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Relationship Between Opioid-Receptor Occupancy and Stimulation of Low-K_m GTPase in Brain Membranes

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Abstract: Treatment of rat brain membranes with the irreversible opioid ligand *cis*-3-methylfentanylisothiocyanate (Superfit) was used to reduce gradually the number of available binding sites for the δ-selective agonist [^{3}H][D-Ser^2,Leu^5]enkephalin-Thr^6 ([^{3}H]DSLET). Subsequently, the correlation between ligand binding and low-K_m GTPase was investigated. Alkylation with 10 μ*M* and 25 μ*M* Superfit inactivated 66% and 71% of high-affinity (K_D, 1 n*M*) binding sites without decreasing the affinity of the remaining sites and the stimulation of low-K_m GTPase by DSLET. Following exposure of the membranes to 50 μ*M* and 75 μ*M* Superfit, ligand binding was confined to the low-affinity (K_D, 20 n*M*) sites. In these membranes, the δ-agonists DSLET and [D-Pen^2,D-Pen^5]enkephalin still stimulated low-K_m GTPase, and these effects were blocked by ICI 174864 (*N,N*-diallyl-Tyr-AIB-AIB-Phe-Leu-OH; AIB, α-aminoisobutyric acid), a δ-selective antagonist. A similar relationship between low-affinity ligand binding and GTPase stimulation was observed following alkylation of the δ-opioid receptor with the nonselective irreversible antagonist β-chlornaltrexamine in the presence of protective concentrations of DSLET. The results reveal spare receptor sites in the coupling of the δ-opioid receptor to low-K_m GTPase in brain and identify low-affinity ligand binding as a functional component in the process. **Key Words:** δ-Opioid receptor—GTP binding protein—Brain GTPase—Receptor-effector coupling—Rat brain membranes. **Clark M. J. et al.** Relationship between opioid-receptor occupancy and stimulation of low-K_m GTPase in brain membranes. *J. Neurochem.* **52,** 1162–1169 (1989).

On the basis of acute inhibition by opiates and a long-term compensatory increase in enzyme activity in NG108-15 neuroblastoma × glioma hybrid cells, the functional coupling of opioid receptor to adenylate cyclase was proposed (Sharma et al., 1975*a,b*). That interaction has since been well documented both in neurally derived transformed cells (Blume et al., 1979; Law et al., 1982) and in brain (Law et al., 1981; Barchfeld et al., 1982). In membrane preparations from these tissues, opiates stimulated a low-K_m GTPase as part of the mechanism that links opioid receptor occupancy to adenylate cyclase inhibition (Koski and Klee, 1981; Barchfeld and Medzihradsky, 1984). The enzymatic hydrolysis of GTP is an important step in the mechanism by which GTP binding proteins regulate the coupling of membrane receptors to adenylate cyclase (Cassel and Selinger, 1978; Hildebrandt et al., 1983). Purified inhibitory GTP binding protein (G_i) from brain has been shown to exhibit low-K_m GTPase activity (Milligan and Klee, 1985).

The effects of opioid agonists on adenylate cyclase and low-K_m GTPase from brain displayed the major characteristics of ligand binding to opioid receptor: stereospecificity and inhibition by antagonists (Barchfeld et al., 1982; Barchfeld and Medzihradsky, 1984). However, information on the quantitative relationship between opioid receptor occupancy and effector responses is spotty. Spare receptors in the inhibition of adenylate cyclase (Fantozzi et al., 1981) and cyclic AMP formation (Law et al., 1983) by opiates in the neuroblastoma × glioma NG108-15 hybrid cells were described, and different receptor reserves for dynorphin in guinea pig ileum and in mouse vas deferens were implied to explain the diverging potency of the opioid peptide in these tissues (Cox and Chavkin, 1983). With other receptor systems, the occupancy theory of effector

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Abbreviations used: β-CNA, β-chlornaltrexamine; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr^6; ICI 174864, *N,N*-diallyl-Tyr-AIB-AIB-Phe-Leu-OH, where AIB = α-aminoisobutyric acid; RSS, residual sum of squares; Superfit, *cis*-3-methylfentanylisothiocyanate.

activation was shown clearly to represent just one of the existing relationships between the degree of receptor occupancy and tissue response: e.g., the inhibitory α_2-adrenergic receptor in human platelets (Lenox et al., 1985), but not the stimulatory β-adrenergic receptor in S49 lymphoma cells (Mahan and Insel, 1985), were shown to have spare ligand binding sites in their coupling to adenylate cyclase. Furthermore, a markedly different receptor reserve was shown for coupling of the muscarinic receptors to adenylate cyclase and phosphoinositide hydrolysis, respectively (Brown and Goldstein, 1987).

Following the characterization of the kinetics of ligand binding to opioid receptors in brain membranes (Fischel and Medzihradsky, 1981, 1986) and brain slices (Barchfeld-Rothschild and Medzihradsky, 1987), we have described the coupling of multiple opioid receptors to brain GTPase (Clark et al., 1986; Clark and Medzihradsky, 1987) and adenylate cyclase (Barchfeld et al., 1982). In some of these studies, we have selectively alkylated opioid receptors to obtain brain membranes specific for a given type of receptor. In the present work, we have used progressive alkylation of the δ-opioid receptor to study the efficacy of ligand binding in stimulating low-K_m GTPase, a process underlying the inhibition of the putative effector, adenylate cyclase. In addition, we have evaluated the significance of high- and low-affinity ligand binding sites in receptor–effector coupling. Initial findings of this work have been presented in a preliminary form (Clark and Medzihradsky, 1986).

MATERIALS AND METHODS

Materials

The δ-selective opiates, DPDPE ([D-Pen^2,D-Pen^5]enkephalin), ICI 174864 (*N,N*-diallyl-Tyr-AIB-AIB-Phe-Leu-OH; AIB, α-aminoisobutyric acid), and Superfit (*cis*-3-methylfentanylisothiocyanate), were obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan. The nonselective-opioid antagonist, β-CNA (β-chlornaltrexamine), was purchased from Research Biochemicals, Inc. (Wayland, MA, U.S.A.), and DSLET ([D-Ser^2,Leu^5]enkephalin-Thr^6) was bought from Cambridge Research Biochemicals (Atlantic Beach, NY, U.S.A.). [γ-^{32}P]GTP (25 Ci/mmol) and [^{3}H]DSLET (30.5 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.) and New England Nuclear Corp. (Boston, MA, U.S.A.), respectively. The biochemicals used in the GTPase assay were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Membrane preparation

Membranes from the cerebrum of Sprague–Dawley rats weighing 200 g were isolated as described (Fischel and Medzihradsky, 1981). The membranes were eventually sedimented at 20,000 *g* and suspended in Tris-HCl (pH 7.4). Aliquots of this suspension, sufficient for experiments on one given day, were frozen at −70°C. Prior to use, the suspension was quickly thawed in a water bath and dispersed in a Dounce homogenizer. Protein concentration in the latter preparation was approximately 0.6 mg/ml.

Receptor alkylation

For direct alkylation (Clark and Medzihradsky, 1987), the suspension of cerebral membranes in Tris-HCl (pH 7.4), adjusted to a protein concentration of 0.35 mg/ml, was incubated with different concentrations of Superfit for 40 min at 25°C in a shaking water bath. After fivefold dilution with Tris-HCl (pH 7.4), the suspension was centrifuged at 20,000 *g* for 15 min at 4°C. The pellet was suspended, using a Dounce homogenizer, in five original volumes of the Tris buffer and centrifuged as described above. The procedure was repeated once more and the pellet suspended in the original volume of Tris-HCl (pH 7.4). The adequacy of this washing procedure to remove unbound alkylator was ascertained by the constancy of [^{3}H]DSLET binding parameters (K_D, B_{max}) after the third centrifugation; subsequent washes yielded superimposable binding curves. The treated membranes either were used fresh or were frozen in aliquots at −70°C. The freezing had no detectable effects on the results.

For protective alkylation (Clark and Medzihradsky, 1987), a suspension of cerebral membranes in Tris-HCl buffer (pH 7.4), adjusted to a protein concentration of 0.35 mg/ml, was incubated with 400 μM DSLET for 5 min at 25°C. After NaCl and β-CNA were added to a final concentration of 120 mM and 25 μM, respectively, the suspension was incubated for 40 min at 25°C and then centrifuged at 20,000 *g* for 15 min at 4°C. The pellet was washed by suspension and centrifugation as described above for the direct alkylation. Aliquots of the membrane suspension were frozen at −70°C.

GTPase assay

The experimental procedure used has been described previously (Clark and Medzihradsky, 1987) and is based on the method of Cassel and Selinger (1976). In principle, the hydrolysis of [γ-^{32}P]GTP was measured in the presence of Mg^{2+} and an ATP regenerating system. The concentration of GTP was either 0.5 μM or 2 μM, the latter corresponding to the 10-fold value of the low-affinity K_m. In separate tubes, the reaction mixture also contained 50 μM GTP to measure the opiate-insensitive, high-K_m GTPase activity. The latter was subtracted from total enzyme activity to yield the opiate-sensitive, low-K_m GTPase activity. The following controls were included routinely in each assay: basal GTPase activity in the absence of opiates, GTP hydrolysis in the absence of membranes, and GTP hydrolysis in the assay medium prior to sample incubation.

Determination of protein

The method of Lowry et al. (1951) was used, with bovine serum albumin as standard.

Ligand binding

The experimental procedure was described in detail previously (Fischel and Medzihradsky, 1981; Clark et al., 1988). The medium, dispensed to 8-ml polypropylene tubes, consisted of 185 μl of membrane suspension (24 μg of protein), 25 μl of [^{3}H]DSLET (to yield final concentrations of 0.2–60 nM), 25 μl of unlabeled DSLET (to give a final concentration of 50 μM) or H_2O, and 25 μl of NaCl (final concentration 120 mM) or H_2O. In some experiments, $MgCl_2$ and GTP were added together with the NaCl to give final concentrations of 5 mM and 0.5 or 2 μM, respectively. In a few experiments, ligand binding was determined in the complete GTPase assay medium, excluding the radiolabeled nucleotide. Following incubation of the membrane suspension for 15 min at 25°C, the assay was started by the addition of radiolabeled and

unlabeled DSLET (the latter was added to the tubes used for the determination of specific binding). After incubation for 65 min at 25°C to reach equilibrium in binding, the samples were quickly filtered and subjected to liquid scintillation counting. Opioid receptor binding of [^{3}H]DSLET was defined as the difference between ligand binding determined in the absence and presence of 50 μM unlabeled DSLET. This concentration maximally displaced the total binding of [^{3}H]DSLET at all the concentrations used in this study (data not shown). Also established and considered was the dependence of specific ligand binding on time and amount of protein.

Statistical analysis of the data

The data for ligand binding was fit to a receptor model with two saturable sites. The binding parameters were estimated by the weighted nonlinear least-square regression program, NONLIN (Metzler, 1969; Fischel and Medzihradsky, 1981, 1986). Initial estimates for the parameters K_{D1}, K_{D2}, B_{max1}, and B_{max2} were provided by a subroutine based on a partitioned reciprocal analysis that corrected for the mutual interaction between the high- and low-affinity binding components (Neal, 1972). Variability in parameter estimates (±SD) was computed from the variance–covariance matrix, and the residual sum of squares (RSS) from the regression. For each experiment, the regression was repeated several times using initial estimates in the range ±5% of the apparent solution. The resulting estimates were then averaged to reduce bias due to convergence limits in the regression.

To ascertain the experimental conditions under which the high-affinity sites of the receptors become blocked, the binding data for alkylated membranes were also fit by a one saturable site regression model. The corresponding RSS values for the two models were compared by the extra sum of squares F statistic: $F = [(RSS_1 - RSS_2)/2]/[RSS_2/\upsilon]$, where υ is the degree of freedom for the two-site model.

Estimates for K_D and B_{max} for each binding site are highly correlated, and reliable values are difficult to obtain especially at low levels of binding (Figs. 1 and 2). Considering the particular importance of B_{max} in this work, the regression analysis for each experiment was repeated, constraining the parameters K_{D1} and K_{D2} to the values obtained with nonalkylated (control) membranes. By eliminating variability in K_D values, the estimates of B_{max} were improved (Table 2). The validity of this approach was confirmed by comparing the RSS of the unconstrained series (Table 1) with that of the constrained series using the variance ratio F statistic (Table 2).

The stimulation of low-K_m brain GTPase was characterized by the parameters K_s and S_{max}. The ligand concentration at which half-maximal stimulation (K_s) occurred was obtained from log-probit plots, relating stimulation of basal enzyme activity to five different ligand concentrations. The K_s estimates were derived from a linear regression analysis. Maximal stimulation of basal low-K_m GTPase (S_{max}) was obtained with

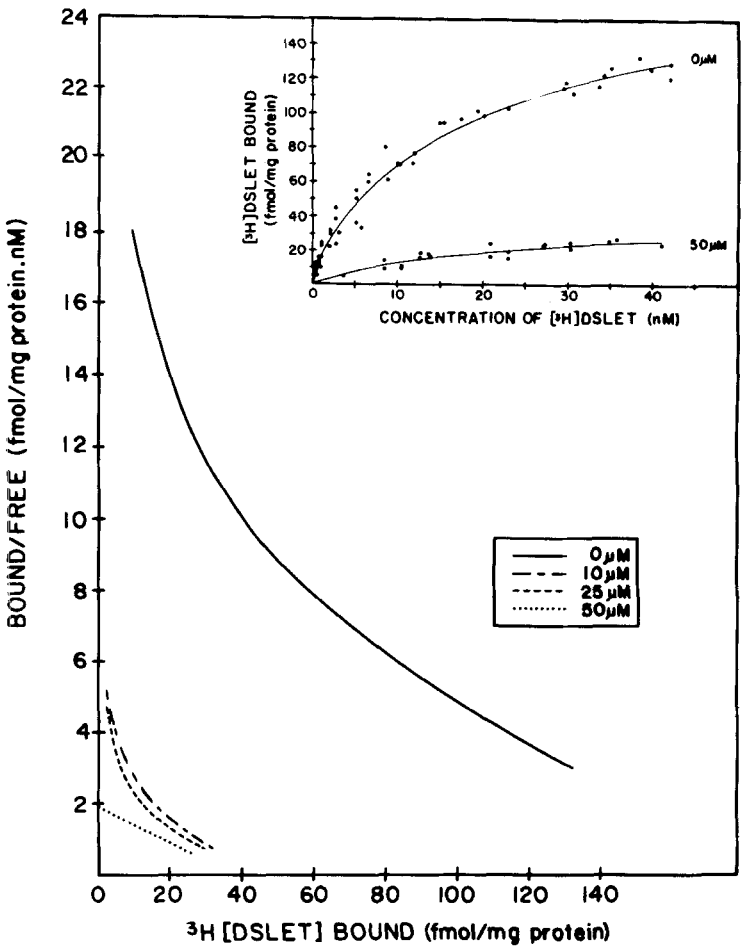

FIG. 1. Equilibrium binding of [^{3}H]DSLET following receptor alkylation. Rat cerebral membranes were treated with Superfit at the indicated concentrations as described under Materials and Methods. Subsequently, the specific equilibrium binding of [^{3}H]DSLET in the presence of 120 mM NaCl was determined. The Scatchard plots were drawn using the mean values for K_D and B_{max} obtained by the constrained nonlinear regression analysis (Table 2). The curves for 10 μM and 25 μM Superfit are not statistically different. Illustrated in the **inset** is the distribution of actual data points of ligand binding around the computer-generated saturation curve using the parameters listed in Table 2. To improve the lucidity of the graph, results obtained with only one of the alkylated membranes (50 μM Superfit) are contrasted with the control.

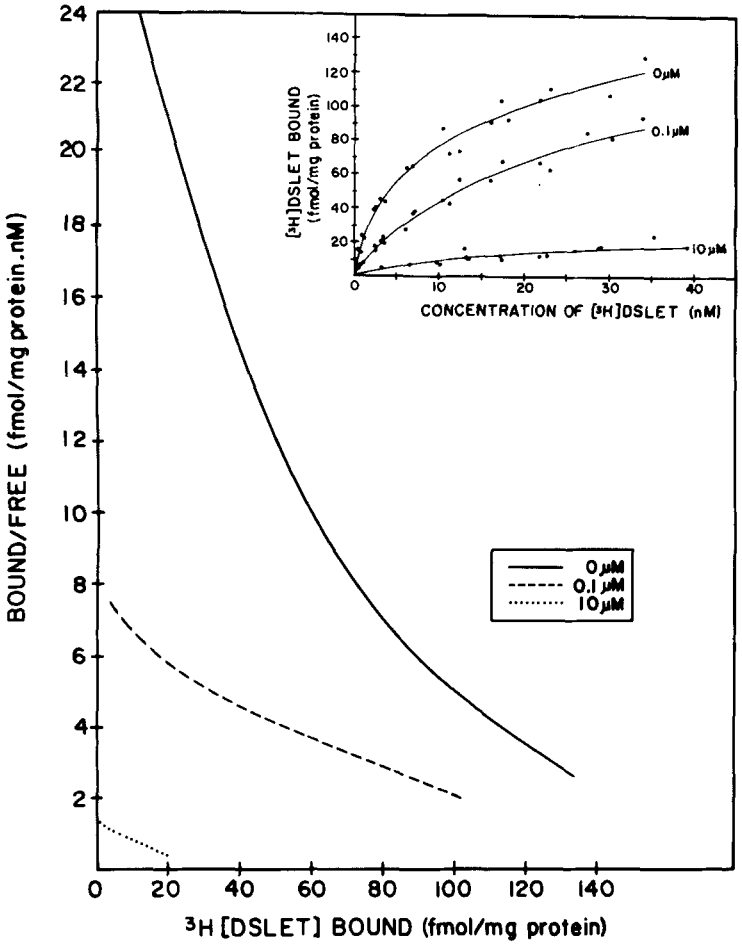

FIG. 2. Equilibrium binding of [^{3}H]DSLET in alkylated membranes: effects of Mg^{2+} and GTP. Rat cerebral membranes were treated with 0.1 μM and 10 μM Superfit as described under Materials and Methods. Subsequently, equilibrium binding of [^{3}H]DSLET in control and alkylated membranes was determined in the presence of 120 mM NaCl, 5 mM $MgCl_2$, and 0.5 μM GTP, the concentrations present in the GTPase assay medium. The Scatchard plots were derived from the mean values for K_D and B_{max} obtained by the constrained nonlinear regression analysis (Table 2). Shown in the **inset** is the distribution of actual data points of ligand binding around the computer-generated saturation curve drawn according to the parameters in Table 2.

gradually increasing ligand concentrations as a well-defined upper limit of enzyme activity. Opioid agonists present at concentrations larger than that yielding S_{max} progressively inhibited GTPase stimulation. Variability of K_s and S_{max} was expressed as standard deviation of the mean determined from replicate experiments (Table 3).

RESULTS

The binding of [^{3}H]DSLET to opioid receptor in cerebral membranes revealed two populations of saturable sites with K_D values of 0.99 nM and 19.63 nM, respectively (Fig. 1 and Table 1). Incubation of the membranes with 10 μM Superfit, an irreversible ligand at the δ-opioid receptor (Smith et al., 1985; Clark and Medzihradsky, 1987), blocked 78% of the binding sites for [^{3}H]DSLET without raising the K_D values of either the high- or low-affinity sites relative to the control (Table 1). Also unaltered by this level of alkylation were the parameters K_s and S_{max}, by which DSLET stimulated basal low-K_m GTPase activity (Table 3). Treatment of the membranes with 25 μM Superfit effectively blocked the high-affinity sites (Table 1); S_{max} was unaltered, but the K_s for DSLET stimulation of GTPase increased twofold (Table 3). Receptor alkylation with 50 μM and 75 μM Superfit abolished the high-affinity sites of ligand binding and raised the K_D of the low-affinity components (Fig. 1 and Table 1). In these membranes, DSLET still stimulated GTPase, although with deteriorating kinetic parameters (Table 3). Enzyme activity was also enhanced by DPDPE, an opioid ligand of high δ selectivity (Clark et al., 1988), with K_s and S_{max} values of 24 μM and 11%, respectively. Confirming earlier results (Clark and Medzihradsky, 1987), the stimulation by both DSLET and DPDPE was inhibited (data not shown) by ICI 174864, a δ-selective antagonist (Cotton et al., 1984). Finally, 100 μM Superfit eliminated both high- and low-affinity ligand-binding sites (Table 1), and DSLET failed to stimulate GTPase activity (Table 3).

By constraining the numerical values for K_D, the significance of the data on the number of high- and low-affinity sites was enhanced (Table 2). The constrained series also gave B_{max} estimates that decreased with increasing Superfit concentrations, as expected by a priori reasoning. The absence of high-affinity sites described by regression was confirmed by the fit of the respective data to a one-site receptor model (Tables 1 and 2).

The δ-opioid receptor was also alkylated with the nonspecific irreversible opioid antagonist β-CNA

TABLE 1. *Parameters of [^{3}H]DSLET equilibrium binding and GTPase stimulation*

Membrane treatment (μM alkylator)	Components added to binding assay[a]	Binding parameters[b] K_{D1}	K_{D2}	B_{max1}	B_{max2}	RSS	n
Superfit							
0	Na^+	0.99 (± 0.22)	19.63 (± 1.22)	13.93 (± 2.44)	170.52 (± 1.97)	42.18	47
10	Na^+	0.25 (± 0.30)	13.63 (± 3.24)	3.65 (± 1.58)	36.63 (± 1.42)	16.63	22
25[c]	Na^+	1.14 (± 1.29)	21.30 (± 9.27)	4.58 (± 3.82)	38.58 (± 3.53)	19.06	22
50[c]	Na^+	1.61 (± 7.37)	27.34 (± 6.79)	1.38 (± 2.69)	41.52 (± 3.13)	10.70	21
75[c]	Na^+	ND[d]	33.43 (± 3.52)	ND	45.69 (± 4.36)	21.08	21
100	Na^+	ND	ND	ND	ND	—	12
0	Na^+, Mg^{2+}, GTP	1.97 (± 0.46)	28.40 (± 11.48)	48.33 (± 11.51)	136.71 (± 12.85)	20.04	23
0.1	Na^+, Mg^{2+}, GTP	1.49 (± 0.68)	38.81 (± 7.60)	9.01 (± 3.56)	171.94 (± 13.24)	6.59	21
10[c]	Na^+, Mg^{2+}, GTP	1.16 (± 5.73)	51.55 (± 22.78)	0.79 (± 1.80)	45.05 (± 9.25)	6.23	16
β-CNA[e]							
25 (+ 400 μM DSLET)	Na^+	ND	24.01 (± 3.05)	ND	61.20 (± 5.62)	0.43	10
25	Na^+	ND	ND	ND	ND	—	8

The binding parameters were obtained from an unconstrained nonlinear regression analysis using a receptor model with two or one binding site as described under Materials and Methods. Shown are parameter means, standard deviations, and total number of data points (n). RSS reflects the variability in the data remaining after the variability due to parameter effects is removed. RSS was typically 1% of the total sum of squares.

[a] The concentrations of NaCl, $MgCl_2$, and GTP were 120 mM, 5 mM, and 0.5 μM, respectively.

[b] K_D and B_{max} values are in units of nM and fmol/mg of protein, respectively.

[c] The data also fit a one-site model.

[d] ND, not detectable.

[e] These experiments were part of the first series shown in this table, with 0 Superfit as the appropriate control.

(Ward et al., 1982) in the presence of 400 μM DSLET as the site-protective ligand (Table 1). The concentration of DSLET was selected to provide submaximal receptor protection. Following treatment, the characteristics of ligand binding and GTPase stimulation were indeed similar to those obtained by alkylation with 50 μM or 75 μM Superfit: total ligand binding was reduced by 72% with only low-affinity sites for [^{3}H]DSLET binding remaining (Tables 1 and 2), and DSLET still stimulated GTPase activity (Table 3). Again, this stimulation was inhibited by ICI 174864 (not shown). However, whereas protective alkylation with 75 μM Superfit adversely affected the parameters of GTPase stimulation, these were similar to control values in membranes alkylated with β-CNA and DSLET (Table 3). In membranes treated with β-CNA alone, saturable ligand binding and GTPase stimulation were undetectable.

Na^+, Mg^{2+}, and GTP are effective modulators of ligand binding to opioid receptor (Blume, 1978; Childers and Snyder, 1980), and Na^+ and GTP are essential for the coupling of opioid receptor to adenylate cyclase (Blume et al., 1979). Therefore, we have investigated the relationship between [^{3}H]DSLET binding and

TABLE 2. *Statistical analysis of ligand binding*

Membrane treatment (μM alkylator)	Binding parameters[a] K_{D1}	K_{D2}	B_{max1}	B_{max2}	Statistical parameters RSS	F	υ
0[b]	1.00	20.00	14.42	170.79	42.24	1.001	43
10	1.00	20.00	4.87	39.52	19.08	1.147	18
25[c]	1.00	20.00	4.20	37.52	19.01	1.003	18
50[c]	1.00	20.00	0.05	37.86	11.05	1.033	17
0[d]	2.00	28.00	47.71	136.86	20.31	1.013	19
0.1	2.00	28.00	5.52	150.36	7.40	1.123	17
10[c]	2.00	28.00	1.51	28.62	6.03	1.033	12

As described under Materials and Methods, the parameters listed below were estimated from a regression analysis with K_D values constrained to the values obtained with nonalkylated membranes (Table 1). Listed are the parameter estimates, the RSS, the F statistic calculated as the ratio of RSS values in Tables 2 and 1, and the degrees of freedom (υ) for the regression.

[a] K_D and B_{max} values are in units of nM and fmol/mg of protein, respectively.

[b] In this series, 120 mM NaCl was present in the binding assay.

[c] Data also fit a one-site model.

[d] In this series, 120 mM NaCl, 5 mM $MgCl_2$, and 0.5 μM GTP were present in the binding assay.

TABLE 3. *GTPase stimulation following receptor alkylation*

Membrane treatment (μM alkylator)	GTPase stimulation	
	K_s (μM)	S_{max} (%)
Superfit		
0	5.6 ± 0.8	23 ± 3
0.1	6.2 ± 0.7	22 ± 2
10	6.2 ± 1.5	21 ± 2
25	11.0 ± 2.8	19 ± 2
75	55.0 ± 7.4	13 ± 3
100	ND[a]	ND
β-CNA[b]		
25 (+400 μM DSLET)	6.5 ± 1.1	29 ± 13
25	ND	ND

The assay of low-K_m GTPase activity was carried out as described under Materials and Methods. Shown are K_s, the concentrations of DSLET yielding half-maximal stimulation of basal enzyme activity, and S_{max}, the maximal stimulation of basal GTPase activity expressed in percent. Basal enzyme activity (mean value 67 pmol/mg protein/min) was not significantly affected by the alkylations. Listed are mean values and standard deviations obtained in three to five experiments carried out in triplicate.

[a] ND, not detectable.

[b] The appropriate control values are the data listed under 0 Superfit.

GTPase stimulation in the presence of identical concentrations of Na^+, Mg^{2+}, and GTP (Fig. 2 and Table 1). Under these conditions, the affinity of ligand binding was decreased (Table 1). It was reduced further when binding was carried out in the GTPase assay medium containing 2 μM, rather than 0.5 μM, GTP: the K_D values for [^{3}H]DSLET were 4.2 nM and 54.4 nM, whereas the K_s for DSLET was 0.6 μM. These experiments also revealed the effectiveness of Superfit at submicromolar concentrations: 0.1 μM alkylator reduced high-affinity [^{3}H]DSLET binding by 81% (Table 1). Nonetheless, under the conditions of either partial or complete abolishment of high-affinity ligand binding, either in the absence or presence of GTPase assay components, the stimulation of low-K_m GTPase was indistinguishable from control (Table 3).

DISCUSSION

In a number of tissues and receptor systems, the total occupancy of ligand binding sites was found not to correlate with the maximum response at the effector level. For example, in human platelets, the elimination of 90% of α_2-adrenergic receptor sites still produced half-maximal inhibition of cyclic AMP accumulation (Lenox et al., 1985), and maximal activation of glucose transport in adipocytes was obtained with about 5% of the high-affinity insulin receptor sites on the cell surface (Kono and Barham, 1971). However, the magnitude of receptor reserve in a tissue can be characteristic of a given ligand, and different at various degrees of receptor response (Kenakin, 1986). The experimental approach frequently used to investigate the existence of spare receptors is to alter the number of receptor sites by their alkylation with irreversible ligands and to measure functional responses of one or more effector components (Brown and Goldstein, 1987) in one (Brown and Goldstein, 1987) or different (Mahan and Insel, 1985; Fisher and Snyder, 1987) tissues. These studies described different muscarinic receptor reserves in various brain regions and cloned neural cells (Fisher and Snyder, 1987), and, most interestingly, for two effector processes in chick heart cells (Brown and Goldstein, 1987). Opioid receptor reserves were implicated in smooth muscle preparations (Cox and Chavkin, 1983), and alkylation by β-CNA of 95% of opiate binding sites in NG108-15 hybrid cells did not alter the extent of inhibition of adenylate cyclase (Fantozzi et al., 1981). In these cells, the ratio of K_D of ligand binding and K_i to inhibit adenylate cyclase was used to describe spare receptor sites for some, but not all, opiates investigated (Law et al., 1983). In a recent preliminary study, both irreversible opioid ligands and exposure of NG108-15 cells to phospholipase were used to demonstrate continued inhibition of adenylate cyclase by opiates despite the substantial decrease in ligand binding (Childers et al., 1986).

In rat cerebral membranes, Scatchard analysis of ligand binding at equilibrium and assessment of the association and dissociation kinetics (Fischel and Medzihradsky, 1981, 1986) have shown the existence of two populations of saturable opioid receptor sites with different binding affinities. Such biphasic binding was described for both opioid agonists (Childers and Snyder, 1980; Nishimura et al., 1984; Fischel and Medzihradsky, 1986) and antagonists (Childers and Snyder, 1980; Fischel and Medzihradsky, 1981; Roth and Coscia, 1984), and shown by computer fitting to correspond to a minimal model of two saturable binding sites (Fischel and Medzihradsky, 1981).

In the present study, the specific binding of [^{3}H]DSLET in the presence of Na^+ was best described by interaction with two populations of binding sites with K_D values of 1 nM and 20 nM. In coupling to brain GTPase, there was a large excess of receptor sites: occupancy of approximately 20% was sufficient to produce maximal stimulation of enzyme activity. Specifically, following the preferential alkylation of the high-affinity sites, GTPase was stimulated apparently by ligand binding to the low-affinity sites. Further alkylation eventually abolished both specific ligand binding and receptor-GTPase coupling. The number of δ-ligand binding sites was also altered by the approach of protective alkylation. Following treatment with β-CNA in the presence of DSLET, only low-affinity binding sites of [^{3}H]DSLET remained, and their occupancy by DSLET stimulated GTPase. The absence of high-affinity ligand binding was confirmed by rigorous statistical analysis. Such treatment of the data was particularly important in view of the increased variability of ligand binding in alkylated membranes with a diminished number of receptor sites.

The δ selectivity of the low-affinity sites was ascertained by two criteria using highly selective ligands (Clark et al., 1988): in alkylated membranes with only these sites present, the δ-opiates DSLET and DPDPE stimulated GTPase, and these effects were blocked by the δ-antagonist ICI 174864. As shown recently, this compound inhibited GTPase stimulation by δ- but not by μ- or κ-opioid agonists (Clark and Medzihradsky, 1987).

Under any of the employed experimental conditions, maximal coupling of the δ-opioid receptor to low-K_m GTPase occurred at submaximal receptor occupancy, and enzyme activity was stimulated in the absence of high-affinity ligand binding sites. In the presence of Na^+, Mg^{2+}, and GTP, the difference between the values for K_D and K_s decreased, and became even smaller if ligand binding was carried out under conditions identical to those of the GTPase assay. The proposition that optimal coupling between the opioid receptor and brain GTPase requires an intact membrane and/or cellular microenvironment, disrupted by the process of membrane isolation, is supported by the strongly impaired inhibition of adenylate cyclase by opioid agonists in brain membranes, in contrast to intact cells (Barchfeld et al., 1982). The dependence of opioid receptor mechanisms on the structural and dynamic characteristics of the plasma membranes has been ascertained and is presently under investigation (Medzihradsky, 1989).

Of particular interest in the present work is the apparent functional role of the low-affinity binding component. In intact cells, the muscarinic acetylcholine receptor (Nathanson, 1983) and β-adrenergic receptor (Pittman and Molinoff, 1980) were present as a single population of binding sites with affinities similar to those of the low-affinity component of these receptors measured in isolated membranes. To explain these findings, the presence in intact cells of two rapidly interconverting forms of the receptor or a single low-affinity form regulated by intracellular GTP was proposed (Nathanson, 1983). In our work, the presence of 0.5 μ*M* GTP decreased the affinity of both receptor binding components (Table 1), and 2 μ*M* GTP-γ-S markedly reduced the number of high-affinity opioid receptor sites (Remmers and Medzihradsky, 1987). Furthermore, the EC_{50} values for the displacement of [^{3}H]etorphine by opiates in slices, but not in isolated membranes, from rat brain striatum were similar to the respective EC_{50} values of these compounds in inhibiting adenylate cyclase (Barchfeld-Rothschild and Medzihradsky, 1987). Thus, our data seem to support the hypothesis that, in contrast to cellular preparations, the δ-opioid receptor in isolated membranes is present to a large extent in an uncoupled high-affinity form. It was suggested previously that the high-affinity receptor sites observed following cell disruption are not fully subject to regulation by guanine nucleotides (Nathanson, 1983). On the other hand, as shown with the β-adrenergic receptor in rat reticulocytes, even in well-preserved intact cells a small fraction of high-affinity sites can be demonstrated along with the predominant low-affinity forms (Porzig, 1982). In our study, the δ-opioid receptor in isolated brain membranes showed strong sensitivity toward components of the GTPase assay medium, including GTP. In freshly prepared membranes suspended in such a medium at 37°C, the difference between the K_D value of ligand binding and K_s of low-K_m GTPase stimulation was markedly decreased. These results are now being complemented by the assessment of opioid receptor–effector coupling in primary neuronal cultures.

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