MOLECULAR PHARMACOLOGY, 28:146–154

Multiple States of Opioid Receptors May Modulate Adenylate Cyclase in Intact Neuroblastoma × Glioma Hybrid Cells

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Received June 13, 1984; Accepted April 29, 1985

SUMMARY

Opioid receptor binding and opioid-mediated inhibition of cAMP accumulation were studied simultaneously in intact NG108-15 cells. The dose-response curves for the biological response were suggestive of positive cooperativity and systematically occurred at lower ligand concentrations than those for the binding of [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE), which were instead shallow and suggestive of a site heterogeneity or of a cooperative phenomenon. Computer modeling of the binding isotherms revealed that the data are best described assuming two binding sites with different affinities for the agonist; the mean ratio between the DADLE concentrations yielding half-maximal occupancy of the high affinity site and half-maximal response was 1.5, but it was 36 when the fractional occupancy of the sum of the two sites was considered. On examining several opioids, no direct correlation was found between high affinity site and biological response; however, several agonists displayed different affinities for the two sites, while the antagonist naloxone and the partial agonist diprenorphine bound to them with identical affinities. Furthermore, naloxone exhibited a good agreement between half-maximal receptor occupancy and K_i in blocking the agonist response. Thus, the binding heterogeneity detectable in intact cells is agonist-specific, and suggests rather that the sites are states of an identical receptor population. When [^{3}H]diprenorphine was used to label the opioid receptors, the competition curves for DADLE were consistent with the existence of an additional, very low affinity state undetectable by direct binding with labeled agonist and, again, not discriminated by naloxone. Multiple affinity states of the opioid receptor in intact cells may reflect its interaction with the effector system in the plasma membrane.

INTRODUCTION

For hormone receptors linked to adenylate cyclase activity, occupancy-effect relationships are usually linear for antagonists, but not for agonists (1, 2). It is now clear that, for these receptors, signal transmission involves at least three components: the receptor site (R), which recognizes the ligand; the catalytic unit (C), converting ATP into cAMP; and a class of regulatory guanine nucleotide-binding proteins (N_s and N_i) which couple C to, respectively, stimulatory or inhibitory R (3–5). Thus, the discrepancy between fractional receptor occupancy and fractional effect may be related to the stoichiometric arrangement and to the different rate constants for the partial reactions regulating the interactions between R, N, and C in the cell membrane. For the β-adrenoceptor in isolated membrane preparations, earlier biochemical studies indicated that a high affinity nucleotide-sensitive binding state and an increase in apparent receptor size (reviewed in Ref. 6) were both induced by the agonist, and were suggested to represent the interaction of the receptor with the nucleotide regulatory component. Computer modeling of β-adrenoceptor-binding isotherms in membranes led to the proposal of a ternary complex resulting from the interaction of the agonist-occupied receptor with an additional membrane component (X) having the characteristics expected for N_s: the intrinsic activities of the agonist were significantly correlated with the affinity constant of X for the liganded receptor, and it was proposed that guanine nucleotides exerted their effect by destabilizing the ternary complex and accelerating the dissociation of both the ligand and regulatory component from R (7, 8). The direct demonstration that the high affinity state for the agonist depends from the interaction of R and N_s has been recently achieved by reconstitution of pure β-adrenoceptor and pure N_s in lipid vesicles (9). However, recent studies of β-adrenoceptor binding in intact cells have provided evidence for agonist-dependent rapid changes in receptor affinity that are not correlated with the intrinsic activity of the ligand and cannot be easily explained by the ternary complex model (10, 11). For receptors inhibiting adenylate cyclase, agonist-specific multiple affinity states (12–14)

This work was supported by the Deutsche Forschungsgemeinschaft.

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and negative heterotropic effects of guanine nucleotides have been described in membrane preparations (15–17). However, fewer studies have directly addressed the question of occupancy-effect relationships for inhibitory receptors in intact cells. Both for the α_2-adrenoceptor of human platelets (18) and for the δ-opioid receptor of neuroblastoma × glioma hybrid cells (19, 20), it was found that agonist-binding curves are located at higher concentrations compared to their respective bioresponse curves.

We have further investigated the interaction of opioid ligands with the δ-receptor of neuroblastoma × glioma hybrid NG108-15 cells by comparing binding and inhibition of cAMP accumulation in intact living cells, employing strictly identical conditions and analyzing the binding isotherms with a computer modeling method (LIGAND) (21) which provides optimal estimates of the binding parameters for any number of ligands reacting simultaneously with any number of receptors.

EXPERIMENTAL PROCEDURES

Materials. Materials were obtained from the following sources: culture media and sera, GIBCO, Karlsruhe, F. R. G.; DADLE[1] and other enkephalin analogs, Peninsula, Belmont,CA; [^{3}H]DADLE and [^{3}H] diprenorphine (40 and 38 Ci/mmol, respectively), Amersham, Dreieich, F. R. G.; Naloxone HCl, Endo, Garden City, NJ; diprenorphine, Reckitt & Colman, Hull, U. K.; Ro 20-1724 Hoffmann-La Roche, Basel, Switzerland. All other materials were acquired from Sigma, München. F. R. G.

Cell culture. NG108-15 cells (synonymous with 108 CC 15 cells), were a gift of Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD). A stock of the cells of identical passage number (No. 21) was divided in sterile glass vials and kept frozen at the temperature of liquid nitrogen. The experiments presented in this paper were performed employing cells of passage numbers between 21 and 30.

The properties of the cells have been described previously (22). Cells were grown in Falcon T75 bottles using DMEM medium, with addition of 5.6 mM glucose and 5% fetal calf serum, and 0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine. Generally, the cells were cultured in a humidified atmosphere at 5% CO_2 and 37° with medium changes every 2 days. For propagation, cells were dislodged from the flasks by firmly tapping the flask on the palm of the hand; this procedure released cells into the medium. Cell viability was routinely measured by the trypan blue exclusion test.

Radioimmunoassay. Intracellular cyclic AMP levels were measured by RIA after extraction of the cells with 0.5 N HCl, neutralization with NaOH, dilution of the samples in sodium acetate (0.1 M, pH 6.), and acetylation of the sample (23).

Parallel determinations of binding and inhibition of cAMP accumulation in intact cells. Experiments were performed using cell suspensions. Confluent monolayers (1–1.5 × 10^7 cells) were collected and suspended in DMEM-HEPES prewarmed at room temperature. Incubations for binding reaction and cAMP accumulation were always started simultaneously with samples of the same batch of cells.

For the binding assay, aliquots of the cell suspensions were distributed in plastic vials, while for the cAMP accumulation assay they were distributed in 96-well microtiter plastic plates (Titertek, Flow). Peptides, drugs, and radioactive ligands were added in the form of concentrated stock solutions as 10% of final reaction volume. Bestatin was present at a final concentration of 10 μM.

Binding reactions and cAMP accumulation were initiated by addition of the cell suspensions (50% of final volume) to both vials and plates. Final cell concentration was 0.5–1 × 10^6 cells ml^{-1} and the total sample volume was 2 ml for binding and 0.1 ml for cAMP accumulation. The incubations lasted 90 min at 20° and were terminated simultaneously; cAMP accumulation was arrested by pipetting an equal volume of cold 1 N HCl into each well, and binding reactions were terminated by vacuum filtration on GF/B glass fiber filters (Whatman). Filters were washed with two sequential 5-ml aliquots of ice-cold phosphate-buffered saline, dried and counted by liquid scintillation spectrometry. The Titertek plates were kept at 0° until being assayed, and then 0.1 ml of 0.9 N NaOH was added to each well, plates were centrifuged to sediment cell debris, and the supernatant was diluted (200×) for cAMP RIA.

Data collection and presentation. In each experiment, four to six ligands were simultaneously tested in binding and cAMP inhibition. Binding of opioid ligands was studied as competition for the binding sites labeled by a tracer concentration of [^{3}H]DADLE (0.15–0.3 nM) or [^{3}H]DIPR (0.25–0.5 nM). Eight to 16 duplicate concentrations of ligand were used to establish the binding isotherms, while 10 to 12 duplicate concentrations were used for the cAMP inhibition dose-response curve. Thus, each experiment, corresponding to a single batch of cells identical in age and growth history, allows for a direct comparison of binding and effect for a given set of ligands.

Computer analysis. The concentration-response curve for both binding and cAMP inhibition were first analyzed with the computer program ALLFIT (24). The program fits families of sigmoid curves to the general logistic equation $y = d + (a - d)/(1 + (x/c)^b)$ where y is the response (radioligand bound or cAMP levels), x is the concentration of the ligand, c is 50% effective concentration of ligand (IC_{50}), b is the slope factor, and a and d are the extrapolated upper and lower limits for the observed value of y when x is 0 or infinite, respectively. The concentration-response curves for sets of ligands obtained in the same batch of cells were simultaneously fitted. Binding data were further analyzed by the computer program LIGAND. This program provides estimates of binding parameters for any number of ligands reacting simultaneously with any number of receptors, by using an exact mathematical model of the ligand-binding system and untransformed data (i.e., bound versus total ligand concentration). The program has been extensively used in several ligand-binding systems, and methods and the general strategy of its applications already have been described (21).

Statistical evaluation. The deviation of the points from their predicted value on the curves was weighted according to the reciprocal of their expected variance. Objective statistical tests for the goodness of fit are incorporated into the programs and were used as described. The choice between different models in LIGAND is based on the "extra sum of square" principle (21) which compares the residual mean square of each fit weighted for the reduction in degree of freedom produced by increasing the number of parameters, using conventional F statistics. The level of significance throughout the study was set at $p \leq 0.05$.

RESULTS

Inhibition of cAMP accumulation in NG108-15 cells: concentration-response curves. Incubation of intact NG108-15 cells with opioids results in a concentration-dependent inhibition of cAMP levels (19, 20). Fig. 1 shows the concentration-response curves of selected opioid ligands in inhibiting cAMP levels.

DADLE was the most potent ligand tested (IC_{50} = 0.77 ± 0.21 nM) while other nonpeptide opioids showed potencies consistent with their known relative δ activity; thus, etorphine was a very potent inhibitor in the cells and so was deprenorphine, a mixed agonist-antagonist,

[1] The abbreviations used are: DADLE, [D-Ala^2,D-Leu^5]enkephalin; Ro 20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DIPR, diprenorphine; DAPHA, [D-Ala^2, des Leu^5]enkephalin amide; DALEA, [D-Ala^2,Leu^5] enkephalin amide; O_{50}, half-maximal occupancy; RIA, radioimmunoassay.

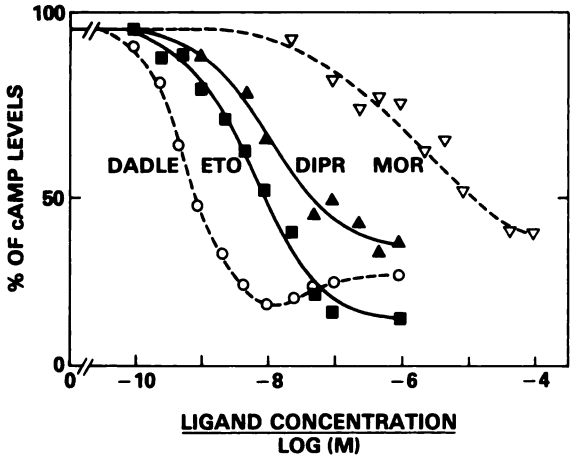

FIG. 1. *Concentration-dependent inhibition of cAMP accumulation in NG108-15 cells by opioids*

Cells were incubated in microtiter plates as described in Experimental Procedures with various concentrations of opioid agonists in the presence of 100 μM Ro 15-1724. cAMP levels represent here total intra- and extracellular amounts and are expressed as percentage of those determined in the absence of opioid (480 ± 45 pmol/10^6 cells). Points are means of triplicate determinations. *ETO*, etorphine; *MOR*, morphine.

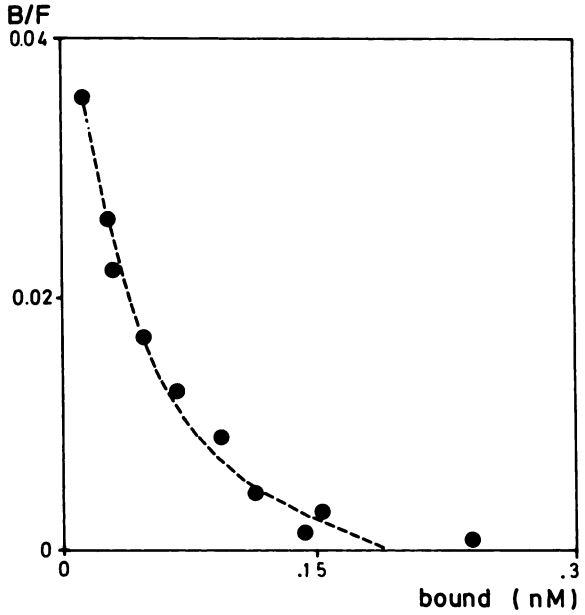

FIG. 2. *Equilibrium binding of [^{3}H]DADLE to intact NG108-15 cells*

The binding of [^{3}H]DADLE was determined as competition of varying concentrations of unlabeled peptide with a fixed (0.23 nM) concentration of monitoring ligand, as described in Experimental Procedures. The resulting curve was analyzed with the computer program LIGAND by sequentially fitting one-site and two-site models. The latter produced a significant reduction in the residual mean squares (F = 12.8, $p < 0.001$). The experimental points (means of triplicate determinations) are here shown together with the computer-fitted line in Scatchard's coordinates. The best fit was obtained, for the experiment presented here, with the following concentrations of binding sites: R1 = 2.1 ± 24% × 10^{-11} and R2 = 1.7 ± 11% × 10^{-10} M; and dissociation constants K_{d1} = 0.52 ± 40 % and K_{d2} = 9.8 ± 22% nM, nonspecific binding N = 0.22 ± 0.06% of the total radioactivity present, where the indicated relative errors are standard deviations of the computer fit. Significant better fits to a two-site model as shown here were obtained in four additional experiments, and the mean parameters estimated for a total of five experiments are reported in Table 1.

while morphine was a weak inhibitor of cAMP accumulation (1.45 ± 0.32 μM).

DADLE showed a slope factor greater than 1 (1.56, 95% confidence limits, 1.4–1.7), while less potent ligands had slope factors slighly lower than DADLE, but greater than 1. In contrast, morphine consistently exhibited a slope factor lower than 1 (0.79 ± 0.09).

Different ligands also displayed characteristic differences in their maximal inhibitory effects; a maximal inhibition of 70–80% was observed for etorphine and DADLE, whereas diprenorphine and morphine inhibited cAMP accumulation by only 40–60%.

Analysis of DADLE-binding isotherms. In every batch of cells, the half-maximal cAMP inhibition produced by DADLE was observed at a lower ligand concentration than the corresponding IC_{50} for binding measured in parallel. Furthermore, in all the batches of cells studied, the steep curves for cAMP inhibition were in contrast with the slope factor of the binding curves, which was significantly lower than 1 (mean = 0.78; confidence limit, 0.85–0.73).

The slopes of the binding isotherms of DADLE suggested heterogeneity of binding sites and/or negative cooperativity. Each curve, therefore, was analyzed further with the computer program LIGAND, by sequentially fitting the data to a one-site or a two-site model. The two-site model was in every case statistically significantly better than the one-site model.

In Fig. 2, a representative binding curve is presented in Scatchard coordinates. The following mean affinities were obtained in five experiments: K_{d1} = 0.998 (0.44–2.26) nM, K_{d2} = 33.9 (7.7–73.6) nM; the total number of receptors was 396 (221–766) fmol/10^6 cells, of which 21% (20–46%) was of high affinity type. When the binding of the same ligand is studied in the plasma membrane fraction of NG108-15 cells using hypotonic buffer, the data are always consistent with a single population of high affinity (1–2 nM) binding sites (not shown). Thus, binding studies in intact cells yield results different from those obtained in isolated membranes. The possibility that extensive degradation of the peptide could occur in intact cells and thus explain the difference between the two preparations was tested by "rebinding" experiments. [^{3}H]DADLE (10 nM) was incubated with cells or medium at 20° for 90 min, and, following acidification to pH 4 with acetic acid, neutralization, and centrifugation, its ability to bind to fresh cells was measured at a final concentration of 1 nM. At least 95% of the tracer incubated with cells compared to that in buffer was still bindable, suggesting there is no extensive DADLE degradation under these conditions.

Comparison between fractional receptor occupancy and fractional biological response. Using the parameters computed above for DADLE, we have calculated the apparent relationship between receptor occupancy and total ligand concentration for both the high and low affinity sites and their sum, assuming that they are independent sites; the corresponding curves for inhibition of cAMP accumulation were converted to fractional effects by scaling

them to corresponding maximal inhibition. The data are plotted side by side in Fig. 3. It appears that the receptor occupancy at the high affinity site (Fig. 3, — —) better agrees with the measured response, though the computer-generated curve still cannot describe the fractional effect (·····). The mean ratio between the concentration of total ligand yielding half-maximal fractional occupancy of the high affinity site and the concentration of total ligand resulting in half-maximal fractional response is then 1.5 (95% confidence limits, 0.7–2.7). On the other hand, the analogous ratio between fractional effect and occupancy to the sum of the two sites is 36 ± 18.

Thus, of the two apparent binding sites detectable when the binding of DADLE is studied in intact cells, only one, the high affinity one, appears in partial agreement with measured response, whereas the relationship between fractional effect and overall receptor occupancy is strongly discrepant (Fig. 3).

Comparison of occupancy and effect for other opioid agonists in intact cells. Binding isotherms for competition of [^{3}H]DADLE binding in parallel with the assessment of the ability to inhibit cAMP accumulation were also obtained, as detailed before, for several opioid peptides and alkaloids, both agonists and antagonists. The binding curves were further analyzed by LIGAND, theoretical occupancies to the different sites were computed, and the relationships between them and the corresponding fractional effects were evaluated. Although the relationship between occupancy and effect was variable and appeared to be a characteristic feature of each ligand, all

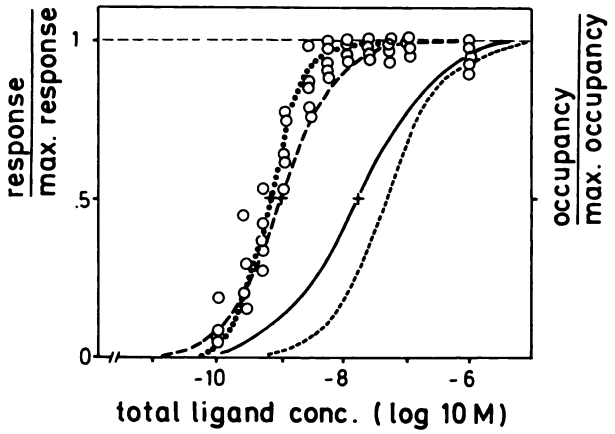

FIG. 3. *Relation between fractional response and fractional occupancy as a function of total DADLE concentration*

The inhibition of cAMP accumulation produced by various concentrations of DADLE measured in five independent experiments was normalized by scaling the data to the best fit maximal response (maximal inhibition of cAMP accumulation) obtained in each experiment. The five concentration-response curves are shown here in a single plot, where empty points are means of duplicate or triplicate determinations and the dotted line passing between them indicates the mean fractional response for the pooled data. Fractional occupancies to the high-affinity (— —), low-affinity (- - -), and sum of the two sites (——) as a function of total ligand concentration were computed using LIGAND, on the basis of the mean of the best fit binding parameters obtained from the five corresponding binding isotherms; experimental points for the binding isotherms are not plotted for clarity. The means for the five paired ratios between half-maximal fractional occupancies and half-maximal fractional effects are reported in the text.

agonists examined so far recognized both sites labeled by [^{3}H]DADLE (Table 1).

Reversal of DADLE effect by naloxone. Naloxone does not affect cAMP accumulation in NG108-15 cells, even at very high concentrations (10^{-4} M), but effectively inhibits the action of opioid agonists (Fig. 4, left panel). The IC_{50} values derived from concentration-response curves of naloxone in reversing several agonists varied between 0.6 and 2 μM.

The slope factor of the curves of naloxone for reversing DADLE effect was higher than 1 (mean, 1.55 ± 0.15; n = 4), whereas naloxone inhibited morphine effect with a slope factor lower than 1 (0.78 ± 0.15, n = 2) (see Fig. 4, left panel).

In parallel with the assessment of the ability of naloxone to reverse agonist effects, its binding as a competitor for [^{3}H]DADLE binding was studied. In three of four experiments, it was impossible to obtain a convergence of the fitting routine without letting the program set equal affinities of naloxone for the two sites labeled by [^{3}H]DADLE. In one experiment, two different affinities were computed; however, they were not significantly different. Thus, naloxone does not discriminate between the two sites labeled by DADLE.

When the occupancy by naloxone of the total number of receptor sites is computed and compared to the corresponding apparent fractional effect (i.e., reversal of

TABLE 1

Affinities of opioid agonists for the two sites labeled by [^{3}H]DADLE and comparison with the corresponding effect

Inhibition of cAMP accumulation and binding, measured as competition for [^{3}H]DADLE, were studied in paired experiments as described in Experimental Procedures. The parameter estimates of the affinity of each ligand (indicated as K_d) for the two sites were obtained using LIGAND, by analyzing the competition curves of DADLE and the corresponding curves of one to three competing ligands simultaneously. On the basis of these best fit estimates, half-maximal occupancies for the sum of the two sites for each ligand were computed as described for DADLE in the legend of Fig. 3. The n values indicate the number of experiments in which the agonist has been tested in paired measurements of binding and activity.

Ligand	Affinity	K_d ratio (K_{d2}/K_{d1})	Response (IC_{50})	Half-maximal total occupancy/ Half-maximal response (O_{50}/IC_{50})
	nM		*nM*	
DADLE (n = 5)				
K_{d1}	0.998 ± 0.26	24	0.77 ± 0.21	36.4
K_{d2}	23.9 ± 8.1			
DALEA (n = 3)				
K_{d1}	3.2 ± 0.6	17	3.3 ± 0.8	10.5
K_{d2}	55.3 ± 9			
DAPHA (n = 3)				
K_{d1}	118 ± 40	17	96 ± 16	10.9
K_{d2}	1984 ± 120			
Etorphine (n = 2)				
K_{d1}	3.1 ± 0.6	20	6.9 ± 1.2	8.9
K_{d2}	62 ± 15			
Morphine (n = 2)				
K_{d1}	225 ± 32	10.5	1450 ± 330	1.54
K_{d2}	2366 ± 158			

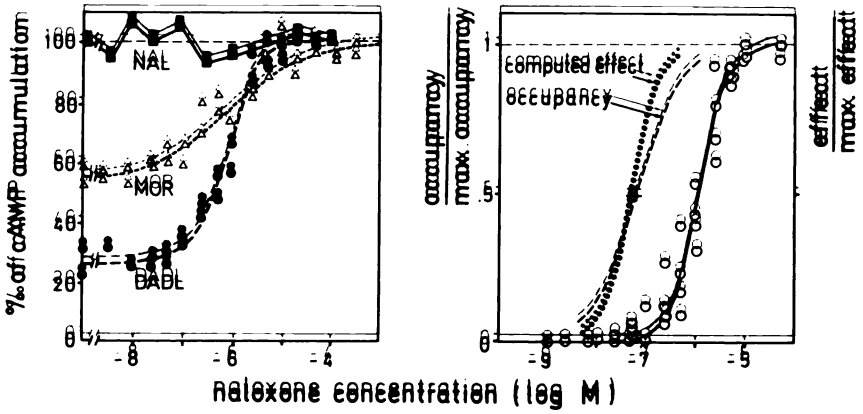

FIG. 4. *Antagonism of opioid ligands by naloxone*

Left, summarization of two independent experiments in which increasing concentrations of naloxone (*NAL*) reversed the maximal inhibition of cAMP accumulation produced by DADLE, 10 nM (●), and morphine (*MOR*), 100 μM (△). Each experimental point is the mean of duplicate determinations, scaled to the amount of cAMP accumulation observed in the absence of ligand. The effect of naloxone itself on cAMP accumulation observed in a separate experiment is also shown in the same graph, where the experimental points (■) (means of triplicate determinations) represent the cAMP accumulation expressed as percentage of the value observed in the absence of ligand. In three additional experiments, a single concentration of naloxone (100 μM) produced insignificant changes in cAMP levels. The absolute levels of cAMP measured in the absence of naloxone ranged in the three experiments shown between 390 and 490 pmol/10^6 cells. Right, concentration-response curves for naloxone reversal of the inhibition of cAMP accumulation produced by 10 nM DADLE obtained in four independent experiments (two of which are also shown at the left) were scaled as fractional blockade of agonist effect and were pooled in a single plot (empty points, each representing duplicate determinations). The corresponding solid line was obtained by averaging the best fit parameters computed by ALLFIT analysis of each curve. The IC_{50} value from this curve was employed to calculate (25) a mean K_i value for naloxone (46.4 ± 16 nM), which, in turn, was used to draw the "true" fractional blockade curve for the antagonist (· · · · ·) by translating the former curve to the new midrange value without altering its slope. In the four experiments shown here, paired determinations of naloxone binding were obtained as competition for the binding sites labeled by [^{3}H]DADLE. As reported in the text, naloxone displayed identical affinities for the two sites labeled by the agonist, and a single K_d value was obtained in each experiment. The mean K_d averaged from the four experiments (59 ± 11 nM) was used to compute the receptor fractional occupancy for naloxone (– – –). Experimental points for the binding are omitted for clarity.

agonist inhibition of cAMP accumulation) obtained in parallel (Fig. 4, right panel), the relationship is inverted as compared to the one obtained for the agonist: half-maximal occupancy is obtained at lower concentrations and it is located to the right of the IC_{50} for the effect.

K_i values were computed (25) for each experiment and pooled to plot the "true" fractional effect isotherms of naloxone (Fig. 4, right panel).

K_i values for naloxone effect were very close to the K_d values measured in the parallel binding assays. The mean ratio K_i/K_d for naloxone reversal of DADLE is 1.23 ± 0.37, a value not significantly different from 1, and the mean ratio K_i/K_d for all the agonists tested is ±1.04 with confidence limits of 0.62–1.45. The antagonism of naloxone for DADLE effect was further investigated by Schild analysis (26). Increasing concentrations of naloxone produced a shift on the right in the concentration-response curves of DADLE for inhibition of cAMP accumulation and did not affect their maximal inhibition. A Schild plot of the data (Fig. 5, left panel) was linear and exhibited a slope not significantly different from 1, suggesting competitive behavior. However, naloxone did also produce a concentration-dependent decrease of the slope factors of the agonist concentration-response curves which became statistically significant at the highest concentration (Fig. 5, right panel) and did not appear to be dependent on differences in equilibrium between agonist and antagonist, since it was observed either by the simultaneous addition of agonist and antagonist or by preincubation with the antagonist and later addition of the agonist. Thus, naloxone exhibits a deviation from strictly competitive behavior in inhibiting DADLE response.

Diprenorphine binding in intact cells. Diprenorphine is a partial agonist in NG108-15 cells (19, 27) (Fig. 1). The binding of this compound was first measured as a competitor for [^{3}H]DADLE binding.

LIGAND analysis of data showed that diprenorphine, similar to naloxone, could not discriminate between the two binding sites labeled by DADLE; the best fits were always obtained by setting the affinities of diprenorphine for the two sites as identical, although the curves were always steeper than predicted from the two-site model. To further clarify this point, binding was studied using [^{3}H]DIPR as monitoring ligand. The association of this tracer to the receptor in intact cells reached a plateau after 20 min of incubation and was stable during the following 70 min (not shown). After 90 min, the dissociation induced by a large excess of unlabeled diprenorphine (1 μM) was consistent with a monoexponential curve, in agreement with the findings of Law *et al.* (19). Both (=)naloxone and diprenorphine competed with this tracer with binding isotherms whose slope factors were not different from 1, and produced identical maximal inhibitions, suggesting that both ligands compete for a single homogeneous class of sites; (+)naloxone, studied between 0.1 and 100 μM, did not produce any significant inhibition of [^{3}H]DIPR binding, indicating that all the tracer is bound to stereospecific sites in the intact cell. However, the agonists DADLE and etorphine inhibited

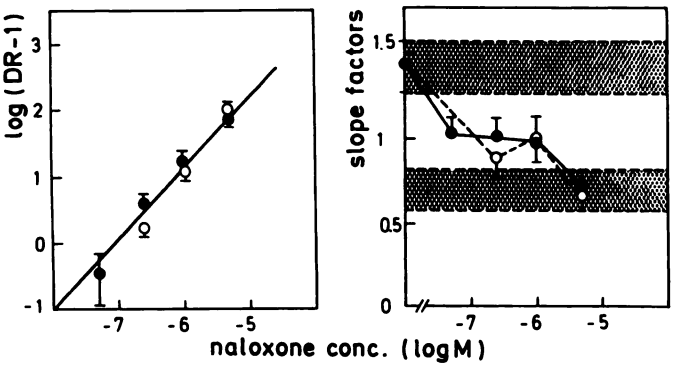

FIG. 5. *Schild analysis of naloxone antagonism of DADLE*

Concentration-response curves for DADLE inhibition of cAMP accumulation in intact NG108-15 cells were obtained in the presence of increasing concentrations of naloxone (0.05, 0.25, 1, and 5 μM); naloxone was added either simultaneously with the agonist (●) or 60 min prior to the addition of the agonist (○), while the total incubation lasted 90 min at 20°. The ratios between the IC_{50} in the presence of the antagonist versus the IC_{50} of the control (*DR*) were computed using the best fit parameters obtained by simultaneous fit of the sets of curves with ALLFIT. The data are plotted here according to Schild (26) (left panel). Unweighed linear regression analysis reveals a slope slightly higher, but not statistically significantly different from 1 (1.09 ± 0.1). The maximal inhibition produced by the agonist was not significantly affected by naloxone, and was 80 ± 6% in the experiment presented here. The computed K_i was 85 ± 27 nM which is close to a K_d of 69 ± 18 nM measured as competition of [^{3}H]DADLE binding in the paired experiment. The slope factors of the DADLE curves, however, appeared to be reduced by increasing naloxone concentration, and the effect was statistically significant as determined with ALLFIT analysis, by observing the deterioration of the fit produced when the slope factors of all the curves were forced to be identical ($p < 0.01$ and 0.03 for the two sets of curves examined). The relationship between slope factors and antagonist concentration is plotted in the right panel; the shaded areas indicate ± standard deviation limits obtained from the computer fit.

[^{3}H]DIPR binding with very shallow isotherms (slope factors of 0.52 ± 0.2, $n = 4$, and 0.65 ± 0.15, $n = 2$, respectively). Experiments were then designed to compare the binding of the three ligands, DADLE, naloxone, and diprenorphine, using both [^{3}H]DIPR and [^{3}H]DADLE as monitoring ligands, and simultaneously assessing their effects on cAMP accumulation. The binding isotherms of the three ligands obtained as competition for each tracer were then simultaneously analyzed with LIGAND as illustrated in Tables 2 and 3. It is clear that when [^{3}H]DIPR is the tracer, the curves of both diprenorphine and naloxone can be modeled satisfactorily with a single class of binding sites (Fig. 1, Table 2) but not the one of DADLE. Thus, to obtain a simultaneous fit of the three curves, it is necessary to introduce two ($p < 0.001$) and even three ($p = 0.007$) binding sites for which both naloxone and diprenorphine share uniform affinities, while DADLE binds to the first two with affinities similar to those measured in [^{3}H]DADLE-binding experiments, and to the third with a lower affinity (fit 3, Table 2). When [^{3}H]DADL is the monitoring ligand, the situation is symmetrical: two sites are necessary ($p < 0.001$) to describe DADLE isotherms, but the curves of the three ligands must be simultaneously fitted with both naloxone and diprenorphine sharing equal affinities for the two sites (fits 1, 3, and 4, Table 3). The curve of diprenorphine, however, still shows a departure from the model (three runs, $p < 0.05$, table 3). Indeed, in three different experiments, the curves of diprenorphine competition for [^{3}H]DADL showed a slight but systematic deviation from unity of the slope factors. The introduction of a third site having the same affinity as the other two for both diprenorphine and naloxone, but inaccessible to the monitoring ligand (fit 5, Table 3), improves dramatically the description of the diprenorphine curve and reduces significantly the overall variance ($p = 0.001$). In fact, when the tracer is an agonist, a major part of low affinity binding is lost in the filtration procedure and the sites apparent from competition of DADLE with [^{3}H]DIPR are underestimated. However, the existence of a third site is still apparent as a shift to the right and an increase in slope factor for the diprenorphine curve.

Relationship between occupancy and effect derived from [^{3}H]DIPR binding. Using the parameters computed from the three-site fit of the data, the fractional occupancies and fractional effects obtained in the corresponding experiments were compared as described before for DADLE. Using the three-site model, the fractional occupancy for DADLE of the high affinity site was again very close to the fractional effect while for diprenorphine the occupancy of the total sum of sites occurred at lower concentrations than the response. The mean ratio of half-maximal occupancy versus half-maximal effect was for this ligand ($n = 3$) 0.35 ± 0.18.

DISCUSSION

We have compared receptor binding and biological response for the opioid agonist DADLE in living, intact cells by use of simultaneous assays and identical conditions. At least three features of the opioid interaction with the δ receptor of NG108-15 cells are at variance with the simple occupancy theory of hormone receptors: the curve for bioresponse is too steep, that for binding is too flat and their relationship is far from linearity.

The binding curves, which were measured in parallel with the inhibition of cAMP accumulation, allowed for a further analysis in terms of the law of mass action. The results of such analysis are clear: curves of agonist binding to the opioid receptor in intact cells are best fitted by a model involving two binding sites. A variety of agonists, including the partial agonist morphine, compete for the two sites labeled by DADLE with different affinities independent of their chemical nature as peptides or alkaloids and not as consequent upon their preference for established classes of opioid receptors (28). However, the antagonist naloxone and the partial agonist diprenorphine do not discriminate between the two sites, but bind to them with identical affinities, i.e., they "see" the sites labeled by [^{3}H]DADLE as a homogeneous receptor preparation. When [^{3}H]diprenorphine is used as a tracer, diprenorphine itself and naloxone compete with this ligand for a single population of sites, but the competition curve of DADLE is shallow, covers more than 3 orders of magnitude, and can be fitted assuming three sites having three different affinities for the agonist, discriminated by neither diprenorphine nor naloxone.

TABLE 2

Ligand analysis of opioid competition for [^{3}H]DIPR

Six competition curves (13 triplicate points) using two labeled ligands ([^{3}H]DIPR and [^{3}H]DADL) and three unlabeled ligands (DADL, diprenorphine, and naloxone) were obtained in parallel in a single batch of cells. Each set of three curves was simultaneously evaluated with LIGAND by sequentially fitting models of increasing complexity or containing different constraints. The set of curves obtained for the competition for [^{3}H]DIPR is illustrated here; the one for [^{3}H]DADL is in Table 3. Ligands are numbered as L1 (monitoring ligand, here DPR, diprenorphine), L2 (DADLE), and L3 (naloxone). Parameter estimates of affinities are indicated as K_{nm}, where n = ligand number, m = site number, and have the dimensions of liters/mol; R = molar site concentration. The root mean square (RMS) reported for each fit is based on the assumption of a constant per cent error in bound ligand concentration. The runs test reported for each curve in each fit indicates, when significant ($p < 0.05$), a poor description of the experimental points by the computed curve. The F value refers to the comparison of each fit with the preceding one. The estimates for the nonspecific binding parameter (which was typically 1.6–2% of the total counts for [^{3}H]DIPR) are not reported since they varied insignificantly across the models. The concentration of [^{3}H]DIPR used was 0.5 nM. Notice that the introduction of a second site (from fit 1 to fit 2) produces a dramatic reduction in the RMS ($F = 329$), but still a significant further improvement ($F = 8$) is achieved with a three-site model, where the affinities for the three sites are shared for both DIPR and naloxone. Removing such constraints (not shown here) did not produce further reductions in the RMS. Similar results were observed in two additional experiments.

	Model (constraints)	K			R	Runs test	RMS (*df*)	*F* (*p* value)
		L1 (DPR)	L2 (DADLE)	L3 (naloxone)				
		M^{-1}		M				
Fit 1	1 site	$K_{11} = 3.5 \pm 1.2 \times 10^7$	$K_{21} = 1.1 \pm 0.3 \times 10^8$	$K_{31} = 8.8 \pm 2 \times 10^6$	$R = 5.8 \pm 2 \times 10^{-9}$	L1 = 2 $p < 0.01$ L2 = 3 $p < 0.01$ L3 = 6 $p > 0.05$	470.5 (33)	
Fit 2	2 sites ($K_{11} = K_{12}$) ($K_{31} = K_{32}$)	$K_{11} = K_{12}$ $4.8 \pm 0.3 \times 10^7$	$K_{21} = 1.8 \pm 0.3 \times 10^8$ $K_{22} = 8.4 \pm 2 \times 10^4$	$K_{31} = K_{32}$ $1.2 \pm 0.7 \times 10^7$	R1 = $3.5 \pm 0.2 \times 10^{-9}$ R2 = $1.6 \pm 0.1 \times 10^{-9}$	L1 = 6 $p > 0.05$ L2 = 4 $p > 0.05$ L3 = 7 $p > 0.05$	22.5 (31)	329 ($p < .0001$)
Fit 3	3 sites ($K_{11} = K_{12} = K_{13}$) ($K_{31} = K_{32} = K_{33}$)	$K_{11} = K_{12} = K_{13}$ $4.9 \pm 0.3 \times 10^7$	$K_{21} = 2.1 \pm 2 \times 10^9$ $K_{22} = 4.7 \pm 2 \times 10^7$ $K_{23} = 5 \pm 2 \times 10^4$	$K_{31} = K_{32} = K_{33}$ $1.2 \pm 0.06 \times 10^7$	R1 = $1.7 \pm 0.5 \times 10^{-9}$ R2 = $1.9 \pm 0.5 \times 10^{-9}$ R3 = $1.4 \pm 0.1 \times 10^{-9}$	L1 = 4 $p > 0.05$ L2 = 8 $p > 0.05$ L3 = 7 $p > 0.05$	15.5 (29)	8 ($p = 0.007$)

TABLE 3

Ligand analysis of opioid competition for [^{3}H]DADLE

Symbols and explanations are as in Table 2. L1 is DADLE, L2 is DIPR, and L3 is naloxone. A two-site model (compare fit 1 versus fit 2) better described the data ($p < 0.001$) but both diprenorphine and naloxone curves can be fitted by sharing identical affinities for the two sites, since sequentially removing the constraint of equal affinities both for diprenorphine (fit 3) and naloxone (fit 4) does not show any improvement in the goodness of the fit. Notice that in all cases the runs test for diprenorphine is poor. The introduction of a third site (fit 5) to which diprenorphine and naloxone bind with affinities identical to those for the others, but undetectable by DADLE ($K_{13} = 0$) reduces significantly the RMS and improves the runs test for diprenorphine.

	Model (constraints)	K			R	Runs test	RMS (*df*)	*F* (*p* value)
		L1 (DADLE)	L2 (DPR)	L3 (naloxone)				
Fit 1	1 site	$K_{11} = 4.2 \pm 0.7 \times 10^8$	$K_{21} = 1.2 \pm 0.2 \times 10^8$	$K_{31} = 2 \pm 0.3 \times 10^7$	$K = 1.8 \pm 0.3 \times 10^{-10}$	L1 = 3 $p < 0.05$ L2 = 3 $p < 0.05$ L3 = 3 $p < 0.05$	284 (29)	
Fit 2	2 sites ($K_{21} = K_{22}$) ($K_{31} = K_{32}$)	$K_{11} = 7.2 \pm 2 \times 10^8$ $K_{12} = 8.5 \pm 7 \times 10^6$	$K_{21} = K_{22}$ $1.2 \pm 0.1 \times 10^8$	$K_{31} = K_{32}$ $1.9 \pm 0.2 \times 10^7$	R = $1.1 \pm 0.2 \times 10^{-10}$ R2 = $6.5 \pm 3 \times 10^{-10}$	L1 = 6 $p > 0.05$ L2 = 3 $p < 0.05$ L3 = 4 $p > 0.05$	105 (27)	12.3 ($p < 0.001$)
Fit 3	2 sites ($K_{31} = K_{32}$)	$K_{11} = 8.8 \pm 4 \times 10^8$ $K_{12} = 3.2 \pm 3 \times 10^7$	$K_{11} = 1.1 \pm 0.3 \times 10^8$ $K_{22} = 1.7 \pm 6 \times 10^8$	$K_{31} = K_{32}$ $2.1 \pm 0.3 \times 10$	R1 = $8.6 \pm 4 \times 10^{-11}$ R2 = $3.2 \pm 0.9 \times 10^{-10}$	L1 = 7 $p > 0.05$ L2 = 3 $p < 0.05$ L3 = 8 $p > 0.05$	122 (26)	−3 ($p = 1$)
Fit 4	2 sites ($K_{21} = K_{22}$)	$K_{11} = 7.4 \pm 2 \times 10^8$ $K_{12} = 8.7 \pm 7 \times 10^6$	$K_{21} = K_{22}$ $1.2 \pm 0.1 \times 10^8$	$K_{31} = 1.8 \pm 0.2 \times 10^7$ $K_{32} = 3.4 \pm 19 \times 10^8$	R1 = $1.1 \pm 0.2 \times 10^{-10}$ R2 = $6.5 \pm 3 \times 10^{-10}$	L1 = 6 $p > 0.05$ L2 = 3 $p < 0.05$ L3 = 8 $p > 0.05$	108 (26)	0.8 ($p = 1$)
Fit 5	3 sites ($K_{21} = K_{22} = K_{23}$) $K_{31} = K_{32} = K_{33}$) ($K_{13} = 0$)	$K_{11} = 6 \pm 1 \times 10^8$ $K_{12} = 9.5 \pm 6 \times 10^6$ $K_{13} = 0$	$K_{21} = K_{22} = K_{23}$ $2.2 \pm 0.3 \times 10^8$	$K_{31} = K_{32} = K_{33}$ $2 \pm 0.1 \times 10^7$	R1 = $1.2 \pm 0.2 \times 10^{-10}$ R2 = $5.2 \pm 2 \times 10^{-10}$ R3 = $1.3 \pm 0.4 \times 10^{-8}$	L1 = 8 $p > 0.05$ L2 = 4 $p > 0.05$ L3 = 8 $p > 0.05$	50 (26)	30.5 ($p < 0.001$)

Thus, the binding heterogeneity for opioid ligands in intact cells appears to be agonist-specific and can hardly be explained with the existence there of fixed classes of opioid receptor types. This suggests rather that the sites are states of an identical population of receptors. A variety of possible artifacts inherent in binding studies performed in intact cells should be ruled out in order to strengthen this suggestion. As described in Results, we didn't detect extensive ligand degradation or severe departures from equilibrium in the present assay conditions. More difficult to rule out is the possibility that tracer and competitor are differentially sequestered into the cells, as might happen with the tracer [^{3}H]diprenorphine, more hydrophobic than the competitor DADLE. The binding of [^{3}H]diprenorphine was completely insensitive to (+)naloxone, suggesting that there is no specific uptake of this ligand into the cell, but it is still possible that [^{3}H]diprenorphine has access to stereospecific sites present in an intracellular compartment (internalized receptors) which are not available for the more hydro-

philic peptide (29). Such a possibility cannot be completely ruled out without the availability of a hydrophilic antagonist for the opioid receptor, yet we consider it less likely since etorphine, an oripavine agonist whose physicochemical properties are presumably very similar to those of diprenorphine, exhibits shallow curves similar to those of DADLE in competing with [^{3}H]diprenorphine.

An interesting outcome of this study is the striking difference between the binding data obtained in intact cells and those derived from membranes incubated in hypotonic buffers, in the absence of sodium and guanine nucleotides. Under such conditions, there is a single class of high affinity sites for both agonist and antagonist opioids (30). It has been recently shown that, when membrane preparations from NG108-15 cell are studied in the presence of sodium, magnesium, and GTP, an agonist-specific binding heterogeneity similar to that described here in intact cells can be detected, although the proportion of states and their affinities depends on the concentration of modulators in the medium (31). Thus, taken together, those studies and the data presented here in intact cells strongly suggest that, when opioid binding is studied under "coupling conditions," the receptor exists in multiple affinity states that possibly reflect its interaction with the membrane component involved in adenylate cyclase inhibition. It is pertinent to ask how these multiple states correlate with the observed biological response, and we have devoted most of this study to such a task. One must be aware of the limits of such comparison. When analyzing the biological effect, it is difficult to go beyond a phenomenological description since intact cells constitute a complex system and no valid assumption of equilibrium can be made for phenomena occurring at the intracellular side of the plasma membrane. Yet living cells offer the distinct advantage that the effector system is present in an unaltered membrane and conceivably exposed to physiological concentrations of co-modulators. Thus, even if descriptive by necessity, data obtained in the intact cell represent an important paradigm to which studies in more simple preparations can be apposed. Three observations, derived from such comparisons, require further discussion: 1) the relationship between fractional occupancy and fractional effect; 2) the antagonism produced by naloxone; and 3) the involvement of multiple states in cyclase inhibition.

For agonists, the relationship between overall occupancy and effect is strongly hyperbolic, whereas for the antagonist occupancy and effect (i.e., fractional blockade of agonist effect) are in close agreement. Thus, the activation of opioid receptors in intact cells exhibits an agonist-specific receptor "spareness" whose extent appears a property of the ligand and might indicate the different degree of coupling produced by different agonists. A large "receptor reserve" was previously reported in this same cell line by Fantozzi *et al.* (20) who showed that it is possible to irreversibly block up to 90% of the existing binding sites without reducing the ability of opioid agonists to inhibit cAMP accumulation. The slope of the bioresponse curve of a highly efficiently coupled ligand such as DADLE is higher than 1, suggesting that cooperativity and/or strong amplification occurs during the coupling mechanism. Since similar steep curves also have been reported (32) in membrane preparations, it is unlikely that they are an artifact of intact cells. Interestingly, when stimulating the low K_m GTPase activity, which results from the interaction of receptor and N_i, DADLE exhibits concentration-response curves with slopes not different from 1 and EC_{50} values at least 10 times higher than those observed here.[2] It is thus possible that the amplification mechanism operates at the level of N_i-cyclase rather than receptor-N_i interaction.

Naloxone exhibits a small but consistent deviation from strictly competitive behavior. In fact, while it affects the concentration-response curves of the agonist with a major shift to the right without changing the maximal effects, it also reduces their slopes in a concentration-dependent manner. This may suggest that the amplification operating at the level of signal transduction is inhibited in the presence of antagonist. Cerione *et al.* (9), by studying the interaction of purified β-adrenoceptors with purified N_s reconstituted in liposomes, have obtained evidence that the antagonist-occupied receptor has a reduced ability compared to the unoccupied receptor to interact with the GTP-binding regulatory component. Such observations might provide a mechanistic explanation for the deviation of an antagonist from pure competitive behavior.

The fractional occupancy computed for the high affinity state is, for peptide agonists, closer to the response. For nonpeptide ligands, especially morphine, the half-maximal occupancy of the high affinity state actually precedes the observed response. Therefore, there is no obvious proportionality between the high affinity state and the biological response induced by the agonist. In contrast, some relationship exists between the ratio of affinities of each ligand for the two states and its apparent "coupling efficiency," i.e., the ratio between half-maximal total occupancy and half-maximal fractional effect. Morphine, which has the lowest ability to discriminate between the two states ($K_{d2}/K_{d1} = 10$), also shows the lowest coupling efficiency ($O_{50}/EC_{50} = 1.6$) compared to DADLE, which exhibits the highest ratio of affinities ($K_{d1}/K_{d2} = 24$) and produces the maximal effect with minimal binding ($O_{50}/EC_{50} = 36$); diprenorphine, which does not discriminate between different affinity states in intact cells, exhibits an even inverted relationship between occupancy and effect ($O_{50}/EC_{50} = <1$). Multiple affinity states have been described for the interaction of dopamine ligands with the D_2-dopamine receptor in pituitary, which is also negatively coupled to adenylate cyclase (14). A recent extension of the ternary complex model to this system reveals basic differences with respect to the β-adrenoceptor: 1) the presence of a "precoupled" complex between receptor and regulatory unit which is reciprocally stabilized or destabilized by, respectively, agonists and antagonists or guanine nucleotides; 2) the existence of a negative correlation between the ability of agonists to discriminate between the two states

[2] L. Vachon, T. Costa, and A. Herz, manuscript in preparation.

(ratio of K_H/K_L) and their capacity to stabilize the ternary complex (ratio of L/M) (3).

Additional studies, however, are necessary before attempting to relate the observations made here in intact cells and results obtained from membrane preparations. Firstly, the affinities for the two states here were computed on the basis of competition for an agonist. When a tracer with antagonistic properties is used, the data suggest an additional third state which is not predicted from the ternary complex model in the present form.

Secondly, even for the more extensively studied β-adrenoceptor, binding data obtained in intact cells under nonequilibrium conditions cannot be easily related to those obtained in membranes at equilibrium (10, 11), since they suggest that, in the intact cell at least initially, the receptor exists in the high affinity state only. The data reported here for opioid receptors were obtained at equilibrium and do not allow discrimination of whether multiple affinity states are preexistent to agonist interaction with the receptor or are time-dependently induced by such event.

Thirdly, as reported by Law *et al.* (34) in this cell line, receptor down-regulation is largely inhibited at the temperature used for our binding assay, but receptor desensitization still occurs, and it could play an important role here.

For instance, it is possible that one of the three states observed represents a nonfunctional, desensitized form of the receptor. Studies with cells desensitized specifically by chronic opioid treatment, or aspecifically by pertussis toxin, are in progress, and may help in the determination of the functional relevance of multiple affinity states of the opioid receptor in intact cells.

ACKNOWLEDGMENTS

We wish to thank Peter J. Munson for his valuable suggestions on the computer analysis of the data and Dr. M. Nirenberg for the gift of the NG 108-15 cell line. We are also deeply indebted to Ursula Bäuerle for her wonderful ability in growing large amounts of healthy cells and to Sabine Kosin who skillfully performed the cAMP radioimmunoassays. We finally thank Dr. Mark J. Millan for stylistic revision of the manuscript and Linda Jones and Heide Roth for the excellent secretarial assistance.

REFERENCES

1. Ross, E. M., M. E. Maguire, T. W. Sturgil, R. L. Biltonen, and A. G. Gilman. Relationship between the β-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* **252:**5761–5775 (1977).
2. Brown, E. M., S. A. Fedak, C. J. Woodard, G. D. Aurbach, and D. Rodbard. β-Adrenergic receptor interactions: direct comparison of receptor interaction and biological activity. *J. Biol. Chem.* **251:**1239–1246 (1976).
3. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284:**17–22 (1980).
4. Gilman, A. G. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49:**533–564 (1980).
5. Gilman, A. G. G proteins and dual control of adenylate cyclase. *Cell* **36:**577–579 (1984).
6. Limbird, L. E. Activation and attenuation of adenylate cyclase. *Biochem. J.* **195:**1–13 (1981).
7. De Lean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255:**1108–1111 (1980).
8. Kent, R. S., A. De Lean, and R. J. Lefkowitz. A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol. Pharmacol.* **17:**14–23 (1980).
9. Cerione, R. A., J. Codina, J. L. Benovic, R. J. Lefkowitz, L. Birnbaumer, and M. G. Caron. The mammalian β-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* **23:**4519–4525 (1984).
10. Toews, M. L. and J. P. Perkins. Agonist-induced changes in β-adrenergic receptors on intact cells. *J. Biol. Chem.* **259:**2227–2235 (1984).
11. Hoyer, D., E. E. Reynolds, and P. B. Molinoff. Agonist-induced changes in the properties of beta-adrenergic receptors on intact S49 lymphoma cells. *Mol. Pharmacol.* **25:**209–218 (1984).
12. Klein, W. L. Multiple binding states of muscarinic acetylcholine receptors in membranes from neuroblastoma × glioma hybrid cells. *Biochem. Biophys. Res. Commun.* **93:**1058–1066 (1980).
13. Hoffman, B. B., T. Michel, T.B. Brenneman, and R. J. Lefkowitz. Interaction of agonists with platelet α-adrenergic receptors. *Endocrinology* **110:**926–932 (1982).
14. De Lean, A., B. F. Kilpatrick, and M. Caron. Dopamine receptor of the porcine anterior pituitary gland: evidence for two affinity states of the receptor discriminated by both agonists and antagonists. *Mol. Pharmacol.* **22:**290–297 (1982).
15. Jacobs, K. H., K. Aktories, and G. Schultz. A nucleotide regulatory site for somatostatin inhibition of adenylate cyclase in S49 lymphoma cells. *Nature (Lond.)* **303:**177–178 (1983).
16. Blume, A. J. Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. USA* **75:**1713–1717 (1978).
17. Smith, S. K., and L. E. Limbird. Solubilization of human platelet α-adrenergic receptor: evidence that agonist occupancy of the receptor stabilizes receptor-effector interactions. *Proc. Natl. Acad. Sci. USA* **78:**4026–4030 (1981).
18. Macfarlane, D. E., and D. C. Stump. Parallel observation of the occupancy of the alpha$_2$-adrenergic receptor in intact platelets and its ability to inhibit the adenylate cyclase. *Mol. Pharmacol.* **22:**574–579 (1982).
19. Law, P. Y., D. S. Hom, and H. H. Loh. Opiate regulation of adenosine 3′,5′-monophosphate levels in neuroblastoma × glioma NG 108-15 hybrid cells: relationship between receptor occupancy and effect. *Mol. Pharmacol.* **23:**26–35 (1983).
20. Fantozzi, R., D. Mullikin-Kilpatrick, and A. J. Blume. Irreversible inactivation of the opiate receptors in the neuroblastoma × glioma hybrid NG108-15 by chlornaltrexamine. *Mol. Pharmacol.* **20:**8–15 (1981).
21. Munson, P. J. LIGAND: a computerized analysis of ligand binding data. *Methods Enzymol.* **92:**543–576 (1983).
22. Hamprecht, B. Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma × glioma cell hybrids in the cell culture. *Int. Rev. Cytol.* **49:**99–170 (1977).
23. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-*O*-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1:**207–218 (1975).
24. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radio-ligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235:**E97–E102 (1978).
25. Waud, D. R., and R. B. Parker. Pharmacological estimation of drug-receptor dissociation constants: statistical evaluation. II. Competitive antagonists. *J. Pharmacol. Exp. Ther.* **177:**13–24 (1971).
26. Arunlakshana, O., and H. O. Schild. Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14:**48–58 (1959).
27. Koski, G., and W. Klee. Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. *Proc. Natl. Acad. Sci. USA* **78:**4185–4189 (1981).
28. Lord, J. A. H., A. A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors *Nature (Lond.)* **267:**495–500 (1977).
29. Staehelin, M., P. Simons, K. Jaeggi, and N. Wigger. CGP-12177: a hydrophilic β-adrenergic radioligand reveals high affinity binding of agonists to intact cells. *J. Biol. Chem.* **258:**3496–3502 (1983).
30. 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).
31. Law, P. Y., D. S. Hom, and H. H. Loh. Multiple affinity states of opiate receptor in neuroblastoma × glioma NG108-15 hybrid cells. *J. Biol. Chem.* **260:**3561–3569 (1985).
32. Sharma, S., M. Nirenberg, and W. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* **76:**5626–5630 (1979).
33. Wreggett, K., and A. De Lean. The ternary complex model: its properties and application to ligand interactions with the D$_2$-dopamine receptor of the anterior pituitary gland. *Mol. Pharmacol.* **26:**214–227 (1984).
34. Law, P. Y., D. S. Hom, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment: a multiple step process. *Mol. Pharmacol.* **22:**1–4 (1982).

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