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Comparative Pharmacological Properties and Functional Coupling of μ and δ Opioid Receptor Sites in Human Neuroblastoma SH-SY5Y Cells

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SUMMARY

The characteristics of μ and δ opioid receptor sites present in human neuroblastoma SH-SY5Y cells were investigated using [D-Ala^2-*N*-methyl-Phe^4-Gly-(01)^5]enkephalin (DAGO) and [2-D-penicillamine, 5-D-penicillamine]enkephalin (DPDPE), which are the most selective radioligands available for μ and δ sites, respectively. Scatchard analysis of the saturation isotherms revealed high affinity binding to a single class of sites for both [^{3}H]DAGO (μ) and [^{3}H]DPDPE (δ). [^{3}H]DAGO labeled twice the number of sites compared to the binding capacity of [^{3}H]DPDPE, yielding a μ/δ ratio of 2:1. Selective suppression of [^{3}H]diprenorphine binding by specific opioid "blocking" ligands also showed a predominance of μ receptors, representing 65–70% of the total opioid sites. Competition binding studies carried out with a series of opiates and opioid peptides displayed higher potencies of μ- and δ-selective ligands in displacing the specific binding of [^{3}H]DAGO and [^{3}H]DPDPE, respectively. The [^{3}H]diprenorphine/agonist competition curves were biphasic, indicating the high and low affinity states of μ and δ receptor sites in SH-SY5Y cells. Guanine nucleotide and sodium had differential effects on the agonist affinity and the proportion of high affinity states of μ and δ receptors. The μ and δ receptor sites were shown to be functionally coupled to adenylate cyclase. All of these data support the independent existence of μ and δ receptor types in human neuroblastoma cells. SH-SY5Y cells, therefore, represent a suitable model for investigating opioid-mediated responses in nerve cell populations.

The sequelae of the concept of "opiate receptor dualism," originally proposed by Martin *et al.* (1) to account for the complex interaction between morphine and its *N*-allyl derivative (nalorphine), have witnessed the accumulation of a variety of biochemical, pharmacological, and anatomical evidence for the heterogeneity of opioid-binding sites in brain and for the presence of specific subtypes in ileum, vas deferens, and murine neuroblastoma cells (for review, see Refs. 2–4). These receptor subtypes include μ (morphine), δ (enkephalin), κ (ketocyclazocine), σ (*N*-allynorphenazocine, SKF-10047), ϵ (β-endorphins), and μ_1 (a common high affinity site for both morphine and enkephalins).

Despite the enormous pharmacological evidence, our understanding of the molecular basis of opioid receptor multiplicity is by no means complete. It is yet to be firmly established whether the different opioid-binding sites are distinct biochemical entities or whether they represent multiple affinity states of the same receptor. Some recent reports indicate the individuality of μ and δ receptor sites on the basis of the differential effects of ions and guanine nucleotides in brain membrane preparations (5, 6). However, the presence of multiple cell types in brain and the complexity of *in vivo* studies, coupled with the cross-reactivity of most of the opioid ligands, limit the degree to which a rigorous interpretation can be made with regard to the molecular nature of receptor giving rise to the multiplicity in binding or response in brain.

Murine neuroblastoma clones and the neuroblastoma × glioma hybrid cells (NG108-15) containing homogeneous δ opioid receptors have proven to be very useful in delineating the pharmacological parameters of δ receptor-ligand interactions and the resultant inhibition of adenylate cyclase activity (7–9). Recently, Frey and Kebabian (10) reported that the mouse 7315c tumor, containing the homogeneous μ opioid receptor, could serve as an excellent system to characterize the coupling of μ receptors with the effector system, resulting in the inhibition of prolactin release and adenylate cyclase activ-

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ABBREVIATIONS: DAGO, [D-Ala^2-*N*-methyl-Phe^4-Gly-01^5]-enkephalin; DADLE, [D-Ala-D-Leu^5]-enkephalin; DSLET, [D-Ser-Leu^5-enkephalyl]-Thr; DTLET, [D-Thr^2-Leu^5]-enkephalin-Thr; DPDPE, [2-D-penicillamine, 5-D-penicillamine]-enkephalin; DPLPE, [2-D-penicillamine, 5-L-penicillamine]-enkephalin; FK-33824, [Tyr-D-Ala^2-Gly-*N*-methyl-Phe^4-Met(0)01]-enkephalin; U-50488H, [*trans*-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrrolidynyl)cyclohexyl)-benzeneacetamine; SKF-10,047, *N*-allylnormetazocine; ICI-174864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH: GPP(NH)p, guanosine 5′-(β,γ-imido)triphosphate; EDTA, ethylene diaminetetraacetate; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PGE_1, prostaglandin E_1; N_i, inhibitory guanine nucleotide-regulatory protein; N_s, stimulatory guanine nucleotide-regulatory protein.

ity. However, if the functional opioid system in brain is a consequence of allosteric interactions among the μ and δ sites (3), the pharmacological significance of the heterogeneity of opioid receptors and the subsequent biochemical events could be better determined in a neuronal clone that contains both μ and δ receptor sites. Such models should be even more important for the human nervous system because it is very difficult to study both *in vivo* and *in vitro* the properties of human neurons.

Recently, we demonstrated the presence of stereospecific, high affinity μ and δ opioid-binding sites in human neuroblastoma, SH-SY5Y cells (11). The parental cell line of SH-SY5Y, known as SK-N-SH, also exhibited μ and δ receptor sites in the ratio of 4.5:1 (12). Although the density and the proportion of these binding sites in SH-SY5Y cell were different from those observed in SK-N-SH (12), the binding parameters of the opioid-binding sites were analogous to those reported in NG108-15 cells (δ) and brain (μ and δ) (see Refs. 2 and 3, for review). The opioid receptors in SH-SY5Y cells are particularly interesting because they offer unique experimental model to investigate the pharmacological significance and functional coupling of both μ and δ receptors in a single neuronal cell type in culture.

In the present communication, we have extended the investigation on the characterization of μ and δ sites in SH-SY5Y cells by using [^{3}H]DAGO and [^{3}H]DPDPE, which are the most selective tritiated ligands available for μ (13) and δ (14) sites, respectively. The characteristics of μ and δ opioid receptors were established by the analysis of saturation kinetics and competition curves, using specific opiates and opioid peptides. The regulation of adenylate cyclase by μ and δ opioids was also studied under identical experimental conditions to elucidate the molecular signals responsible for physiological events initiated by μ and/or δ opioid receptor occupancy.

Materials and Methods

Chemicals. [^{3}H]DAGO (47.7 Ci/mmol), [^{3}H]DPDPE (33.6 Ci/mmol), [^{3}H]naloxone (42.7 Ci/mmol), and [α-^{32}P]ATP (34 Ci/mmol) were purchased from New England Nuclear (Boston, MA) [^{3}H]Etorphine (46 Ci/mmol) and [^{3}H]diprenorphine (41 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL), RPMI 1640 and fetal bovine serum were from Gibco (Grand Island, NY). GTP and Gpp(NH)p were purchased from Boehringer Mannheim. The unlabeled opioid alkaloids and peptides were obtained from the following sources: D-Pro^4-morphiceptin, DAGO, and DTLET, Peninsula Laboratories DSLET and dynorphin$_{(1\text{-}13)}$, Sigma Chemical Co.; DPDPE and DPLPE, Armand Frappier, Quebec; ICI-174864, Cambridge Research Biochemicals Ltd.; ethylketocyclazocine, Sterling Winthrop; naloxone, Endo Lab.; FK-33824, a gift from Dr. D. Romer, Sandoz; U-50488H, a gift from The Upjohn Co.; etorphine and diprenorphine, gifts from the National Institute on Drug Abuse; and levorphanol and dextrorphan, gifts from Hoffmann-La Roche.

Cell culture. The initial stock of human neuroblastoma SH-SY5Y cells was a generous gift of Dr. K. H. Sonnenfeld (Mt. Sinai School of Medicine, New York, NY). The cells were grown at 37° in a humidified atmosphere of 5% Co_2 in air, in tissue culture flasks (75 cm^2, Falcon) containing RPMI 1640, fetal bovine serum (15%), penicillin (50 IU/ml), and streptomycin (50 μg/ml). For experiments, confluent monolayers were harvested using 1 mM EDTA in Hanks' salt solution, washed with serum-free medium, and used for membrane preparation.

Receptor binding assays. Initially, opioid receptor binding was carried out with both intact cells and membrane preparations. We observed that the receptor density obtained in cell membranes was significantly higher than that in the intact cells and, in addition, the data from cell membrane binding were less variable. Therefore, the binding assays were performed in the membranes, as described previously (11). Briefly, the cells were homogenized in sucrose buffer (0.32 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4) and centrifuged at 800 $\times g$ for 10 min at 4°. The resultant supernatant fraction was centrifuged at 100,000 $\times g$ for 60 min at 4° and the pellet was washed twice with the assay buffer (50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4). The final pellet was suspended in the assay buffer (1–2 mg of protein/ml) and used for receptor binding.

Opioid receptor binding was carried out by incubating the membrane (150–200 μg of protein/assay) in 0.5 ml final volume. For [^{3}H]DAGO and [^{3}H]DPDPE and other opioid peptide binding, bacitracin (50 μg/ml) and bovine serum albumin (1 mg/ml) were included in the assay. Nonspecific binding was defined as the fraction of radioligand that remained in the presence of 5 μM levorphanol or other drugs (indicated in the text). After incubation for 60 min at 25°, the reaction was terminated by rapid cooling and filtration over Whatman GF/B filters under vacuum. Filters were washed three times with 5 ml of cold Tris-HCl (50 mM)/EDTA (1 mM), pH 7.4, dried, and placed in scintillation cocktail (Scint. A). Radioactivity was determined at least 8 hr later in a Beckman LS-5801 apparatus, which corrected for the counting efficiency of each individual sample (50%).

Measurement of adenylate cyclase activity. Adenylate cyclase activity in the membrane preparations was determined by measuring the production of cyclic AMP from [α-^{32}P]ATP. The membrane preparation was similar to that used for opioid binding assays, except that 1 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride were included in the homogenizing buffer. The enzyme activity was determined in 150 μl of reaction mixture containing the following, in final concentration: 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM cyclic AMP, 0.5 mM isobutylmethylxanthine, 10 mM creatine phosphate, 10 units of creatine phosphokinase, 1 mg/ml bovine serum albumin, 1 mM [α-^{32}P]ATP (1 μCi/assay), and 50–100 μg of membrane protein, with or without additions such as GTP (10 μM), NaCl (50 mM), PGE_1 (10 μM), and the test drugs. Assays were carried out at 37° for 10 min, and the reaction was terminated by the addition of 150 μl of 13% trichloracetic acid. Recovery was monitored with the addition of 0.3 ml of water containing 10,000 cpm of [^{3}H]cyclic AMP. The separation of [^{32}P]cAMP from [^{32}P]ATP was accomplished by sequential elution over Dowex and alumina columns as described by White and Karr (15). Recovery was consistently greater than 70%. Proteins were determined according to the method of Lowry *et al.* (16).

Data analysis. Binding parameters for each labeled ligand (K_D and B_{max}) and homo- or heterogeneous competition curves were analyzed with the assistance of BDATA and CDATA (EMF Software, Knoxville, TN), utilizing a computerized iterative nonlinear least squares curve-fitting program to determine the simultaneous contribution of total binding of one or more independent sites. The statistical difference between the one-site and two-site receptor models was estimated by comparing the residual variance between the predicted and actual data points. The relationship between IC_{50} and the equilibrium dissociation constant (K_D) in the antagonist/agonist competition studies was established using the equation of Cheng and Prusoff, i.e., $K_D = IC_{50}/1+(L)/(K_L)$, where L is the concentration of free labeled ligand and K_L is the equilibrium dissociation constant of the labeled ligand determined by Scatchard analysis, as described earlier (17).

Results

Equilibrium binding studies. The specific binding of [^{3}H]DAGO and [^{3}H]diprenorphine attained equilibrium within 20 min of incubation at 25°, while maximum binding of [^{3}H]DPDPE was achieved after 30–35 min. Therefore, all subsequent incubations for the three ligands were carried out for 60 min at 25°, at which time there was no evidence of any degradation of labeled or unlabeled ligands.

The specific binding of opioid agonists, [^{3}H]DAGO and [^{3}H]DPDPE, was saturable and readily reversible (Fig. 1). Scatchard transformation of the saturation isotherm indicated that a single-site model was most consistent with the experimental data, having average correlation coefficients (r) of 0.97 and 0.93 for [^{3}H]DAGO and [^{3}H]DPDPE, respectively (Fig. 1). Our previous investigations on the differentiation of μ and δ sites in SH-SY5Y cells were carried out with [^{3}H]DADLE as δ probe (11). D-Pro^4-morphiceptin (10^{-5} M) was used to block the cross-reactivity of [^{3}H]DADLE with the μ sites; and in the absence of D-Pro^4-morphiceptin, the computer-assisted curves best fitted the experimental data in terms of [^{3}H]DADLE binding with approximately equal affinity to two classes of sites, μ and δ (11). However, the high affinity binding of [^{3}H]DAGO and [^{3}H]DPDPE, with a K_D of 0.95 ± 0.15 nM and 1.20 ± 0.14 nM, respectively, did not show any cross-reactivity at the concentration range employed (0.1–10 nM) and further established the independent existence of μ and δ sites in SH-SY5Y cells. The receptor densities (B_{max}) for [^{3}H]DAGO and [^{3}H]DPDPE were 76 ± 6 fmol/mg of protein and 35 ± 4 fmol/mg of protein, yielding a μ/δ ratio of 2:1 (Fig. 1). The specific binding of both the ligands at the 2–2.5 nM concentration, which was used for most of the competitive displacement studies, was 70–80% in the presence of either 5 μM levorphanol or 1 μM unlabeled DAGO or DPDPE.

The presence and relative proportion of μ and δ sites in SH-SY5Y cells were further characterized using [^{3}H]diprenorphine, a nonselective opiate antagonist. [^{3}H]Diprenorphine showed concentration-dependent and saturable specific binding (Fig. 2). Scatchard analysis suggested high affinity binding with a dissociation constant (K_D) of 0.62 ± 0.05 nM and a B_{max} of 175 ± 11 fmol/mg of protein (Fig. 2). It is of interest to mention that [^{3}H]DAGO (μ) and [^{3}H]DPDPE (δ) specifically labeled 43% and 20%, respectively, of the maximum number of binding sites labeled by [^{3}H]diprenorphine (Figs. 1 and 2). The antagonist is known to label μ, δ and κ sites in brain with approximately equal affinity (2). However, the remaining 40% of sites labeled by [^{3}H]diprenorphine should not be assumed as κ sites per se, since [^{3}H]ethylketocylcazocine did not exhibit any significant specific binding in the presence of saturating concentration of μ- and δ-specific ligands (data not shown).

Selective inhibition of [^{3}H]diprenorphine binding. Selective effectors which mask one or more types of sites could be used in order to discriminate between the binding sites labeled by nonspecific opioid ligands. Selective suppression of [^{3}H]diprenorphine (Fig. 3) or [^{3}H]etorphine (not shown) by the submaximal and the "blocking" concentrations of specific opioid(s) yielded the ratio of μ and δ sites as 2:1 in SH-SY5Y cells. At low concentrations (10 nM), DAGO and DPDPE inhibited [^{3}H]diprenorphine binding by 48% and 23%, respectively (Fig. 3). Displacement of the antagonist binding was increased up to 70% and 34% by the higher concentration (100 nM) of DAGO and DPDPE, but the μ/δ ratio remained the same as that observed at lower (10 nM) concentrations (Fig. 3). The relevance of differential "blockade" of specific receptor subtypes by high and low concentrations of DAGO and DPDPE will be considered later in conjunction with the observed K_D values of the same ligands in displacing [^{3}H]diprenorphine binding.

The pure κ agonist, U-50488H, was relatively ineffective in displacing [^{3}H]diprenorphine binding (<10% inhibition). However, both dynorphin$_{(1\text{-}13)}$ and ethylketocyclazocine (ligands with preference to κ sites in brain) inhibited the antagonist binding with high affinity (Fig. 4), which would most likely arise due to the apparent cross-reactivity of these ligands with μ and δ sites, as reported earlier for brain opioid receptors (18). The stereospecific nature of opioid-binding sites in SH-SY5Y cells was evident by a several hundredfold higher potency of levorphanol (IC_{50}, 2.2 nM), as compared to its inactive isomer, dextrorphan (IC_{50}, 820 nM) in displacing [^{3}H]diprenorphine binding (Fig. 4).

Competitive inhibition of [^{3}H]DAGO and [^{3}H]DPDPE binding. Displacement studies with various opiates and opioid peptides were performed to establish the ligand binding selectivity at μ and δ receptor sites in SH-SY5Y cells. The high affinity [^{3}H]DAGO-binding sites displayed the characteristics

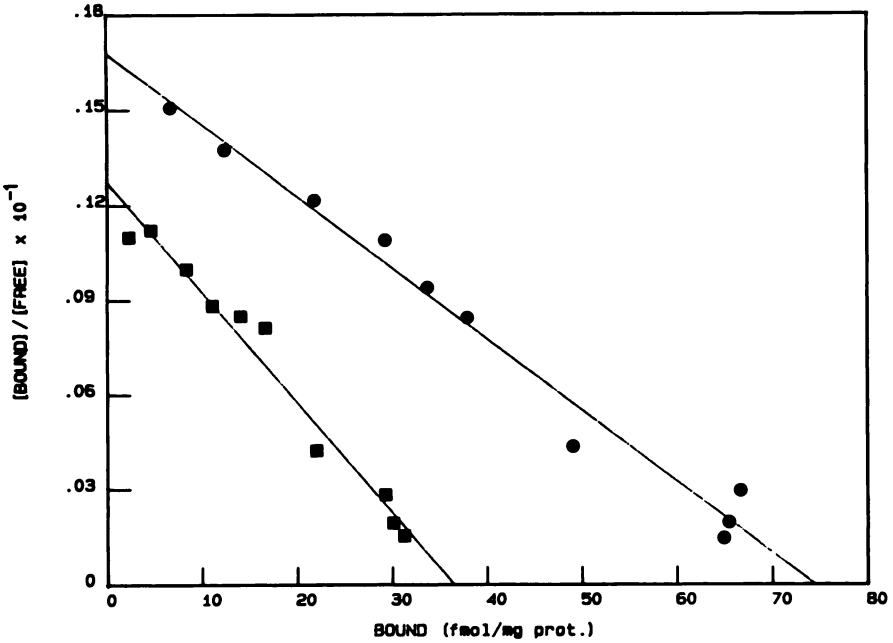

Fig. 1. Scatchard analysis of [^{3}H]DAGO and [^{3}H]DPDPE binding (0.1–10 nM) to SH-SY5Y cell membranes. The specific binding of [^{3}H]DAGO and [^{3}H]DPDPE was saturable and the data best represented a single-site model for the binding sites. Data are the mean of three independent experiments, each performed in triplicate (SE < 10%). ●, [^{3}H]DAGO; ■, [^{3}H]DPDPE.

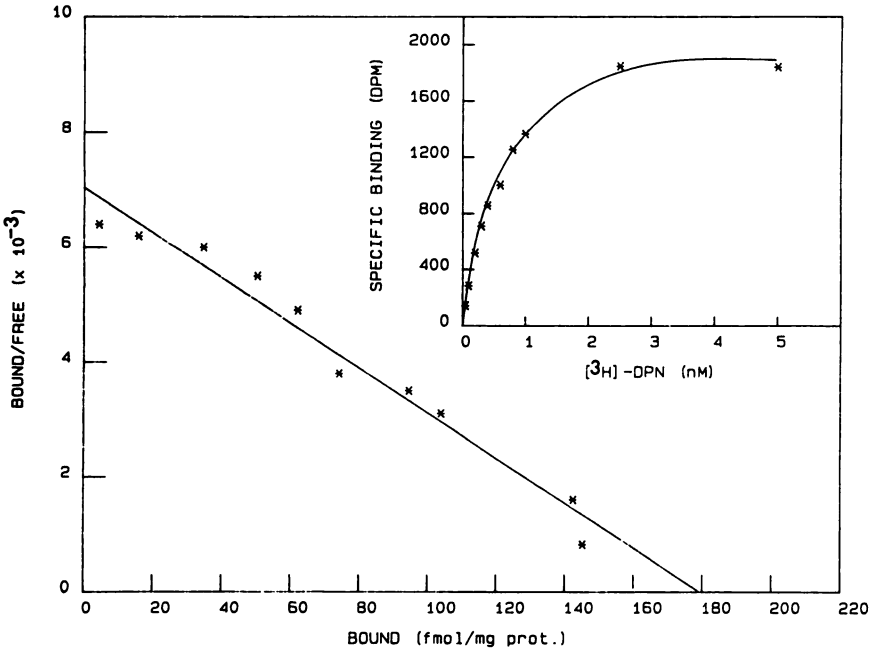

Fig. 2. Scatchard analysis of [^{3}H]diprenorphine binding (0.05–5.0 nM) to SH-SY5Y cell membranes. [^{3}H] diprenorphine binding was saturable (*inset*) and the data were best fitted to a one-site model, representing binding to different opioid receptor sites with equal affinity. The K_D and B_{max} values were 0.60 nM and 177 fmol/mg of protein. Each point is the mean of triplicate determinations (SE < 10%).

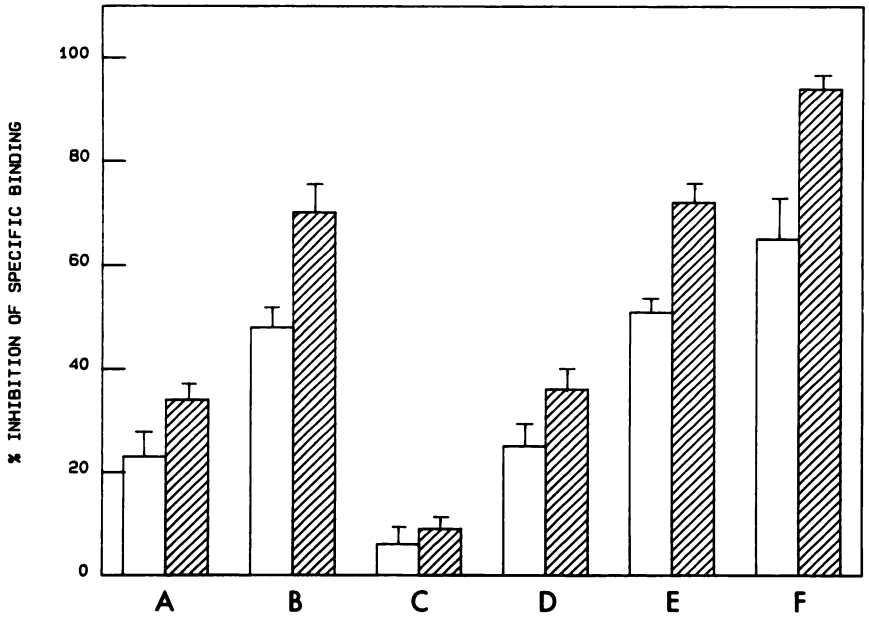

Fig. 3. Selective inhibition of the specific binding of [^{3}H]diprenorphine (0.5 nM) by μ (DAGO)-, δ (DPDPE)-, and κ (U-50488H)-selective ligands. [^{3}H]Diprenorphine was incubated with 10 nM (□) or 100 nM (▨) concentrations of specific opioids, and binding assays were carried out as described in Materials and Methods. The sequence of additions was: DPDPE (A), DAGO (B), U-50488H (C), DPDPE + U-50488H (D), DAGO + U-50488H) (E), DPDPE + DAGO (F). Values are means ± standard errors of triplicate determinations from two separate experiments.

of the μ opioid receptor. Table 1 summarizes the binding potencies of a series of opiates, opioid peptides, and related drugs in displacing [^{3}H]DAGO and [^{3}H]DPDPE binding. The rank order of potency for μ- and δ-selective ligands to displace [^{3}H]DAGO binding was FK-33824 > naloxone > D-Pro^4-morphiceptin ~ DTLET > DSLET > DPDPE (Table 1). DPDPE did not inhibit [^{3}H]DAGO binding significantly until the concentration exceeded 1×10^{-6} M. Although D-Pro^4-morphiceptin and DTLET appeared equipotent in competing with [^{3}H] DAGO binding, the selectivity of D-Pro^4-morphiceptin for μ sites was evident when displacement studies were performed for the inhibition of [^{3}H]DPDPE binding to δ sites (Table 1).

The δ-selective ligands, DTLET, DSLET and DPLPE displayed significantly higher affinities (IC_{50}, 3–4 nM) than FK-33824, naloxone, DAGO, and D-Pro^4-morphiceptin (μ selective) in displacing [^{3}H]DPDPE binding (Table 1). It should be pointed out that, unlike DAGO and FK-33824, D-Pro^4-morphiceptin was relatively ineffective in displacing [^{3}H]DPDPE binding to δ receptors even up to 2×10^{-6} M concentration. Therefore, the morphiceptin analogue appears to be a more selective μ ligand, whereas DAGO may be considered as the most specific μ agonist in terms of affinity and efficacy. The displacement curves of [^{3}H]DAGO against μ-specific ligands or of [^{3}H]DPDPE against δ-specific ligands were better fitted to a one-site model in most cases, suggesting that the radioligands bound to a single type of opioid receptors at the concentration employed (2–2.5 nM).

The κ-selective agonist, U-50488H, could not elicit any significant displacement of either [^{3}H]DAGO or [^{3}H]DPDPE binding up to 5×10^{-6} M concentration (Table 1).

Dynorphin$_{(1\text{-}13)}$ and ethylketocyclazocine, which have relatively higher affinity for κ sites in brain (18), displaced [^{3}H] DAGO at concentrations slightly lower than that required to inhibit [^{3}H]DPDPE binding (Table 1). ICI-174864, a δ antag-

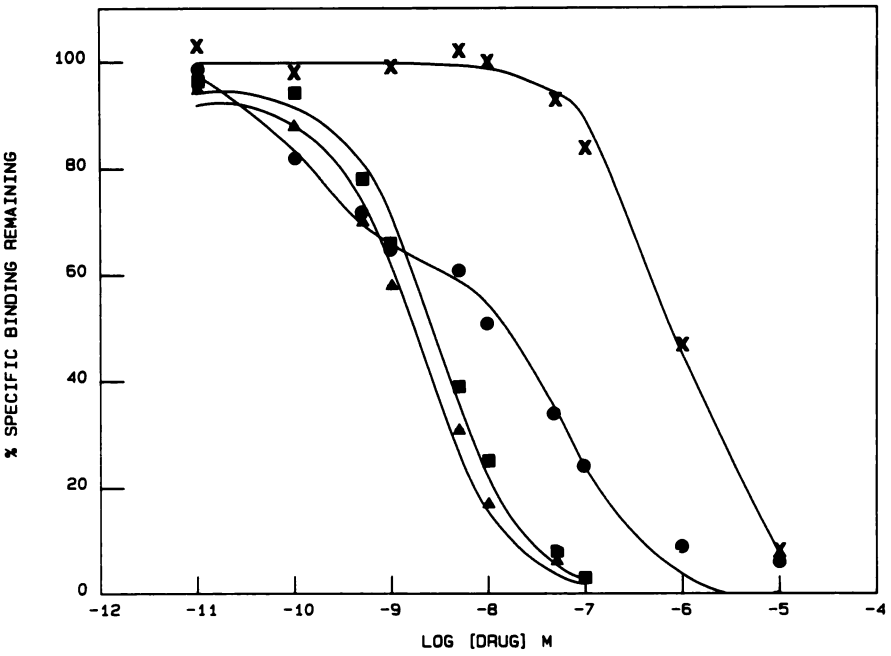

Fig. 4. Displacement of [^{3}H]diprenorphine binding to SH-SY5Y cell membranes by different opioid ligands. [^{3}H]Diprenorphine (0.5 nM) was incubated with various concentrations (10^{-11}–10^{-5} M) of drugs and the specific binding was determined as described in Materials and Methods. ▲, levorphanol; ×, dextrorphan; ●, dynorphin$_{(1-13)}$; ■, ethylketocyclazocine. Values represent means of triplicate determinations from three experiments (SE < 5%).

TABLE 1

Relative potencies of opiates and opioid peptides in displacing the specific binding of [^{3}H]DAGO and [^{3}H]DPDPE

SH-SY5Y cell membranes were prepared and the binding of [^{3}H]DAGO and [^{3}H]DPDPE was carried out as described in Materials and Methods. [^{3}H]DAGO or [^{3}H]DPDPE (2 nM) was incubated with different concentrations (10^{-11}–10^{-4} M) of the drugs at 25° for 60 min. Data represent the mean ± standard error of three independent experiments, each performed in triplicate.

Drugs	IC_{50}	
	[^{3}H]DAGO (2 nM)	[^{3}H]DPDPE (2 nM)
	nM	
FK-33824	2.2 ± 0.17	160 ± 23
D-Pro^4-morphiceptin	35.5 ± 2.8	>5000
DAGO		745 ± 62
Naloxone	7.8 ± 1.3	105 ± 8
DTLET	24.5 ± 2.8	3.4 ± 0.41
DSLET	68.7 ± 3.8	4.1 ± 0.36
DPDPE	1530 ± 167	
DPLPE	1750 ± 202	3.2 ± 0.44
ICI-174864	>5000	350 ± 27
U-50488H	>5000	>5000
Ethylketocyclazocine	5.5 ± 1.4	13 ± 1.6
Dynorphin$_{1-13}$	8 ± 1.5	11 ± 1.8
Diprenorphine	0.5 ± 0.04	0.6 ± 0.07

onist, was at least 20-fold more potent in displacing [^{3}H]DPDPE-binding sites as compared to [^{3}H]DAGO binding (Table 1). In contrast, diprenorphine, a nonselective opioid antagonist, inhibited both [^{3}H]DAGO and [^{3}H]DPDPE binding with comparable affinity.

Modulation of opioid binding by guanine nucleotide and sodium. We have shown in the preliminary report (11) that GTP and sodium decreased the association and increased the dissociation of ^{3}H-labeled opioid agonist (DADLE) in SH-SY5Y cell membranes. Initial experiments with selective radioligands, [^{3}H]DAGO and [^{3}H]DPDPE (2.5 nM concentration), showed that the nucleotide and sodium could exert significant inhibition of steady state binding of agonist at μ and δ sites; the presence of both the modulators [100 μM Gpp(NH)p and 100 mM NaCl] lowered the agonist binding by 80–85%, whereas the specific binding of the antagonist, [^{3}H]diprenorphine, was unaffected (data not shown).

In order to determine the nature of Gpp(NH)p- and sodium-induced inhibition of agonist binding at each receptor subtype, we employed two approaches: 1) analysis of saturation binding of [^{3}H]DAGO and [^{3}H]DPDPE, and 2) displacement experiments using the antagonist, [^{3}H]diprenorphine, in the presence and absence of guanine nucleotide and sodium.

Scatchard analysis of [^{3}H]DAGO binding in the presence of Gpp(NH)p (100 μM) revealed a 2-fold decrease in the agonist affinity at μ sites with a small reduction in the number of binding sites (Table 2). In sharp contrast, the affinity of [^{3}H]DPDPE at δ sites remained unaffected in the presence of guanine nucleotide, while the receptor density (B_{max}) was reduced significantly. The inhibition induced by sodium was also remarkably different at μ and δ receptors. The maximum number of binding sites for [^{3}H]DPDPE was decreased more than 2-fold with no change in K_D value in the presence of sodium. In contrast, sodium caused a comparable reduction in both affinity and B_{max} value obtained with [^{3}H]DAGO at μ sites (Table 2).

The differential regulation of agonist binding at μ and δ sites was further characterized using [^{3}H]diprenorphine/agonist competition studies. Displacement of the antagonist binding by DAGO (μ) yielded a biphasic curve; computer-assisted analysis

TABLE 2

Saturation binding parameters of [^{3}H]DAGO (μ) and [^{3}H]DPDPE in the presence and absence of Gpp(NH)p and sodium

Different concentrations (0.1–10 nM) of [^{3}H]DAGO and [^{3}H]DPDPE were incubated in the absence (control) and presence of Gpp(NH)p (100 μM) or NaCl (100 mM), and the Scatchard analysis was performed as described in Materials and Methods. K_D and B_{max} values are given in nM and fmol/mg of protein, respectively. Data are means ± standard errors of three independent experiments.

Assay conditions	[^{3}H]DAGO		[^{3}H]DPDPE	
	K_D	B_{max}	K_D	B_{max}
Control	0.95 ± 0.15	76 ± 6	1.20 ± 0.14	36 ± 4
+Gpp(NH)p	1.97 ± 0.13	66 ± 7.3	1.38 ± 0.12	25.5 ± 3.6
+NaCl	2.12 ± 0.17	45.6 ± 5.7	1.42 ± 0.18	16 ± 2.8

revealed the existence of two agonist affinity states in the proportion of 65% (high) and 35% (low) (Fig. 5). The dissociation constants of DAGO at high (K_H) and low (K_L) affinity states were 0.74 nM and 165 nM, respectively (the means ± SE from three independent experiments were 0.81 ± 0.12 nM and 148 ± 32 nM, respectively). Gpp(NH)p caused a significant increase in the K_H (4.9 nM), whereas sodium decreased the agonist affinity more significantly at the low affinity site; the proportion of the high affinity site was also reduced by sodium (Fig. 5).

DTLET, a δ-preferring agonist, also differentiated two distinct populations of agonist affinity states of opioid receptors in almost the same proportion as observed with DAGO (Fig. 6). The K_D values of DTLET at high (K_H) and low (K_L) affinity sites were 2.6 nM and 65 nM, respectively (means ± SE from three independent experiments were 2.3 ± 0.7 nM and 62 ± 6 nM, respectively). In the presence of Gpp(NH)p, the K_H for DTLET remained unchanged, whereas the B_H was reduced to 43% (Fig. 6). Sodium, however, caused a significant reduction in the K_H and B_H values, showing further rightward shift in the displacement curve. Addition of both Gpp(NH)p and sodium in either case resulted in a monophasic curve representing the low affinity state of the opioid receptor.

Since the specific binding of [^{3}H]diprenorphine would represent both μ and δ receptors in SH-SY5Y cells, it was considered appropriate to determine the high and low affinity states of individual opioid receptors in competition binding studies with [^{3}H]diprenorphine under conditions of radioligand binding to a single type of opioid receptor. Interestingly, the displacement of [^{3}H]diprenorphine binding (in the presence of 100 nM DPDPE to block δ receptors) by various concentrations of DAGO yielded the binding parameters, similar to those obtained in the absence of the blockade of δ receptors (K_H 1.2 nM, K_L 128 nM; B_H 62%, B_L 38%) (data not shown). Similarly, when the cross-reactivity of [^{3}H]diprenorphine to μ receptors was blocked by 100 nM DAGO, K_H and B_H values of DTLET were found to be nearly identical to those described in Fig. 6. However, the affinity of DTLET at low affinity sites was reduced (from 65 nM to 173 nM) in the presence of DAGO (data not shown). This observation was not unexpected because of the apparent cross-reactivity of DTLET with μ receptor sites (6).

Opioid inhibition of adenylate cyclase activity. Opioid agonists, D-Ala^2-Met^5-enkephalinamide and etorphine, inhibited adenylate cyclase activity in SH-SY5Y cells, and naloxone could reverse the inhibition of enzyme activity (19). In light of the current observations regarding the presence of independent μ and δ sites in SH-SY5Y cells, together with the availability of more selective ligands, it would be of interest to determine whether only μ or δ or both receptor subtypes are involved in the modulation of cyclic AMP levels in SH-SY5Y cells.

The basal activity of adenylate cyclase in SH-SY5Y cell membrane preparations varied from 15 pmol/min/mg of protein to 40 pmol/min/mg of protein in six different experiments. However, the percentage of stimulation of the cyclase activity by PGE_1 (10 μM) in the presence of GTP and NaCl (10 μM and 50 mM, respectively) was highly reproducible (2–2.5-fold increase in the enzyme activity). Omission of GTP and Na^+ drastically reduced the stimulation of adenylate cyclase by PGE_1 (from 250% to 30%) and abolished the inhibition caused by opioid agonists, in agreement with the previous observations in NG108-15 cells with regard to the absolute requirement of these endogenous effectors for the inhibition of enzyme activity (20).

Opioid agonists, etorphine, DAGO, and DPDPE, inhibited the adenylate cyclase activity (15–25%) in a concentration-dependent manner (Fig. 7). However, the efficacy of DAGO was significantly higher than that of DPDPE, indicating a difference in the coupling of μ and δ receptor subtypes to the adenylate cyclase system. D-Pro^4-morphiceptin and DTLET also caused the reduction in adenylate cyclase activity, and naloxone (10^{-6} M) could completely reverse the inhibition induced by μ- and δ-specific ligands (Table 3). ICI-174864 (10^{-5} M), a δ-selective antagonist, completely abolished the inhibition

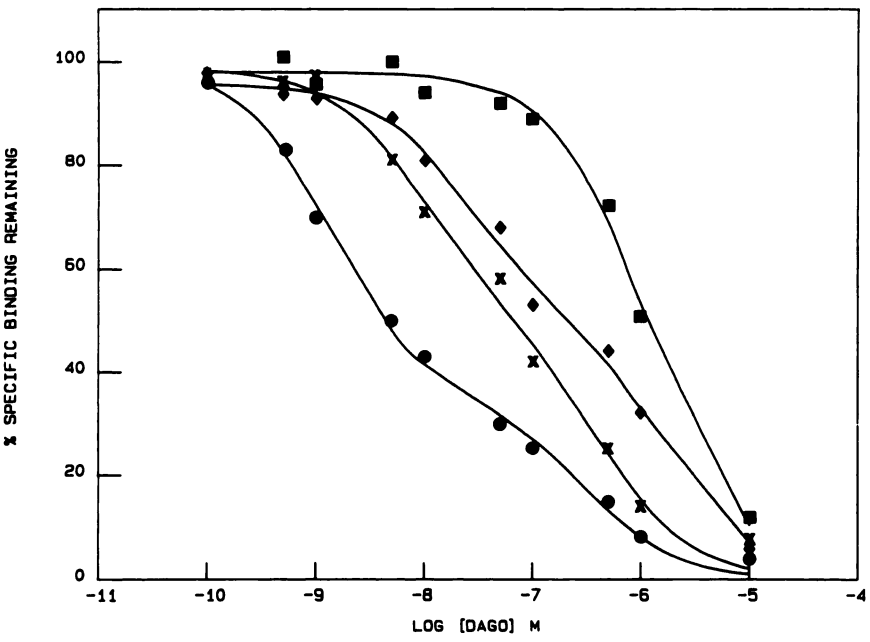

Fig. 5. Competition of [^{3}H]diprenorphine with DAGO in the presence and absence of Gpp(NH)p and NaCl. [^{3}H]Diprenorphine (0.5 nM) was incubated in the presence of various concentrations of DAGO alone (●), DAGO + 100 μM Gpp(NH)p (×), DAGO + 100 mM NaCl (♦), and DAGO + Gpp(NH)p + NaCl (■). DAGO differentiated two distinct populations of μ opioid receptors. The binding parameters were as follows: K_H and K_L (K_D), 0.74 nM and 165 nM (control), 4.9 nM and 218 nM [Gpp(NH)p], and 1.3 nM and 764 nM (NaCl). B_H and B_L (B_{max}), 65% and 35% (control), 48% and 52% [Gpp(NH)p], and 43% and 57% (NaCl). Addition of both Gpp(NH)p and NaCl (■) caused drastic reduction in the agonist affinity (K_D 654 nM) and the competition curve represented single sites of low affinity. Data are means of triplicate determinations (SE < 10%).

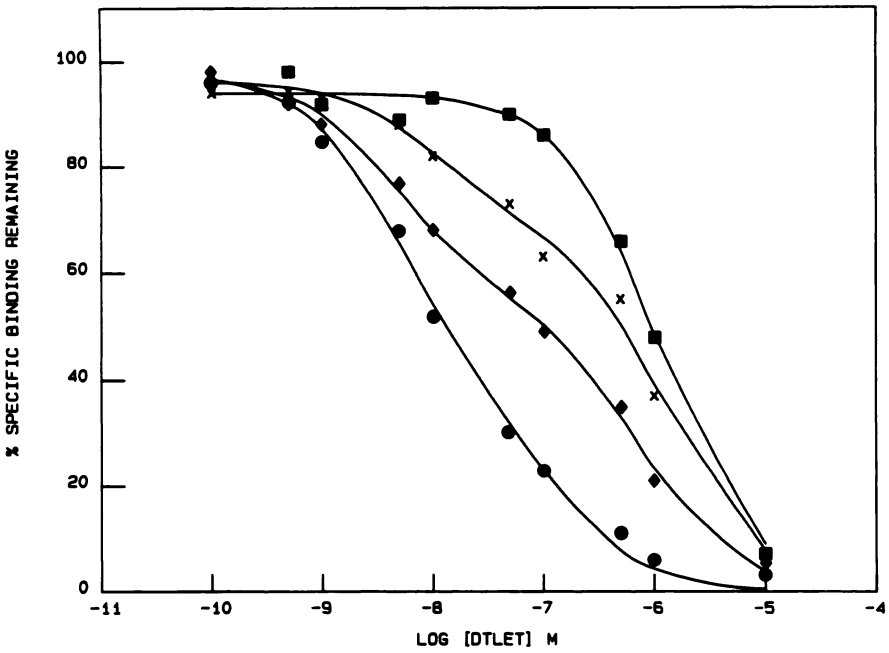

Fig. 6. Competition of [^{3}H]diprenorphine with DTLET in the presence and absence of Gpp(NH)p and NaCl. Without Gpp(NH)p or NaCl (control), the competition curve was better represented by a two-site model. The binding parameters under different conditions were as follows: K_H and K_L (K_D), 2.6 nM and 65 nM (●, control), 2.8 nM and 809 nM [♦, Gpp(NH)p], and 10.4 nM and 709 nM (×, NaCl). B_H and B_L (B_{max}), 60% and 40% (control), 44% and 56% [Gpp(NH)p], and 32% and 68% (NaCl). Gpp(NH)p and NaCl, when added together, caused almost complete conversion of high affinity sites to low affinity sites and the K_D value of DTLET was 545 nM (■). Data represent average values of triplicate determinations (SE < 10%).

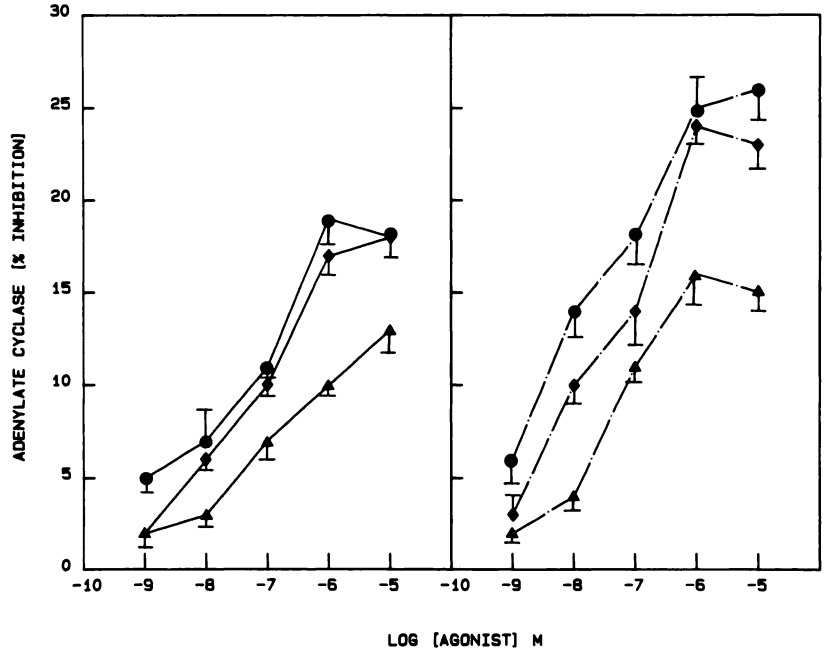

Fig. 7. Dose-related inhibition of basal (*left*) and PGE_1-stimulated (*right*) adenylate cyclase activity in the presence of GTP (10 μM) and NaCl (50 mM) by etorphine (●), DAGO (♦), and DPDPE (▲). In the absence of opioids, the basal and PGE_1-stimulated enzyme activities were 30 ± 3.5 pmol/min/mg of protein and 71 ± 4.3 pmol/min/mg of protein, respectively. Data are means ± SE (*bars*) of triplicate determinations from a representative experiment.

caused by DPDPE, but the effects of DTLET and μ-specific agonists were partially blocked.

In view of the partial attenuation of DAGO-induced inhibition by ICI-174864 (10^{-5} M), we reexamined the level of adenylate cyclase activity in the presence of DAGO or DPDPE (10^{-6} M), with or without increasing concentrations of the δ-selective antagonist. ICI-174864 attenuated DPDPE-induced inhibition at concentrations as low as 0.75–1 μM, whereas the partial antagonism of DAGO-induced inhibition was noticed over and above 7.5–10 μM (data not shown). The κ agonist, U-50488H, did not cause any inhibition of adenylate cyclase activity in SH-SY5Y cells under the incubation conditions employed (Table 3).

Discussion

The results described in the present study provide strong evidence for the existence of independent μ and δ receptor sites in human neuroblastoma SH-SY5Y cells (11). The characterization of receptor dualism in the human neuroblastoma cells was made on the basis of 1) direct saturation binding experiments with [^{3}H]DAGO, [^{3}H]DPDPE, and [^{3}H]diprenorphine; 2) the rank order of displacement of labeled agonist and antagonist binding by a series of opiates and opioid peptides; 3) the differential effects of guanine nucleotide and sodium ion on the affinity states of μ and δ receptors; and 4) the coupling of μ and δ receptors to the adenylate cyclase system.

Both [^{3}H]DAGO and [^{3}H]DPDPE showed high affinity and saturable specific binding, and the equilibrium dissociation constants for μ and δ sites obtained from Scatchard analysis were comparable to those observed in brain and NG108-5 cells (5–7, 14). Competition binding studies clearly demonstrated the higher potencies of a number of μ and δ ligands in displacing

TABLE 3

Inhibition of PGE_1-stimulated adenylate cyclase activity by μ and δ opioid agonists in SH-SY5Y cell membranes

Adenylate cyclase activity was determined in the presence of PGE_1 (10 μM), GTP (10 μM), and NaCl (50 mM). The agonists and antagonists (naloxone and ICI-174864) were used at 10^{-6} M and 10^{-6} M concentrations, respectively. Data represent the mean and standard error of triplicate determinations. Despite the day-to-day variation in the basal activity, the qualitative results were consistent among three experiments averaged in this manner.

Assay conditions	Adenylate cyclase activity		
	−	+ Naloxone	+ ICI-174864
	pmol/min/mg protein		
Without opioid agonists (control)	62 ± 3.4		
DAGO	48 ± 2.2	59 ± 2.8	54 ± 3.6
D-Pro⁴-morphiceptin	53 ± 2.5	63 ± 2.5	54 ± 1.8
DPDPE	53 ± 1.5	61 ± 2.5	59 ± 3.7
DTLET	52 ± 1.7	59 ± 2.6	56 ± 4.1
U-50488H	63 ± 2.8	61 ± 2.4	58 ± 3.3

[^{3}H]DAGO and [^{3}H]DPDPE binding, respectively. The spectrum of opioid receptor subtypes determined with a nonselective opiate antagonist, [^{3}H]diprenorphine, in the presence of specific opioid "blocking" ligands (100 nM) showed a preponderance of the μ receptor, representing about 70% of the total opioid sites. Direct saturation binding experiments also indicated that [^{3}H]DAGO labeled twice the number of sites compared to the binding capacity of [^{3}H]DPDPE. However, the B_{max} values for [^{3}H]DAGO and [^{3}H]DPDPE constituted only 43% and 20% of the binding capacity of [^{3}H]diprenorphine. It is of interest to note that the blockade of [^{3}H]diprenorphine binding by lower concentrations (10 nM) of DAGO and DPDPE also yielded similar proportions of the μ and δ receptor sites, i.e., 47% and 23% of the total sites labeled by the antagonist.

Taken together, these observations would tend to suggest that higher concentrations of DAGO and DPDPE may cross-react with the additional site(s) labeled by [^{3}H]diprenorphine in SH-SY5Y cell membranes, although the same higher concentration (100 nM) of DAGO has been used earlier to specifically block the μ sites for characterizing the κ (18) and σ (21) receptor sites labeled by [^{3}H]ethylketocyclazocine and [^{3}H] SKF-10,047, respectively. DPDPE has also been reported to be very specific for δ sites, with selectivity better than that of DSLET and DTLET (14). Despite these reports, the possibility of additional sites being labeled by [^{3}H]diprenorphine should also be considered. In view of the poor potency of U-50488H against [^{3}H]diprenorphine binding and the very low specific binding of [^{3}H]ethylketocyclazocine in the presence of DAGO and DPDPE, the κ receptor is an unlikely candidate. The parental cell line SK-N-SH also appears to contain an additional type of diprenorphine-binding site, yet to be characterized (12). Whether these diprenorphine-binding sites belong to the family of opioid receptors in brain or whether they represent the desensitized opioid receptors specific to tumor cells remains to be ascertained. The latter possibility is relevant to the recent reports on the regulation of tumor (neuroblastoma) growth by opioid antagonist (22). The opioid receptor agonists involved in the control of tumorigenic events and cell differentiation are not yet known (23).

Another plausible reason for such observations could be the existence of different affinity states of μ and δ sites in SH-SY5Y cells. It has recently been shown that, when displacement studies using [^{3}H]diprenorphine and δ agonist (DADLE and DTLET) was carried out with intact NG108-15 cells (24) or cell membranes (25) and guinea pig cortex membranes (26), an agonist-specific binding heterogeneity representing high and low affinity δ sites could be detected. However, the low affinity sites revealed by displacement studies could not be identified by a labeled agonist, since the range of detectable binding above nonspecific was substantially less than necessary to characterize the low affinity site (26). In SH-SY5Y cell membranes, the computer-assisted analysis of competition curves for agonists and [^{3}H]diprenorphine also revealed high and low affinity states of both μ and δ opioid receptors. It is reasonable to assume that, in saturation binding experiments, [^{3}H]DAGO and [^{3}H]DPDPE (0.1–10 nM concentration) labeled only the high affinity state of μ and δ sites, respectively.

The regulation of opioid agonist binding by guanine nucleotides and sodium is well established in brain and NG108-15 cells (6, 25–28). In SH-SY5Y cell membranes, guanine nucleotide and sodium decreased the association and increased the dissociation of [^{3}H]DADLE in a concentration-dependent manner (11). Further experiments with selective agonists revealed that the μ and δ receptors were under differential regulation by guanine nucleotide and sodium.

Saturation binding of [^{3}H]DAGO and [^{3}H]DPDPE showed that Gpp(NH)p and sodium reduced the μ agonist binding by lowering the affinity whereas, for δ receptors, the main effect was a substantial decrease in the maximum number of binding sites. The regulation of high affinity sites for μ and δ receptors by sodium and Gpp(NH)p in displacement studies also indicated a separate coupling paradigm for the two receptor subtypes. The binding parameters for μ and δ in brain, in mouse 7315c tumor, and in NG108-15 cells (6, 25–28) obtained from saturation binding and displacement studies also showed differential alterations in the presence of guanine nucleotide and sodium. Taken together, these results suggest that the μ and δ receptor sites identified in SH-SY5Y cells are not the multimeric forms of the same receptor but, rather, represent separate structural entities with molecular organization similar to that of NG108-15 and brain opioid receptors.

It has been suggested that sodium-induced inhibition of agonist binding may arise either due to the alteration in receptor conformation through association-dissociation, resulting in steric hindrance of available binding sites, or due to the modulation of the coupling of guanine nucleotide-regulatory protein (N_i) with the receptor (28). The negative heterotropic effect of guanine nucleotides on agonist interaction with receptor coupled to the adenylate cyclase system through guanine nucleotide-regulatory protein (N_i) is well characterized (29). Pertussis toxin-catalyzed ADP ribosylation has been shown to impair the interaction of N_i with the opioid receptor-ligand complex, resulting in a decrease in agonist affinity as well as the potency of Gpp(NH)p to inhibit the agonist binding in NG108-15 cells (30). The sensitivity of both μ and δ receptor subtypes to Gpp(NH)p and sodium in SH-SY5Y cells, therefore, suggests the functional coupling of human neuroblastoma opioid receptors to the effector molecule(s).

Since SH-SY5Y cells contain more than one type of opioid receptor, it can be argued that the observed affinity states in [^{3}H]diprenorphine/agonist competition studies are due to the cross-reactivity of opioid ligands, i.e., the high and low affinity states for DAGO binding represent μ and δ receptors, respectively. However, such an assumption appears unlikely because:

1) if the low affinity sites for DAGO and high affinity sites for DTLET represent the δ receptors, the regulation by guanine nucleotide and Na^+ should be similar in the two states, which is not the case, and 2) both DAGO and DTLET differentiated the high and low affinity states in displacing [^{3}H]diprenorphine binding, even under conditions of antagonist binding to a single type of receptor. For the same reasons, the high affinity sites may not represent the μ_1 sites: the blocking concentration (100 nM) of either DAGO or DPDPE would not allow the expression of μ_1 sites. Those observations should provide further insights into the functional coupling of opioid receptors in relation to the pharmacological actions of the ligands exposing the apparent heterogeneity of binding sites.

Several reports have suggested a functional relationship of adenylate cyclase with δ opioid receptors in brain and in NG108-15 cells (Ref. 3 and references therein). However, the opioid-mediated inhibition of adenylate cyclase in NG108-15 cells (containing a homogeneous population of δ receptors) should not be extrapolated to other subtypes of opioid receptors. It must be determined whether μ and/or any other subtype of opioid receptor could also elicit similar coupling with adenylate cyclase. In human SH-SY5Y cells, both μ- and δ-specific agonists caused the inhibition of adenylate cyclase, but the potency of DAGO (μ) was more than that of DPDPE (δ). Naloxone attenuated the inhibition of cyclase activity induced by DAGO and DPDPE. Conversely, ICI-174864, a δ-specific antagonist (up to 10^{-6} M concentration) selectively abolished the effects of DPDPE without altering the μ-mediated response, suggesting the independent coupling of the two receptor subtypes in SH-SY5Y cells. Recent reports have also shown the ability of μ agonists to inhibit adenylate cyclase activity in mouse 7315c tumor (10) and brain (31). The naloxone-reversible inhibition of cyclic AMP levels (12–20%) by both morphine and enkephalinergic ligands (10^{-6} M) has also been reported in the parental cell line, SK-N-SH (12).

The observed differences in the efficacy of μ- and δ-specific opioids should not be accounted for by the difference in the receptor densities, as suggested earlier for the opiate regulation of adenylate cyclase activity in NG108-15 and N18TG2 cells (32). Since guanine nucleotides and Na^+, known to be essential for eliciting opioid-mediated inhibition of adenylate cyclase (20), have differential effects on μ and δ receptors in SH-SY5Y cells, the difference in efficacy would most likely arise due to the differences in the receptor-adenylate cyclase coupling process. Further studies with pertussis toxin and [^{32}P]NAD^+ to determine the independent interaction of μ and δ receptors with guanine nucleotide-regulatory protein (N_i) are required to elucidate the mechanism of specific opioid-mediated regulation of cyclic AMP levels in SH-SY5Y cells.

It may be pointed out that the maximum obtainable inhibition of PGE_1-stimulated adenylate cyclase in SH-SY5Y cell membranes was moderate (25%) as compared to murine neuroblastoma cells (8, 9, 32), which may be, in part, due to the weak stimulation of cyclase activity by PGE_1 (2–2.5-fold in SH-SY5Y versus 10–50-fold in NG108-15 cells and cell membranes). If the PGE_1 receptor itself or the receptor-N_s coupling that mediates cyclase stimulation is perturbed in isolated membranes of SH-SY5Y cells, agents which stimulate cyclase by acting directly on N_s (forskolin) or catalytic subunit (NaF) may be a better choice to monitor the cyclase inhibition in the human neuroblastoma cells.

In summary, the present studies provide the first detailed characterization of μ and δ opioid receptors in human neuroblastoma SH-SY5Y cells. Furthermore, differential regulation of agonist affinity states by guanine nucleotide and sodium as well as the coupling of receptors with the adenylate cyclase system represent the classical opioid receptor-effector interactions observed in brain. The human neuroblastoma SH-SY5Y cells, therefore, should serve as a valuable experimental system to elucidate the interactions of μ and δ opioid receptors and the physiological significance of the coupling of either receptor subtype to the effector system.

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