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European Journal of Pharmacology 378 (1999) 323–330

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Differential binding properties of oripavines at cloned μ- and δ-opioid receptors

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Received 8 February 1999; received in revised form 17 April 1999; accepted 25 June 1999

Abstract

This study examines the possibility that oripavine opioid receptor agonists bind equally to both high and low affinity states of the μ-opioid receptor. Studies were performed in C6 cells expressing μ- or δ-opioid receptors; high and low agonist affinity states of the receptors were defined by the absence and presence, respectively of Na^+ ions and the GTP analog Gpp(NH)p. At the μ-opioid receptor dihydroetorphine and etorphine were full agonists, buprenorphine had moderate efficacy while diprenorphine was an antagonist. At the δ-opioid receptor, dihydroetorphine, etorphine, and diprenorphine had moderate efficacy while buprenorphine was an antagonist. The binding affinities of the oripavines at the μ-opioid receptor decreased only one to 2-fold in the presence of NaCl and Gpp(NH)p. In contrast, decreases in oripavine affinity at the δ-opioid receptor correlated with δ-opioid receptor efficacy. The ability of oripavine agonists to bind with high affinity to the low agonist affinity state of the ν-opioid receptor may explain the high potencies of these compounds in vivo.

Keywords: Oripavine; Opioid receptor; Ligand binding; Efficacy; Buprenorphine; Etorphine

1. Introduction

Opioid receptors are members of the seven transmembrane domain receptor superfamily that are coupled to pertussis toxin sensitive G-proteins (e.g., Dhawan et al., 1996). Evidence from numerous studies shows that the presence of sodium ions and guanine nucleotides reduces agonist binding to opioid receptors while the binding of antagonists is unaffected (Childers and Snyder, 1980; Puttfarcken et al., 1986; Werling et al., 1986; Emmerson et al., 1996). The shift in affinity caused by sodium ions and guanine nucleotides correlates with agonist efficacy (e.g., Childers and Snyder, 1980). These findings support a conformational selection model of agonist action whereby agonist binding to the high affinity state of the receptor shifts the equilibrium in favor of this state, and away from the low affinity form, thereby invoking a response (e.g., Kenakin, 1997).

Buprenorphine, an oripavine derived from thebaine, is a moderate efficacy, μ-opioid agonist with analgesic effects that are 25-times more potent than morphine (Cowan et al., 1977a,b). From a receptor interaction viewpoint, buprenorphine is of interest because, unlike other opioid agonists, its binding affinity is not affected by the presence of sodium ions (Villiger, 1984; Rothman et al., 1995). However, these conclusions were made from studies performed using rat brain membranes that contain a heterogeneous population of opioid receptors. Consequently it is unclear whether the binding properties of buprenorphine are indicative of its binding only to the μ-opioid receptor. For example, buprenorphine acts as an antagonist at the κ-opioid receptor (Leander, 1987; Negus and Dykstra, 1988; Toll et al., 1998) and has no agonist action at the δ-opioid receptor (Toll et al., 1998). Therefore, antagonist binding of buprenorphine to non-μ-opioid receptors could explain the lack of a Na^+ and guanine nucleotide induced shift.

In order to elucidate the binding properties of buprenorphine to specific opioid receptors, cloned μ- and δ-opioid receptors expressed in C6 glioma cells (C6 mu) which do not endogenously express any opioid receptors (Klee and Nirenberg, 1974) were used. During the course of these experiments, it was established that other oripavines with opioid agonist properties also bind to the μ-opioid receptor

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00460-4

in a Na^+-insensitive fashion. The oripavines examined in addition to buprenorphine were the agonists, dihydroetorphine (Kamei et al., 1995) and etorphine (Blane et al., 1967; Walker et al., 1998), and the antagonist, diprenorphine (Dewey and Harris, 1971; Traynor et al., 1987). The degree of relative agonist efficacy of the compounds was compared in the same cell systems using the [^{35}S]GTPγS binding assay. (Traynor and Nahorski, 1995; Emmerson et al., 1996; Clark et al., 1997; Alt et al., 1998).

The findings of this study show that opioid receptor agonist oripavines bind to the cloned μ-opioid receptor in a fashion that is insensitive to the presence of the non-hydrolysable GTP analog, Gpp(NH)p, while sodium ions produce a slight shift in dihydroetorphine and etorphine binding affinities. This insensitivity may be specific to the μ-opioid receptor since at the cloned δ-opioid receptor expressed in C6 cells (C6 delta) oripavine binding is sensitive to Na^+ and guanine nucleotides.

2. Materials and methods

2.1. Chemicals

[^{3}H]Diprenorphine (45 Ci/mmol and 58 Ci/mmol), [^{3}H]naloxone (54.6 Ci/mmol), [^{3}H]naltrindole (33 Ci/mmol) and [^{35}S]GTPγS, (guanosine-5′-*O*-(3-thio)triphosphate, 1250 Ci/mmol) were purchased from DuPont NEN (Boston, MA). DAMGO ([Tyr-D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin), pertussis toxin, Gpp(NH)p (5′-guanylylimido-diphosphate) and GDP (guanosine diphosphate) were purchased from Sigma (St. Louis, MO). DPDPE ([D-Pen^2-D-Pen^5]enkephalin) and SNC-80 ((+)-4-((α-*R*)-α-[(2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxylbenzyl)-*N*,*N*-dimethylbenzamide) were gifts from Dr. Henry Mosberg (University of Michigan, Ann Arbor, MI) and Dr. Kenner C. Rice (National Institutes of Health, Bethesda, MD), respectively. The following drugs were provided as gifts: morphine sulfate from Mallinckrodt (St. Louis, MO), butorphanol tartrate from Bristol Myer Squibb (Wallingford, CT), pentazocine base from Sterling-Winthrop Research Institute (Rensselaer, NY) and nalbuphine HCl from Dupont Merck (Wilmington, DE). Buprenorphine HCl, dihydroetorphine HCl, diprenorphine HCl, etorphine HCl, fentanyl HCl, methadone HCl and naloxone HCl were obtained from the National Institute on Drug Abuse (Rockville, MD). Tissue culture materials were from Gibco Life Sciences (Gaithersberg, MD).

2.2. Cell culture

C_6 glioma cells stably transfected with the cDNA of either the rat μ- (C6 mu; Emmerson et al., 1996) or rat δ- (C6 delta; Clark et al., 1997) opioid receptor were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and either harvested or subcultured when confluency was reached.

2.3. Cell membrane preparation

Cells were removed from culture flasks using harvesting buffer (0.68 mM EDTA, 0.15 M NaCl and 20 mM HEPES, pH 7.4) and collected by centrifugation (3 min at $500 \times g$). Cell pellets were resuspended in 50 mM Tris–HCl, pH 7.4 (for binding assays) or Buffer A (20 mM HEPES, 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.4 for [^{35}S]GTPγS assays) and homogenized for 4 s at 20,000 rpm using a Tissue Tearor (Model 985-370, Biospec Products). Crude membranes were separated by centrifugation (15 min at $40{,}000 \times g$) then resuspended in Tris buffer and centrifuged once more. The final pellets were resuspended in Tris buffer and stored at −80°C in aliquots of 1.0 ml (0.5–1.5 mg/ml protein; Lowry et al., 1951). For pertussis toxin treatment cells were incubated with 100 ng/ml of pertussis toxin for 24 h before harvest. Membranes from pertussis toxin treated cells were prepared as described above.

2.4. Receptor binding

[^{3}H]Diprenorphine (0.2 nM), [^{3}H]naloxone (1.0 nM) and [^{3}H]naltrindole (0.2 nM) were used for competition binding assays. Cell membrane protein (25–50 μg) was incubated with appropriate radioligand in either 50 mM Tris–HCl, pH 7.4 or 50 mM Tris–HCl, pH 7.4 with 100 mM NaCl and 50 μM Gpp(NH)p in a final sample volume of 1 ml at 25°C for 2 h. At least eight concentrations of non-labeled ligands were used to generate competition binding curves. Determination of maximal number of receptors (B_{max}) was performed using varying concentrations of [^{3}H]diprenorphine (0.008–4 nM) or [^{3}H]naltrindole (0.01–3 nM). An excess amount of unlabeled naloxone (10 μM) was used to define non-specific binding. Binding assays were terminated by rapid filtration using a Brandel cell harvester. Samples were collected on glass fiber filters (Schleicher and Schuell #32, Keene, NH), washed three times with 3 ml ice-cold Tris–HCl buffer and quantified by liquid scintillation counting. Each experiment was performed in duplicate and repeated at least three times.

2.5. [^{35}S]GTPγS binding

Opioid agonist-stimulated [^{35}S]GTPγS binding was performed as described by Traynor and Nahorski (1995). Cell membranes (25–50 μg protein) were incubated in Buffer A with at least eight concentrations of agonist, GDP (10 μM) and [^{35}S]GTPγS (100 pM) for 1 h at 30°C in total volume of 1 ml. Maximum stimulation of [^{35}S]GTPγS binding was defined with 10 μM fentanyl (for the μ-receptor) and with 10 μM SNC-80 (for the delta receptor). Assays were terminated by rapid filtration using a Brandel cell harvester to collect membranes on glass fiber filters (Schleicher and Schuell #32, Keene, NH). After washing

three times with 3 ml ice-cold of Buffer A [^{35}S]GTPγS retained on the filters was quantified by liquid scintillation counting. Each [^{35}S]GTPγS binding experiment was performed in duplicate and repeated at least three times using membranes from different passages of cells in order to reduce artifacts due to the cell preparation.

2.6. Data analysis

Nonlinear regressions of the data for both ligand and [^{35}S]GTPγS binding were generated using Graphpad Prism, version 1.02 (GraphPad, San Diego, CA). Receptor binding experiments were analyzed using one site binding and K_i values were calculated with the Cheng and Prusoff equation (Cheng and Prusoff, 1973). EC_{50} values for the [^{35}S]GTPγS binding assay were determined using fixed slope sigmoidal dose response curve analysis.

3. Results

3.1. [^{3}H]Diprenorphine binding

The C6 mu cells used in this study expressed μ receptors at a level of 435 ± 75 fmols mg^{-1} protein as determined with [^{3}H]diprenorphine, with a K_d for [^{3}H]diprenorphine of 0.13 ± 0.01 nM. The number of receptors in C6 delta cells defined with [^{3}H]naltrindole was 955 ± 54 fmols mg^{-1} protein, with a K_d for [^{3}H]naltrindole of 0.01 ± 0.003 nM. Wild-type C6 cells demonstrated no specific binding of [^{3}H]diprenorphine at radioligand concentrations of 0.001 to 5 nM, at a protein level which afforded 80% specific binding in the C6 mu cells.

3.2. [^{35}S]GTPγS binding

Maximal stimulation of the binding of [^{35}S]GTPγS to membranes of C6 mu cells, defined with 10 μM fentanyl, varied between 40 and 100% above basal for membranes from different cell passages, but was consistent within a passage. The maximum stimulation varied from 69.6 to 99.6 fmol/mg protein across passages.

The oripavines, dihydroetorphine and etorphine, stimulated binding of [^{35}S]GTPγS to C6 mu cell membranes (Fig. 1) affording 112% and 109% of the maximal determined with 10 μM fentanyl (Table 1). This stimulation was also greater than DAMGO and morphine which gave 98% and 91% of the fentanyl response, respectively (Table 1). In contrast, buprenorphine maximally afforded only

Table 1
Relative activity of opioid receptor agonists and antagonist at the μ-opioid receptor measured by [^{35}S]GTPγS binding
Agonist stimulated [^{35}S]GTPγS binding was measured as described in Section 2. The activity of each agonist is expressed as percent of 10 μM fentanyl stimulated [^{35}S]GTPγS binding. 95% Confidence intervals are given in parentheses for the EC_{50} value of each compound. % Maximum stimulation values given are mean ± S.E.M. of three experiments carried out in duplicate.
N.A. = not applicable.

	EC_{50} (nM)	Maximal stimulation (% 10 μM fentanyl)
Dihydroetorphine	0.26 (0.20–0.35)	112 ± 3
Etorphine	0.31 (0.26–0.37)	109 ± 2
DAMGO	34.3 (21–58)	101 ± 8
Fentanyl	13.2 (8.5–21)	100
Morphine	65.8 (41–99)	98 ± 5
Methadone	19.2 (12–31)	91 ± 4
Buprenorphine	0.21 (0.09–0.42)	51 ± 2
Pentazocine	117 (46.9–298)	33 ± 3
Butorphanol	7.20 (2.8–19)	30 ± 2
Nalbuphine	1.70 (0.53–5.5)	17 ± 2
Diprenorphine	N.A.	8 ± 2[a]
Naloxone	N.A.	−0.55 ± 1.6[a]

[a] Stimulation achieved at 10 μM ligand concentration.

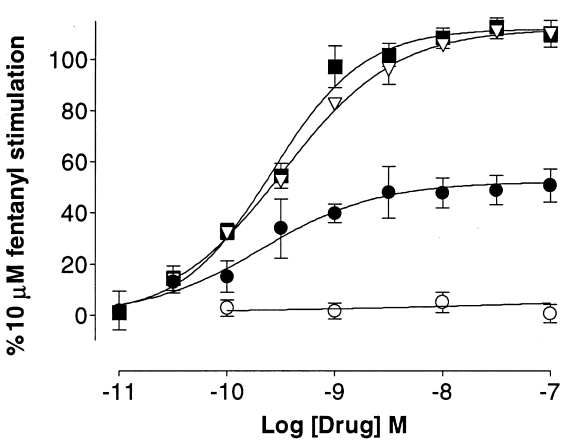

Fig. 1. Oripavine stimulation of [^{35}S]GTPγS binding to membranes of C6 mu cells. Cell homogenates were incubated with various concentrations of dihydroetorphine (■), etorphine (∇), buprenorphine (●) and diprenorphine (○) for 1 h at 30°C. Binding of [^{35}S]GTPγS is expressed as percent of 10 μM fentanyl stimulation. Points are means ± S.E.M. of three experiments performed in duplicate.

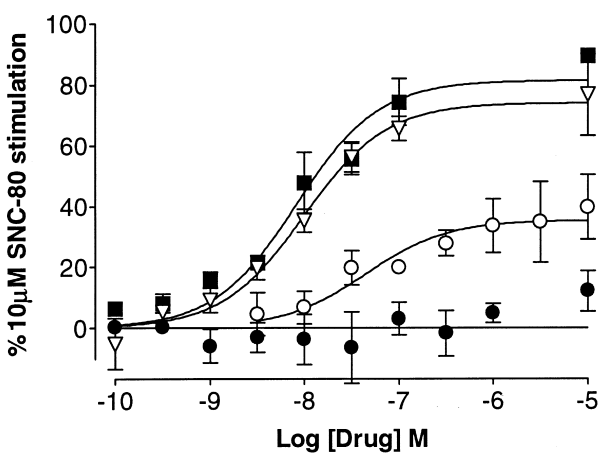

Fig. 2. Oripavine stimulation of [^{35}S]GTPγS binding to membranes of C6 delta cells. Cell homogenates were incubated with various concentrations of dihydroetorphine (■), etorphine (∇), diprenorphine (○) and buprenorphine (●) for 1 h at 30°C. Binding of [^{35}S]GTPγS is expressed as percent of 10 μM SNC-80 stimulation. Points are means ± S.E.M. of three experiments carried out in duplicate.

Table 2
Activity of opioid receptor agonists and antagonists at the δ-opioid receptor measured by [^{35}S]GTPγS binding
Agonist stimulated [^{35}S]GTPγS binding was measured as described in Section 2. The activity of each agonist is expressed as percent of 10 μM SNC-80 stimulated [^{35}S]GTPγS binding. 95% Confidence intervals are given in parentheses for the EC_{50} value of each compound. % Maximum stimulation values given are mean ± S.E.M. of three experiments carried out in duplicate.
N.A. = not applicable.

	EC_{50} (nM)	Maximal stimulation (% 10 μM SNC-80)
SNC-80	9.0 (5.8–14.0)	100
DPDPE	83.0 (44–156)	94 ± 4
Dihydroetorphine	8.3 (5.1 ± 13.5)	81 ± 5
Etorphine	10.0 (6.2–16.1)	74 ± 4
Diprenorphine	113 (34–375)	36 ± 4
Buprenorphine	N.A.	12 ± 7[a]
Naltrindole	N.A.	6 ± 3[a]

[a]Maximum stimulation achieved at 10 μM ligand concentration.

51% while diprenorphine (10 μM) gave less than 10% of the maximal fentanyl response (Fig. 1 and Table 1). The EC_{50} values of fentanyl, DAMGO and morphine were 13.2, 34.3 and 65.8 nM, respectively while those of etorphine (0.31 nM), dihydroetorphine (0.26 nM) and buprenorphine (0.21 nM) were all similar and at least 40-fold more potent than fentanyl (Table 1).

At the δ-opioid receptor a maximal stimulation of 57–70% [^{35}S]GTPγS binding above basal (representing 69.3–340 fmols bound/mg protein, depending on the cell passage) was achieved with 10 μM SNC-80. At the δ-opioid receptor, dihydroetorphine and etorphine were of moderate efficacy, giving 81% and 74% of the maximum effect produced by 10 μM SNC-80 (Fig. 2, Table 2). Diprenorphine and buprenorphine exchanged relative efficacies compared with their activities at the μ-opioid receptor, with diprenorphine now giving an partial agonist response of 35% while buprenorphine, at a concentration of 10 μM, only gave 12% stimulation. Furthermore, the potencies of etorphine and dihydroetorphine (EC_{50} values of 10.0 nM and 8.3 nM) were similar to the EC_{50} of 9.0 nM obtained for the δ-opioid receptor agonist SNC-80 (Table 2).

3.3. Competitive ligand binding

The specific binding of [^{3}H]diprenorphine to the μ receptor expressed in C6 mu cells represented 80.8 ± 1.0% ($n = 98$) of total [^{3}H]diprenorphine binding, as defined by displacement with naloxone. The affinities (as K_i values) of various opioids for the μ-opioid receptor, determined by competition binding assays in the presence or absence of NaCl and the non-hydrolysable GTP analog Gpp(NH)p, using [^{3}H]diprenorphine as the radioligand are given in Table 3. The K_i values for fentanyl, morphine and methadone were affected more by the presence of NaCl and Gpp(NH)p (with a 22.0-fold decrease in binding affinity) than the moderate efficacy compounds pentazocine (7.2-fold), butorphanol (2.8-fold) and nalbuphine (1.5-fold) or the antagonist naloxone (0.3-fold). In contrast affinities of the highly efficacious oripavine agonists etorphine and dihydroetorphine were changed only 1- to 2-fold (Table 3), while the affinity of partial agonist buprenorphine was not altered. Surprisingly binding of the peptide agonist DAMGO was shifted only 8-fold in the presence of Gpp(NH)p and NaCl (Table 3). All of the displacement curves had slopes not significantly different from unity.

Table 3
Affinities of opioids at the μ-opioid receptor determined by competition assays against [^{3}H]diprenorphine
Competition binding of opioids was performed by incubating cell membrane homogenates for 2 h at 25°C with various amount of unlabeled ligand in the presence of [^{3}H]diprenorphine (0.2 nM) with and without 100 mM NaCl and 50 μM Gpp(NH)p as described in Section 2. K_i values were determined from three experiments, each carried out in duplicate. 95% Confidence intervals for each K_i value are given in parentheses.

	K_i (Tris–HCl)	K_i (with NaCl and Gpp(NH)p)	L/H ratio[a]
	(nM)		
Agonists			
Fentanyl	5.0 (4–6)	110 (96–127)	22.0
Morphine	11.2 (8.8–15)	244 (195–307)	21.8
Methadone	12.8 (8.8–19)	266 (164–431)	20.8
DAMGO	7.6 (6.9–8.3)	61 (49–75)	8.0
Pentazocine	21.9 (17–28)	158 (135–184)	7.2
Etorphine	0.22 (0.18–0.27)	0.51 (0.40–0.65)	2.3
Dihydroetorphine	0.21 (0.18–0.26)	0.28 (0.23–0.34)	1.3
Butorphanol	2.6 (2.0–3.2)	7.3 (6.5–8.2)	2.8
Nalbuphine	8.6 (7.8–9.5)	13 (12–14)	1.5
Buprenorphine	0.74 (0.63–0.88)	0.40 (0.34–0.48)	0.5
Antagonists			
Naloxone	6.1 (4.9–7.6)	1.8 (1.6–2.1)	0.3
Diprenorphine	0.15 (0.10–0.21)[b]	0.11 (0.04–0.18)[b]	0.7

[a]L/H ratio = K_i value of ligand in 50 mM Tris–HCl with 100 mM NaCl and 50 μM/K_i value of ligand in 50 mM Tris–HCl, pH 7.4 Gpp(NH)p.
[b]K_d value determined by saturation binding.

Table 4
Affinities of opioids at the μ-opioid receptor determined by competition assays against [^{3}H]naloxone
Competition binding of opioids was performed by incubating cell membrane homogenates for 2 h at 25°C with various amount of unlabeled ligand in the presence of [^{3}H]naloxone (1.0 nM) with and without 100 mM NaCl and 50 μM Gpp(NH)p as described in Section 2. K_i values were determined from three experiments, each carried out in duplicate. 95% Confidence intervals for each K_i value are given in parentheses.

	K_i (Tris–HCl)	K_i (with NaCl and Gpp(NH)p)	L/H ratio[a]
	(nM)		
Agonists			
Fentanyl	0.67 (0.47–0.93)	118 (95–147)	176
Morphine	2.3 (1.9–2.8)	140 (118–168)	62
DAMGO	0.98 (0.74–1.3)	70.1 (58.9–83.7)	72
Etorphine	0.28 (0.22–0.36)	0.98 (0.76–1.27)	3.5

[a] L/H ratio = K_i value of ligand in 50 mM Tris–HCl with 100 mM NaCl and 50 μM/K_i value of ligand in 50 mM Tris–HCl, pH 7.4 Gpp(NH)p.

It is possible that the lack of affinity shift with the oripavines and the reduced shift with DAMGO was because the labeled ligand [^{3}H]diprenorphine is an oripavine. Competition binding studies were therefore repeated using [^{3}H]naloxone. In Tris–HCl buffer alone, the affinities of fentanyl ($K_i = 0.67$ nM), morphine ($K_i = 2.3$ nM) and DAMGO ($K_i = 0.98$ nM) for the μ-opioid receptor were five to 8-fold higher than those obtained in competition assays with [^{3}H]diprenorphine, but the affinity of etorphine ($K_i = 0.28$ nM) remained the same (Table 4). However, the affinities of fentanyl, morphine and DAMGO determined against [^{3}H]naloxone binding to the μ-opioid receptor in the presence of NaCl and Gpp(NH)p were much reduced showing large shifts for fentanyl (176-fold), morphine (62-fold) and now DAMGO (72-fold), while there was still only a minimal shift with etorphine (3.5-fold).

In an attempt to determine the relative roles of G-protein coupling and sodium ions in modulating the binding of oripavines to the μ-opioid receptor, studies were repeated using membranes from cells treated with pertussis toxin to uncouple receptors from G-protein. The affinity of fentanyl binding to the μ-opioid receptor was reduced just 2-fold by this treatment, from 5.0 nM in membranes from control cells to 10.7 nM in membranes from pertussis toxin treated cells. Unlike fentanyl, the binding of etorphine ($K_i = 0.22$ nM) was not altered by pretreatment of the cells with pertussis toxin.

The oripavines bound to the δ-opioid receptor with affinities two- to 5-fold lower than those obtained at the μ-opioid receptor. In Tris–HCl buffer, dihydroetorphine, etorphine, diprenorphine and buprenorphine gave K_i values comparable to those of the highly efficacious delta agonist SNC-80 ($K_i = 0.82$ nM; Table 5). Unlike their interaction with the μ-opioid receptor, large shifts to lower affinity were obtained for dihydroetorphine, etorphine and diprenorphine in the presence of NaCl and Gpp(NH)p (Table 5). The degrees of shift observed were in line with those of the selective δ-opioid receptor agonists DPDPE, SNC-80 and the antagonist naltrindole.

4. Discussion

The oripavines dihydroetorphine and etorphine stimulated [^{35}S]GTPγS binding to membranes from C6 mu cells to a higher degree than the highly efficacious agonists DAMGO, fentanyl and morphine. The relative efficacy of moderately efficacious agonists at this receptor was in the order buprenorphine > pentazocine > butorphanol > nalbuphine, while diprenorphine and naloxone were antagonists in these cells confirming and extending previous findings (Emmerson et al., 1996).

Previous studies have shown that the presence of sodium ions and guanine nucleotides changes the binding of opioid receptor agonists differentially according to their efficacies (Childers and Snyder, 1980; Ishizuka and Oka, 1984; Emmerson et al., 1996). The degree of shift in affinity values of non-peptide opioid ligands at the μ-opioid receptor produced by the presence of Na^+ and Gpp(NH)p

Table 5
Affinities of opioids at the δ-opioid receptor determined in competition assays against [^{3}H]naltrindole
Competition binding of opioids was performed by incubating cell membrane homogenates for 2 h at 25°C with various amount of unlabeled ligand in the presence of [^{3}H]naltrindole (0.2 nM) with and without 100 mM NaCl and 50 μM Gpp(NH)p as described in Section 2. K_i values were determined from three experiments, each carried out in duplicate. 95% Confidence intervals for each K_i value are given in parentheses.

	K_i (Tris–HCl)	K_i (with NaCl and Gpp(NH)p)	L/H ratio[a]
	(nM)		
DPDPE	8.6 (6.6–11)	738 (521–1044)	85.4
SNC-80	0.82 (0.65–1.0)	32 (25–41)	39.0
Dihydroetorphine	0.81 (0.71–0.94)	26 (22–31)	32.3
Etorphine	1.1 (0.86–1.3)	34 (26–52)	31.3
Diprenorphine	0.45 (0.37–0.56)	1.9 (1.6–2.3)	4.3
Buprenorphine	1.1 (0.93–1.4)	0.94 (0.72–1.2)	0.8
Naltrindole	0.039 (0.023–0.054)[b]	0.031 (0.023–0.039)[b]	0.8

[a] L/H ratio = K_i value of ligand in 50 mM Tris–HCl with 100 mM NaCl and 50 μM Gpp(NH)p/K_i value of ligand in 50 mM Tris–HCl, pH 7.4.
[b] K_d value determined by saturation binding.

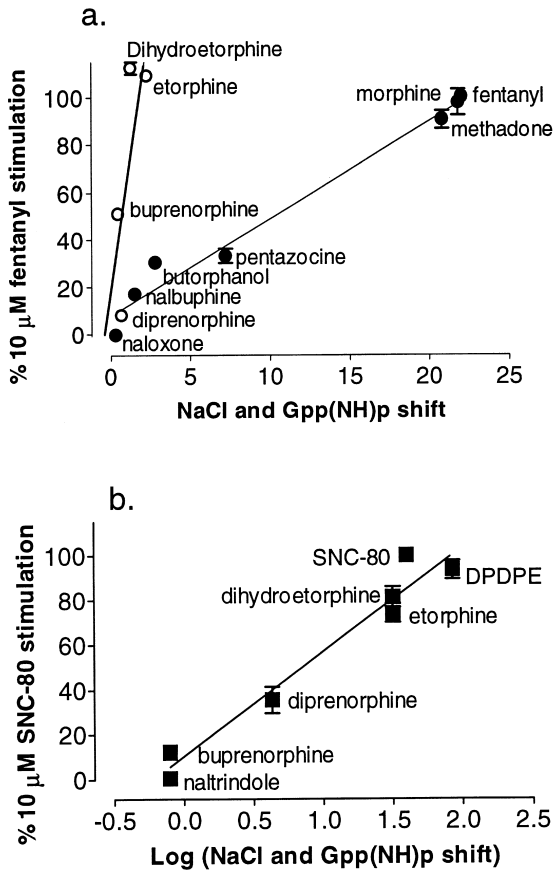

Fig. 3. Correlation of stimulation of [^{35}S]GTPγS binding by opioids with shifts in binding affinity induced by 100 mM NaCl and 50 μM Gpp(NH)p at (a) the mu opioid receptor and (b) the delta opioid receptor.

showed a high correlation (slope = 4.1 ± 0.3; $r^2 = 0.98$; Fig. 3a) with the ability of the ligands to stimulate [^{35}S]GTPγS binding, but only if the oripavines and the peptide DAMGO, were excluded. Indeed, the oripavines formed a second group of opioids (r^2 of 0.57) (Fig. 3a). Consequently, the efficacies of the oripavines do not relate to the differences in their affinities for the μ-opioid receptor under conditions promoting high and low agonist affinity states. Data from several studies supports this finding. For example, Childers and Snyder (1980) showed the presence of NaCl and GTP decreased the binding affinity of etorphine in rat brain homogenates four-times less than that of morphine and 10-times less than the endogenous peptide ligands [Met^5]enkephalin and [Leu^5]enkephalin. In addition, etorphine binds with the same affinity to SH-SY5Y cells and membranes from these cells (Toll, 1992).

The difference between the binding of oripavines and other opioids must be explained by the interaction of sodium ions and guanine nucleotides on the ligand-receptor-G-protein complex. Gpp(NH)p, as a non-hydrolyzable form of GTP induces an accumulation of receptors in a low affinity, uncoupled conformation. This might also be achieved with pertussis toxin pretreatment (Kurose et al., 1983). However, pertussis toxin pretreatment only reduced the binding affinity of fentanyl slightly, while the affinity of etorphine remained the same, indicating that the binding of etorphine does not depend on the coupling state of the receptor (Jauzac et al., 1983). This agrees with previous studies that show opioid receptor agonist binding is either not altered following pertussis toxin treatment (Wuster et al., 1984) or is shifted only 10-fold (Toll, 1992) in the absence of sodium ions. Thus, while pertussis toxin treatment prevents activation of G protein (Traynor and Nahorski, 1995) it alone does not necessarily alter agonist binding. Therefore, sodium ions appear to be the major determinant in the large shift in fentanyl binding affinity, in agreement with findings that sodium is needed to form a low affinity state of the opioid receptor (Childers and Snyder, 1980; Ishizuka and Oka, 1984; Wong et al., 1994), and is responsible for the small observed shift in oripavine binding affinity. A direct interaction of sodium ions with opioid receptors is supported by mutagenesis studies (Kong et al., 1993).

The nature of the radioactively labeled ligand, rather than NaCl or Gpp(NH)p, may be responsible for the observed results. [^{3}H]Diprenorphine is an oripavine, and the present studies have established that oripavines interact with the μ-opioid receptor in a manner different from other opioids. To test for this, binding studies were repeated using [^{3}H]naloxone. The affinities of the non-oripavine ligands for the μ-opioid receptor determined by competition with [^{3}H]diprenorphine were consistently lower than those determined using [^{3}H]naloxone. In addition the binding of DAMGO determined against [^{3}H]naloxone shifted 71-fold when sodium and Gpp(NH)p were included in the buffer, but when [^{3}H]diprenorphine was used as the radioligand, the shift was only 8-fold. This may explain why DAMGO does not fit the correlation between μ-opioid receptor binding affinity and [^{35}S]GTPγS stimulation (Fig. 3a). Shifts for morphine and fentanyl were also greater when using [^{3}H]naloxone as the radioligand. The difference in K_i values determined from experiments with [^{3}H]diprenorphine and [^{3}H]naloxone could arise by [^{3}H]diprenorphine binding to sites which do not recognize [^{3}H]naloxone. However, no binding of [^{3}H]diprenorphine is seen in C6 wild-type cells showing binding is only to the expressed mu receptor in the C6 mu cells, 80% of the diprenorphine binding is specific and there is no evidence of heterogeneity from the competition studies. Presumably therefore the difference arise from a differential interaction of the two ligands ([^{3}H]diprenorphine and [^{3}H]naloxone) with the μ-opioid receptor (Rodriguez et al., 1992). Indeed, Bot et al. (1998) have shown the affinity of diprenorphine and buprenorphine is reduced 125- and 165-fold, respectively when the histidine-297 residue in the sixth transmembrane domain of the μ-opioid receptor is changed to an asparagine. In contrast, the same mutation only changes the affinity of naloxone and morphine by 3.5- and 2.4-fold, respectively. However, this does not readily explain the reduced sodium effects on oripavine binding

since the lack of an affinity change was observed when either [^{3}H]diprenorphine or [^{3}H]naloxone were used as the radiolabeled ligand, while the opioids fentanyl and morphine showed large shifts using either radioligand.

Although buprenorphine and etorphine are highly lipophilic (Kosterlitz et al., 1975), this property of the oripavines does not seen to be a major determinant in their observed binding properties. For example, fentanyl (partition coefficient = 2.76; F. Medzihradsky, personal communication), has a very similar lipophilicity to that of etorphine (partition coefficient of 2.79; Medzihradsky et al., 1992). However, in the present experiments, the affinity of fentanyl was 18-fold more sensitive to the presence of sodium ions and guanine nucleotides than etorphine. Furthermore, the hydrophilic agonist morphine (partition coefficient = −0.21) and the lipophilic agonist methadone (partition coefficient of 1.82; Medzihradsky et al., 1992) have the same 22-fold shift in binding affinity in the presence of Na^+ and guanine nucleotides. Therefore, it appears there is no correlation between the lipophilic nature of the compounds and the reduction in binding affinity in the presence of sodium and guanine nucleotides.

In contrast to findings at the μ-opioid receptor, ligand and [^{35}S]GTPγS binding experiments at the δ-opioid receptor show that the oripavines correlate well with other δ-opioid receptor ligands in terms of their relative ability to bind the receptors in the absence and presence of NaCl and Gpp(NH)p (Fig. 3b). However, the correlation is different from that seen at the μ-opioid receptor since the relationship of Na^+ and Gpp(NH)p shift with agonist efficacy at the δ-opioid receptor was best fitted on a log scale, while the correlation at the μ-opioid receptor was best fitted on a linear scale. This may indicate a difference in the way in which Na^+ and guanine nucleotides alter agonist binding at the two types of opioid receptor. For example, Puttfarcken et al. (1986) and Werling et al. (1986) have shown that sodium affects the binding of agonists at μ-opioid receptors by lowering binding affinities without affecting the number of agonist binding sites. In contrast, Na^+ decreased the binding of agonists to the δ-opioid receptor by apparently reducing the B_{max} rather than lowering agonist binding affinity.

Similar to the binding of oripavines at the μ-opioid receptor, the binding of the highly efficacious selective agonist BW373U86 to the δ-opioid receptor is not affected by the presence of sodium ions and guanine nucleotides. Childers et al. (1993) suggest this may be due to the slow dissociation rate of the bound ligand. This hypothesis is reinforced by the reduced effectiveness of the δ-opioid receptor antagonist naltrindole to reverse the established agonist effects of BW373U86 compared with reversal of the peptide agonist DSLET (Childers et al., 1993). In the case of the oripavines, however, dissociation kinetics cannot adequately explain the lack of sensitivity to sodium ions at the mu opioid receptor. Although the partial agonist buprenorphine is known to have a slow dissociation rate (Hambrook and Rance, 1976), the rapid dissociation of etorphine in the presence of Na^+ and Gpp(NH)p (Kurowski et al., 1982) and the reversibility and short duration of action of dihydroetorphine (Wang et al., 1995) suggest the dissociation kinetics of oripavines are not important in determining the lack of Na^+ and guanine nucleotide effects.

In conclusion, the data show that the oripavine agonists have approximately equal affinity for the low affinity and high affinity conformations of the μ-opioid receptor, but not the δ-opioid receptor. The high affinity states of G-protein coupled receptors are considered to be the functional state (for review, see Birnbaumer et al., 1990) and a high affinity state of the receptor is responsible for stimulation of [^{35}S]GTPγS binding (Traynor and Nahorski, 1995; Emmerson et al., 1996). However, the predominant form of the receptor in vivo is a low agonist affinity state (Carroll et al., 1995). The conformational selection model of agonist activation suggest that by binding to a high affinity site an agonist shifts the equilibrium in favour of a high affinity, coupled form of the receptor. The present results suggest that a purely conformational selection model of agonist receptor interaction (Kenakin, 1997) is not adequate to explain the action of oripavine agonists at the mu receptor since this would predict etorphine and dihydroetorphine to be low efficacy agonists as they recognize different affinity states approximately equally. Instead, by binding to the low affinity state, the oripavine agonists may convert the receptor into a higher affinity coupled state, leading to subsequent agonist effect. If so this may extrapolate to the binding of oripavine agonists to low affinity states of the μ-opioid receptor in vivo and explain why in ex-vivo experiments etorphine shows selectivity for the μ-opioid receptor (Richards and Sadee, 1985) and also help explain the extraordinary potency of these compounds.

Acknowledgements

This work was supported by National Institute of Drug Abuse Grants DA00254 and DA 02265 and NIGMS (GM07767).

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