Thermodynamic Analysis of Rat Brain Opioid Mu-Receptor-Ligand Interaction

P. ZEMAN[1], G. TÓTH[2] and R. KVETŇANSKÝ[1]

1 *Institute of Experimental Endocrinology, Centre of Physiological Sciences, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Czechoslovakia*
2 *Institute for Radioisotopes, Biological Research Centre of Hungarian Academy of Sciences, POB 521, 6703 Szeged, Hungary*

Abstract. Opioid mu-receptors are membrane bound receptors. The mechanism by which they transduce their biological effect into the inner compartment of the postsynaptic cell is still not fully understood. The present study was attempted to the measurement of changes of the thermodynamic parameters of the receptor — agonist/antagonist interaction. We have set up the binding assays of a mu-receptor agonist (^{3}H-dihydromorphine) as well as an antagonist (^{3}H-naloxone). The saturation isotherms of both ligands have been assayed at various temperatures and from the resulting K_D values the standard changes of Gibbs energy, enthalpy and entropy have been calculated. While the binding of the mu-receptor agonist ^{3}H-dihydromorphine appears to be entropy driven ($\Delta S^0 = 230\,J\,mol^{-1}\,K^{-1}$) and endothermic ($\Delta H^0 = 19\,kJ\,mol^{-1}$), the binding of the mu-receptor antagonist ^{3}H-naloxone is apparently driven by a decrease of standard enthalpy ($\Delta H^0 = -27\,kJ\,mol^{-1}$; i.e. the reaction is exothermic) and is also characterized by an increase of standard entropy ($\Delta S^0 = 76\,J\,mol^{-1}\,K^{-1}$). The maximal number of ^{3}H-naloxone binding sites has to be determined by incubation at 0—4 °C. The present data do not support the view that opioid mu-receptors transduce their biological signal through the adenylatecyclase system by a mechanism similar to beta-adrenergically stimulated adenylatecyclase.

Key words: Receptor — Temperature dependence — Naloxone — Dihydromorphine — Thermodynamic parameters

Introduction

Although there has been rapid development of knowledge concerning opioid peptide synthesis and degradation in the brain and other tissues, progress in

understanding the mechanism(s) by which endogenous opioid peptides transmit their biological signal was not that fast.

Evidence for the existence of multiple types of opioid receptors even predates the biochemical identification of opioid receptors in mammalian brain in 1973 (Portoghese 1965; Martin 1967). The question of how many types of opioid receptors do exist is still controversial (Akil et al. 1984; Goldstein and James 1984; Adler 1983). The solubilization and isolation of opioid receptors has been only partially successfull. Itzhak et al. (1984) and Gioannini et al. (1982) have found that the molecular weight of mu- and delta-opioid receptors is similar ($4—4.5 \times 10^5$) while that of kappa-receptors is significantly higher ($7.5—8.5 \times 10^5$) indicating the biochemical relatedness of mu- and delta-, but not kappa-receptors. Nevertheless, Newman and Barnard (1984) found that a mu-subunit of the opioid receptor exists, can be alkylated specifically and has a molecular weight of 58 k. Both groups have solubilized opioid receptors from mammalian brain by the use of digitonin in the presence of different cations.

The mechanism(s) by which opioid receptors transduce their biological response into the inner compartment of the postsynaptic cell after agonist binding is not fully understood (Miller 1984). It seems likely that delta-opioid receptors upon agonist binding inhibit adenylate cyclase activity through coupling with the inhibitory regulatory protein N_i (Blume 1983; Koschel and Münzel 1984). The decreased Ca^{2+} influx through voltage-sensitive calcium channels has also been proposed as a possible effector mechanism of opioid receptors (Miller 1984).

At present the best studied hormone-sensitive adenylatecyclase is undoubtedly the beta-adrenergic receptor. Weiland et al. (1979) have measured the thermodynamic parameters of the interaction of beta-adrenergic receptors with various agonists and antagonists. The authors came to the conclusion that agonist binding of beta-adrenergic receptor is largely enthalpy-driven with only a small entropy component. The binding of beta-agonist is associated with a large decrease in enthalpy permitting a highly unfavourable decrease in entropy which accompanies it. According to these results the pattern of changes of thermodynamic parameters of beta-adrenergic agonist/antagonist interaction is compatible with a model, where the decrease of entropy associated with agonist binding reflects a fast isomerisation step occurring in the binding protein of the beta-receptor. This putative isomerisation most probably leads to the activation of the effector mechanism, i.e. to coupling of the receptor proteins resulting in catalytic unit C activation (production of cAMP). Both agonist and antagonist binding starts with a "simple" hydrophobic adsorption, but the putative isomerisation step is agonist-specific and reverses the overall entropy balance, thereby causing agonist binding to be enthalpy driven.

Only the mu-receptor of Martin's classification (Martin et al. 1976) is

identical with the mu-receptor described in direct binding experiments. For the mu-opioid receptor there are several labelled ligands available, allowing the determination of the number and affinity of mu-receptors by the use of both agonist and antagonist binding. We have set up the binding assays for the mu-receptor agonist (^{3}H-dihydromorphine; ^{3}H-DHM) and antagonist (^{3}H-naloxone; ^{3}H-NLX) by adapting standard binding procedures (Pasternak et al. 1975; Naber et al. 1981; Gianoulakis 1983). The binding of the antagonist ^{3}H-NLX was assayed in the presence of a high concentration of Na^+ ions. The presence of this ion increases the binding affinity of antagonists and strongly inhibits the binding of agonists to opioid receptors (Pert and Snyder 1974; Simon and Hiller 1978).

The aim of the present study was to measure the changes of the thermodynamic parameters of the mu-opioid receptor agonist/antagonist interaction. The binding of ^{3}H-DHM (in the absence of Na^+) was observed to be entropy driven, while to the decrease of standard Gibbs energy of naloxone binding (in the presence of Na^+) confers a decrease of standard enthalpy as well as a standard entropy increase. These data indicate that the binding protein is transformed to a less ordered state upon agonist binding. By comparing the present results with the data reported by Weiland et al. (1979) it is evident that if the binding of a mu-receptor agonist is to be followed by stimulation of adenylatecyclase, the mechanism has to be different from the beta-adrenergically stimulated adenylatecyclase. Hence, our results support the view that the effector mechanism coupled with mu-opioid receptors is most probably not stimulation of adenylatecyclase system.

Materials and Methods

Experimental animals and tissue preparation.
Rats of Wistar strain (Velaz, ČSSR) weighing 200—300 g were maintained in the local animal room under standard conditions (max. 6 per cage, light : dark = 12 : 12 hours with lights on from 6 a.m., constant temperature 23 °C, with free access to standard chow and water) for at least one week prior to the experiments. Animals were decapitated and the brains removed within 1 min after sacrifice. The brain tissue was immediatelly transferred into chilled (0—4 °C) homogenization buffer (0.27 mol . l^{-1} sucrose, 50 mmol . l^{-1} TRIS-HCl pH 7.4).

Total particulate fraction ("membranes") preparation.
The membranes were isolated according to a standard procedure used for opioid receptor binding (Pasternak et al. 1975; Naber et al. 1981; Pfeiffer et al. 1984). All steps of the preparation procedure were accomplished at 0—4 °C, unless stated otherwise.

The tissue was homogenised in 30 vol. (w/v of the wet tissue) of homogenization buffer by means of an Ultraturrax homogenizer (Janke und Kunkel AG, FRG) for 20 s at 75 % of maximal speed while constantly cooling the sample. The homogenate was centrifuged at $17 \times 10^3 \times g$, 30 min. The

sediment was resuspended in the initial volume of homogenization buffer by Ultraturrax (under identical conditions). The suspension was then incubated in a thermostated water bath at 37 °C, 30 min, while gently shaking. This step was followed by a second centrifugation ($17 \times 10^3 \times g$; 30 min) and the resulting sediment was rehomogenized in 5 vol (v/w of the original wet tissue weight) of 50 mmol.l^{-1} TRIS HCl buffer pH 7.4. This final preparation (membranes) was either used fresh, or quickly frozen by dipping the tubes into a mixture of ethanol — dry ice and stored at − 20 °C for 2 weeks maximally. In an aliquot of the membrane preparation the protein content was measured (Lowry et a. 1951).

^{3}H-DHM binding assay

The number and affinity of mu-opioid receptors were measured from saturation analysis of ^{3}H-DHM binding according to Gianoulakis (1983). The binding occurs in 50 mmol.l^{-1} TRIS-HCl buffer pH 7.4. The incubation time varied from 60 min at 10 °C to 30 min at 30 °C. The incubation was performed under subdued light. For the determination of one saturation isotherm 7 concentrations of ^{3}H-DHM were used (0.1—3 nmol.l^{-1}; 250—1000 µg prot tube; concentrations are given as final concentrations in the reaction mixture). Nonspecific binding was determined in the presence of 10^{-6} mol.l^{-1} levallorphan. The final volume of the reaction mixture was 0.5 ml. The reaction was stopped by adding 5 ml of cold (0—4 °C) washing buffer (50 mmol.l^{-1} TRIS-HCl buffer pH 7.4) followed by immediate filtration through Whatman glass fibre filters (Whatman GF B, Millipore, USA) presoaked in 0.5 % bovine serum albumin in the washing buffer, to reduce filter binding of the label. A Whatman 1225 Filtration Manifold (Millipore, USA) connected to a standard vacuum oil pump was used. Tubes and filters were washed by additional 2 × 5 ml of washing buffer. The filtration of one sample was accomplished in less than 20 s. The filters were transferred into scintillation vials and dried overnight in stream of air at 40 °C. Toluen based scintillation fluid was added (SLT 41) and radioactivity was counted (RackBeta 1217; LKB, Sweden).

^{3}H-NLX binding assay

The affinity and number of opioid mu-receptors was also measured from saturation analysis of ^{3}H-NLX binding (Pasternak and Snyder 1974; Simon et al. 1973) using the modification of Naber et al. (1981). Binding occurred in 50 mmol.l^{-1} TRIS-HCl buffer pH 7.4 containing 0.1 mol.l^{-1} NaCl, 400—1000 µg prot tube. The incubation time varied from 60 min at 1 °C to 30 min at 35 °C. For the determination of a saturation curve 7 concentrations of ^{3}H-NLX (0.1—2.0 nmol.l^{-1}) were used. Nonspecific binding was determined in the presence of 10^{-6} mol.l^{-1} of nonlabelled NLX. The final volume of the reaction mixture was 1 ml. The reaction was terminated by adding 5 ml of the same washing buffer (see "^{3}H-DHM binding assay") followed by immediate filtration through Whatman GF B filters presoaked in 0.5 % BSA in the washing buffer. The filters were then washed and treated exactly in the same way as described for ^{3}H-DHM binding.

Results calculation and chemicals

The values of specific binding for both ^{3}H-DHM and ^{3}H-NLX binding were transformed into the Scatchard plot (Scatchard 1949). This was linear for both ligands in the concentration range used in our studies and the B_{MAX} and K_D values were calculated by means of the least squares method. The standard error of their estimation was calculated according to Zivin and Waud (1982). A Van't Hoff plot was constructed ($\ln K_D$ vs T) and the value of ΔH^0 was calculated from the slope of this plot by linear regression analysis ($\Delta H^0 = -R \,.\, \mathrm{tg}\, \alpha$). The standard change of Gibbs energy was calculated from the equation $\Delta G^0 = RT \ln K_D$ and the standard change of entropy can be evaluated from the equation $\Delta S^0 = \dfrac{\Delta H^0 - \Delta G^0}{T}$.

All chemicals used in this study were from Lachema, ČSSR, p.a. grade, unless otherwise stated. ^{3}H-DHM was purchased from NEN, USA. ^{3}H-NLX was synthetised by dr.G.T. Unlabelled NLX and levallorphane tartarate are generous gifts of Endo Labs Inc., USA, and Hoffman – La Roche, Switzerland, respectively.

Results

A representative result of a saturation isotherm of ^{3}H-DHM binding is shown in Fig. 1. The Scatchard plot is linear (with Hill coefficient $n_H = 1.18$) suggesting that within the used ^{3}H-DHM concentration range this ligand binds to a single class of noncooperating binding sites with high affinity — the mu-receptor.

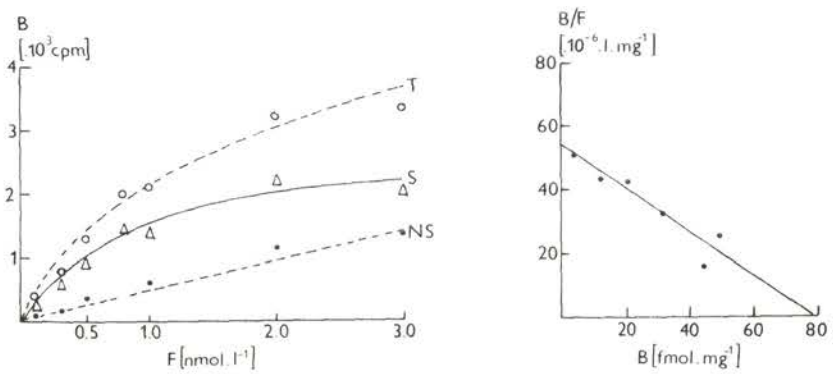

Fig. 1. Saturation isotherm and its Scatchard transformation of ^{3}H-dihydromorphine binding. For experimental conditions see, Materials and Methods. The parameters of Scatchard analysis are following: $n = 6$, $r = -0.9411$, $p < 0.01$, $K_D = 1.4 \pm 0.4$ nmol.l^{-1}, $B_M = 77 \pm 9$ fmol.mg^{-1} prot. T — total binding; NS — nonspecific binding; S — specific binding (S = T − NS). The Scatchard plot is constructed of specific binding values.

A representative result of a saturation experiment of ^{3}H-NLX binding is shown in Fig. 2. The binding of this labelled ligand in the concentration range used in our experiments results in a linear Scatchard plot (with Hill coefficient $n_H = 0.98$). In agreement with the results of ^{3}H-DHM binding it seems therefore probable, that ^{3}H-NLX in the presence of a high Na^+ concentration binds to a single class of noncooperating binding sites — the mu-receptor. With the use of both binding assays it was not possible to detect any heterogenity of mu-opioid receptors.

The dependence of the affinity of the opioid receptor (K_D) on temperature is shown in Fig. 3 (Van't Hoff plot). Saturation isotherms of ^{3}H-DHM binding have been measured at 10, 15, 20, 25 and 30 °C; (Fig. 3). Specific binding of

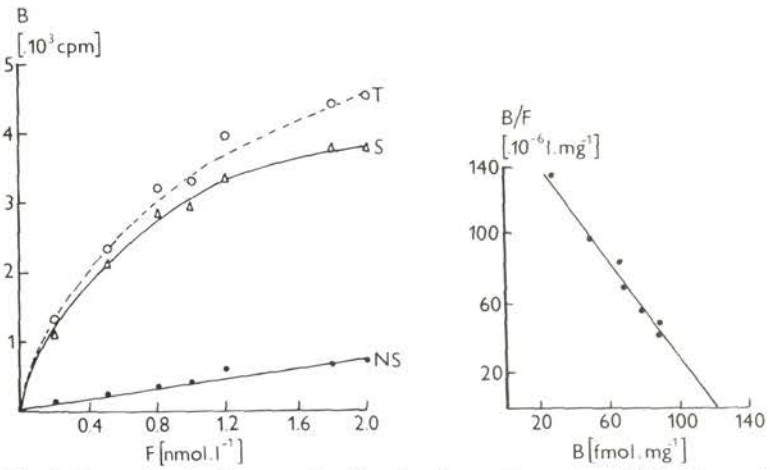

Fig. 2. Saturation isotherm and its Scatchard transformation of ^{3}H-naloxone binding. For experimental conditions see Material and Methods. The parameters of Scatchard analysis are following: $n = 7$, $r = -0.9928$, $p < 0.001$, $K_D = 0.72 \pm 0.04$ nmol.l^{-1}, $B_M = 121 \pm 3$ fmol.mg^{-1} prot. T – total binding; NS – nonspecific binding; S – specific binding (S = T – NS). The Scatchard plot is constructed of specific binding values.

^{3}H-DHM below 10 °C was not detected. The value of B_{MAX} of ^{3}H-DHM binding did not vary significantly with temperature (data not shown). The same experiment was performed with the use of ^{3}H-NLX binding at the following temperatures: 1, 10, 15, 25, 30, 35 °C (Fig. 3). In both cases i.e. ^{3}H-DHM and ^{3}H-NLX binding, the Van't Hoff plot yielded a significant straight line; $p < 0.01$. Unlike the ^{3}H-DHM binding, the maximal number of mu-receptors evaluated from ^{3}H-NLX binding decreased exponentially with temperature (Fig. 4). The calculated thermodynamic parameters for both ligands binding are presented in Table 1. The standard decrease of Gibbs energy is similar for both ligands; $-46 \ldots -51$ kJ.mol^{-1} (10 ... 30 °C) for ^{3}H-DHM binding and $-47 \ldots -51$ kJ.mol^{-1} (1 ... 35 °C) for ^{3}H-NLX binding. The standard change of enthalpy is positive for ^{3}H-DHM binding ($\Delta H^0 = 19$ kJ.mol^{-1}) and therefore the reaction is endothermic, while the standard change of enthalpy of ^{3}H-NLX binding is negative ($\Delta H^0 = -27$ kJ.mol^{-1}; exothermic reaction). The absolute values of ΔH^0 are for both ligands different, what is indicating a differential entropy contribution to the changes of Gibbs energy. Hence, although an increase in standard entropy coupled with the binding of both opioid ligands has been observed, the ΔS^0 value that accompanies ^{3}H-DHM binding is almost 3 times that of ^{3}H-NLX binding ($\Delta S^0 = 230$ J.mol^{-1}K^{-1} and $\Delta S^0 = 76$ J.mol^{-1}.K^{-1}, resp.). It is clear, therefore, that the binding of the mu-opioid agonist is entropy driven, whereas to the standard Gibbs energy of binding of the mu-antagonist confers both an increase of standard entropy as well as a decrease of standard enthalpy.

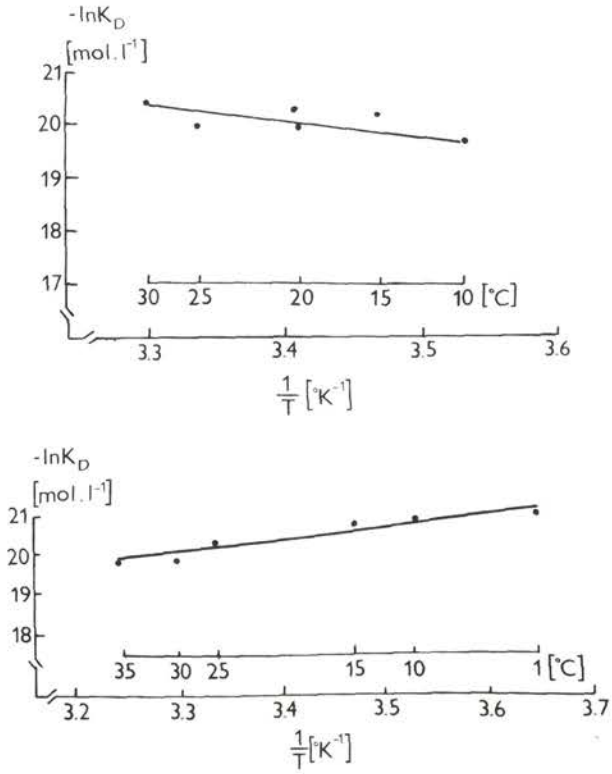

Fig. 3. Van't Hoff plot of ^{3}H-dihydromorphine (upper graph) and ^{3}H-naloxone (lower graph) binding.

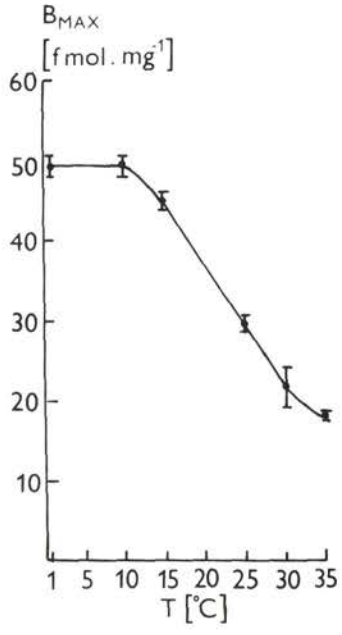

Fig. 4. Temperature dependence of maximal number of binding sites (B_{MAX}) as determined from saturation analysis of ^{3}H-naloxone binding at given temperatures. The ln B_{MAX} vs temperature plot is a significant straight line ($p < 0.01$).

Table 1. Thermodynamic parameters of binding of:

a) ^{3}H-dihydromorphine	$\Delta G^0 = -46 \ldots -51$ kJ . mol^{-1} (10—30 °C) $\Delta H^0 = 19$ kJ . mol^{-1} $\Delta S^0 = 230$ J . mol^{-1} . K^{-1}
b) ^{3}H-naloxone	$\Delta G^0 = -47 \ldots -51$ kJ . mol^{-1} (1—35 °C) $\Delta H^0 = -27$ kJ . mol^{-1} $\Delta S^0 = 76$ J . mol^{-1} . K^{-1}

Discussion

A binding assay for assessing the affinity and number of mu-opioid receptors has been set up by the use of a mu-receptor agonist (^{3}H-DHM) and an antagonist (^{3}H-NLX). As can be seen from the data presented in Figs. 1 and 2 the saturation isotherms of binding for both ligands result in a linear Scatchard plot. This finding is in apparent contrast with the early works of Pasternak and Snyder (1974). These authors have found that dihydromorphine and naloxone binding yield nonlinear Scatchard plots, the high affinity binding representing mu- and low affinity binding delta-receptors (Zukin and Zukin 1981). We have made attempts to find experimental conditions under which both ligands bind to only one class of binding sites. As can be seen from data of Figs. 1 and 2 such conditions were found with the use of low concentrations of labelled ligand (0.1 — 3 nmol . l^{-1} and 0.1 — 2 nmol . l^{-1} for ^{3}H-DHM and ^{3}H-NLX resp.). Under these conditions both ligands apparently bind to a single class of noncooperating binding sites, the mu-opioid receptor.

There are data in the literature suggesting the heterogenity of mu-receptors (Nishimura et al. 1983; Wolozin and Pasternak 1981; Rothman et al. 1983). Nevertheless, it is not possible to detect different types of mu-receptors under the experimental conditions used in the present study.

The time curve of ^{3}H-NLX binding at various temperatures (data not shown) suggests that significantly different B_{MAX} estimates can be obtained depending on the incubation temperature. Measurement of saturation isotherms at various temperatures has confirmed that the B_{MAX} value of ^{3}H-NLX binding decreases exponentially (the plot ln B_{MAX} vs. temperature is a significant straight line) with increasing temperature (Fig. 4). Currently there is no explanation for this effect of temperature on ^{3}H-NLX binding at molecular level, but this result stresses the necessity to assay the maximal number of ^{3}H-NLX binding sites at 0—4 °C.

Although the opioid receptors represent a very intensively studied field of neurochemistry, the effector mechanism(s) by which different types of opioid receptors transmit their biological signal is still unclear (Miller 1984). It seems likely that delta-receptor stimulation leads to the inhibition of prostaglandin-stimulated adenylatecyclase and that binding to this type of opioid receptors leads to increased GTP hydrolysis (Blume 1983; Cooper et al. 1982). These findings fit in well with the model in which the agonist stimulation of the delta-receptor is followed by coupling of the receptor complex with the N_i regulatory protein (Koschel and Münzel 1984). Nevertheless, it remains possible that the effect of opioids on adenylatecyclase is involved in the mediation of some of the chronic, but not acute actions of opiates (Miller 1984).

If coupling with adenylate cyclase may occur after mu-receptor agonist

stimulation, according to the model of Weiland et al. (1979) a decrease of standard entropy would be expected, i.e. the transition of the binding protein to a more ordered state due to coupling with the N_i (or N_S) regulatory protein provoked by ^{3}H-DHM binding. On the contrary, we have found that the binding of this agonist to neural membranes is accompanied by a large increase of standard entropy, which renders the binding to be entropy-driven. The binding of beta-adrenergic agonist is enthalpy-driven with a highly unfavourable decrease of entropy (Weiland et al. 1979).

The presented results are in good agreement with the findings of Hitzemann et al. (1985). These authors have measured the thermodynamic parameters of ^{3}H-etorphine (an opioid agonist) and ^{3}H-diprenorphine (an opioid antagonist) binding. Although these ligands were chosen by the authors because they bind equally well and with high affinity to various classes of opioid receptors (mu, delta and kappa), the pattern of changes of thermodynamic parameters is identical to that found in the present study. This is probably attributable to 2 factors: a) Hitzemann et al. (1985) also use rat brain membranes where mu-receptors are predominant (Werling et al. 1985; Robson et al. 1985) b) they used the ligands in low concentration (0.01—8 nmol . k^{-1}) similar to our study. These 2 factors may explain the linear Scatchard plot of the binding of ^{3}H-etorphine and ^{3}H-diprenorphine. This is typical for binding to one class of receptors, in this case most probably to the mu-opioid receptor, although the binding to several noncooperating binding sites with similar affinity cannot be excluded. These authors came to the same conclusion that agonist stimulation of the mu-opioid receptor leads to a reaction which is fundamentally different from that which accompanies beta-receptor — agonist interaction. The results from beta-adrenergic receptor agonist/antagonist binding should not be generalized as there is no reason to assume that an entropy increase is incompatible with agonist action. Increased standard entropy (i.e. decrease of order of the binding protein and/or it's microenvironment) as a driving force of agonist binding is contradictory to coupling of the binding protein with another entity in the membrane (e.g. N_i). The present results therefore support the findings of Hitzemann et al. (1985) that other effector mechanisms might be connected with the action of opioid ligands through mu-receptors (Miller 1984; Koschel and Münzel 1985). An intriguing, yet only speculative, possibility, that the marked decrease of ordering of the binding protein upon agonist binding reflects opening or closing of an ionic chanel, as suggested e.g. by Mitchell and Anderson (1985), remains open.

In agreement with the results of Hitzemann et al. (1985) we have found that the change of standard Gibbs energy for both agonist and antagonist binding to mu-opoid receptors is negative (decreasing), confirming that the binding reaction at given temperatures occurs spontaneously. The standard enthalpy

change is positive for the mu-agonist binding, but negative for antagonist binding. (The binding of agonist is endothermic, while that of antagonist exothermic.) Their absolute values are different from the value of standard Gibbs energy, the change of standard entropy contributing significantly to Gibbs energy of binding of both ligands.

We have found an increase of standard entropy for the binding of both ligands, but the value for ^{3}H-DHM binding is approx. 3 times the value for ^{3}H-NLX binding.

Hence we conclude: 1) Since we have found an exponential decrease of ^{3}H-NLX binding with increasing temperature, it is necessary to estimate the maximal number of ^{3}H-NLX binding sites at 0—4 °C. 2) The binding of the mu-receptor agonist is entropy driven ($T\Delta S = 68\,\mathrm{kJ\,.\,mol^{-1}}$; $\Delta H^0 > 0$). The Gibbs energy of antagonist binding is composed of a decrease of standard enthalpy ($\Delta H^0 = -27\,\mathrm{kJ\,.\,mol^{-1}}$) as well as an increase of standard entropy ($T\Delta S^0 = 20\,\mathrm{kJ\,.\,mol^{-1}}$). These findings suggest the importance of the energy which is released due to the decrease of ordering of the system folowing agonist binding. 3) The pattern of changes of thermodynamic values of mu-opioid receptor agonist/antagonist interaction is fundamentally different from the values for beta-adrenergic receptor — ligand interaction found by Weiland et al. (1979). If the opioid mu-receptor interacts with adenylate-cyclase, the mechanism has to be different from beta-adrenergically stimulated adenylate cyclase, or, what seems more likely, the effector mechanism of mu-receptors in the rat brain membranes is not represented by this enzymatic system.

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