

Opiate Regulation of Adenosine 3':5'-Cyclic Monophosphate Level in Neuroblastoma × Glioma NG108-15 Hybrid Cells

Relationship between Receptor Occupancy and Effect

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SUMMARY

Opiate receptor binding and opiate agonist inhibition of cyclic AMP accumulation were measured with intact neuroblastoma × glioma NG108-15 hybrid cells under identical conditions. [³H]Diprenorphine bound to intact hybrid cells with an apparent K_D value of 1.96 ± 0.04 nM. The maximal number of binding sites on the NG108-15 cells was determined to be $345,000 \pm 3,300$ receptors/cell. *Mu* and *delta* ligands competitively inhibited [³H]diprenorphine binding. Apparently, cyclic AMP accumulation in hybrid cells was regulated by a single population of opiate receptors. This was concluded from observations that the pA_2 values of naloxone to antagonize *mu*, *kappa*, and *delta* ligands were identical and that the *sigma* partial agonist, *N*-allylnormetazocine, competitively interacted with the receptor sites of the *delta* agonist, Leu⁵-enkephalin. When opiate inhibition of the prostaglandin E_1 -stimulated accumulation of cyclic AMP was determined, it was observed that the intrinsic activities of the opiate ligands were nonidentical. All opioid peptides tested have intrinsic activities ≥ 0.9 when compared with etorphine. Opiate alkaloids, with the exception of etorphine, ketocyclazocine, ethylketocyclazocine and GPA 1657, have partial agonist activities. Because of the putative *delta* nature of the receptor in the neuroblastoma cell line, opioid peptides, as a group, were more potent than the opiate alkaloids in the displacement of [³H]diprenorphine binding and in the inhibition of cyclic AMP level increase. When the ratio of the K_d and K_i values of opiate ligands was determined, opioid peptides, with the exception of β -endorphin, have a ratio greater than 1. Thus, opioid peptides did not require all receptors for maximal activity. With the exception of etorphine and the benzomorphans, opiate alkaloids required full occupancy of the receptor for their maximal activity ($K_d/K_i \approx 1$). As the *mu* characteristic increased in the opioid peptides, the K_d/K_i ratio of the peptides approached unity. Hence, the efficiency of coupling between a homogeneous population of opiate receptor and the effector is determined by the receptor-ligand complex. Thus, it must follow that the opiate receptor in neuroblastoma × glioma NG108-15 cells undergoes conformational changes after binding of agonists.

INTRODUCTION

Although the existence of opiate receptors in neural tissue has long been postulated and subsequently demonstrated (1-3), the exact molecular mechanism of opiate receptor action is unknown. Attempts to fit a theoretical model with observed animal data have been unproductive. Only with *in vitro* models was there some success in model fitting. Creese and Snyder (4), using guinea pig ileum, suggested that opiate receptor action followed the

classical drug receptor model; i.e., the opiate effect could be accounted for by the agonist's affinity for its binding sites. Because several opiate alkaloids showed a linear correlation between binding affinity and potency to inhibit the twitch of the guinea pig ileum, Creese and Snyder (4) concluded that the concept of Stephenson (5) and Ariens *et al.* (6) of intrinsic activity, or the maximal response to the drugs, was not necessary to account for opiate action in this model system.

However, in another model system, neuroblastoma × glioma NG108-15 hybrid cells, the results have been equivocal. In this hybrid neuronal cell line, the high-affinity, stereospecific opiate receptor inhibits adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC

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4.6.1.1] (7-9). Considering the relative affinities of various opiate ligands in receptor binding assays [K_d (10)] and their relative potencies in the inhibition of adenylate cyclase activity assays [K_i (11)], the opiate receptor in the neuroblastoma cell line is probably *delta* in nature. When analysis of the binding and of inhibition caused by several opiate alkaloids was carried out, Sharma *et al.* (9) reported a correlation between the amount of opiate required to half-saturate the binding sites and that required to produce half-maximal inhibition. Thus, it appeared that opiate receptor action in NG108-15 cells also followed the classical model for drug-receptor action. But recently, Fantozzi *et al.* (12) reported that D-Ala²,Met⁵-enkephalinamide inhibition of adenylate cyclase did not require all of the receptor found in the hybrid cells. D-Ala²,Met⁵-enkephalinamide inhibition remained unaltered after 95% of the opiate binding sites were irreversibly inactivated with an antagonist, chlornaltrexamine. Hence, the possibility of "spare" receptor exists in the hybrid cells. The classical model for drug-receptor action cannot account for such an observation. Other receptor models, such as the cooperativity model (13) or the aggregation-disaggregation model (14), must be considered as alternatives for opiate receptor action in NG108-15 hybrid cells.

The discrepancy between the coupling efficiency, defined as the ratio of K_d/K_i , of opiate alkaloids and opioid peptides could be due to the *delta* nature of the receptor in hybrid cells. Possibly, the *delta* ligands could promote the receptor to a better conformational fit for the effector, adenylate cyclase, than the opiate alkaloids. If this is the case, then, all *delta* ligands should have K_d/K_i ratios greater than that of the *mu* and *kappa* ligands. Therefore, in the present communication, the affinities (K_d) of various opiate alkaloids and opioid peptides were compared with their respective potencies to inhibit the production of cyclic AMP (K_i). In order to measure K_d and K_i values under identical conditions, opiate receptor binding and cyclic AMP accumulation were determined with intact cells. The characteristics of opiate binding to intact cells and the apparent presence of a single class of opiate receptor in this cell line are discussed in the present report.

MATERIALS AND METHODS

Cell culturing. The initial stock cultures of neuroblastoma × glioma NG108-15 cells were generous gifts of Dr. B. Hamprecht (Physiologischem Institut des Universitat, Wurzburg, Federal Republic of Germany). The hybrid cells were cultured in Dulbecco's modified Eagle's medium containing 0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine plus 10% fetal calf serum in a humidified atmosphere of 10% CO₂ and 90% air. The cells were detached from the growing surface by the addition of saline D for propagation or experimentation, as described previously (15). Cells with passage number between 20 and 30 were used in all of the studies.

Measurement of cyclic AMP levels. Cyclic AMP levels in NG108-15 cells were carried out by determining the [³H]cyclic AMP formed from the intracellular ATP pools prelabeled with [³H]adenine, as described previously (15). The cyclic AMP formation assays were carried out

with 0.5×10^6 cells/0.5 ml of KRHB² at pH 7.4, incubated at 37° for 20 min. After addition of the internal standard [³²P]cyclic AMP, the radioactive cyclic AMP was separated from other ³H-labeled nucleotides by the double-column chromatographic methods outlined by White and Karr (16). The opiate agonists' ability to inhibit cyclic AMP accumulation in NG108-15 cells was determined as described previously (15). The potency of opiate antagonists was determined by measuring the etorphine potency to inhibit cyclic AMP accumulation in the presence and in the absence of known amounts of these antagonists. The K_e of antagonist was then calculated from the equation for competitive inhibitors, which is

$$K_{I(\text{etorphine})}^{\text{App}} = K_{I(\text{etorphine})}(1 + [I_0]/K_e) \quad (1)$$

where I_0 is the initial concentration of antagonist present in the assay.

Opiate receptor binding. Opiate receptor binding with intact cells was carried out by incubating 10^6 cells in suspension in 1.0 ml of KRHB at pH 7.4 at 37° for 20 min with [³H]diprenorphine. Nonspecific binding was determined by the addition of 10 μM levorphanol in the binding mixtures. The incubations were terminated by collecting the cells on Whatman GF-B filters, and excess radioactivity was removed by washing the filters three times with 5 ml of KRHB at 0°. After incubating at 20° overnight in 5 ml of Liquiscint (National Diagnostics, Somerville, N. J.), the radioactivity on the filters was determined by liquid scintillation counting. The K_d values of various opiate ligands were determined from the IC₅₀ values of the respective ligand to displace the [³H]diprenorphine specific binding. Identical solutions of opiates were used in the opiate receptor binding and adenylate cyclase assays. The K_D values were calculated from the IC₅₀ values based on the equation of competitive displacement,

$$K_d = \text{IC}_{50}/(1 + [[^3\text{H}]\text{DIP}]/K_d[^3\text{H}]\text{DIP}) \quad (2)$$

where [³H]DIP = [³H]diprenorphine.

Materials. [³H]Adenine (16 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). [³H]Diprenorphine (9 Ci/mmole) and [α -³²P]ATP (17 Ci/mmole) were supplied by Amersham (Arlington Heights, Ill.). [³²P]cyclic AMP was prepared by enzymatic conversion of [α -³²P]ATP with NG108-15 hybrid cell's adenylate cyclase. All of the reagents used in the present studies were purchased from Sigma Chemical Company (St. Louis, Mo.). ZK62711 was a generous gift from Schering (Berlin, Federal Republic of Germany). All opiate alkaloids were generous gifts from Dr. T. M. Cho (University of California, San Francisco). Opioid peptides were purchased from Peninsula Laboratories, (San Carlos, Calif.). The D-Ala² analogues and the N-CH₃-D-Ala² analogues of enkephalins were generous gifts from Wellcome Laboratories (Research Triangle Park, N. C.).

² The abbreviations used are: KRHB, Krebs-Ringer-Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer; PGE₁, prostaglandin E₁.

RESULTS

Characteristics of opiate binding to intact cells. The presence of high-affinity stereoselective opiate binding sites in NG108-15 membrane has been demonstrated unequivocally (17, 18). Because Na^+ and GTP inhibited agonist binding to the receptor (18), the putative opiate antagonist diprenorphine was used as the radioactive tracer in our studies. [^3H]Diprenorphine binding to intact cells was observed to be saturable and stereoselective.

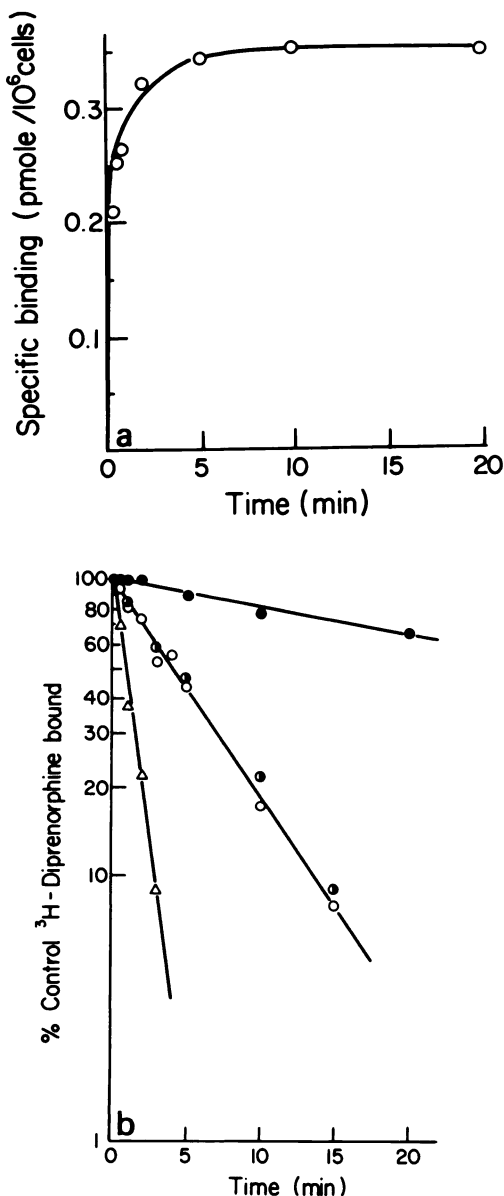


FIG. 1. Time course of [^3H]diprenorphine binding to intact neuroblastoma \times glioma NG108-15 hybrid cells

a. The time-dependent specific binding of [^3H]diprenorphine (4 nM) to intact hybrid cells ($10^6/\text{ml}$) was determined as described in the text. b. The dissociation rates of [^3H]diprenorphine from intact cells were determined at 0° (●), 24° (○), and 37° (△). [^3H]Diprenorphine (4 nM) was incubated with 10^6 cells/ml for 20 min at 37°. The mixtures were then equilibrated to the desired temperature prior to dissociation. At zero time, dissociation was initiated by the addition of levorphanol (final concentration, 10 μM). Dissociation of [^3H]diprenorphine from intact cells at 24° was carried out by the addition of levorphanol (○) or by 1:100 dilution of the ligand cell mixtures (○).

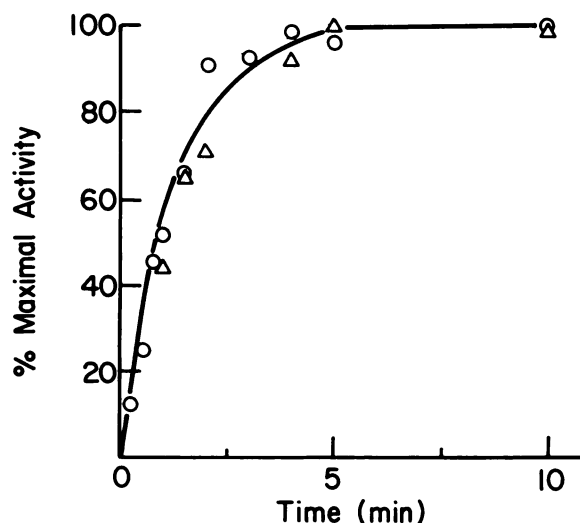


FIG. 2. Time course of morphine reversal of [^3H]diprenorphine binding and morphine inhibition of PGE₁-stimulated cyclic AMP production

The ability of 100 μM morphine to reverse [^3H]diprenorphine binding to intact cells (○) and to inhibit PGE₁-stimulated cyclic AMP production (△) were determined at various times. Reversal of [^3H]diprenorphine binding was carried out by the addition of morphine to binding mixtures after 10-ml incubation of the cells with [^3H]diprenorphine at 37°. An identical concentration of morphine was added to the reaction mixtures at 37° in which the cyclic AMP accumulation had been carried out in the presence of 10 μM PGE₁ for 10 min. The morphine activity at various times was expressed as the percentage of maximal displacement of [^3H]diprenorphine binding or the percentage of maximal inhibition of cyclic AMP formation.

With 4 nM [^3H]diprenorphine, the binding attained equilibrium within 5 min of incubation at 37° (Fig. 1a). The amount of [^3H]diprenorphine bound remained constant during 20 min of incubation at 37°. Similar to opiate binding to brain and hybrid cell membranes (17, 18), [^3H]diprenorphine binding to intact NG108-15 cells was reversible. At all three temperatures in which dissociation rates were determined (0°, 24°, and 37°), [^3H]diprenorphine associated according to simple first-order kinetics (Fig. 1b). As predicted, the dissociate rate, k_{-1} , was dependent on temperature. The $t_{1/2}$ values were determined to be 2.0, 9.3, and 82.5 min for 37°, 24°, and 0°, respectively (Fig. 1b). Identical dissociation rates were observed when dissociations were initiated by the addition of 10 μM levorphanol or by 1:100 dilution of receptor-ligand complex (Fig. 1b). Apparently, the sites labeled by [^3H]diprenorphine were the opiate receptors involved in regulation of [^3H]cyclic AMP accumulation: the rate of 100 μM morphine to displace [^3H]diprenorphine bound and the rate of the same concentration of morphine to inhibit PGE₁-stimulated cyclic AMP accumulation were identical (Fig. 2).

When the specific opiate binding was determined with 0.4–20 nM [^3H]diprenorphine, a linear Scatchard analysis (19) of the binding data was obtained. Figure 3 summarizes the binding data obtained from four separate passages of cells and the Scatchard analysis of these data. Under the present conditions, i.e., intact cells in KRHB at pH 7.4 and at 37°, [^3H]diprenorphine has a K_d value of 1.96 ± 0.04 nM. This value was dramatically larger

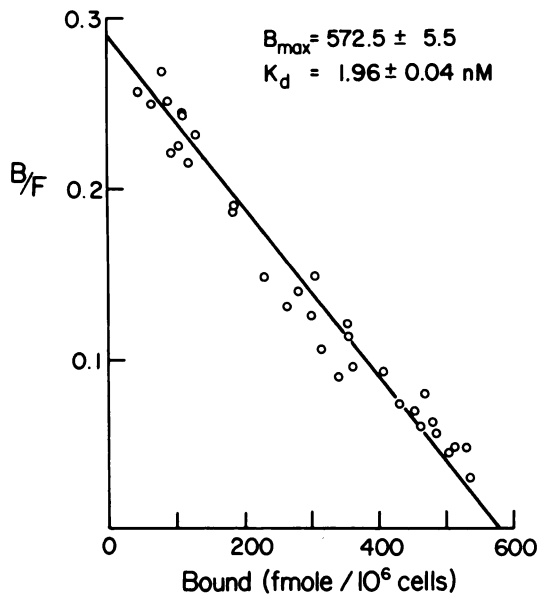


FIG. 3. Scatchard analysis of [^3H]diprenorphine binding to intact neuroblastoma \times glioma NG108-15 cells at 37°

The plot summarizes the data obtained from binding experiments carried out with four separate passages of cells.

than that obtained with opiate binding in neuroblastoma membrane preparations and low-salt conditions (10). The possibility that Na^+ in KRHB and intracellular GTP decreased [^3H]diprenorphine binding suggests that this opiate ligand is not a pure antagonist in the NG108-15 hybrid cell. Nevertheless, the amount of [^3H]diprenorphine maximally bound, which was 572.5 ± 5.5 fmole/ 10^6 cells, was identical with the previously reported opiate receptor density (10, 12, 17, 18). This is equivalent to $345,000 \pm 3,300$ receptor molecules/cell.

It is possible that [^3H]diprenorphine is labeling some sites which cannot be competitively displaced with either *mu*, *kappa*, or *delta* ligands. In order to utilize the

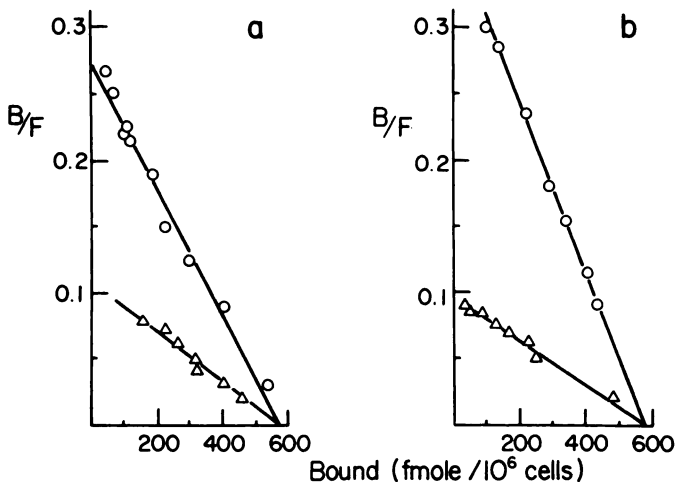


FIG. 4. Scatchard analysis of [^3H]diprenorphine binding to intact NG108-15 cells in the presence of (a) 20 nM *Met*⁵-enkephalin (Δ) or (b) 500 nM levorphanol (Δ)

[^3H]diprenorphine binding in the absence of added opiates (\circ) was carried out with the same passage of cells used in a and b.

formula in Eq. 2 for the calculation of K_d values, competitive inhibition of [^3H]diprenorphine binding with nonradioactive ligands must be fulfilled. As shown in Fig. 4, specific [^3H]diprenorphine binding in the presence of either *Met*⁵-enkephalin or levorphanol, putative *delta* and *mu* agonists, displayed competitive inhibition. There was a decrease in the receptor affinity for the ^3H -ligand without alteration in the number of binding sites. The K_d value of [^3H]diprenorphine binding to these whole cell preparations was determined to be 2.1 nM . In the presence of 20 nM *Met*⁵-enkephalin or 500 nM levorphanol, the slope of Scatchard plots increased to 4.5 nM and 8.6 nM , respectively. The K_d values for *Met*⁵-enkephalin and levorphanol (17.5 nM and 160 nM , respectively) were calculated from the following equation:

$$K_d^{\text{APP}} = K_d(1 + [L_0]/K_d^L)$$

where K_d^{APP} and K_d were apparent dissociation constants of [^3H]diprenorphine in the presence and absence of a known initial free concentration of ligand L_0 having a dissociation constant K_d^L .

Intrinsic activity of opiate ligands to inhibit cyclic AMP accumulation. Opiate agonists inhibit the basal or

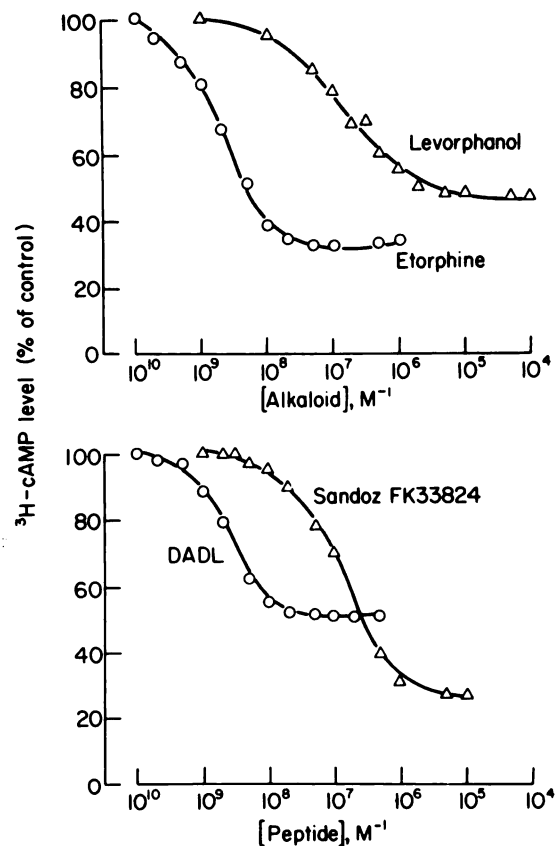


FIG. 5. Dose-dependent inhibition of cyclic AMP accumulation by opiate agonists

The [^3H]cyclic AMP levels in the presence of PGE_1 ($5 \mu\text{M}$) and various concentrations of etorphine, levorphanol, *D-Ala*²,*D-Leu*⁵-enkephalin (*DADL*) and Sandoz FK33824 were determined as described under Materials and Methods. The amount of [^3H]cyclic AMP produced during a 20-min incubation at 37° in the absence of any opiate was $1.29 \pm 0.08 \times 10^6$ cpm/ 10^6 cells ($n = 3$). The activities of the opiate agonists were measured with four different passages of cells.

PGE₁-stimulated adenylate cyclase activity in a dose-dependent manner (7-9). Those results were obtained by measuring adenylate cyclase activity in membrane preparations or by direct measurement of the intracellular cyclic AMP level with the protein kinase regulatory component. In the current studies, cyclic AMP levels in NG108-15 cells were determined by measuring the conversion of intracellular radioactive ATP pools, labeled with [³H]adenine, to [³H]cyclic AMP. Because the basal level of [³H]cyclic AMP formed was low, 1000 cpm/3-4 × 10⁵ cells, the PGE₁-stimulated increase in cyclic AMP level was measured in order to facilitate accurate measurements of opiate activities. As shown in Fig. 5, the PGE₁-stimulated cyclic AMP formation was inhibited by the opiate agonists in a dose-dependent manner. The slopes of the four opiate agonists' dose-response curves in Fig. 5 were dissimilar (see Table 3). Thus, apparently, the putative *delta* agonists inhibited cyclic AMP accumulation in a positive cooperative manner (Hill coefficient > 1), whereas the putative *mu* agonists did not have cooperativity in their regulation of cyclic AMP level.

Of the four opiate agonists represented in Fig. 5, etorphine possesses the highest potency in inhibiting PGE₁-stimulated conversion of ATP to cyclic AMP. There was an apparent difference among the intrinsic activities of these four opiates. However, these differences in intrinsic activities could be due to variance in the maximal responses among different passage numbers. Within the passage number of cells used, 20-30, the maximal inhibitory level of etorphine varied from 60% to 70%. Thus, in order to compare the intrinsic activities of various opiate ligands, the maximal responses to various opiate ligands in one passage number were normalized to that of etorphine in the same passage number.

When the maximal responses to various opiate alkaloids and peptides were examined in three consecutive passage numbers of cells, etorphine and Sandoz FK33824 possessed, respectively, the greatest intrinsic activity

among opiate alkaloids and opioid peptides tested (Table 1). Since the intrinsic activities of the D-Ala² analogues of enkephalin were not significantly different from that of Leu⁵- and Met⁵-enkephalin, and the enkephalins' intrinsic activities were not significantly different from that of Sandoz FK33824, it was concluded that all of the opioid peptides are agonists in the regulation of cyclic AMP level in NG108-15 cells. A limit of 0.9 is set as the lower limit for agonist intrinsic activity.

Of all of the opiate alkaloids tested, only four compounds have intrinsic activities greater than 0.90 and, hence, can be classified as agonists. They are etorphine, ketocyclazocine, ethylketocyclazocine, and GPA 1657. The *mu* ligands, such as morphine and levorphanol, are only 60%-70% as efficacious as etorphine or the opioid peptides in inhibiting cyclic AMP accumulation in the NG108-15 cells. Compared with etorphine, these opiate alkaloids are partial agonists in this receptor system.

As a group, opiate alkaloids which are partial agonists and antagonists in other assaying systems also have lower intrinsic activities than their agonist counterparts, e.g., cyclazocine-ketocyclazocine, *N*-allylnormetazocine-metazocine, GPA 2163-GPA 1657, nalorphine-morphine, and naloxone-oxymorphone (Table 1). Of all of the ligands tested, only naloxone and GPA 2163 are pure antagonists in the opiate inhibition of cyclic AMP accumulation in the hybrid cells. Diprenorphine, an antagonist in whole animal studies but a partial agonist in guinea pig ileum (20), possesses agonistic activity. Diprenorphine has 70% of etorphine's intrinsic activity. This partial agonistic property of diprenorphine could account for this ligand's lower affinity for the receptor in the present assaying conditions, as compared with that in membrane preparations (10). Also, the partial agonistic activity of diprenorphine could explain the sensitivity of [³H]diprenorphine binding to guanine nucleotide concentration (18) and diprenorphine's ability to stimulate GTPase activity (21).

TABLE 1

Intrinsic activity of various opiate ligands to inhibit cyclic AMP accumulation in neuroblastoma × glioma NG108-15 hybrid cells

Intrinsic activity (*I*) of each opiate ligand was defined as the PGE₁-sensitive adenylate cyclase activity maximally inhibited by the respective ligand. The intrinsic activity thus determined was normalized with that of etorphine, which was equal to 64.6 ± 2.1% (*n* = 3). The values represent the average intrinsic activity determined from three separate passage numbers of hybrid cells.

Opiate alkaloids	<i>I</i> ± SEM	Opioid peptides	<i>I</i> ± SEM
Etorphine	1.00	Sandoz FK33824	1.01 ± 0.01
Ketocyclazocine	0.97 ± 0.02	β-Endorphin	0.99 ± 0.01
Ethylketocyclazocine	0.96 ± 0.01	α-Endorphin	0.98 ± 0.01
GPA 1657	0.96 ± 0.01	D-Ala ² ,Met ⁵ -Enkephalin	0.98 ± 0.01
Diprenorphine	0.70 ± 0.04	D-Ala ² ,Met ⁵ -Enkephalinamide	0.98 ± 0.01
Metazocine	0.69 ± 0.03	Dynorphin, 1-13	0.96 ± 0.01
Morphine	0.69 ± 0.03	Met ⁵ -Enkephalin	0.95 ± 0.02
Oxymorphone	0.68 ± 0.03	Leu ⁵ -Enkephalin	0.94 ± 0.02
Levorphanol	0.65 ± 0.06	<i>N</i> -CH ₃ -D-Ala ² -D-Met ⁵ -Enkephalin	0.94 ± 0.03
Cyclazocine	0.62 ± 0.07	<i>N</i> -CH ₃ -D-Ala ² ,D-Leu ⁵ -Enkephalin	0.95 ± 0.01
Dihydromorphine	0.51 ± 0.06	D-Ala ² ,D-Met ⁵ -Enkephalin	0.91 ± 0.01
Oxilorphan	0.48 ± 0.06	D-Ala ² ,D-Leu ⁵ -Enkephalin	0.92 ± 0.04
<i>N</i> -Allylnormetazocine	0.40 ± 0.08		
Levallorphan	0.37 ± 0.01		
Nalorphine	0.18 ± 0.06		
Naloxone	0		
GPA 2163	0		

Single population of opiate receptors regulating cyclic AMP level. The differences among the intrinsic activities of various opiate ligands could be caused by the presence of multiple opiate receptors in NG108-15 cells. In the regulation of adenylate cyclase and intracellular cyclic AMP levels by other receptors, maximal activity with the agonist was reported to be dependent on receptor concentration (22, 23). Possibly, the percentage of the total opiate receptors in various multiple receptor populations could determine the maximal activity of the *mu*, *sigma*, *kappa*, and *delta* agonists tested. The data summarized in Fig. 6 and Table 2 support the notion of a single population of opiate receptors regulating cyclic AMP formation in hybrid cells. As shown in Fig. 6a, with either 2 μM or 20 μM *N*-allylnormetazocine (a *sigma* receptor ligand) present in the assay mixtures, there was a parallel shift of the Leu⁵-enkephalin dose-response curve. An additive effect of *N*-allylnormetazocine inhibition and the inhibition produced by low concentrations of Leu⁵-enkephalin was not observed (Fig. 6a). The dose-dependent curves of enkephalin in the presence of *N*-allylnormetazocine are those of the computer-generated curves of agonist activity in the presence of a fixed concentration of a partial agonist acting on the same sites (13). Receptor theory predicts that, if the two drugs are acting at the same sites, a plot of equal responses produced by concentration $X_{\text{Leu-Enk}}$ of Leu⁵-enkephalin on its own and concentration of $X_{\text{Leu-Enk}}^1$ in the presence of *N*-allylnormetazocine should be linear (13). As shown in Fig. 6b, the plots of equieffective concentrations of Leu⁵-enkephalin with and without 2 μM or 20 μM *N*-allylnormetazocine yielded straight lines with correlation coefficients of 0.995 and 0.986, respectively. Therefore, *N*-allylnormetazocine, the *sigma* receptor ligand, inhibits cyclic AMP accumulation in NG108-15 cells at the same receptor site as Leu⁵-enkephalin, the *delta* receptor ligand.

The pA_2 values of naloxone to reverse agonistic effects have been used to determine the heterogeneity of the receptor and/or the manner of ligand receptor interaction (24). If there are multiple opiate receptors present in NG108-15 hybrid cells, then the naloxone pA_2 values to reverse the agonistic effect of the *mu*, *kappa*, and *delta* ligands would be dissimilar. As summarized in Table 2, naloxone pA_2 values to antagonize levorphanol (*mu*), ketocyclazocine (*kappa*) and Leu⁵-enkephalin (*delta*) were identical. Furthermore, the slopes of the Schild plot of these three ligands did not significantly deviate from unity (Table 2), as predicted for competitive inhibitors. Hence, naloxone competitively inhibited the action of these three opiate ligands, and regulation of cyclic AMP level by *mu*, *kappa*, and *delta* opiate agonists is mediated via the same population of receptors.

Relative affinity (K_d) of opiate ligands. In measuring the K_d values of the opiate ligands, competitive inhibition of [³H]diprenorphine by all of the ligands was assumed. From the experimental data summarized in Fig. 4, competitive kinetics was observed with *mu* and *delta* ligands. Thus, the apparent dissociation constants were calculated from the IC_{50} values using Eq. 2.

The receptor in the neuroblastoma cells is reported to be *delta* in nature (10, 11). Therefore, the opioid peptides

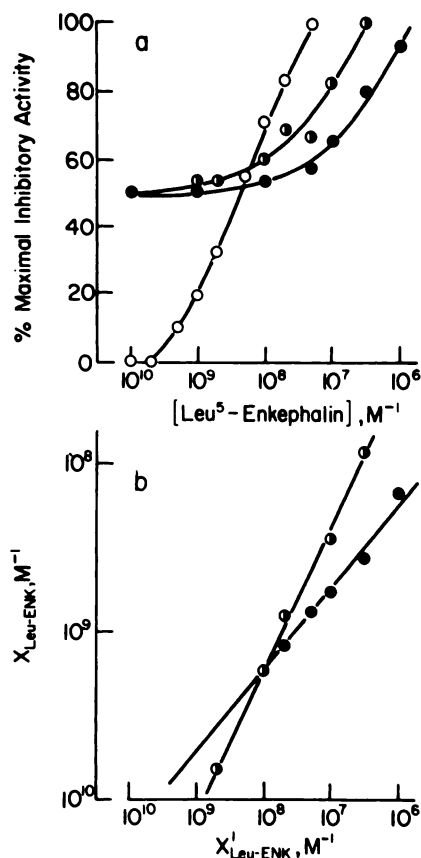


FIG. 6. Leu⁵-Enkephalin inhibition of cyclic AMP accumulation in the presence of *N*-allylnormetazocine (SKF 10047)

a. The ability of various concentrations of Leu⁵-enkephalin to inhibit the PGE₁-stimulated cyclic AMP formation in the presence of 0 (○), 2 μM (●), or 20 μM (●) SKF 10047 was determined. The activity of a concentration of Leu⁵-enkephalin was expressed as maximal inhibitory percentage, which was $63.0 \pm 3.7\%$.

b. Plot of equieffective concentrations of Leu⁵-enkephalin without ($X_{\text{Leu-Enk}}$) and with ($X_{\text{Leu-Enk}}^1$ 2 μM (●) or 20 μM (●) SKF 10047, from a.

should have the highest affinity for the opiate receptor in NG108-15 cells. Among all of the peptides tested, D-Ala²,Met⁵-enkephalin had the highest potency in displacing [³H]diprenorphine binding (Table 3). As a group, the D-Ala² analogues of enkephalin were more potent than enkephalin in displacing [³H]diprenorphine binding.

TABLE 2

The pA_2 values of naloxone to antagonize opiate agonists' regulation of cyclic AMP level in NG108-15 cells

The IC_{50} values of the three opiate agonists to inhibit PGE₁-stimulated cyclic AMP production were determined in the presence of various concentrations of naloxone (10 nM–500 nM). The pA_2 values were determined from the Schild plots (24). The values represent the average pA_2 values with their standard deviation. The numbers in parentheses represent the numbers of different passages of hybrid cells used.

Opiate agonist	pA_2	Slope
Leu ⁵ -Enkephalin ($n = 3$)	7.43 ± 0.04	1.05 ± 0.15
Ketocyclazocine ($n = 3$)	7.35 ± 0.09	1.22 ± 0.16
Levorphanol ($n = 4$)	7.38 ± 0.11	0.85 ± 0.15

With an increase in *mu* characteristics in the opioid peptides, the affinity for the opiate receptor in NG108-15 cells decreased. The affinity of β -endorphin was less than that of enkephalin (Table 3). The peptide Sandoz FK33824, which is an analogue of the pentapeptide and which has affinity for the *mu* receptor (25), has relatively low affinity for the receptor in hybrid cells. The derivative of casomorphin, Tyr-Pro-Phe-Pro-NH₂, or morphiceptin, which has been suggested to recognize the *mu* receptor specifically (25), did not have detectable affinity for the opiate receptor in this cell line (Table 3). All of the Hill coefficients of all peptides to displace [³H]diprenorphine were significantly lower than 1.0 (Table 3). Thus, opioid peptides either interact with the receptor in a negatively cooperative manner or there are binding sites of different affinities for the peptides in NG108-15 hybrid cells.

When the binding affinity of the opiate alkaloids was determined, it was clear that the alkaloids had lower affinities for the receptor than the opioid peptides. This

is to be expected, because most of the alkaloids tested are *mu*, *kappa*, and *sigma* ligands. Only alkaloids (e.g., etorphine), which have affinity for the *delta* receptor (26), interact with the opiate receptor in NG108-15 cells with high affinity. The *mu* agonists have lower affinity than the benzomorphans (Table 3). As noted with the [³H]diprenorphine binding (Fig. 3), the affinities of opiate alkaloids binding to intact cells were much lower than those obtained with membrane preparations in a buffer with low ionic strength. The relatively low affinities of these agonists could be due to the presence of Na⁺ in KRHB and intracellular GTP, the two agents which have been reported to decrease receptor affinity for the opiate agonists (18). As with the opioid peptides, the opiate alkaloids displaced [³H]diprenorphine binding with Hill coefficients significantly lower than 1.0 (Table 3). The only opiate alkaloids which have Hill coefficients approaching unity are metazocine and its *N*-allyl analogue.

Relative potency (K_i) of various opiate ligands. The K_i values of various opiate agonists to inhibit cyclic AMP

TABLE 3

The *K_d* and *K_i* values of various opiate alkaloids and opioid peptides

The *K_d* and *K_i* of the opiate ligands were determined as described under Materials and Methods. The *K_d* values were calculated from the IC₅₀ values of the opiates to displace 4.5–5.0 nM [³H]diprenorphine. The *K_i* values represent the IC₅₀ values of the opiates to inhibit PGE₁-stimulated cyclic AMP formation. Identical opiate ligand solutions were used in both assays. *K_d* and *K_i* values were obtained from the linear regression analysis of the Hill plots drawn from 12 concentrations of opiate agonists and antagonists. As for the opiate ligands which are known antagonists (*), *K_i* was determined from the shift of the etorphine dose-response curve by a fixed concentration of opiates. *n_H'* and *n_H* are the Hill coefficients of the [³H]diprenorphine displacement curves and inhibition of cyclic AMP accumulation curves, respectively. Sandoz FK33824 is D-Ala²-N-CH₃-Phe⁴-Met⁵(O)al enkephalin. The values represent the average of three dose-dependent curves \pm standard error of the mean from three separate passage numbers of cells.

Opiate alkaloids	<i>K_d</i> nM	<i>n_H'</i>	<i>K_i</i> nM	<i>n_H</i>	<i>K_d/K_i</i>
Etorphine	22.2 \pm 2.27	0.64 \pm 0.04	2.20 \pm 0.74	1.38 \pm 0.04	10.1 \pm 1.2
Diprenorphine	1.96 \pm 0.04	—	2.10 \pm 0.30	—	1.08 \pm 0.03
Levallorphan	22.3 \pm 2.7	0.50 \pm 0.032	24.4 \pm 2.8	0.72 \pm 0.02	0.96 \pm 0.05
Cyclazocine	51.9 \pm 8.3	0.74 \pm 0.02	38.8 \pm 14.8	0.86 \pm 0.01	1.16 \pm 0.07
Ethylketocyclazocine	91.5 \pm 21.3	0.79 \pm 0.02	40.6 \pm 1.2	0.85 \pm 0.03	2.26 \pm 0.06
Ketocyclazocine	95.8	0.87	50.3 \pm 13.7	0.98 \pm 0.07	2.02 \pm 0.30
Naloxone*	61.1 \pm 21.7	0.53 \pm 0.01	46.2 \pm 5.8	—	1.41 \pm 0.18
<i>N</i> -Allylnormetazocine	162.0 \pm 10.8	0.92 \pm 0.01	108.0 \pm 17.0	0.91 \pm 0.11	1.63 \pm 0.33
GPA 1657	158.0 \pm 16.1	0.65 \pm 0.02	109.0 \pm 11.2	0.85 \pm 0.03	1.74 \pm 0.27
GPA 2163*	160.0 \pm 10.8	0.61 \pm 0.04	101.0 \pm 24.3	—	1.67 \pm 0.20
Levorphanol	249.0 \pm 29.5	0.69 \pm 0.04	153.0 \pm 11.0	0.95 \pm 0.03	1.63 \pm 0.19
Nalorphine*	372.0 \pm 51.3	0.76 \pm 0.01	190.0 \pm 22.6	0.76 \pm 0.04	1.89 \pm 0.53
Oxilorphan	627.0 \pm 150	0.76 \pm 0.09	—	0.81 \pm 0.08	1.84 \pm 0.53
Metazocine	548.0 \pm 8.2	0.90 \pm 0.03	336.0 \pm 140.0	1.08 \pm 0.08	2.38 \pm 1.00
Dihydromorphine	768.0 \pm 166	0.70 \pm 0.04	911.0 \pm 31.0	0.80 \pm 0.24	0.84 \pm 0.17
Oxymorphone	1740 \pm 244	0.84 \pm 0.04	1510	0.76	1.15 \pm 0.16
Morphine	5340 \pm 388	0.85 \pm 0.03	3390 \pm 296	0.82 \pm 0.01	1.41 \pm 0.44
Fentanyl	1480	—	—	—	—
D-Ala ² ,Met ⁵ -Enkephalin	7.0 \pm 2.1	0.72 \pm 0.03	0.89 \pm 0.27	1.16 \pm 0.11	6.04 \pm 0.90
D-Ala ² ,D-Met ⁵ -Enkephalin	7.3 \pm 0.9	0.78 \pm 0.04	2.42 \pm 0.63	1.14 \pm 0.17	5.07 \pm 0.71
D-Ala ² ,D-Leu ⁵ -Enkephalin	10.0 \pm 1.5	0.651	2.10 \pm 0.16	1.54 \pm 0.15	4.81 \pm 0.40
Met ⁵ -Enkephalin	15.0 \pm 1.7	0.59 \pm 0.08	3.90 \pm 1.50	1.34 \pm 0.08	5.30 \pm 2.10
Leu ⁵ -Enkephalin	20.2 \pm 10.1	0.72 \pm 0.04	3.40 \pm 0.57	1.24 \pm 0.07	5.14 \pm 0.34
D-Ala ² ,Met ⁵ -Enkephalinamide	31.8 \pm 7.0	0.50 \pm 0.03	6.95 \pm 0.61	1.12 \pm 0.08	4.53 \pm 0.74
α -Endorphin	302.8 \pm 44.6	0.62 \pm 0.02	14.5 \pm 0.40	1.14 \pm 0.05	21.0 \pm 3.4
<i>N</i> -CH ₃ -D-Ala ² ,D-Met ⁵ -Enkephalin	831.8 \pm 93.3	0.67 \pm 0.02	63.1 \pm 2.41	0.97 \pm 0.06	13.2 \pm 1.50
<i>N</i> -CH ₃ -DAla ² ,D-Leu ⁵ -Enkephalin	1054.9 \pm 48.6	0.69 \pm 0.07	71.7	1.11 \pm 0.11	14.7 \pm 0.69
Dynorphin, 1–13	952.9 \pm 282.9	0.65 \pm 0.04	86.0 \pm 17.6	0.98 \pm 0.14	6.66 \pm 1.44
β -Endorphin	121.8 \pm 30.1	0.85 \pm 0.07	95.5 \pm 22.5	1.93 \pm 0.15	1.26 \pm 0.05
Sandoz FK33824	2460 \pm 164	0.68 \pm 0.04	154.9 \pm 28.2	0.96 \pm 0.08	15.9 \pm 1.05
Tyr-Pro-Phe-Pro-NH ₂	>10,000	>10,000	—	—	—

accumulation further indicate that the opiate receptor in the hybrid cells is *delta* in nature. Enkephalin and its analogues possessed the greatest potency in inhibition of cyclic AMP formation (Table 3). The D-Ala² analogues of enkephalin were more potent than Met⁵- or Leu⁵-enkephalin in the present assay condition. The K_i values determined for Met⁵- and Leu⁵-enkephalin are analogous to those reported by Wahlstrom *et al.* (11). With an increase of *mu* character in the opioid peptides, the potency of the peptide decreased. β -Endorphin was less potent than α -endorphin, which was itself less potent than enkephalin in regulating cyclic AMP level. Sandoz FK33824 was the least potent active opioid tested. Morphiceptin, at concentrations up to 1 mM, was inactive in the inhibition of cyclic AMP accumulation in NG108-15 cells (Table 3). There are some opioid peptides which inhibit cyclic AMP formation in a positive cooperative manner ($n_H > 1$). β -Endorphin exhibited the largest cooperativity. Opioid peptides which do not inhibit cyclic AMP formation in a positively cooperative manner are the *N*-CH₃-D-Ala² analogues of enkephalin, dynorphin and Sandoz FK33824 (Table 3).

Of all of the alkaloids tested, only etorphine was as potent as enkephalin in the inhibition of cyclic AMP accumulation (Table 3). Benzomorphans, which are putative *kappa* ligands, were as potent as the endorphins in the regulation of cyclic AMP level. The *mu* agonists possessed relatively low potency (Table 3). Morphine was the least potent alkaloid among all of the opiates tested. Although fentanyl has greater affinity than morphine for the opiate receptor in NG108-15, fentanyl concentrations up to 1 mM did not significantly inhibit the PGE₁-stimulated increase in cyclic AMP level. Of all of the opiate alkaloids tested, only etorphine regulated the cyclic AMP level in a positively cooperative manner, $n_H > 1$ (Table 3).

In determining the K_e values of various opiate alkaloids, it was observed that the K_e values of the partial agonists are identical with their K_i values. The concentrations of diprenorphine, levallorphan, nalorphine, and *N*-allylnormetazocine required to antagonize the etorphine inhibition by 50% and the concentrations required to inhibit maximally the PGE₁-stimulated increase in cyclic AMP level are identical. Thus, these data suggest that the antagonistic and agonistic activities of these opiate alkaloids were mediated via the same receptor.

Coupling efficiency of the opiate ligands. When the K_d and K_i (or K_e in the case of antagonist) ratios of the opiate ligands were determined, opiate agonist regulation of cyclic AMP level appeared to involve the notion of spare receptor. As summarized in Table 3, opiate alkaloids (with the exception of etorphine) are partial agonists in NG108-15 cells and have coupling efficiencies less than 2. Benzomorphans have K_d/K_i ratios of 2. The similarity in the K_d and K_i values of opiate alkaloids has been reported by Sharma *et al.* (9). The K_d/K_e ratios of putative antagonists were close to 1. Furthermore, alkaloids which have similar molecular structure have analogous coupling efficiency. Etorphine, which has equal affinity for all of the opiate receptor subtypes (26), was the only alkaloid which had a coupling efficiency ratio greater than 10.

On the other hand, the K_d/K_i ratio of the opioid peptides, with the exception of β -endorphin, were greater than 2.0 (Table 3). The K_d/K_i ratio of D-Ala²,Met⁵-enkephalinamide obtained through the current studies was similar to that obtained by Fantozzi *et al.* (12) with an irreversible antagonist, chlornaltrexamine. Enkephalins and their D-Ala² analogues have analogous K_d/K_i ratios. The coupling efficiency ratio of β -endorphin was similar to that of the *mu* and *kappa* agonists (Table 3). Sandoz FK33824 had a coupling efficiency distinctly different from that of *mu* agonists and a ratio similar to that of etorphine. Slight alteration of the peptide structure could alter the coupling efficiency greatly. *N*-Methylation of the tyrosine residue significantly enhanced the coupling efficiency of the D-Ala²,D-Met⁵- or D-Ala²,D-Leu⁵-enkephalins ($p < 0.005$). As the chain length of the peptide increased from the pentapeptide to β -endorphin, the coupling efficiency decreased (Table 3). α -Endorphin had the greatest K_d/K_i ratio. With the exception of β -endorphin, the full opioid peptide cellular effect in NG108-15 cells, i.e., regulation of cyclic AMP level, did not require occupation of all of the receptors present in the cellular membrane.

DISCUSSION

In the present studies, we have been successful in measuring opiate ligand binding and the subsequent opiate agonist effect, i.e., inhibition of cyclic AMP accumulation under identical conditions. In order to minimize the effect of Na⁺ and GTP on opiate agonist binding and to optimize these two agents' effects on opiate receptor regulation of adenylate cyclase (27), opiate receptor binding and adenylate cyclase measurements were determined at 37° with intact cells. Under these conditions, [³H]diprenorphine interacted with a single class of opiate binding sites in NG108-15 cells. This was deduced from the linear Scatchard analysis (Fig. 3) and the competitive inhibition kinetics of *mu* and *delta* ligands displacing [³H]diprenorphine binding (Fig. 2). However, these data could also be explained by equal affinity of diprenorphine for multiple opiate receptors (25). This hypothesis was unlikely because identical pA₂ values of naloxone in antagonizing the *mu*, *kappa*, and *delta* agonists' effect were observed (Table 2). Naloxone has been reported to have higher affinity for *mu* than *delta* opiate receptor (26). The data summarized in Fig. 6 further substantiate the conclusion that cyclic AMP formation in NG108-15 cells is regulated by a single type of opiate receptor. The inhibition of cyclic AMP accumulation by Leu⁵-enkephalin, a *delta* agonist, and by *N*-allylnormetazocine, a putative *sigma* agonist, is not additive. The dose-dependent inhibition curve of Leu⁵-enkephalin in the presence of a fixed concentration of *N*-allylnormetazocine is identical with that generated by computer for an agonist and a partial agonist competing for the same binding sites (13). The equieffective plots derived from these data are straight lines (Fig. 6b). Therefore, from all of these data, it can be concluded that the opiate receptor population which regulates cyclic AMP level in NG108-15 is a homogeneous one. It follows that the apparent K_d values of all opiate ligands can be calculated from the IC₅₀ values

to displace [³H]diprenorphine using the competitive inhibitor equation derived from the law of mass action.

If the opiate agonist inhibition of cyclic AMP accumulation in NG108-15 hybrid cells is mediated via a single receptor subtype, then the question of a "spare receptor" in the mechanism of opiates can be addressed. According to Fantozzi *et al.* (12), an opioid peptide's inhibition of adenylate cyclase involved a "spare receptor." This is confirmed by our K_d/K_i ratio determination. All opioid peptides, which are putative *delta* ligands, have coupling efficiencies greater than 4 (Table 3). The K_d/K_i value of D-Ala²,Met⁵-enkephalinamide was analogous to that reported by Fantozzi *et al.* (12). The difference in K_d and K_i values could result from the variable degradation rates of the peptides in these two assays. This conclusion is unlikely because (a) identical conditions were used in K_d and K_i measurements; (b) the proteolytic enzyme inhibitor, 1,10-*O*-phenanthroline, was present in both assays, and (c) a K_d/K_i ratio of 16 was observed with Sandoz FK33824, a metabolically stable analogue of enkephalin (Table 3).

Another possible explanation for the observed differences in K_d and K_i values is that they could be caused by opiate receptor desensitization during the 20-min incubation at 37°. Reduction of agonists' affinity during receptor desensitization has been reported with other receptors (26). Prolonged treatment of NG108-15 cells with opiate agonists produced receptor desensitization and down-regulation of opiate receptor (27, 28). Thus, dissimilarities in K_d/K_i ratios could reflect different desensitization kinetics among various opiate agonists in the K_d and K_i determinations. However, this explanation appears not to be the cause of the divergent K_d/K_i ratios observed. In our studies, we observed identical apparent K_d values for [³H]diprenorphine when incubations were carried out at 37° for 5, 10, or 20 min. Although opiate inhibition of cyclic AMP accumulation could be reduced by opiate agonist exposure, minimal reduction of opiate activity was observed after 1 hr of incubation of 10 nM etorphine (27). In this respect, neuroblastoma × glioma NG108-15 hybrid cells are slightly different from neuroblastoma N4TG1 cells. When N4TG1 cells were incubated with a concentration of opioid peptides which saturated all receptors, maximal reduction of [¹²⁵I]D-Ala²,D-Leu⁵-enkephalin binding was observed within 1 hr (28). Incubation of NG108-15 cells with 10 nM etorphine for 1 hr at 37° produced no detectable alteration in [³H]etorphine binding to the membrane preparation.³ Furthermore, the K_i values obtained in current studies were similar to those reported by Wahlstrom *et al.* (11) and those in our earlier studies (15) in which a 10-min incubation period was used. Hence, probably there was minimal desensitization of the opiate receptor during the 20-min incubation period. In support of our findings, using an irreversible antagonist, Fantozzi *et al.* (12) obtained an identical discrepancy in K_d and K_i values of D-Ala²,Met⁵-enkephalinamide. Therefore, the observed differences in K_d and K_i values among the opiates reflect the acute and not the chronic effect of the compounds.

When the K_d/K_i ratio is greater than 1, it indicates

that full cellular effect could be attained without complete occupancy of the receptor. Since all putative *delta* ligands have coupling efficiency greater than 4, the full effect of *delta* ligands was observed with minimal occupancy of the binding sites. Opiate alkaloids and β -endorphin, the putative *mu* agonist, all have K_d/K_i ratios ≤ 2 . This observation suggests that in a putative *delta* receptor system such as the hybrid cell line, full cellular response to *mu* agonists requires complete occupancy of the receptor. Because there is a single population of opiate receptor involved in the regulation of the cyclic AMP level in NG108-15 cells (Table 2; Fig. 6), the difference in *delta* and *mu* ligands' coupling efficiency ratios implies a difference in *delta* and *mu* ligand-receptor complexes. Apparently, *delta* ligands can convert the receptor in hybrid cells to a coupling state which better fits the effector, whereas the *mu* ligand receptor complex is not in the most optimal coupling state. Hence, one can conclude that the opiate receptor in the hybrid cell must undergo some form of conformational changes after ligand binding. One can also conclude that, unlike guinea pig ileum (4), the intrinsic activity of the ligand (in addition to the ligand's affinity for the receptor) plays a role in the opiate receptor's action in NG108-15 hybrid cells.

Accordingly, the K_d/K_i values of various opioid compounds could be used to classify the nature of the drugs. From our studies, all opiates tested could be classified into two groups—one which has coupling efficiency greater than 2 (the *delta* agonists) and the other which has K_d/K_i ratio less than 2 (the *mu* and *kappa* agonists). Etorphine, which has been suggested to have equal affinity for all of the multiple opiate receptors (29), has a K_d/K_i ratio similar to those of the *delta* agonists. The putative *mu* peptide, Sandoz FK33824, which has comparatively low affinity for the receptor, possesses the K_d/K_i ratio of a *delta* agonist. Thus, Sandoz FK33824 receptor complex must be analogous to that of a *delta* agonist. By comparing the relative affinity for radioactive *delta* or *mu* ligand binding, Chang *et al.* (29) concluded that *N*-allylnormetazocine, the putative *sigma* agonist, is a *delta* agonist. Apparently, from our K_d/K_i ratio and the intrinsic activity measurement (Table 1), *N*-allylnormetazocine at best is a partial agonist in the NG108-15 hybrid cell opiate receptor system. Clearly, measurement of opiate ligand's affinity for the binding site alone could not accurately predict the pharmacological or physiological action of the drug. Although diprenorphine binding affinity was not shifted greatly by Na⁺ (30) and, hence, is a putative antagonist, in the current studies diprenorphine clearly possessed agonistic properties (Table 1). This is supported by observations that diprenorphine binding is sensitive to guanine nucleotide (18) and that diprenorphine could increase GTPase activity (21). Fentanyl, which has affinity for the receptor in NG108-15 cells, could not regulate the cyclic AMP level in NG108-15 cells (Table 3). Therefore, classification of opiate drugs by their affinities in the presence or absence of Na⁺ in receptor binding assay could not accurately predict their cellular action.

In conclusion, by carrying out opiate receptor binding assays under physiological conditions, we have observed

³ P. Y. Law, D. S. Hom, and H. H. Loh, manuscript in preparation.

possible conversion of receptor states promoted by agonists. For opiate alkaloids which are *mu* and *kappa* agonists, and also for β -endorphin, the affinity for the receptor correlates with the potency to inhibit cyclic AMP accumulation. But for the opioid peptides which are *delta* agonists and for alkaloids which have affinity for the *delta* receptor, the effect requires minimal occupancy of the binding sites. This possible agonist-induced receptor conformational change was illustrated further by positive cooperativity ($n_H > 1$) in some agonists' inhibition of cyclic AMP formation (Table 3). Because the opiate receptor appears to undergo conformational changes and, hence, affinity state alteration, binding of the radioactive ligands could alter the subsequent binding of the nonradioactive ligands. This could account for the $n_H < 1$ for all of the opiate ligands in displacing [^3H] diprenorphine (Table 3). Although a single population of receptor exists, multiple affinities could be obtained in binding experiments.

REFERENCES

- Goldstein, A., L. Lowney, and B. K. Pal. Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1742-1747 (1971).
- Pert, C. B., and S. H. Snyder. Opiate receptor: demonstration in nervous tissue. *Science (Wash. D. C.)* **179**:1011-1014 (1973).
- Simon, E. J., J. M. Hiller, and I. Edelman. Stereospecific binding of the potent narcotic analgesic ^3H -etorphine to rat brain homogenate. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1947-1949 (1973).
- Creese, I., and S. H. Snyder. Receptor binding and pharmacological activity of opiates in guinea pig intestine. *J. Pharmacol. Exp. Ther.* **194**: 205-219 (1975).
- Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol.* **71**:379-393 (1979).
- Ariens, E. J., J. M. van Rossum, and P. C. Koopman. Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs. *Arch. Int. Pharmacodyn. Ther.* **127**:459-478 (1960).
- Hamprecht, B. Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *Int. Rev. Cytol.* **49**:99-170 (1977).
- Traber, J., K. Fischer, S. Latzin, and B. Hamprecht. Morphine antagonizes action of prostaglandin in neuroblastoma \times glioma hybrid cells. *Nature (Lond.)* **253**:120-122 (1975).
- Sharma, S., M. Nirenberg, and W. Klee. Morphine receptor as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 590-594 (1975).
- Chang, K. J., R. J. Miller, and P. Cuatrecasas. Interaction of enkephalin with opiate receptors in intact cultured cells. *Mol. Pharmacol.* **14**:961-970 (1978).
- Wahlstrom, A., M. Brandt, L. Moroder, E. Wunsch, G. Lindeberg, U. Ragnarsson, L. Terenius, and B. Hamprecht. Peptides related to β -lipotropin with opioid activity: effects on level of adenosine 3':5'-cyclic monophosphate in neuroblastoma \times glioma hybrid cells. *F. E. B. S. Lett.* **77**:28-32 (1977).
- Fantozzi, R., D. Mullikin-Kilpatrick, and A. J. Blume. Irreversible inactivation of the opiate receptors in the neuroblastoma \times glioma hybrid NG108-15 by chlornaltrexamine. *Mol. Pharmacol.* **20**:8-15 (1981).
- Colquhoun, D. The relation between classical and cooperative models for drug action, in *Drug Receptors* (H. P. Rang, ed.). Macmillan, London, 149-181 (1973).
- Rodbell, M. The role of hormone receptors and GTP regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17-22 (1980).
- Law, P. Y., J. E. Koehler, and H. H. Loh. Comparison of opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 and neuroblastoma \times glioma NG108-15 hybrid cell lines. *Mol. Pharmacol.* **21**:483-491 (1982).
- White, A. A., and D. B. Karr. Improved two-step method for the assay of adenylate and guanylate cyclase. *Anal. Biochem.* **85**:451-460 (1978).
- Klee, W. A., and M. Nirenberg. A neuroblastoma \times glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **71**:3474-3477 (1974).
- Blume, A. J. Opiate binding to membrane preparations of neuroblastoma \times glioma hybrid cells NG108-15: effect of ions and nucleotides. *Life Sci.* **22**:1843-1852 (1978).
- Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660-672 (1949).
- Kosterlitz, H. W., J. A. H. Lord, and A. J. Watt. Morphine receptor in the myenteric plexus of the guinea-pig ileum, in *Agonist and Antagonist Actions of Narcotic Analgesic Drugs* (H. W. Kosterlitz, H. O. J. Collier, and J. E. Villarreal, eds.). University Park Press, Baltimore, 45-61 (1971).
- Koski, G., and W. Klee. Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4185-4189 (1981).
- Insel, P. A., and M. Stoolman. Radioligand binding to β -adrenergic receptors of intact cultured S49 cells. *Mol. Pharmacol.* **14**:549-561 (1978).
- Terasaki, W. L., J. Linden, and G. Brookes. Quantitative relationship between β -adrenergic receptor number and physiologic responses as studied with a long-lasting β -adrenergic antagonist. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6401-6405 (1979).
- Schild, H. O. PA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**:189-206 (1947).
- Chang, K. J., E. Hazum, and P. Cuatrecasas. Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4141-4145 (1981).
- Lefkowitz, R. J., M. R. Wissels, and J. M. Stadel. Hormones, receptors and cyclic AMP: their roles in target cell refractoriness. *Curr. Top. Cell. Regul.* **17**:205-230 (1980).
- Law, P. Y., D. S. Hom, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma \times glioma NG108-15 hybrid cells after chronic opiate treatment: a multiple-step process. *Mol. Pharmacol.* **22**:1-4 (1982).
- Chang, K. J., R. W. Eckel, and S. G. Blanchard. Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells. *Nature (Lond.)* **296**:446-448 (1982).
- Chang, K. J., E. Hazum, and P. Cuatrecasas. Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative kappa and sigma agonists). *Proc. Natl. Acad. Sci. U. S. A.* **77**:4469-4473 (1980).
- Blume, A. J., D. Lichtstein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5626-5630 (1979).

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