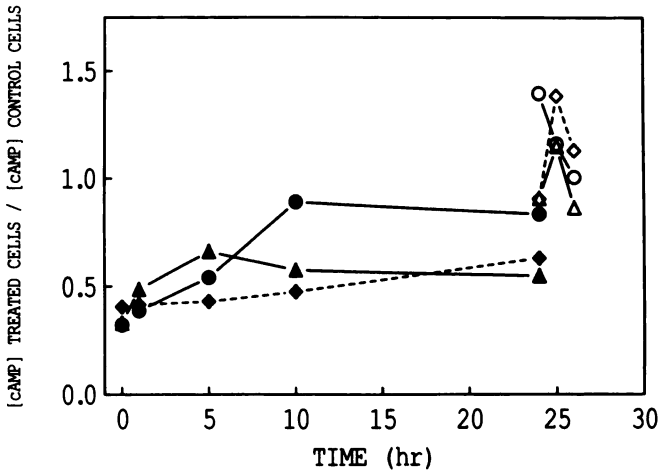

Fig. 2. Time-dependent changes in cellular cAMP during long term exposure to opioids. The concentration of cAMP was determined after the incubation of SH-SY5Y cells with 10 μM levels each of DAMGO (●), morphine (◆), and profadol (▲) at 37° for various lengths of time. *Open symbols*, levels of cAMP after withdrawal of the corresponding opioid by thorough washing of the cells. The depicted data represent the means obtained in two or three experiments carried out in duplicate.

Inhibition of adenylate cyclase after long term opioid treatment. The exposure of SH-SY5Y cells to different opioids for 24 hr distinctly affected the pattern of subsequent inhibition by DAMGO of cAMP formation (Fig. 3; Table 2). Exposure to potent agonists such as DAMGO, levorphanol, or PL017 caused a rightward shift and decreased the I_{max} of the DAMGO dose-response curve (Fig. 3A). Other opioids either had no effect (profadol and morphiceptin; Fig. 3C) or shifted the curve slightly (morphine and nalbuphine; Fig. 3B). Chronic treatment with the δ-selective agonist DPDPE did not affect the acute response to DAMGO. In addition, long term exposure of the cells to DAMGO in the presence or absence of the δ-opioid antagonist ICI 174864 produced identical right- and downward shifts in the DAMGO dose-response curve. On the other hand, long term treatment with DAMGO plus naloxone resulted in acute DAMGO responses identical to those obtained with opioid-naive cells (results not shown).

Complete removal of ligands after their incubation with the cells was ascertained. Successful washout of the opioid was assessed by the addition of naloxone; stimulation of basal adenylate cyclase activity by the antagonist would have been considered evidence for incomplete removal of the agonist. In addition, incubation of the cells with naloxone for 5 min before the washout procedure did not alter the results obtained in the absence of this treatment.

Stimulation of low-K_m GTPase after long term opioid treatment. As a result of prolonged exposure of SH-SY5Y cells to various opioids, the ability of DAMGO to stimulate low-K_m GTP hydrolysis in the subsequently isolated membranes was diminished (Table 2). In analogy to their effects on adenylate cyclase, DAMGO and levorphanol strongly increased

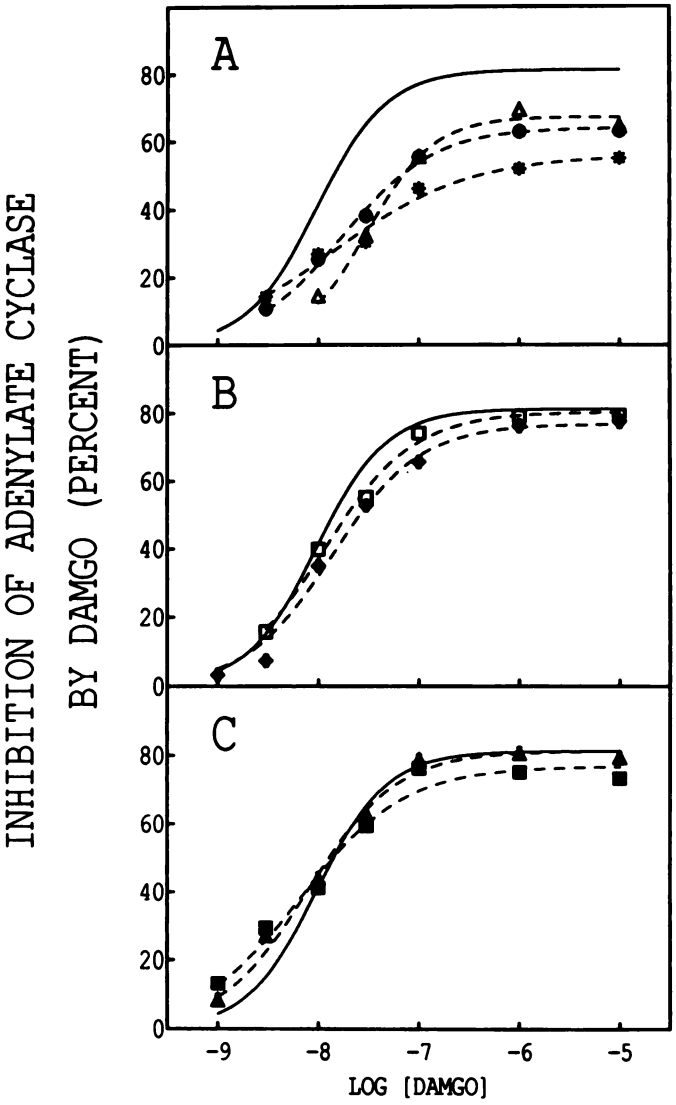

Fig. 3. Inhibition of adenylate cyclase by DAMGO after long term exposure of cells to opioids. Shown are the effects in SH-SY5Y cells incubated for 24 hr with 10 μM levels each of DAMGO (●), levorphanol (△), or PL017 (*) (A), morphine (◆) or nalbuphine (□) (B), or morphiceptin (■) or profadol (▲) (C). After the treatment, the cells were washed free of the given opioid, and the acute inhibition of adenylate cyclase by DAMGO was determined. *Solid lines without symbols*, inhibition produced by DAMGO in opioid-naive cells (shown in Fig. 1). Plotted is the acute inhibition of adenylate cyclase, relative to the activity measured in untreated cells. The results are the means of three or four experiments run in duplicate, and the *curves* were drawn according to the nonlinear regression analysis described in Experimental Procedures. The corresponding parameters and data variability are listed in Table 2.

the K_s for DAMGO stimulation of GTPase, whereas morphine, nalbuphine, and profadol produced only minor changes. The degree of GTPase desensitization was greater than that of adenylate cyclase; the potent agonists shifted the K_s >10-fold, whereas the K_i was affected 2–3 fold (Table 2). Opioid tolerance was also reflected in the increased K_s of levorphanol-induced stimulation of GTPase after pre-exposure of the cells to levorphanol or DAMGO (data not shown).

Ligand binding after opioid exposure. Membranes from cells treated with opioid displayed a reduced level of specific [^{3}H]naltrexone binding. Potent agonists such as levorphanol and DAMGO down-regulated the μ receptor by 91% and 80%, respectively, whereas exposure to morphiceptin reduced binding by only 33% (Fig. 4). As revealed by Scatchard analysis, the attenuated [^{3}H]naltrexone binding was the result of a reduced number of sites (Fig. 4, *inset*); in control and treated membranes the B_{max} values were 250 and 107 fmol/mg of protein, respectively, with no change in affinity (K_d, 0.15 versus 0.18 nM). Cells treated with DAMGO just before membrane isolation showed no reduction in [^{3}H]naltrexone binding (data not shown).

In SH-SY5Y cells, receptor down-regulation could not be separated from effector desensitization; after exposure to 10 μM DAMGO for 5 hr at 25°, instead of 24 hr at 37°, both the adenylate cyclase response (IC_{50} increased by 36.8%) and [^{3}H]naltrexone binding (reduced by 36%) were affected, to a remarkably similar extent (Fig. 5).

Relationship between acute and long term opioid effects. Based on the dose-response curves, both desensitization and receptor down-regulation were related to agonist potency. The potency of a given opioid in acutely inhibiting adenylate cyclase correlated well with that of DAMGO after chronic exposure to opioids (Fig. 6). On the other hand, no relation was apparent between the maximal inhibition of adenylate cyclase by an agonist and the K_i (Fig. 5, *inset*) or I_{max} of DAMGO (data not shown). The alkylation of opioid receptors with β-CNA reduced [^{3}H]naltrexone binding by 50% but had minimal effects on the order with which agonists exerted their maximal effects; the I_{max} for morphiceptin was slightly reduced, placing it below that for morphine (Table 3).

The significance of signal strength, and of agonist potency, in effector desensitization was examined by exposing the cells to increasing concentrations of DAMGO. Although to a different extent, all concentrations of the opioid (including 10 nM) depressed the acute inhibitory action of DAMGO on adenylate cyclase. The shift of the dose-response curve was proportional to the concentration of DAMGO used in the pretreatment (Fig. 7). The effect of reduced signal in the induction of opioid tolerance was further examined by pretreating the cells with pertussis toxin (Fig. 8). Although the inactivation of G protein attenuated the maximum inhibition of adenylate cyclase by DAMGO, from 75% to 38%, the IC_{50} value remained virtually unchanged (8.4 nM versus 7.7 nM). After prolonged exposure to DAMGO, cells pretreated with pertussis toxin still demonstrated receptor down-regulation to an extent identical to that in untreated cells (Fig. 8).

Discussion

By compensating for the initial attenuation of cAMP concentration and by overshooting the normalized concentration upon withdrawal of the agonist, the SH-SY5Y cells treated with μ-selective opioids displayed the phenomena of tolerance and dependence. A potential contribution by δ receptors to the results obtained was ruled out by the lack of effects of the δ-opioids DPDPE and ICI 174864. Long term exposure of the cells to opioid agonists affected both ligand-receptor and receptor-effector interactions; a reduced number of opioid binding sites was accompanied by desensitization of low-K_m GTPase stimulation and adenylate cyclase inhibition. The greater increase in K_s, relative to K_i, indicated extensive uncoupling of μ-opioid receptor from G protein, suggesting amplification of the signal from transducer to effector. Previous findings have indicated a loose coupling between δ-opioid-sensitive G protein

TABLE 2

Inhibition of adenylate cyclase and stimulation of low-K_m GTPase by DAMGO after long term exposure of cells to opioids

The experimental conditions for the assay of adenylate cyclase were as described in the legend to Fig. 3. GTPase activity was determined in isolated membranes after exposure of the cells to 10 μM levels of a given opioid, thorough washing, and hypotonic lysis. All the listed parameters were determined by nonlinear regression analysis, as described in Experimental Procedures. Shown are mean ± standard error values and *n*, the number of observations obtained in the number of experiments given in parentheses.

Opioid used for pretreatment	Adenylate cyclase inhibition by DAMGO			GTPase stimulation by DAMGO		
	K_i	I_{max}	*n*	K_a	S_{max}	*n*
	nM	%		nM	%	
DAMGO	18.0 ± 2.0	64 ± 1.5	28 (5)	740 ± 210.0	28 ± 2.0	15 (3)
DPDPE	7.0 ± 1	76 ± 3.0	8 (2)	—*	—	—
Levorphanol	31.0 ± 4.0	67 ± 2.2	16 (3)	1000 ± 420.0	59 ± 6.9	18 (3)
Morphiceptin	6.7 ± 1.5	77 ± 3.8	18 (3)	—	—	—
Morphine	14.0 ± 1.6	77 ± 1.9	22 (4)	150 ± 92.0	58 ± 9.3	16 (3)
Nalbuphine	12.0 ± 1.4	80 ± 2.0	20 (3)	155 ± 50.0	40 ± 3.8	12 (2)
PL017	15.0 ± 4.4	56 ± 3.2	20 (3)	—	—	—
Profadol	7.9 ± 0.9	81 ± 1.8	20 (3)	140 ± 93.0	50 ± 9.5	25 (4)
DAMGO (without pretreatment)	9.7 ± 0.9	81 ± 0.8	32 (5)	71 ± 6.2	49 ± 1.0	34 (5)

* —, Not investigated.

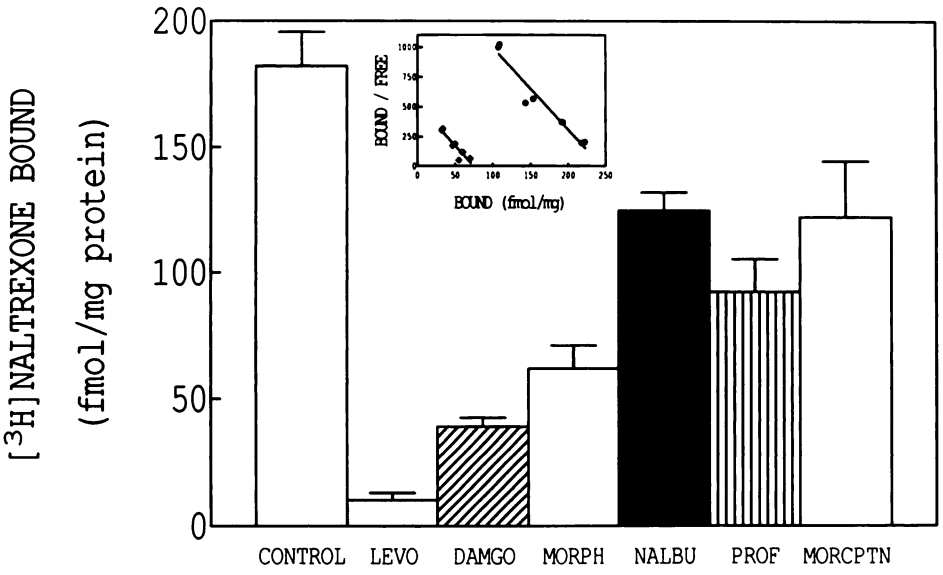

Fig. 4. Binding of [^{3}H]naltrexone in membranes of SH-SY5Y cells after their long term exposure to opioids. The cells were incubated at 37° for 24 hr with 10 μM levels each of levorphanol (*LEVO*), DAMGO, morphine (*MORPH*), nalbuphine (*NALBU*), profadol (*PROF*), or morphiceptin (*MORCPTN*). Subsequently, membranes were prepared by hypotonic lysis and the binding of 1 nM [^{3}H]naltrexone was determined. Shown are mean values and standard errors obtained in three experiments. *Inset*, Scatchard plot of [^{3}H]naltrexone binding in membranes of control cells (●) and of cells treated with 10 μM DAMGO at 37° for 5 hr (■). The results presented were replicated three times, and the binding parameters, determined by SYSTAT as described in Experimental Procedures, are listed in Results.

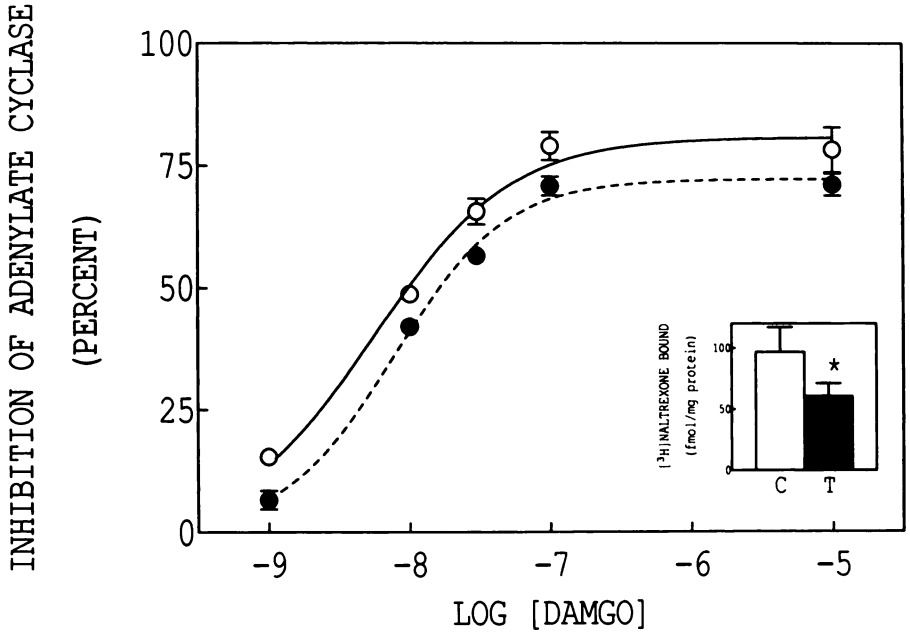

Fig. 5. Effector desensitization and receptor down-regulation after short-time, low-temperature exposure of SH-SY5Y cells to DAMGO. The inhibition of adenylate cyclase by DAMGO in SH-SY5Y cells was measured before (○) and after (●) their incubation with 10 μM DAMGO for 5 hr at 25°, followed by thorough washing to remove residual opioid. The results are expressed as inhibition of enzyme activity, relative to that in the absence of opioid. Shown are the means and standard errors of three experiments run in duplicate, The *curves* were drawn according to the nonlinear regression analysis described in Experimental Procedures. *Inset*, binding of 1 nM [^{3}H]naltrexone in membranes from control cells (□) and those treated for 5 hr at 25° with 10 μM DAMGO (■). Shown are the means and standard errors from three experiments. * Denotes significance at $P < 0.05$.

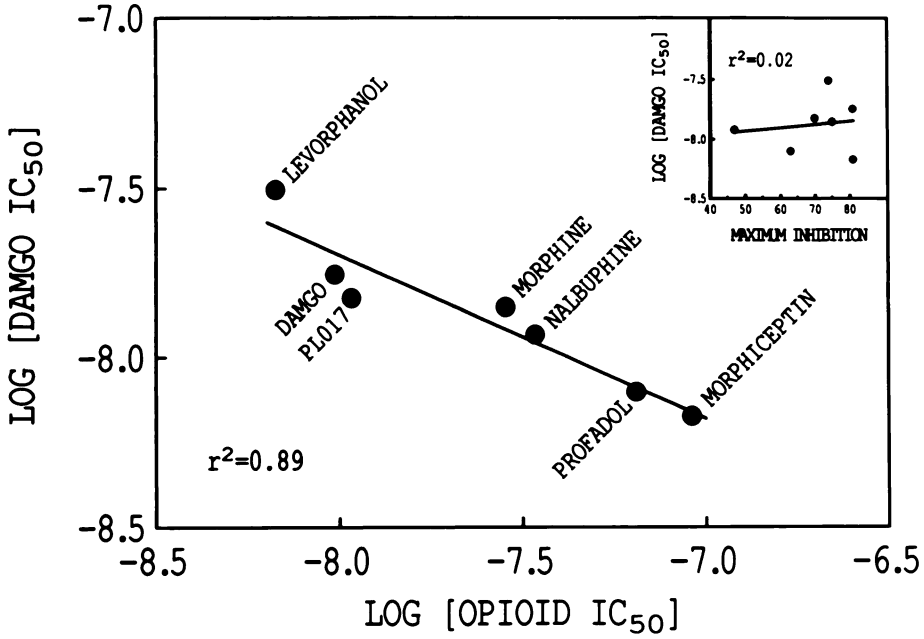

Fig. 6. Relationship between the potency of various opioids to acutely inhibit adenylate cyclase and the potency of DAMGO after exposure of SH-SY5Y cells to the opioids. Plotted is the IC_{50} for acute inhibition of adenylate cyclase by DAMGO in cells after their exposure for 24 hr to various opioids (shown in Fig. 3 and Table 2) as a function of the IC_{50} for acute enzyme inhibition by the corresponding opioid (shown in Fig. 1 and Table 1). Linear regression analysis indicated a correlation coefficient of $r^2 = 0.89$. *Inset*, IC_{50} for acute inhibition of adenylate cyclase by DAMGO after 24-hr treatment of cells with the various opioids as a function of the maximum acute enzyme inhibition caused by these opioids. The correlation coefficient was $r^2 = 0.02$.

TABLE 3

Inhibition of adenylate cyclase by opioids before and after receptor alkylation

Cells were treated with 1 nM β-CNA for 45 min, rinsed, and exposed to opioid as described in Experimental Procedures. Listed is the inhibition produced by 50 μM levels of the given opioid, relative to that produced by 50 μM DAMGO. This concentration of the μ-opioid inhibited adenylate cyclase in untreated and alkylated cells by 62.0 ± 1.3% (four experiments) and 54.7 ± 3.7% (four experiments), respectively. The depicted ratios were obtained from the mean values of three experiments displaying a statistical variability similar to that shown in Table 1.

Opioid	Inhibition of adenylate cyclase relative to that by DAMGO	
	−β-CNA	+β-CNA
DAMGO	1.0	1.0
Levorphanol	0.91	1.0
Morphine	0.93	0.98
Morphiceptin	0.99	0.82
Nalbuphine	0.58	0.37
Profadol	0.77	0.69
PL017	0.86	0.75

and adenylate cyclase (27). However, in comparisons of the degree of desensitization of GTPase and adenylate cyclase, it should be realized that these enzyme activities were measured in isolated membranes and intact cells, respectively.

Although the results of this study confirmed some of the earlier observations made with chronic opioid treatment of NG108–15 neuroblastoma × glioma cell hybrids (1, 28), 7315c rat tumor cells (6), or SH-SY5Y cells (10, 11), they revealed significant differences in the mechanisms of opioid tolerance. In 7315c cells, the initial attenuation of cellular cAMP concentrations was followed by desensitization after 5 hr of treatment with the opioid. The effect was accompanied by uncoupling of receptor from G protein, as indicated by the loss of sensitivity of agonist binding to guanine nucleotides and by the shift from high to low affinity ligand binding. Only after a 24-hr exposure to morphine was a reduction in receptor number observed (6). Similar results were described for NG108–15 cells; desensitization preceded down-regulation (28). In previous studies with SH-SY5Y cells, receptor down-regulation upon chronic morphine treatment was minimal (11) or not investigated (10). In contrast, in the study described here receptor down-regulation and receptor-effector desensitization in SH-SY5Y cells were pronounced but were not separable phenomena. The concurrent occurrence of the two processes was not the result of a rapid recovery of effector regulation by the opioids, as shown by the incomplete restoration of cAMP levels after the exposure of cells to DAMGO for a relatively short time and at low temperature. On the other hand, even under these conditions the increase in IC_{50} of adenylate cyclase corresponded to the extent by which the binding of [^{3}H]naltrexone was attenuated.

The discrepancy in the mechanisms of opioid tolerance observed in the two μ receptor-expressing cell lines, 7315c and SH-SY5Y, could be related to the nature of the agonist used to induce tolerance; morphine, which was used in the 7315c cells, was not a fully effective agonist in the present study. Thus, in contrast to such partial agonists, potent opioids such as DAMGO may convert the receptor to a conformation that allows both desensitization and down-regulation to occur rapidly. Supporting this contention is the observation made by Yu *et al.* (11) that the 24-hr treatment of SH-SY5Y cells with morphine desensitized the inhibition of adenylate cyclase but reduced the number of ligand binding sites only marginally. Had these authors measured antagonist rather than agonist (DAMGO) binding, their results are likely to have been identical to those observed for morphine in this study. SK-N-SH cells, from which the SH-SY5Y cell line was derived, treated with the full muscarinic agonist carbachol also demonstrated parallel receptor down-regulation and desensitization of phosphatidylinositide hydrolysis (29). In the NG108–15 hybrids, effector desensitization and down-regulation, induced with the full agonist etorphine, were distinctly observable (28), indicating that in these cells sequestration of opioid receptors in response to long term opioid treatment occurs less efficiently than in differentiated SH-SY5Y neuroblastomas.

The induction of tolerance by various agonists provided additional evidence for divergent mechanisms of opioid tolerance in the SH-SY5Y cells and NG108–15 cells. In the latter cells, both receptor down-regulation and desensitization of

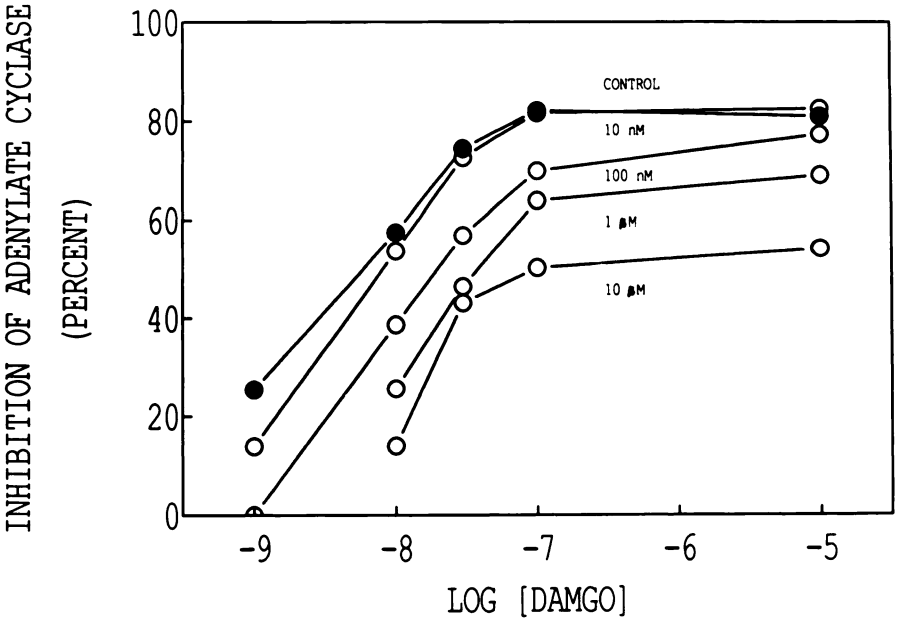

Fig. 7. Inhibition of adenylate cyclase in SH-SY5Y cells by DAMGO after long term treatment with various concentrations of DAMGO. Plotted is the percentage of inhibition of cAMP formation by DAMGO in control cells and in cells exposed to DAMGO at the concentrations listed. Shown are results of a representative experiment replicated three times. The *curves* were drawn according to the nonlinear regression analysis described in Experimental Procedures.

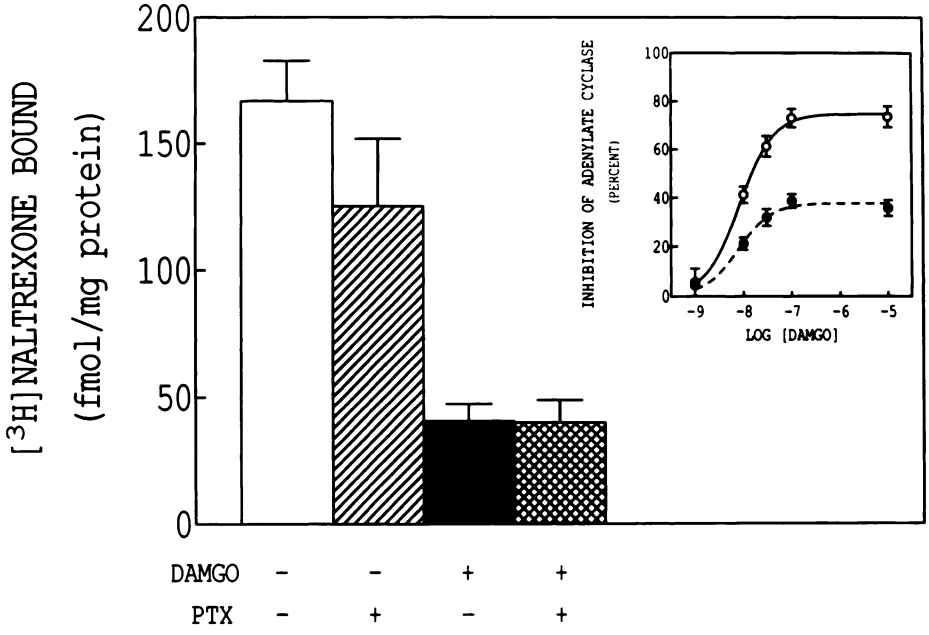

Fig. 8. Down-regulation of opioid receptors in SH-SY5Y cells treated with pertussis toxin and exposed long term to DAMGO. Plotted are the results of 1 nM [^{3}H]naltrexone binding in control cells (□), cells treated for 24 hr with pertussis toxin (▨), cells incubated for 24 hr with 10 μM DAMGO (■), or cells incubated with pertussis toxin followed by DAMGO (■). Shown are the mean values and standard errors obtained in three experiments. *Inset*, inhibition of adenylate cyclase by DAMGO in control (○) and pertussis toxin-treated cells (●). Shown are the means and standard errors from three experiments. The *curves* were drawn according to the nonlinear regression analysis described in Experimental Procedures.

adenylate cyclase inhibition correlated with the intrinsic activity of agonists, defined as the maximal inhibition of cAMP levels by a given opioid. Consequently, only compounds with high intrinsic activity were capable of receptor down-regulation (28). In contrast, in the SH-SY5Y cells long term opioid treatment desensitized receptor-effector coupling and reduced antagonist binding to an extent that was related to the potency of the agonist in acutely inhibiting cAMP formation. On the other hand, the relative maxima of adenylate cyclase inhibition proved to be poor predictors of agonist efficacy in inducing tolerance. The large role of agonist potency suggests that the induction of an active high affinity receptor conformation by agonists represents an essential step in the mechanism producing desensitization. Evidence for agonist-elicited conformational activation of receptors has been provided, e.g., the ability of agonists to promote phosphorylation (induction of an active receptor conformation) of purified β-adrenergic receptor correlated with their ability to stimulate adenylate cyclase in a reconstituted system (14), and conformational changes in muscarinic receptors were related to the efficacy of various agonists (30).

To eliminate the influence of possible spare receptors (31), the effector response to opioid action was also assessed after the partial inactivation of receptors, an approach introduced by Furchgott (32) to reveal the true efficacy of agonists. Although receptor alkylation with the irreversible opioid antagonist β-CNA decreased the number of available receptors by 50%, the maximal inhibition of adenylate cyclase did not correlate with the ability of a given agonist to induce opioid tolerance. The requirement for maximal signal strength in producing opioid tolerance was further tested by partial inactivation of G protein or use of a series of agonist concentrations

to develop cellular tolerance under conditions of differential receptor occupancy. In either of these circumstances, the importance of acute agonist potency and the concurrent nature of desensitization and down-regulation were evident. However, the extent of desensitization did depend on the level of receptor occupancy by DAMGO. At equieffective concentrations of opioids (causing 40% acute inhibition of adenylate cyclase), only DAMGO (at 10 nM) and levorphanol (at 5 nM) produced a minor shift in the dose-response curve of DAMGO.

Interestingly, the results of the pertussis toxin treatment also diverged from the theoretical expectation. According to the receptor-transducer model proposed by Kenakin and Morgan (33), reduction in the amount of G protein should shift the dose-response curve substantially toward higher ligand concentrations before decreasing the maximal effector response. However, in the pertussis toxin-treated SH-SY5Y cells the maximal inhibition of adenylate cyclase by a given agonist was reduced with no change in the corresponding IC_{50}. As shown recently, the increased density of 5-hydroxytryptamine$_{1A}$ receptors, transfected into NIH-3T3 cells, enhanced the maximal inhibition of adenylate cyclase without affecting the corresponding IC_{50} (34). Other discrepancies between established models of agonism and experimental observations have been highlighted recently (16). On the other hand, the relationship between agonist potency and ability to induce tolerance observed in the present study is accomodated by the rate theory of agonism (35). Based on this model, full agonists have fast dissociation rates and, thus, are capable of stimulating many receptors, whereas slowly dissociating ligands exhibit reduced activity. Such a role for binding frequency in the regulation of adenylate cyclase has been demonstrated for epinephrine in S49 lymphoma cells (36). In that study, the rapid on-off processes of ligand binding and dissociation, resulting in a short half-life of the epinephrine-β-adrenergic receptor complex, were shown to account (together with receptor mobility) for the observed activation of the enzyme. By combining this notion with the theory of "induced fit" (15), one could predict that opioids such as morphiceptin have a rapid dissociation rate yet a weak ability to induce conformational activation, resulting in a maximal signal but little tolerance. In contrast, PL017, a derivative of morphiceptin with a slower off rate, should induce a limited number of receptors into an optimally active state. Furthermore, at a concentration of agonist insufficient to occupy many receptors, only some receptors are converted into the excited conformation, explaining why lower concentrations of DAMGO produced less desensitization. Clearly, additional studies are needed to substantiate this hypothesis.

Although in the cellular model described here acute potency of an opioid to inhibit the effector, adenylate cyclase, was the major correlate of its ability to induce tolerance, the role of drug potency in opioid tolerance assessed *in vivo* appears to be equivocal. Behavioral work suggested an inverse relationship between drug potency and the magnitude of opioid tolerance (37), but this hypothesis was subsequently challenged by findings based on the analgesic actions of opioids (38). There is an obvious difficultly in comparing *in vitro* observations with those made in a whole organism, with the latter presenting such complicating factors as the route of drug administration, drug distribution and clearance, drug metabolism, and the method used to assess effector response. Moreover, discrepancies in the definitions of the applied terminology, e.g., efficiency, efficacy, intrinsic efficacy, and potency, represent an additional hinderance to consolidation of the phenomena of opioid tolerance observed with systems at various levels of biological complexity. Efforts are necessary to bridge the gap between the various interpretations by attempting to accomodate a plausible relationship between receptor, transducer, and effector.

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Note Added in Proof. Recent evidence showed that in SH-SY5Y cells exposed to DAMGO for 24 hr the content of $G_{\alpha o}$, the subtype of G protein that preferentially mediates the coupling of μ opioid receptor to adenylyl cyclase in these cells, was significantly decreased (39).

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Send reprint requests to: Fedor Medzihradsky, Ph.D., Department of Biological Chemistry, University of Michigan Medical School, Medical Science I, Ann Arbor, MI 48109–0606.