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Apparent thermodynamic parameters of ligand binding to the cloned rat μ-opioid receptor

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Abstract

The apparent thermodynamic parameters of binding of ten ligands to the cloned rat μ-opioid receptor stably expressed in Chinese hamster ovary (CHO) cells were investigated. For every ligand, the K_d or K_i values at 0°C, 12°C, 25°C and 37°C were determined, a van't Hoff plot was generated and $\Delta H^{o\prime}$, $\Delta S^{o\prime}$ and $-T\Delta S^{o\prime}$ and $\Delta G^{o\prime}$ were calculated. Changes in free energy ($\Delta G^{o\prime}$) ranged from −10.35 to −15.65 kcal/mol. The binding of sufentanil, ohmefentanyl, diprenorphine and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-penicillamine-Thr-NH_2 (CTAP) was endothermic ($\Delta H^{o\prime} > 0$) and driven by an increase in entropy ($-T\Delta S^{o\prime} = -13.08$ to -18.57 kcal/mol). The binding of naltrexone was exothermic ($\Delta H^{o\prime} = -12.56$ kcal/mol) and essentially enthalpy-driven. The binding of morphine, methadone, pentazocine, [D-Ala^2, NMePhe^4, Gly-ol]enkephalin (DAMGO) and Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017) was exothermic ($\Delta H^{o\prime} = -3.53$ to -9.95 kcal/mol) and occurred with an increase in entropy ($-T\Delta S^{o\prime} = -2.48$ to -7.92 kcal/mol). Plots of enthalpy versus entropy and enthalpy versus free energy were linear, although enthalpy–entropy compensation was not evident. The entropy changes were not correlated with apparent lipophilicity of the compounds. These results suggest that: (1) opioid ligands bind to the μ receptor by specific mechanisms, unrelated to lipid solubility; (2) the mechanism of binding is not universally different for peptide and non-peptide ligands; (3) the nature of binding does not a priori determine intrinsic activity. The results reveal a novel differentiation of opioid ligands into two groups (group 1: ohmefentanyl, sufentanil, diprenorphine, CTAP and PL017; group 2: naltrexone, morphine, methadone, DAMGO, pentazocine), based on two distinct relationships between enthalpy versus free energy of binding, the details of which are yet to be elucidated.

Keywords: μ-Opioid receptor; Thermodynamics; Temperature dependence; Receptor binding

1. Introduction

The temperature dependence of the drug-receptor interaction can reveal characteristics that are beyond the resolution of the dissociation constant determined at only a single temperature. Analysis of the temperature dependence of the drug-receptor interaction leads to the determination of quantities that are analogous to thermodynamic parameters and the literature suggests that such an analysis leads to quantities for change in free energy ($\Delta G°$), enthalpy ($\Delta H°$) and entropy ($\Delta S°$) that are reasonable and are consistent with what is known about the interaction (see reviews, Testa et al., 1987; Hitzemann, 1988; Raffa and Porreca, 1989). Whatever the relationship between these calculated values and true thermodynamic parameters, the quantities relate to fundamental characteristics of the drug-receptor interaction. Because of the uncertain relationship to true thermodynamic quantities, the values calculated from the present set of experiments are referred to as 'apparent' thermodynamic parameters. This approach can offer insight into the nature of the interaction between drug and receptor and begin to elucidate the chemical forces underlying the interaction and the mechanistic explanation of concepts such as affinity and efficacy. For example, early studies of β-adrenoceptors suggested that agonists and antagonists may demonstrate different thermodynamic modes, with antagonist binding being entropy-driven while agonist binding was enthalpy-driven, presumably as part of the signal transduction process (Weiland et al., 1979, 1980). This generalization might not hold true for other G-protein coupled receptors, including opioid receptors (Hitzemann et al., 1985; Zeman et al., 1987; Borea et al., 1988; Raffa et al., 1992, 1993; Bourhim

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et al., 1993; Wild et al., 1994; Fabian et al., 1996; Maguire and Loew, 1996).

The existence of at least three types of opioid receptors —μ-, δ- and κ- has been demonstrated (Pasternak, 1988). μ-opioid receptors mediate many effects of opiates and opioids, including modulation of pain perception and euphoria (Pasternak, 1988). Development of tolerance and dependence to opiates is mediated largely by the μ-opioid receptor (Pasternak, 1988). Following the cloning of the mouse δ-opioid receptor (Kieffer et al., 1992; Evans et al., 1992), several laboratories reported cloning of the μ-opioid receptor (Chen et al., 1993; Wang et al., 1993) (for a review, Knapp et al., 1995 and references therein). A splice variant of the μ receptor was reported with sequence variation at the last few amino acids of the C-terminal domain (Bare et al., 1994; Zimprich et al., 1995). Deduced amino acid sequences of these clones display the motif of seven transmembrane helices, characteristics of G protein-coupled receptors.

Thermodynamic analyses of interactions of opiates and opioids with opioid receptors have been reported (see Table 4). The temperature dependence of dissociation constants of ligands was determined using radioligand receptor binding technique or isolated tissue bioassay. With the exception of one study (Maguire and Loew, 1996) in which the cloned mouse δ receptor was used, all other studies utilized systems containing multiple opioid receptors, such as brain membranes. Binding of [^{3}H]dihydromorphine and [^{3}H]DAMGO to the μ receptor in brain or adrenal medulla membranes was endothermic and entropy-driven (Zeman et al., 1987; Borea et al., 1988; Bourhim et al., 1993; Fabian et al., 1996). For the δ receptor, an increase in entropy is the major determinant for the binding of [D-Ala^2,D-Leu^5]enkephalin (DADLE) (Borea et al., 1988; Bourhim et al., 1993), deltorphin B (Fabian et al., 1996), Tyr-Ticψ[CH$_2$NH]Phe-Phe-OH (TIPP(ψ)) (Maguire and Loew, 1996) and (+)-4-[(*aR*)-*a*-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80) (Maguire and Loew, 1996). Binding of [D-Pen^2,D-Pen^5]enkephalin to the cloned mouse δ-opioid receptor was endothermic and entropy-driven (Maguire and Loew, 1996), whereas its binding to the δ-opioid receptor in the mouse vas deferens was exothermic and enthalpy-driven (Raffa et al., 1992). While binding of naltrindole to the cloned mouse δ-opioid receptor (Maguire and Loew, 1996) and the δ-opioid receptor in membranes of the mouse brain or NG108-15 cells (Wild et al., 1994) was entropy-driven, its binding to the mouse spinal cord δ-opioid receptor was enthalpy-driven (Wild et al., 1994), consistent with the heterogeneity of δ-opioid receptors (Wild et al., 1994). For the κ-opioid receptor, binding of [^{3}H]ethylketocyclazocine was endothermic and driven by an increase in entropy (Borea et al., 1988; Bourhim et al., 1993). Binding of naloxone or diprenorphine to opioid receptors in rat brain membranes or the mouse vas deferens was predominantly driven by a decrease in enthalpy (Hitzemann et al., 1985; Zeman et al., 1987; Raffa et al., 1992; Fabian et al., 1996), while an increase in entropy is the predominant driving force of etorphine binding (Hitzemann et al., 1985). Hence, the thermodynamic parameters reflect the ligand- and tissue-selectivity of opioid receptor interactions.

To date, there is no thermodynamic analysis of ligand binding performed on the cloned μ-opioid receptor. The present study was carried out to investigate thermodynamics of binding of ten structurally diverse agonists and antagonists to the cloned rat μ-opioid receptor stably expressed in CHO cells. Five nonpeptide agonists (sufentanil, ohmefentanyl, morphine, methadone and pentazocine), two peptide agonists (DAMGO and PL017), two nonpeptide antagonists (naltrexone and diprenorphine) and one peptide antagonist (CTAP) were examined.

2. Materials and methods

2.1. Materials

[^{3}H]Diprenorphine (60.5 Ci/mmol) was purchased from NEN (Boston, MA). DAMGO and PL017 were purchased from Phoenix Pharmaceuticals (Mountain View, CA). Sufentanil and naloxone were gifts from the Janssen Research Foundation (Beerse, Belgium) and DuPont/Merck (Wilmington, DE), respectively. Ohmefentanyl was obtained from Shanghai Institute of Materia Medica (Shanghai, China). Naltrexone, methadone, pentazocine, morphine and CTAP were provided by the National Institute on Drug Abuse.

2.2. Stable expression of the μ-opioid receptor in CHO cells

The details of establishing CHO cell lines stably expressing the cloned rat μ-opioid receptor (Chen et al., 1993) were published previously (Chen et al., 1995).

2.3. Membrane preparation

Membranes were prepared from CHO cells as described previously (Li et al., 1993). Protein contents of membranes were determined by the bicinchoninic acid method of Smith et al. (1985) with bovine serum albumin as the standard.

2.4. Receptor binding assay

Opioid receptor binding was conducted with [^{3}H]diprenorphine in TEL buffer (50 mM Tris–HCl, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid and 5 μM leupeptin, pH 7.4). Naloxone (1 μM) was used to define nonspecific binding. The time to reach a steady state was determined at 0°C, 12°C, 25°C and 37°C to be

approximately 180, 90, 60 and 20 min, respectively. Saturation experiments were performed with various concentrations of [^{3}H]diprenorphine (ranging from 0.02 nM to 3 nM) to determine its K_d and B_{max} at each assay temperature as described (Xue et al., 1994; Zhu et al., 1996). Competitive inhibition of [^{3}H]diprenorphine binding was performed with 0.15 nM [^{3}H]diprenorphine and various concentrations of unlabeled ligands. Binding assays of each ligand at different temperatures were carried out at the same time. Binding was conducted in a volume of 1 ml with about 3 μg protein. Incubation was terminated by rapid filtration through Whatman GF/B filter, pre-soaked with 0.2% polyethyleneimine and 0.1% bovine serum albumin in 50 mM Tris–HCl (pH 7.4) for at least 1 h, followed by three washes with ice-cold 50 mM Tris–HCl buffer. Radioactivity retained on the filters was determined by liquid scintillation counting. Binding data were analyzed with the EBDA program (McPherson, 1983).

2.5. Calculation of thermodynamic parameters

The change in the standard Gibbs free energy ($\Delta G°$) was calculated using the following equation:

$$\Delta G° = -RT \ln(K_i) \quad (1)$$

where R is the ideal gas constant (1.99 cal/mol K), T (K) is the temperature in Kelvin units, and K_i (M) is the calculated equilibrium dissociation constant for a ligand at temperature T.

Using the relation between change in free energy and change in enthalpy and entropy

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

the variation of K_i with temperature is described by the integrated form of the equation of van't Hoff

$$\ln K_i = (\Delta H°/R)(1/T) - \Delta S°/R \quad (3)$$

Therefore, a plot of $\ln K_i$ against $1/T$ gives a theoretically straight line with slope $\Delta H°/R$. The change in enthalpy of binding ($\Delta H°$) was obtained from $R \times$ slope. The entropy change ($\Delta S°$) was obtained from $1/T(\Delta H° - \Delta G°)$.

For specified reference conditions (the 'standard' state) the above parameters are designated as $\Delta G°$, $\Delta H°$ and $\Delta S°$. The standard state usually is taken to be 25°C, 1 atm pressure and all components at unit activity (sometimes stated as 1.0 M concentration). In addition, the standard state requires that the hydrogen ion concentration also be 1.0 M, i.e., pH = 0. When thermodynamic parameters are determined at other than pH = 0 (as in these experiments) they are designated by their primed counterparts: $\Delta G°'$, $\Delta H°'$ and $\Delta S°'$ (although it is commonplace to see the primes omitted).

2.6. Determination of apparent drug lipophilicity

Apparent drug lipophilicity was determined by a conventional partition method between 1-*n*-octanol phase and

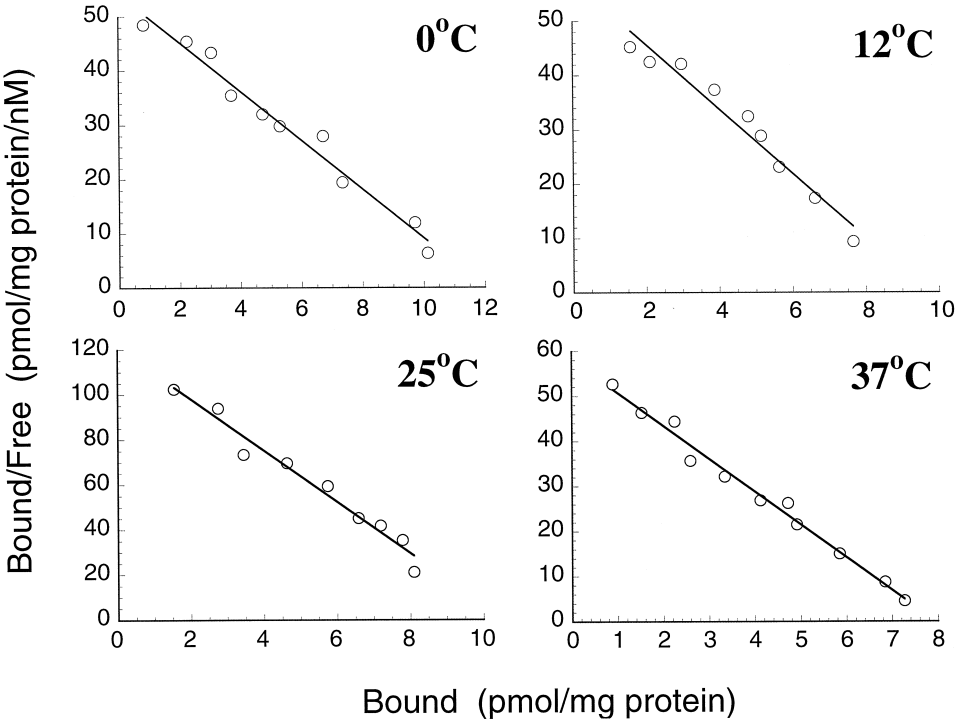

Fig. 1. Scatchard plots of [^{3}H]diprenorphine binding to the μ opioid at 0°C, 12°C, 25°C and 37°C. Saturation binding of [^{3}H]diprenorphine (ranged from 0.03 nM to 3 nM) to the cloned rat μ opioid receptor expressed stably in CHO cells was measured indicated temperatures as described in Section 2. Each figure represents one of the three experiments performed.

Table 1
K_d and B_{max} values of [^{3}H]diprenorphine binding to the cloned rat μ opioid receptor at 0°C, 12°C, 25°C and 37°C

Temperature	[^{3}H]diprenorphine binding	
	K_d (nM)	B_{max} (pmol/mg protein)
0°C	0.20 ± 0.01	10.83 ± 0.63
12°C	0.13 ± 0.02	9.50 ± 0.20[a]
25°C	0.08 ± 0.01	8.95 ± 0.83[a]
37°C	0.20 ± 0.04	8.14 ± 0.39[a]

Binding was performed as described in Section 2. Each value represents mean ± S.E.M. of three experiments in duplicate.
[a] $P > 0.05$ as compared to 0°C by Student's *t*-test.

potassium phosphate buffer (50 mM, pH 7.4) phase. Octanol was saturated with buffer before use. In brief, a certain amount of a drug (from 100 to 400 μg, variable from drug to drug) was dissolved in 4 ml 1-*n*-octanol in a 20-ml test tube. An equal volume of phosphate buffer was added into the tube. The tube was capped, sealed and shaken horizontally at 300 rpm on a gyrotory shaker (New Brunswick Scientific) at room temperature for 30 min. The mixture was then centrifuged at 3000 rpm for 10 min. Concentration of drug at 1-*n*-octanol phase was determined by UV spectrometry at 254 nm. Calibration curves were constructed by serial dilutions in 1-*n*-octanol of the drug under study. Due to poor solubility, morphine, pentazocine and methadone were dissolved in phosphate buffer before partition. Pentazocine concentration was determined at 280 nm instead because of its weak absorbance at 254 nm. The partition coefficient values were then calculated as the following:

$$\text{lipophilicity} = \frac{\text{concentration of drug in the octanol phase}}{\text{concentration of drug in the buffer phase}}$$

3. Results

3.1. Time course of [^{3}H]diprenorphine binding to μ-opioid receptor at 0°C, 12°C, 25°C and 37°C

Time courses of [^{3}H]diprenorphine (0.15 nM) binding to the cloned rat μ-opioid receptor at indicated temperatures were investigated to determine the time that ligand-receptor binding reaches equilibrium. [^{3}H]diprenorphine binding reached a steady state at approximately 180, 90, 60 and 20 min at 0°C, 12°C, 25°C and 37°C, respectively. All other binding experiments were carried out for 180, 90, 60 and 20 min at 0°C, 12°C, 25°C and 37°C, respectively.

3.2. Scatchard analysis of [^{3}H]diprenorphine binding to μ-opioid receptor at various temperatures

Saturation experiments were performed for binding of [^{3}H]diprenorphine (ranging from 0.02 nM to 3 nM) to the cloned rat μ-opioid receptor at 0°C, 12°C, 25°C and 37°C. Scatchard analysis shows that only one binding site is present at each assay temperature (Fig. 1). Increasing the temperature had no significant effect on the K_d or B_{max} values of [^{3}H]diprenorphine binding to the cloned rat μ-opioid receptor (Table 1).

3.3. Effect of temperature on the K_i values of ligand binding to μ-opioid receptor

Competitive inhibition of [^{3}H]diprenorphine binding by each of ten ligands was carried out at 0°C, 12°C, 25°C and 37°C. The pH of the TEL binding buffer was adjusted to 7.4 at each incubation temperature to assure that only

Table 2
K_i values of opioid ligands binding to the cloned rat μ opioid receptor at 0°C, 12°C, 25°C and 37°C

Ligand	K_i (nM)			
	0°C	12°C	25°C	37°C
Nonpeptide agonist				
Sufentanil	0.32 ± 0.10	0.23 ± 0.05	0.19 ± 0.02	0.16 ± 0.03
Ohmefentanyl	0.017 ± 0.005	0.016 ± 0.002	0.009 ± 0.003	0.009 ± 0.002
Morphine	1.6 ± 0.26	2.1 ± 0.95	4.8 ± 0.89	14.4 ± 2.0
Methadone	1.6 ± 0.34	1.6 ± 0.17	5.1 ± 1.70	10.1 ± 0.8
Pentazocine	8.3 ± 0.79	15.6 ± 1.1	28.6 ± 7.5	47.6 ± 8.0
Peptide agonist				
DAMGO	1.5 ± 0.29	2.3 ± 0.78	3.5 ± 0.97	10.9 ± 1.0
PL017	7.9 ± 2.15	7.1 ± 0.63	6.1 ± 0.59	20.6 ± 3.4
Nonpeptide antagonist				
Naltrexone	0.088 ± 0.017	0.21 ± 0.05	0.43 ± 0.10	1.60 ± 0.18
Diprenorphine (K_d)	0.20 ± 0.04	0.13 ± 0.02	0.08 ± 0.01	0.20 ± 0.01
Peptide antagonist				
CTAP	4.2 ± 1.0	1.9 ± 0.66	2.1 ± 0.56	3.1 ± 0.77

Competitive inhibition of [^{3}H]diprenorphine binding (0.15 nM) by an opioid ligand was performed at 0°C, 12°C, 25°C and 37°C as described in Section 2. Data were derived from three separate experiments in duplicate and expressed as mean ± S.E.M.

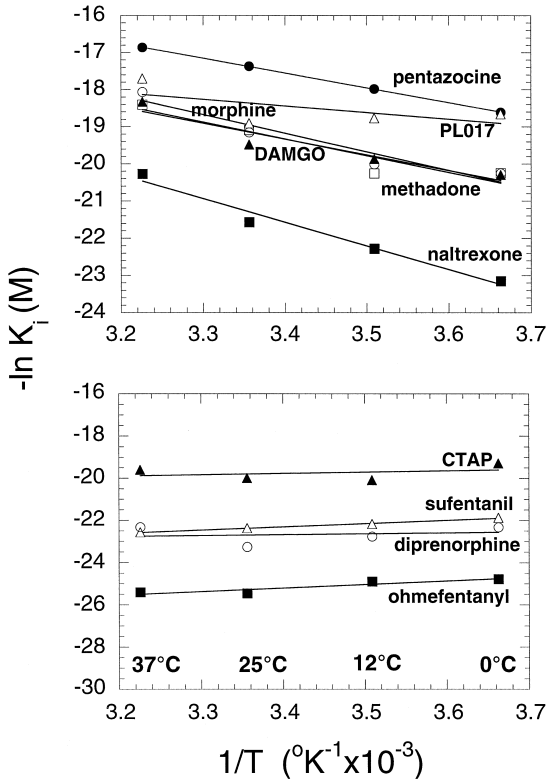

Fig. 2. van't Hoff plots of ln K_i versus $1/T$ for ligand binding to the cloned rat μ opioid receptor. (A) morphine, methadone, pentazocine, DAMGO, PL017 and naltrexone; (B) ohmefentanyl, sufentanil, CTAP and diprenorphine. K_i values (mean) and temperatures listed in Table 2 are used for this plot.

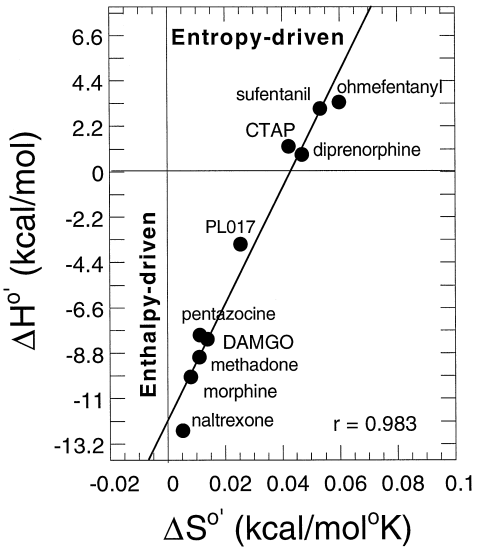

Fig. 3. An extrathermodynamic plot of $\Delta H^{o\prime}$ and $\Delta S^{o\prime}$ pairs calculated from the van't Hoff plot for each ligand. Linear regression analysis yielded a straight line ($r = 0.982$) with a slope of 281.95 and a y-intercept of -12.08.

temperature effects were observed. The K_i values for each ligand at various temperatures are listed in Table 2. K_i values of morphine, methadone, pentazocine, DAMGO and naltrexone increased with the increase in temperature, while those of ohmefentanyl, sufentanil, PL017, CTAP and diprenorphine remained fairly constant at the four temperatures.

3.4. Thermodynamic parameters of ligand binding to the μ-opioid receptor

van't Hoff plots (Fig. 2) of ln K_i versus $1/T$ for all ligands were constructed from their K_i or K_d values determined at 0°C, 12°C, 25°C and 37°C. $\Delta H^{o\prime}$, $\Delta S^{o\prime}$ and $-T\Delta S^{o\prime}$ and $\Delta G^{o\prime}$ at 37°C were calculated (Table 3). Change in free energy ($\Delta G^{o\prime} = -RT$ ln K_i at 37°C) of binding of ten ligands ranged from -11.42 to -15.21

Table 3
Thermodynamic parameters for ligand binding to the cloned rat μ opioid receptor

Ligand	$\Delta S^{o\prime}$ (kcal/mol K)	$\Delta H^{o\prime}$ (kcal/mol)	$-T\Delta S^{o\prime}$ (kcal/mol)	$\Delta G^{o\prime}$ (kcal/mol)
Nonpeptide agonist				
Sufentanil	0.0532	3.06	−16.49	−13.43
Ohmefentanyl	0.0599	3.36	−18.57	−15.21
Morphine	0.0080	−9.95	−2.48	−12.43
Methadone	0.0111	−8.99	−3.44	−12.43
Pentazocine	0.0113	−7.93	−3.49	−11.42
Peptide agonist				
DAMGO	0.0139	−8.14	−4.32	−12.46
PL017	0.0255	−3.53	−7.92	−11.45
Nonpeptide antagonist				
Naltrexone	0.0053	−12.56	−1.65	−14.21
Diprenorphine	0.0469	0.84	−14.54	−13.70
Peptide antagonist				
CTAP	0.0422	1.24	−13.08	−11.84

Calculations were performed according to the following equations: $\Delta H^{o\prime} = R \times$ (slope of van't Hoff plot), $\Delta G^{o\prime} = -RT$ ln K_i (at 37°C), $\Delta S^{o\prime} = (\Delta H^{o\prime} - \Delta G^{o\prime})/T$ (at 37°C).

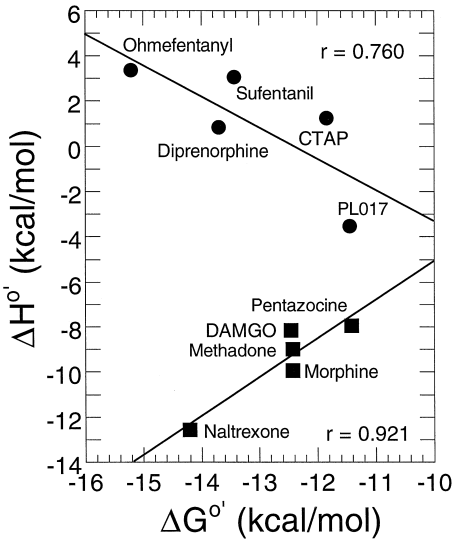

Fig. 4. An extrathermodynamic plot of $\Delta H^{o\prime}$ and $\Delta G^{o\prime}$ pairs for the same ligands as shown in the figure. This plot yielded two straight lines ($r = 0.921$ and 0.760). One line had a positive slope of 1.72 and a y-intercept of 12.12. The other line had a negative slope (-1.38) and y-intercept (-17.12).

kcal/mol. Binding of sufentanil, ohmefentanyl, diprenorphine and CTAP to the cloned rat μ-opioid receptor was endothermic ($\Delta H^{o\prime} > 0$) and driven by an increase in entropy ($-T\Delta S^{o\prime}$ at 37°C ranged from -13.08 to -18.57 kcal/mol). In contrast, binding of naltrexone was exothermic ($\Delta H^{o\prime} = -12.56$ kcal/mol at 37°C) and essentially enthalpy-driven. Binding of morphine, methadone, pentazocine, DAMGO and PLO17 was exothermic ($\Delta H^{o\prime}$ ranged from -3.53 to -9.95 kcal/mol) and occurred with an

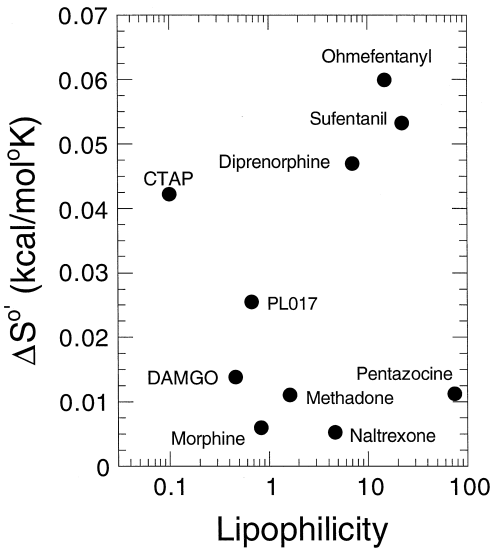

Fig. 5. Relationship between apparent lipophilicity and $\Delta S^{o\prime}$ of ten ligands examined. Lipophilicity of ten ligands was determined as described in Section 2 and plotted versus $\Delta S^{o\prime}$ listed in Table 3. There is no linear relationship between the two parameters.

increase in entropy ($-T\Delta S^{o\prime}$ at 37°C ranged from -2.48 to -7.92 kcal/mol).

3.5. Extrathermodynamic plots

A plot of $\Delta H^{o\prime}$ and $\Delta S^{o\prime}$ pairs for each ligand yielded a straight line ($r = 0.982$) with slope = 281.95 and y-intercept of -12.08 (Fig. 3).

A plot of $\Delta H^{o\prime}$ and $\Delta G^{o\prime}$ pairs for each ligand yielded two straight lines ($r = 0.921$ and 0.760). One had a positive slope (1.72) and y-intercept (12.12). The other had a negative slope (-1.38) and y-intercept (-17.12) (Fig. 4).

3.6. Relationship between apparent lipophilicity and change in entropy

Apparent lipophilicities of ten ligands examined were determined and their relationship to $\Delta S^{o\prime}$ was investigated (Fig. 5). There appeared to no linear relationship between log (lipophilicity) and $\Delta S^{o\prime}$ ($r = 0.194$). When the three peptide ligands were excluded, there was still no linear relationship ($r = 0.405$).

4. Discussion

In this study, the changes in the Gibbs free energy ($\Delta G^{o\prime}$) of binding of all ten ligands examined ranged from -10.35 to -15.65 kcal/mol, consistent with $\Delta G^{o\prime}$ values obtained in other thermodynamic studies of drug-receptor binding processes (Hitzemann et al., 1985; Zeman et al., 1987; Borea et al., 1988; Raffa et al., 1992, 1993; Bourhim et al., 1993; Wild et al., 1994; Fabian et al., 1996; Maguire and Loew, 1996). Binding of sufentanil, ohmefentanyl, diprenorphine and CTAP to the μ-opioid receptor was endothermic and driven by an increase in entropy. Binding of naltrexone was exothermic and essentially enthalpy-driven, whereas binding of morphine, methadone, pentazocine, DAMGO and PL017 was exothermic and occurred with an increase in entropy. There was no apparent distinction between agonists and antagonists nor between peptides and nonpeptides in the relative contribution of the enthalpic or entropic component to the free energy of binding. The enthalpy-free energy plot of the data from the present study divides the compounds into two distinct groupings. One group is characterized by a positive slope, whereas the other group is characterized by a negative slope. The first group contains the compounds naltrexone, methadone, morphine, DAMGO and pentazocine. The second group contains ohmefentanil, sufentanil, CTAP and diprenorphine. PL017 appears to be part of the latter group, although it might occupy an intermediate position. Contained within each group is at least one agonist, one antagonist, one peptide and one non-peptide ligand. Hence the differentiation is not based on the peptidic nature or

intrinsic activity (efficacy) of the ligand. The explanation of this distinctive grouping remains to be elucidated.

In regard to the finding that the thermodynamic parameters obtained in this study do not correlate with the conventional pharmacological designation of agonist vs. antagonist or antinociceptive potencies of these ligands, there are several possibilities. One is that the cloned μ-opioid receptor expressed in CHO cells is not coupled to second-messenger transduction processes in precisely the same manner or to the same extent as in situ in mammalian tissues. Another possibility is that it is not surprising that the change in free energy does not correlate with agonist vs. antagonist designation or antinociceptive potency because $\Delta G = f\{\ln(K_d)\}$ and there is no correlation between affinity and intrinsic activity. The lack of correlation between enthalpy- or entropy-change likewise suggests that these parameters individually do not account for differences in pharmacologic efficacy. Hence, the binding of opioid ligands to the μ-opioid receptor and the activation of those receptors appears to be distinguishable phenomena. According to this view, it could be speculated that the activation of the receptors, as opposed to the occupation of the receptors, might occur in an all-or-none fashion independent of the energetics of binding. In either case, the similar nature of the binding does suggest, however, that the ligands are binding to either the same domain(s) of the receptor or to portions that possess a similar energetic environment.

In the thermodynamic analysis of ligand binding to a particular receptor, some criteria should be met in the experimental design (Raffa and Porreca, 1989). These include (1) the binding reaction should reach equilibrium at each assay temperature; (2) binding to a single receptor class should be measured; (3) multiple temperatures should be used. In this study, we have attempted to fulfill these requirements. The interval for binding to reach equilibrium was determined at each temperature. The cloned rat μ-opioid stably expressed in CHO cells was used, where the presence of only one receptor type is assured. Binding of each ligand was carried out at four temperatures (0°C, 12°C, 25°C and 37°C).

Ideally one should use the K_d values of each radiolabeled ligand to determine thermodynamic parameters of its binding. However, in reality, only a few ligands are available in radiolabeled forms. To the best of our knowledge, among the ligands examined, only [^{3}H]diprenorphine and [^{3}H]DAMGO are available in radiolabeled forms. In addition, it should be noted that a principal goal of the present study was to survey a large number of diverse opioid receptor ligands, encompassing several chemical classes, peptides and non-peptides, with a spectrum of intrinsic activities. In order to do so expeditiously, it was a necessary compromise to determine K_i, rather than K_d, values. The K_i value of DAMGO at room temp determined by competitive inhibition of [^{3}H]diprenorphine binding was similar to the K_d value of [^{3}H]DAMGO at the same temperature. We, therefore, opted to use K_i values in competitive inhibition of [^{3}H]diprenorphine binding for determination of thermodynamic parameters. One potential pitfall is that although in many cases, K_i values are similar to K_d values, the possibility can not be excluded that some ligands may have K_i values in competitive inhibition of [^{3}H]diprenorphine binding which are different from its K_d values, since K_d is determined from direct interaction of the radiolabeled ligand with the receptor, while K_i is derived from competitive inhibition of binding of another ligand. In addition, in order to examine a large number of ligands expeditiously, we did not discriminate the high and low affinity states of the agonists. K_i values determined in this study are similar to published results (Chen et al., 1993; Wang et al., 1993; Thompson et al., 1993). The thermodynamic parameters obtained thus represent a composite for the binding of the agonists to both states. There are inherent limitations to the lack of discrimination of high and low affinity states for the agonists. However, the major conclusions would not have been altered, as seen by the results for the antagonists (which have a single affinity state). Among the three antagonists examined, the binding of naltrexone (non-peptide) was exothermic and essentially enthalpy-driven, while the binding of diprenorphine (non-peptide) and CTAP was endothermic and driven by an increase in entropy. There was no consistent pattern for peptide vs. non-peptide binding or for binding within three antagonists. The possible subtle interaction energetics that might be observed by evaluating separately high- and low-affinity states of the agonists will be explored in future work.

The B_{max} value of [^{3}H]diprenorphine binding to the μ-opioid receptor was not significantly affected by varying temperature from 0°C to 37°C. There appears to be a trend of decrease in B_{max} as the temperature increases although the decrease is not statistically significant. This may be due to enhanced activities of proteases at higher temperatures. Since the affinity of [^{3}H]diprenorphine is fairly constant within the range of 0°C to 37°C, it is a good radiolabeled ligand for competitive inhibition binding assays.

Thermodynamic analyses of ligand binding to opioid receptors are summarized in Table 4. Previous studies utilized rat brain membranes, bovine adrenal medulla membranes or guinea pig brain membranes (Borea et al., 1988; Bourhim et al., 1993; Fabian et al., 1996), whereas we used the cloned rat μ-opioid receptor expressed in CHO cells. Our finding that binding of the peptide μ agonist DAMGO to the cloned μ-opioid receptor occurred with a decrease in enthalpy as the predominant factor was different from previous observations that binding of DAMGO was endothermic and driven by a large increase in entropy in the guinea pig brain (Borea et al., 1988), bovine adrenal medulla (Bourhim et al., 1993) or rat brain (Fabian et al., 1996). Similarly, in our study, binding of the non-peptide agonist morphine was exothermic and a

Table 4
Summary of thermodynamic parameters of ligand binding to opioid receptors in the literature

Ligand	Preparation	ΔH°	ΔS°	Reference
Agonist				
Morphine	cloned rat μ receptor	< 0	> 0	This study
Methadone	cloned rat μ receptor	< 0	> 0	This study
Pentazocine	cloned rat μ receptor	< 0	> 0	This study
PL017	cloned rat μ receptor	< 0	> 0	This study
Sufentanil	cloned rat μ receptor	> 0	> 0	This study
Ohmefentanyl	cloned rat μ receptor	> 0	> 0	This study
DAMGO	cloned rat μ receptor	< 0	> 0	This study
[^{3}H]DAMGO	rat brain	> 0	> 0	(Fabian et al., 1996)
	guinea-pig brain	> 0	> 0	(Borea et al., 1988)
[^{3}H]DAMGO (+0.1 μM [D-Ser^2,Leu^5]enkephalin,Thr^6)	bovine adrenal medulla	> 0	> 0	(Bourhim et al., 1993)
[^{3}H]β-Endorphin	rat brain	> 0	> 0	(Nicolas et al., 1982)
[^{3}H]Etorphine	rat brain	> 0	> 0	(Hitzemann et al., 1985)
[^{3}H]Etorphine (+1 μM morphiceptin +0.1 μM [D-Ser^2,Leu^5]enkephalin,Thr^6)	bovine adrenal medulla	> 0	> 0	(Bourhim et al., 1993)
[^{3}H]Etorphine (+5 μM DADLE)	bovine adrenal medulla	> 0	> 0	(Bourhim et al., 1993
[^{3}H]DADLE	guinea-pig brain	> 0	> 0	(Borea et al., 1988)
[^{3}H]DADLE (+1 μM morphiceptin)	bovine adrenal medulla	> 0	> 0	(Bourhim et al., 1993)
[D-Pen^2, D-Pen^5] enkephalin	mouse vas deferens	< 0	> 0	(Raffa et al., 1993)
	cloned mouse δ receptor	< 0	> 0	(Maguire and Loew, 1996)
SNC80	cloned mouse δ receptor	< 0	> 0	(Maguire and Loew, 1996)
[^{3}H]Deltorphin-B	rat brain	> 0	> 0	(Fabian et al., 1996)
[^{3}H]Ethylketocyclazocine (+0.1 μM DAMGO + 0.1 μM DADLE)	guinea-pig brain	> 0	> 0	(Borea et al., 1988)
[^{3}H]Ethylketocyclazocine (+5 μM DADLE)	bovine adrenal medulla	> 0	> 0	(Bourhim et al., 1993)
[^{3}H]Dihydromorphine	rat brain	> 0	> 0	(Fabian et al., 1996)
	rat brain	> 0	> 0	(Zeman et al., 1987)
Antagonist				
Naltrexone	cloned rat μ receptor	< 0	> 0	This study
CTAP	cloned rat μ receptor	> 0	> 0	This study
[^{3}H]Diprenorphine	cloned rat μ receptor	> 0	> 0	This study
	rat brain	< 0	> 0	(Hitzemann et al., 1985)
[^{3}H]Naloxone	rat brain	< 0	> 0	(Zeman et al., 1987)
Naloxone	rat brain	< 0	> 0	(Fabian et al., 1996)
	mouse vas deferens	< 0	< 0	(Raffa et al., 1992)
[^{3}H]Naltrindole	mouse brain	> 0	> 0	(Wild et al., 1994)
	mouse spinal cord	< 0	> 0	(Wild et al., 1994)
	NG 108-15 cell	> 0	> 0	(Wild et al., 1994)
	cloned mouse δ receptor	> 0	> 0	(Maguire and Loew, 1996)
TIPP(ψ)	cloned mouse δ receptor	< 0	> 0	(Maguire and Loew, 1996)

decrease in enthalpy was the major determinant, whereas binding of dihydromorphine to opioid receptors in rat brain membranes was endothermic and driven by an increase in entropy (Zeman et al., 1987; Fabian et al., 1996). This difference may be due to the different systems used. In addition, there might be multiple subtypes of the μ-opioid receptor in the brain (Pasternak and Wood, 1986) and binding to opioid receptors other than the μ-opioid receptor is likely, particularly for dihydromorphine. Moreover, the possibility cannot be excluded that different lipid compositions of membranes may affect interaction of ligands with receptors.

Binding of the antagonist naltrexone to the μ-opioid receptor was exothermic and essentially enthalpy-driven. This finding is consistent with previous reports that binding of naloxone, a congener of naltrexone, in the rat brain (Zeman et al., 1987; Fabian et al., 1996) or the mouse vas deferens (Raffa et al., 1992) was exothermic with a decrease in enthalpy as the major driving force. Although both diprenorphine and naltrexone are antagonists and have the basic epoxymorphinan structure, the thermodynamic parameters of their binding were different. Binding of diprenorphine was endothermic and entropy-driven, whereas that of naltrexone was essentially enthalpy-driven. The structural difference may render the two ligands to orient differently in the binding pocket.

Bree et al. (1986) found a modest parabolic relationship between the lipophilicity of the twenty β-adrenoceptor ligands and the increase in entropy in their binding to the receptor. Testa et al. (1987) found that a positive linear correlation between the lipophilicity of fifteen antagonists and four agonists of the dopamine D_2 receptor and the increase in entropy in the formation of ligand-receptor complex. No such correlation was found for the opioid ligands investigated in the present study. It is worth noting that the apparent lipophilicity values determined herein

were not traditional lipophilicity values used in chemistry based upon the contribution of non-ionized form alone. Rather, they were apparent lipophilicity values at physiological pH of 7.4, which were contributed from both ionic and neutral forms. It was our attempt to rigorously correlate this lipophilicity characteristics with thermodynamic parameters calculated from our binding results, which were obtained at pH 7.4.

A decrease in enthalpy was the primary driving force for the binding of morphine, methadone, pentazocine and DAMGO in the present studies and binding of naltrexone was essentially enthalpy-driven. A decrease in enthalpy suggests interactions such as hydrogen bonding, ion-pairing and van der Waals interactions. It has been shown that the C3–OH, the aromatic ring A, and N17 of epoxymorphinan compounds (including morphine and naltrexone) are important for high affinity binding to opioid receptors (for a review, Casy and Parfitt, 1986). In addition, each of piperidines (e.g., sufentanil), heptylamines (e.g., methadone) and benzomorphans (e.g., pentazocine) requires an amine nitrogen for activities (for a review, Casy and Parfitt, 1986). Prior to the cloning of opioid receptors, several models of opioid receptors were constructed based on the structure-activity relationship of ligands (for example, Feinberg et al., 1976; Goldblum and Loew, 1991). More recently, molecular modeling of the cloned μ-opioid receptor was reported (for example, Strahs and Weinstein, 1997). Each of these models included an anionic site that interacts with the protonated amine nitrogen, a proton-accepting site that interacts with the phenolic group and one or two lipophilic sites that interact with the aromatic rings.

Binding of sufentanil, ohmefentanyl, diprenorphine and CTAP was endothermic and entropy-driven. Binding of PL017 occurred with an increase in entropy as the major factor. Binding of morphine, methadone, pentazocine and DAMGO took place with an increase in entropy, although a decrease in enthalpy played a more important role. It has been thought that an increase in entropy could be brought about by at least two factors. First, ordered water molecules on the extracellular side of the membranes are perturbed and water molecules surrounding the ligand and around the receptor binding site are expelled. Second, the breaking of hydrogen or van der Waals bonds leads to the receptor assuming a more open configuration so that there is greater freedom of movement in the unfolded ligand-receptor complex. The present results suggest that these or similar interactions with the μ-opioid receptor or its surroundings might play an important role in formation of a ligand-receptor complex for sufentanil, ohmefentanyl, diprenorphine, CTAP and PL017.

It is not uncommon to find a linear relationship between enthalpy change (ΔH°) and entropy change (ΔS°) for a reaction series (sometimes called the 'isokinetic' or the 'isoequilibrium' effect) (for review see Gilli et al., 1994). When this occurs, enthalpy–entropy compensation is said to exist—i.e., that changes in enthalpy are compensated by changes in entropy (or vice versa) such that free energy change $\Delta G^\circ \approx$ constant. Full enthalpy–entropy compensation exists when $\delta\,\Delta G^\circ = 0$ and hence, $\delta\,\Delta H^\circ = T\,\delta\,\Delta S^\circ$. In practice, the linear relationship obtained may take the form $\delta\,\Delta H^\circ = \beta\,\delta\,\Delta S^\circ + \alpha$, where β does or does not equal T. A complication with the interpretation of enthalpy–entropy compensation occurs in situations in which ΔH° and ΔS° are not measured independently, but are derived from linear van't Hoff plots. The estimates of enthalpy and entropy contain uncertainty and these estimates are highly correlated with each other. In such situations, the inherent imprecision associated with these types of experiments can lead to statistical errors that propagate in enthalpy–entropy plots to the point that interdependence gives rise to an apparent linear relationship (see e.g., Petersen, 1964; Exner, 1973; Krug et al., 1976a,b,c; Tomlinson, 1983). Hence, a linear enthalpy–entropy relationship does not a priori mean that compensation exists. However, Krug et al. (1976c) have shown that uncorrelated errors are achieved in a plot of enthalpy vs. free energy calculated at the harmonic mean of the experimental temperatures. The requisite assumptions are generally met by the experimental paradigm. In regard the present data, true chemical compensation does not appear to exist, but the linear extrathermodynamic plots are consistent with the analysis of drug-receptor interaction of Gilli et al. (1994). If a linear relationship exists due to chemical factors (rather than to the propagation of measurement errors), then the van't Hoff plot must show a concurrence (the lines must intersect) at some temperature ($1/\beta$) (for details, see Krug, 1980). Such is not the case for the present data. Likewise, in the enthalpy–entropy plot statistical methods (Krug et al., 1976a,b,c) are based on the null hypothesis that $\beta = T_{hm}$ (where T_{hm} is the harmonic mean of the experimental temperatures), but the value of β for the present data (252 K) is not sufficiently different from T_{hm} (291 K). These results mirror those of Gilli et al. (1994), who analyzed 186 independent experiments performed on 136 ligands binding to 10 biological receptors. The ΔH° and ΔS° pairs were found to be correlated in a linear fashion, exhibiting an apparent enthalpy–entropy compensation effect over a broad range of pharmacologic interactions. Whether or not concurrence was present in the collective van't Hoff plot was not reported, but β was deemed too close to the T_{hm} to satisfy a condition of true compensation. Likewise, ΔH° and ΔG° for the entire set of data were uncorrelated. Therefore the authors concluded that chemical compensation did not exist over this broad range of reactions. However, the authors proposed that two factors had influence on this outcome. Both of their postulations seem of particular importance to the interpretation of drug-receptor interactions. First, the perceived enthalpy–entropy compensation might be a consequence of the relatively narrow range of K_d values of the ligands investigated, limited at the upper end by the unlikeliness of

using ligands with K_d values greater than 100 μM and at the lower end by the unavailability of ligands with K_d values less than about 10 pM. Second, the authors model the drug-receptor interaction as the transfer of drug molecule from one cage (within the solvent space) to another cage (within the receptor space). The receptor space is probably already filled with water (solvent) molecules, hence the drug will displace the water molecules from the binding site cavity. In this model the release of water molecules, which is generally considered the main molecular interpretation of $\Delta S°$ values, essentially is a redistribution of hydrogen bonds. The authors previously showed that $\Delta H°$ values are most likely determined by the energetic balance of the hydrogen bond arrangements before and after binding (Gilli and Borea, 1991). Hydrogen bond rearrangement, then, could underlie both $\Delta H°$ and $\Delta S°$ in drug-receptor interactions and an interrelationship between $\Delta H°$ and $\Delta S°$ seems plausible, indeed likely. Grunwald and Steel (1995) further show that solvent reorganization (the transfer of solvent molecules between the bulk of the solvent and the solvation shells of the solutes) gives rise under common conditions in liquid solutions to a propensity toward enthalpy–entropy compensation and that the data of Gilli et al. (1994) are one such example. They argue that $\beta \neq T_{hm}$ is not a proof that compensation must be due to correlated errors. The advantage of the Gilli et al. (1994) data in this regard is the diversity of ligands and binding sites examined (ten membrane receptors, two enzymes and a DNA site). The present results, although essentially superimposable to that obtained by Gilli et al. (1994) are not as extensive. Hence, the question of enthalpy–entropy compensation for the present results remains an open one.

In conclusion, this study is the first to investigate thermodynamics of ligand binding to the cloned μ-opioid receptor. A strength of this study is the structural diversity of the ligands examined. Our results differentiate two distinct groups of ligands according to their thermodynamics of binding. This is a novel distinction based on energetics of binding, rather than on chemical nature or efficacy.

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