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Binding Thermodynamics at the Human Neuronal Nicotine Receptor

Pier Andrea Borea,‡ Katia Varani,* Stefania Gessi,* Paola Gilli† and Gastone Gilli†*

*Department of Clinical and Experimental Medicine, Pharmacology Unit; and †Department of Chemistry, University of Ferrara, 44100 Ferrara, Italy

ABSTRACT. The thermodynamic parameters ΔG^o (standard free energy), ΔH^o (standard enthalpy) and ΔS^o (standard entropy) of the binding equilibrium of eleven ligands (six agonists and five antagonists) to the neuronal nicotinic receptor were determined by affinity measurements carried out on human thalamus membranes at six different temperatures (0, 10, 20, 25, 30, 35°) and ΔG vs. T plot analysis. Affinity constants were obtained by saturation experiments for [^{3}H]-cytisine, a ganglionic nicotinic agonist, or its displacement in inhibition assays for the other compounds. The ΔG vs T plots appeared to be reasonably linear in the full temperature range for most of the compounds investigated (equilibrium heat capacity change, $\Delta C_p^o \approx 0$), with the exception of the three agonists cytisine, nicotine and methylcarbachol (ΔC_p^o of the order of $-720 \div -1610$ J mol^{-1} K^{-1}). Thermodynamic parameters were in the range $-53.3 \leq \Delta H^o \leq -28.9$ kJ mol^{-1} and $-41 \leq \Delta S^o \leq 69$ J mol^{-1} K^{-1} for agonists, and $8.7 \leq \Delta H^o \leq 68.2$ kJ mol^{-1} and $99 \leq \Delta S^o \leq 311$ J mol^{-1} K^{-1} for antagonists, indicating that agonistic binding was both enthalpy- and entropy-driven, while antagonistic binding was totally entropy-driven. Agonists and antagonists were, therefore, thermodynamically discriminated. Experimental results were discussed with particular regard to the following points: 1) reasons why membrane receptors displayed unusually low values of ΔC_p^o; 2) possible reasons for the phenomenon of thermodynamic discrimination between agonists and antagonists particularly in connection with ligand-gated ion channel receptors; and 3) the origin of the recurrent phenomenon of enthalpy-entropy compensation which has been observed for neuronal nicotinic receptor ligands as well as for all membrane receptors studied thus far. BIOCHEM PHARMACOL **55**;8:1189–1197, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. neuronal nicotine receptor; [^{3}H]-cytisine; receptor binding; thermodynamic discrimination; human thalamus; thermodynamic parameters

nAChRs§ are a family of ligand-gated cation channels having a pentameric structure composed of α and β subunits [1], and recent molecular biology studies have identified the genes coding for eight α (α_2–α_9) and three β subunits (β_2–β_4) in neuronal tissue [2, 3]. Nicotinic binding sites in the brain can be labelled by several agonist ligands, including [^{3}H]nicotine [4], [^{3}H]acetylcholine [5], and [^{3}H]*N*-methylcarbamylcholine [6], that appear to label a same agonist recognition site with high affinity (K_d in the range 3–12 nM), thereby making it possible to characterize pharmacological properties and the anatomical distribution of these receptors [7, 8]. More recently, the ganglionic nicotinic agonist cytisine, an alkaloid found in the seeds of *Laburnum anagyroides*, has been tritium-labelled, and its binding characteristics determined in rat brain [9]. The ligand appears to recognize a single binding site with high affinity (0.4–1.0 nM) and relatively little nonspecific binding, an aspect which makes it well suited for characterization of tissues with low receptor densities. In particular, Hall *et al.* [10] have evaluated cytisine binding features in several subregions of the postmortem human brain, showing that thalamus exhibits the highest receptor density.

The present paper deals with the binding thermodynamics of six agonists and five antagonists at the human thalamus nicotinic receptor. Thermodynamic parameters of the binding equilibrium of drugs to their receptors have aroused increasing interest in recent years due to the additional information they may provide on molecular binding mechanisms. Receptor binding assays performed at a single temperature provide a quantitative measure of the ability of a drug to interact with a given receptor through the determination of $K_a = 1/K_d$, but do not provide complete information on the binding equilibrium at molecular level. In particular, the K_a value permits the calculation of ΔG^o of the equilibrium by the relationship $\Delta G^o = -RT \ln K_a = RT \ln K_d$, but not that of its two

‡ Corresponding author: Prof. Pier Andrea Borea, Department of Clinical and Experimental Medicine, Pharmacology Unit, University of Ferrara, via Fossato di Mortara 17-19, 44100 Ferrara, Italy. Tel. (+)39-532-291214; FAX (+)39-532-291205.

§ *Abbreviations:* B_{max}, receptor density; ΔC_p^o, equilibrium heat capacity change; ΔG^o, standard free energy; ΔH^o, standard enthalpy; ΔS^o, standard entropy; E-E, enthalpy-entropy; $GABA_A$, γ-amino-butyric acid; GPC receptors, G protein-coupled receptors; IC_{50}, inhibitor concentration displacing 50% of the labelled ligand; K_a, association constant; K_d, dissociation constant; K_i, inhibitory binding constant; LGIC, ligand-gated ion channels; L_{max}, total concentration of ligand added; nAChR, neuronal nicotinic receptor.

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components defined by the Gibbs equation, $\Delta G^o = \Delta H^o - T\Delta S^o$, where ΔH^o and ΔS^o are the equilibrium standard enthalpy and entropy, respectively. The separate determination of the enthalpic and entropic contributions seems to be, at present, quite opportune for a number of reasons. Firstly, different ligands may bind to the same receptor with similar affinity constants (similar ΔG^o values) but with very different ΔH^o and ΔS^o terms. Second, it has recently been shown that thermodynamic properties can sometimes discriminate between agonists and antagonists, in the sense that binding of agonists may be enthalpy driven and that of antagonists entropy driven, or vice versa. This effect, which has been called thermodynamic discrimination [11], was first observed by Weiland *et al.* [12] for β-adrenoceptors and has only recently been confirmed in other receptor systems such as adenosine A_1 and A_{2A} [11, 13], $GABA_A$ [14] and 5-HT_3 receptors [15, 16]. It may have practical applications, because it makes it possible to discriminate drug pharmacological profiles *in vivo* through binding experiments *in vitro*, and may also have theoretical implications, suggesting as it does the existence of two different modalities of drug-receptor binding (i.e. thermodynamically discriminated or nondiscriminated). This in turn suggests that, in such cases, the interaction of agonists with the receptor is thermodynamically distinct from that of antagonists, although the mechanistic implications of this are unclear.

MATERIALS AND METHODS

Reagents

[^{3}H]-Cytisine hydrochloride (specific activity = 39.7 Ci/mmol) and Aquassure were obtained from DuPont/NEN (−)-Nicotine di-*d*-tartrate, arecoline hydrobromide, carbamylcholine chloride (carbachol), (+)-epibatidine-L-tartrate, methylcarbamylcholine chloride (methylcarbachol), dihydro-β-erythroidine hydrobromide, (+)-tubocurarine chloride, succinylcholine chloride, tetraethylammonium chloride and hexamethonium dichloride were purchased from Research Biochemical International (RBI), Amersham Italia.

Membrane Preparation

Human thalamus samples utilized in these experiments were dissected from postmortem brains of neurologically normal individuals. The tissues were frozen in liquid nitrogen, stored at −70° and subsequently homogenized in 50 mM of Tris-HCl buffer (pH 7.0 at room temperature) containing 120 mM of NaCl, 5 mM of KCl, 1 mM of $MgCl_2$, and 2.5 mM of $CaCl_2$. The homogenates were centrifuged at 40,000 *g* for 10 min and the pellets resuspended by a Polytron disrupter (PTA 10 probe, setting 5.30 sec) in the same ice-cold buffer. The membranes were centrifuged and the pellets stored at −70°. The protein concentration was determined according to a Bio-Rad method [17], with bovine albumin as reference standard.

Receptor Binding Assays

Binding assays were performed in a thermostat at 0, 10, 20, 25, 30 and 35 (±0.1)° essentially according to Pabreza *et al.* [9]. The binding of [^{3}H]cytisine to human thalamus was measured in a total volume of 250 μL containing 50 mM of Tris-HCl, 120 mM of NaCl, 5 mM of KCl, 1 mM of $MgCl_2$, and 2.5 mM of $CaCl_2$. All buffer solutions were adjusted to maintain a constant pH of 7.4 at any desired temperature. In saturation experiments, 100 μL of membrane homogenate (500 μg of protein/mL) were incubated in duplicate with 8–10 different concentrations of [^{3}H]cytisine in the range 0.08–8 nM. In competition experiments carried out to determine IC_{50} values of 1 nM of [^{3}H]cytisine was incubated in duplicate with 6–8 different concentrations of each of the agonists or antagonists examined. Incubation times ranged from 150 min at 0° to 15 min at 35° in order to allow equilibrium to be reached (data not shown). Nonspecific binding, defined as the binding in the presence of 10 μM (−)-nicotine di-*d*-tartrate, was *ca.* 10% of the total binding. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass-fibre filters; the incubation mixture was diluted with 3 mL of ice-cold incubation buffer, rapidly vacuum filtered, and the filter washed three times with 3 mL of incubation buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 Spectrometer (efficiency 55%). K_i were calculated from IC_{50} values according to the Cheng and Prusoff equation (see below). The LIGAND weighted nonlinear least-square curve fitting program [18] was used for computer analysis of saturation and inhibition experiments.

Calculations

For a generic binding equilibrium $L + R \rightleftharpoons LR$ (L = ligand, R = receptor, and LR = ligand-receptor complex), the affinity constant is calculated as $K_a = [LR]/([L][R]) = [LR]/([L_{max}–LR][B_{max}–LR]) = 1/K_d$, where $[L_{max}]$ = total concentration of ligand added, $[B_{max}]$ = total concentration of binding sites, and K_d = dissociation constant. As $[LR]/[L_{max}–LR] = [Bound]/[Free] = [B_{max}]\ K_a – K_a[Bound]$, the K_a and B_{max} values can be obtained, in saturation experiments, from the slope and intercept of the Scatchard plot [*Bound*]/[*Free*] vs [*Bound*]. In inhibition experiments, K_i values can be calculated from the IC_{50} values according to the equation: $K_i = IC_{50}/(1 + [C^*]/K_d^*)$ where [C*] is the concentration of the radioligand added, and K_d^* its dissociation constant [19].

Thermodynamic Parameter Determination

Equilibrium thermodynamical parameters and their corresponding standard deviations have been calculated by two different methods:

Method A. The observed ΔG values have been fitted by a quadratic expression [20]

TABLE 1. Equilibrium binding parameters at six different temperatures expressed as: 1) dissociation constant, K_d (nM), and B_{max} (fmol/mg protein) for compound 1 ([^{3}H]cytisine) derived from saturation experiments to human thalamus nicotinic receptors; 2) inhibitory constants, K_i (nM), for compounds 2–11 obtained by displacing 1 nM of [^{3}H]cytisine from the same receptors

T(K)=	273	283	293	298	303	308
Agonists						
[^{3}H]cytisine	K_d = 0.75 (0.03)	1.19 (0.09)	1.44 (0.06)	1.88 (0.03)	2.77 (0.09)	3.19 (0.04)
	B_{max} = 32 (3)	33 (2)	33 (3)	32 (2)	33 (3)	33 (3)
Epibatidine	0.34 (0.01)	0.51 (0.05)	1.0 (0.11)	1.31 (0.06)	1.74 (0.07)	1.96 (0.15)
Nicotine	8.2 (0.3)	12.3 (0.6)	15.1 (0.5)	19.4 (0.5)	34.9 (1.6)	42.9 (1.0)
Methylcarbachol	8.7 (0.5)	23.5 (0.9)	33.5 (1.0)	48.5 (2.3)	93 (4.7)	147 (8.9)
Carbachol	272 (6)	410 (6)	544 (12)	728 (12)	1,160 (31)	1,410 (67)
Arecoline	326 (9)	560 (10)	669 (7)	874 (14)	1,160 (83)	1,600 (58)
Antagonists						
Dihydro-β-erythroidine	1,033 (88)	933 (33)	669 (46)	413 (9)	349 (16)	307 (4)
Tubocurarine	13,600 (833)	11,200 (611)	8,370 (188)	7,770 (146)	6,980 (560)	6,010 (577)
Succinylcholine	27,200 (1,114)	24,300 (882)	20,920 (577)	18,450 (797)	15,700 (1,202)	14,667 (882)
Hexamethonium	330,000 (10,000)	261,000 (5,700)	251,000 (5,700)	236,000 (3,330)	221,000 (5,700)	205,000 (2,900)
Tetraethylammonium	543,000 (29,600)	243,000 (14,500)	75,000 (2,900)	485,000 (2,280)	26,200 (1,330)	21,470 (1,270)

Values are means of at least four experiments. Standard errors are in parentheses.

$$\Delta G = A + BT + CT^2 \quad (1)$$

for which it is easy to show that

$$\Delta H = (\partial(\Delta G/T)/\partial(1/T)_p = A - CT^2 \quad (1')$$
$$\Delta S = -(\partial \Delta G/\partial T)_p = -B - 2CT$$
$$\Delta C_p = (\partial \Delta H/\partial T)_p = -2CT$$

Because experimental measurements are performed in a narrow range around T^o = 298.15 K, equilibrium parameters ΔG^o, ΔH^o and ΔS^o are better obtained by interpolation in this range [21], i.e.

$$\Delta G = A' + B'(T - T^o) + C'(T - T^o)^2 \quad (2)$$

for which:

$$\Delta G^o = A' \qquad \sigma(\Delta G^o) = \sigma(A')$$
$$\Delta H^o = A' - B'T^o \qquad \sigma(\Delta H^o)$$
$$= [\sigma^2(A') + T^{o2}\sigma^2(B')]^{1/2} \quad (2')$$
$$\Delta S^o = -B' \qquad \sigma(\Delta S^o) = \sigma(B')$$
$$\Delta C_p^o = -2C'T^o \qquad \sigma(\Delta C_p^o) = 2T^o\sigma(C')$$

The condition $C = C' = 0$ (i.e. $\Delta C_p^o = 0$) corresponds to the well-known case of a linear van't Hoff plot for which $\Delta H = \Delta H^o$ and $\Delta S = \Delta S^o$ at all temperatures. This condition is considered to be verified whenever the second-order C' coefficient in Eqn. 2 is statistically not significant.

Method B. Assuming a priori that $\Delta C_p^o = 0$, data can also be interpolated by the van't Hoff plot method around T^o:

$$\ln K_a = A'' + B''(1/T - 1/T^o) \quad (3)$$
$$= -\Delta G^o/RT^o - \Delta H^o/R(1/T - 1/T^o)$$

In this case

$$\Delta G^o = -A''RT^o \quad \sigma(\Delta G^o) = RT^o\sigma(A'')$$
$$\Delta H^o = -B''R \qquad \sigma(\Delta H^o) = R\sigma(B'') \quad (3')$$
$$\Delta S^o = (\Delta H^o - \Delta G^o)/T^o$$
$$\sigma(\Delta S^o) = [\sigma^2(\Delta H^o) + \sigma^2(\Delta G^o)]^{1/2}/T^o$$

Interpolation of experimental data (Table 1) by Eqn. 2′ has shown that C', and then ΔC_p^o, is significantly different from zero only for three compounds, namely cytisine, nicotine and carbachol, displaying C_p values in the range $-720 \div -1610$ J mol^{-1} K^{-1}. All other compounds were assumed to have ΔC_p^o values indistinguishable from zero and calculations for them were carried out by both Eqn. 2 and 3, obtaining results in agreement within 0.2 standard errors. Final calculated values are given in Table 2.

It is to be noted that values of ΔS^o should be corrected for the effect of dilution from the standard state (ligand concentration = 1 M) to the experimental conditions (ligand concentration approaching zero). Such a correction, called cratic correction, has been evaluated by Kauzmann [22] to be $\Delta S_{CR}^o = -R \ln (1/55.6) = 33.4$ J mol^{-1} K^{-1} at T = 298 K, and unitary standard entropies are defined as $\Delta S_u^o = \Delta S^o + \Delta S_{CR}^o$. Though ΔS_u^o is certainly a more correct quantity than ΔS^o, the latter is used in Table 2 and in the following discussion to make present results more directly comparable with those reported in the literature.

RESULTS

Table 1 reports (in the first two lines) the K_d and B_{max} values derived from saturation experiments of [^{3}H]cytisine

TABLE 2. Thermodynamic parameters for the binding equilibrium of agonists and antagonists to human thalamus nicotinic receptors

Ligand	ΔG^o (kJ mol^{-1})	ΔH^o (kJ mol^{-1})	ΔS^o (J mol^{-1} K^{-1})	$-T\Delta S^o$ (kJ mol^{-1})	ΔC_p^o (J mol^{-1} K^{-1})
Agonists					
Cytisine	−49.53 (0.07)	−28.9 (1.6)	69 (5)	−20.6 (1.5)	
	−49.63 (0.07)	−34.4 (2.4)	51 (8)	−15.2 (2.4)	−720 (240)
Epibatidine	−50.74 (0.08)	−37.3 (1.9)	45 (6)	−13.5 (2.0)	—
Nicotine	−43.45 (0.12)	−32.8 (2.7)	36 (9)	−10.6 (3.0)	
	−43.69 (0.09)	−45.4 (3.2)	−6 (1)	1.8 (0.3)	−1610 (400)
Methylcarbachol	−41.15 (0.12)	−53.3 (2.8)	−41 (9)	12.2 (3.0)	—
Carbachol	−34.68 (0.08)	−33.0 (2.0)	5 (7)	−1.6 (2.1)	
	−34.86 (0.07)	−42.5 (2.3)	−26 (8)	7.7 (2.4)	−1190 (240)
Arecoline	−34.33 (0.07)	−29.6 (1.7)	16 (6)	−4.7 (1.8)	—
Antagonists					
Dihydrobetaerithroidine	−36.20 (0.10)	26.9 (2.3)	212 (8)	−63.1 (2.4)	—
Tubocurarine	−29.18 (0.06)	16.2 (1.4)	152 (5)	−45.4 (1.5)	—
Succinylcholine	−27.08 (0.06)	13.0 (1.3)	134 (4)	−40.1 (1.2)	—
Hexamethonium	−20.73 (0.03)	8.7 (0.7)	99 (2)	−29.4 (0.6)	—
Tetraethylammonium	−24.63 (0.08)	68.2 (2.0)	311 (7)	−92.8 (2.1)	—

ΔG^o, ΔH^o, ΔS^o and ΔC_p^o values are given at $T = 298.15$ K. Standard errors are in parentheses.

binding to human thalamus nicotinic receptors performed at the six chosen temperatures together with the inhibitory binding constants, K_i, for the other ten ligands measured by displacement of [^{3}H]cytisine as labelled ligand. Figure 1 displays a typical saturation experiment for [^{3}H]cytisine binding obtained at 0°; the corresponding Scatchard plot (inset) is essentially linear ($r = 0.98$), suggesting a single class of binding sites ($K_d = 0.74 \pm 0.03$ nM; $B_{max} = 32 \pm 3$ fmol/mg protein). This was also confirmed by the computer analysis of data [18], which failed to show a significantly better fit to a two-site model. Similar results were obtained at all temperatures ($0.97 \leq r \leq 0.99$) for both saturation and competition experiments; representative Scatchard plots for [^{3}H]cytisine binding at three different temperatures are shown in Fig. 2. While K_d and K_i values

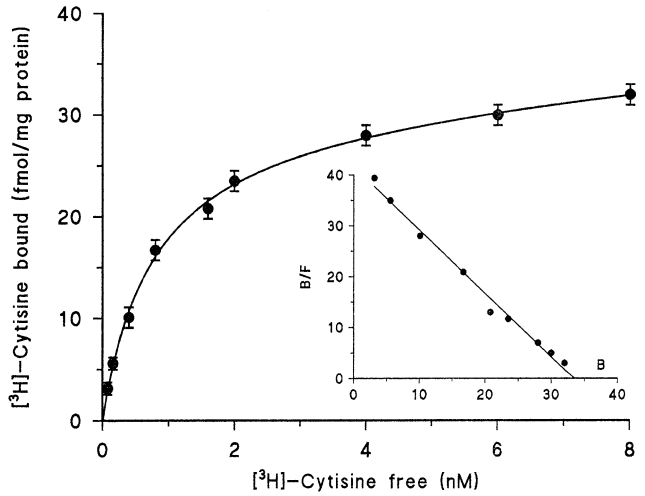

FIG. 1. Saturation of [^{3}H]cytisine binding to human nicotine receptors. Values are means ± SE of four separate experiments performed in duplicate. The corresponding Scatchard plot is shown in the inset.

are widely temperature-dependent (*vide infra*), B_{max} values are quite independent of temperature, suggesting a substantial stability of the effective receptor population at all temperatures studied. The dependence of ΔG values on temperature is displayed in Figs. 3 and 4 for agonists and antagonists, respectively. All plots appear to be reasonably linear in the full temperature range (0 ÷ 35°). A more accurate statistical analysis detected, however, a significant curvature, indicating that $\Delta C_p^o \neq 0$ for the agonists cytisine, nicotine and methylcarbachol. Table 2 reports final thermodynamic parameters obtained by both linear and quadratic fitting for these three compounds, and linear fitting for all other compounds investigated; standard errors are in the range 0.7 ÷ 3.2 kJ mol^{-1} for ΔH^o, 1 ÷ 9 J mol^{-1} K^{-1} for ΔS^o and 0.03 ÷ 0.12 kJ mol^{-1} for ΔG^o. ΔG^o values range from −50.74(8) to −34.33(7) kJ mol^{-1} for agonists and −36.20(10) to −20.73(3) kJ mol^{-1} for antagonists, showing that agonists are much better binders than antagonists, at least in the present series of compounds. Figure 5 summarizes the results in the form of a $-T\Delta S^o$ vs ΔH^o scatter plot ($T = 298$ K). It shows that antagonists clustered in the endothermic region ($8.7 \leq \Delta H^o \leq 68.2$ kJ mol^{-1}) with large positive entropy values ($-92.8 \leq -T\Delta S^o \leq -29.4$ kJ mol^{-1}); their binding is therefore to be classified as totally entropy-driven. Conversely, agonistic binding is mostly or totally enthalpy-driven ($-53.3 \leq \Delta H^o \leq -28.9$ kJ mol^{-1}; $-20.6 \leq -T\Delta S^o \leq 12.2$ kJ mol^{-1}). Agonists and antagonists are therefore thermodynamically discriminated.

DISCUSSION

Three points seem of particular interest in the discussion of the present data, namely the values of ΔC_p^o, the thermo-

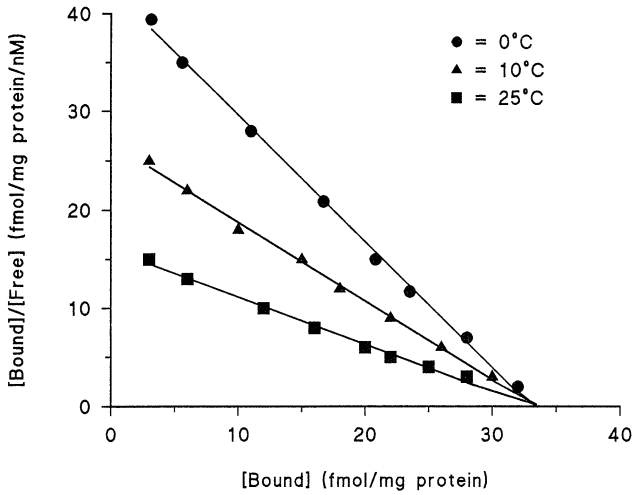

FIG. 2. Representative Scatchard plots for [^{3}H]cytisine binding to human nicotine receptors at 0, 10, and 25°. The linearity of the plots is indicative of the presence of a single class of high affinity binding sites at all temperatures investigated.

dynamic discrimination of agonists from antagonists, and the recurrent phenomenon of E-E compensation.

Present analysis shows that only three out of eleven compounds display ΔC_p^o values significantly different from zero, at least at the significancy level achievable by the use of data collected at six temperatures. This reflects the general situation in the field of membrane receptors, where the curvature of the van't Hoff ($\ln K_a$ vs $1/T$) or ΔG vs T plots is so slight that it is usually neglected, with a very limited number of exceptions such as insuline [23] and 5-HT$_3$ receptors [16]. It seems, therefore, that the binding of most ligands to membrane receptors occurs with null or minimal C_p^o changes, at variance with other processes involving biological macromolecules, such as binding of many biomolecules to enzymes, protein folding, and protein-protein association [24, 25], for which ΔC_p^o values are mostly large and negative, a fact which has often been interpreted as due to the combined action of the hydrophobic effect (reduction of the hydrophobic surface exposed to the solvent) and, to a lesser extent, of the lowering of the vibrational temperature of the protein tightened by the binding of the ligand [24]. It is not easy to understand the reason for the different behaviour (if any) of membrane receptors, though it might be tentatively associated with the more hydrophilic nature of most membrane receptor ligands. In this context, it is to be noted that both transfer of small molecules to their pure liquids and folding of proteins display heat-capacity changes which follow the empirical relationship

$$\Delta C_p^o \approx 1.3\Delta A(np) - 0.59\Delta A(p) (\text{J mol}^{-1}\,\text{K}^{-1})$$

where $\Delta A(np)$ and $\Delta A(p)$ are the changes of solvent-accessible molecular surface area (expressed in Å^2) of non-polar (np) and polar (p) nature, respectively [26, 27]. This model is clearly able to interpret phenomena occurring without heat-capacity changes as a compensation effect between hydrophobic and hydrophilic water-exposed surfaces on the ligand (and/or on the receptor binding site) during the binding process, particularly when the membrane receptor ligands are, as in the present case, relatively small molecules protonated at physiological pH.

From a general point of view, there are good reasons for believing that ΔC_p^o values for binding to membrane receptors should be low, and this because large variations in water-exposed surfaces (those thought to produce the largest C_p changes) would perturb the delicate balance between hydrophobic and hydrophilic parts believed to control the receptor protein position across cell membrane. Therefore, our capability of determining such probably low ΔC_p^o values relies on the quality of data, which should be sound enough to detect a slight statistically significant curvature in the van't Hoff or ΔG^o vs T plots. The statistical analysis carried out on present data shows that the standard error on C_p [$\sigma(C_p)$] is never smaller than 200–250 J mol^{-1} K^{-1}; because a reliable significance level should be of the order of magnitude of 3σ, this implies that the ΔC_p level of detectability is, at least for the present experimental conditions, not smaller than 600–750 J mol^{-1} K^{-1}.

The fact that antagonist and agonist binding is found to be driven by different forces (entropic and mostly enthalpic, respectively) is a common phenomenon. Out of ten membrane receptors studied thus far, only three do not show thermodynamic discrimination (D_2 dopamine [28], 5-HT$_{1A}$ [29], and benzodiazepine receptors [30]), whereas the other seven do. The latter are the three GPC β-adrenergic [12], A_1 and A_{2a} adenosine [31] receptors, and the LGIC GABA$_A$ [14], glycine [32], 5-HT$_3$ serotonin [15] and present nACh nicotinic receptors. Most of the discriminated receptors are characterized by more entropic agonistic binding with the exception of β-adrenergic and present

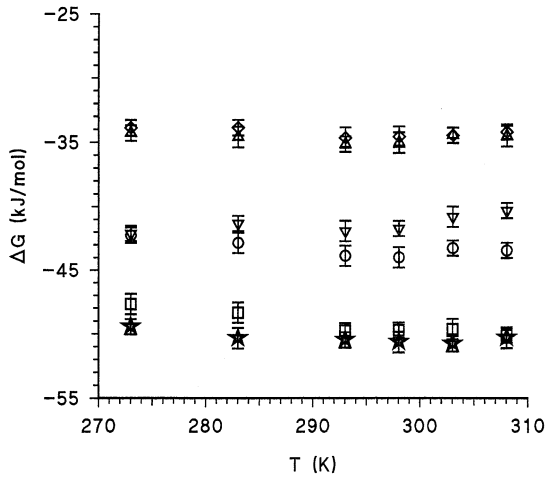

FIG. 3. Plots showing the effect of temperature on the free energy, ΔG, of the receptor-binding equilibrium for the six agonists investigated. ΔG values are means (± SE) of three separate experiments (★ = epibatidine; ■ = cytisine; ● = nicotine; ▼ = methylcarbachol; ▲ = carbachol; ◆ = arecoline).

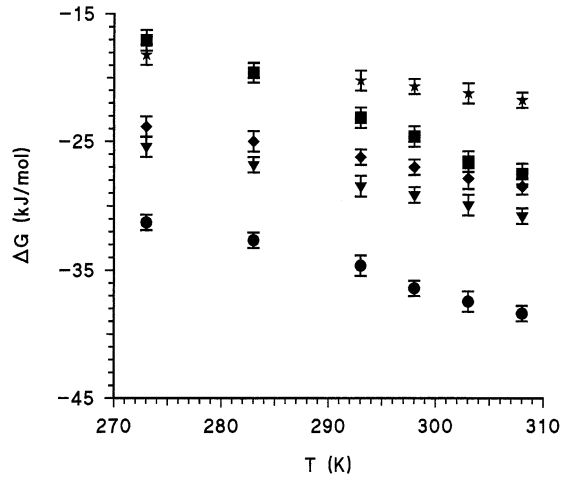

FIG. 4. Plots showing the effect of temperature on the free energy, ΔG, of the receptor-binding equilibrium for the five antagonists investigated. ΔG values are means (± SE) of three separate experiments (● = dihydro-β-erythroidine; ▼ = tubocurarine; ◆ = succinylcholine; ■ = tetraethylammonium; ★ = hexamethonium).

neuronal nicotinic receptors for which the discrimination is reversed. E-E scatter plots for all LGIC receptors studied thus far are collected in Fig. 6, and seem to suggest that thermodynamic discrimination is common to all channel receptors, albeit with rather different intercluster distance. This hypothesis clearly needs to be validated by future studies on the missing P_{2x} purinergic and glutamatergic receptors.

When data for the four receptors are reported on a common ΔH^o vs ΔS^o scatter plot (Fig. 7), it becomes apparent that all points are arranged on a same diagonal band encompassed between the two dashed lines which

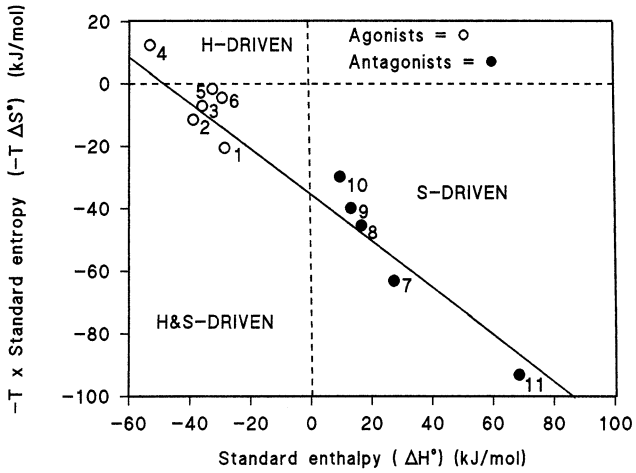

FIG. 5. $-T\Delta S^o$ vs ΔH^o (kJ mol^{-1}; T = 298.15 K) scatter plot for human nicotine receptor agonists (o) and antagonists (●). 1 = cytisine; 2 = epibatidine; 3 = nicotine; 4 = methylcarbachol; 5 = carbachol; 6 = arecoline; 7 = dihydro-β-erythroidine; 8 = tubocurarine; 9 = succinylcholine; 10 = hexamethonium; 11 = tetraethylammonium.

represent the loci of points defined by the limiting K_d values of 100 μM and 100 pM. The correlation equation

$$\Delta H^o(\text{kJ mol}^{-1}) = -35(2)–279(13)\Delta S^o(\text{kJ mol}^{-1}\,\text{K}^{-1}) \quad (4)$$

$$(n = 43;\ r = 0.955;\ s = 8.88;\ P \leq 0.001)$$

is that expected for a case of E-E compensation, $\Delta H^o = \beta\Delta S^o$, with compensation temperature of 278 K. This equation is strictly similar to that given by Gilli *et al.* [33] for a larger set of thermodynamic parameters concerning the binding of 186 compounds to ten different membrane receptor systems (not including data of Fig. 6 and Table 3), i.e.

$$\Delta H^o(\text{kJ mol}^{-1}) = -40(2)–278(4)\Delta S^o(\text{kJ mol}^{-1}\,\text{K}^{-1})$$

$$(n = 186;\ r = 0.981;\ s = 2.06;\ P \leq 0.001)$$

It is generally admitted [34–36] that E-E compensation effects are to be ascribed to a same property of the solvent (the only constant in the problem) rather than to intrinsic properties of the drug-receptor binding phenomenon. Following Grunwald and Steel [36], the binding equilibrium can be subdivided into two different processes

$$L + R \rightleftharpoons LR \quad (5)$$

$$s_L A/L + s_R A/R \rightleftharpoons s_{LR} A/LR + (s_L + s_R – s_{LR})\, A/A \quad (6)$$

with L = ligand, R = receptor, LR = receptor-ligand complex, A = solvent (in this case water), and s_L, s_R and s_{LR} = mean number of A molecules in the solvent cage of L, R, and LR, respectively. Moreover, A/A denotes an A molecule having only other A molecules in its solvent shell, and A/L, A/R and A/LR indicate A molecules having all A molecules in their shell except for one molecule of L, R and LR, respectively.

Equation (5) refers to the nominal binding reaction, abstracting from solvent effects, with thermodynamic parameters ΔG_{nom}, ΔH_{nom} and ΔS_{nom}. Equation (6) indicates the concomitant solvent-environment rearrangement reaction with parameters ΔG_{env}, ΔH_{env} and ΔS_{env} and in any association process will generate new molecules of A/A because of the reduction of the ligand and protein surface exposed to the solvent. It has been shown [36] that $\Delta G_{env} = 0$ at all temperatures, so that it must be

$$\Delta H^o_{env} – T\Delta S^o_{env} = \Delta G^o_{env} = 0$$

and then

$$\Delta H^o_{env} = T\Delta S^o_{env} \quad (7)$$

In other terms, the environmental parts of enthalpy and entropy are *always* related with compensation temperature

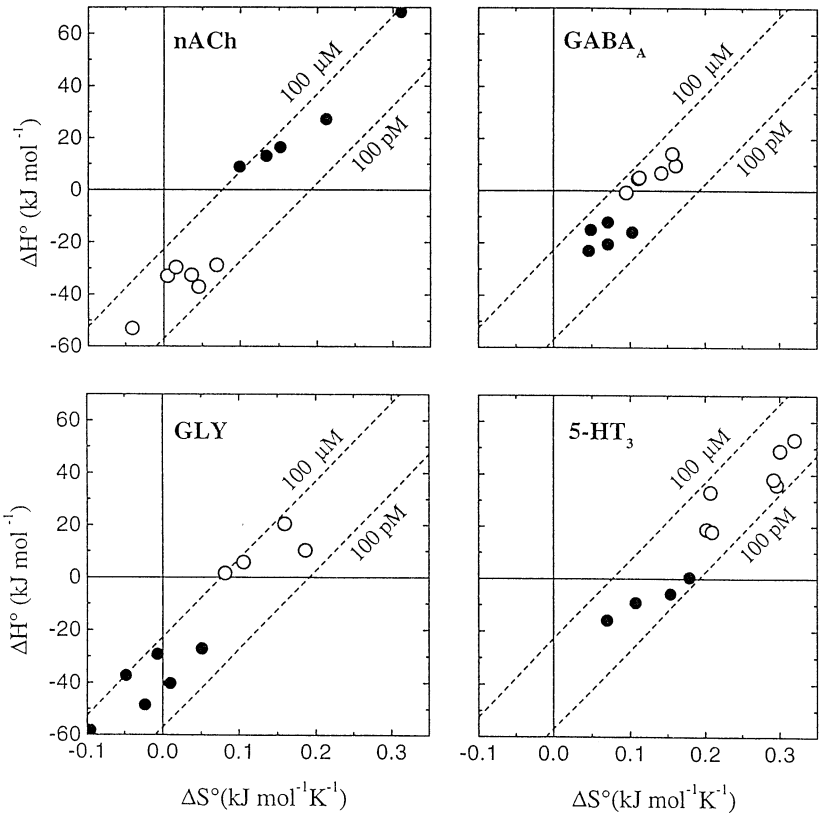

FIG. 6. Comparison of the ΔH^o vs ΔS^o scatter plots for the four LGIC receptors studied thus far. The two diagonal dashed lines are the loci of points for the limiting K_d values of 100 pM and 100 μM (o = agonists; ● = antagonists).

$\beta = \langle T_{exp} \rangle$, which is the mean experimental temperature. When the solvent is able to make reasonably strong interactions with itself ($A \ldots A$) and with the reaction partners ($A \ldots L$, $A \ldots R$, $A \ldots LR$), which is the case for hydrogen-bonding solvents such as water, and the dispersion of ΔG^o_{nom} values is intrinsically small (the case for ligand-receptor binding, for which $10^{-4} \geq K_d \geq 10^{-10}$ M), the variance of ΔH_{env} of Eqn. (6) can be much greater than that of ΔH^o_{nom} of Eqn. (5) because of the large number of water molecules usually displaced during the binding process [37]. In such a case, Eqn. (7) turns out to be nearly satisfied because $\Delta H^o = \Delta H^o_{nom} + \Delta H^o_{env} \approx \Delta H^o_{env}$ and $\Delta S^o = \Delta S^o_{nom} + \Delta S^o_{env} \approx \Delta S^o_{env}$, and the E-E compensation phenomenon is observed. Accordingly, in the ΔH^o vs ΔS^o scatter plot of Fig. 7, experimental points are arranged in a band whose width is the expression of the lack of intrinsic E-E compensation between nominal enthalpic and entropic terms, though a small nominal compensation can also be expected because stronger drug-receptor interactions are necessarily associated with a decrease in entropy. What is not easy to understand is why agonists and antagonists may be discriminated in a thermodynamic sense (that is, located in two disjointed regions of the E-E compensation band) if the compensation itself is mainly to be ascribed to a simple rearrangement of water molecules bearing little relationship with either binding affinity or intrinsic activity properties. This problem has already been debated for the β-adrenergic [12] and adenosine A_1 receptors [11], both of which belong to the class of GPC receptors, without a comprehensive explanation being found, because not all GPC receptors are actually thermodynamically discriminated [29, 38].

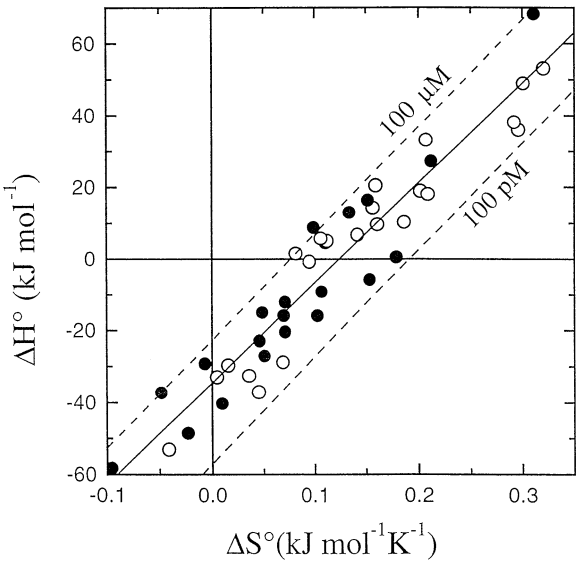

FIG. 7. Cumulative ΔH^o vs ΔS^o scatter plots for the four LGIC receptors of Fig. 6 (o = agonists; ● = antagonists).

A new hypothesis can now be suggested in connection with LGIC receptors. It was shown above that, while ΔG^o values are most probably determined by the features of the ligand-receptor binding process, global ΔH^o and ΔS^o values are expected to be markedly affected by the rearrangements occurring in the solvent. On these grounds, thermodynamic discrimination in ligand-gated ion channels becomes understandable if we recognize that the observed ΔH^o (and compensation-related ΔS^o) values are determined by both specific binding of the ligand and the variation in water-accessible receptor surface which occurs as a consequence of channel opening.

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References

1. Role LW, Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. *Curr Opin Neurobiol* **2:** 254–264, 1992.
2. Sargent PB, The diversity of neuronal nicotinic acetylcholine receptors. *Annu Rev Neurosci* **16:** 403–443, 1993.
3. Elgoyhen AB, Johnson D, Boulter J, Vetter D and Heinemann SF, Cloning and functional expression of alpha 9: A novel acetylcholine-gated ion channel. In: *International Symposium on Nicotine: The Effects of Nicotine on Biological Systems II* (Eds. Clarke PBS, Quick M, Thuran K and Adlkoffer F), p. 7. Birkhaüser Verlag, Basel, 1994.
4. Romano C and Goldstein A, Stereospecific nicotine receptors on rat brain membranes. *Science* **210:** 647–649, 1980.
5. Schwartz RD, Mc Gee R and Kellar KJ, Nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine in rat brain. *Mol Pharmacol* **22:** 56–62, 1982.
6. Boska P and Quiron R, [^{3}H]*N*-methylcarbamylcholine, a new radioligand specific for nicotinic acetylcholine receptors in brain. *Eur J Pharmacol* **139:** 323–333, 1987.
7. Schwartz RD and Kellar KJ, *In vivo* regulation of [^{3}H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J Neurochem* **45:** 427–433, 1985.
8. Marks MJ, Stitzel JA and Collins AC, Time-course study of the effects of chronic nicotine infusion on drug response and brain receptors. *J Pharmacol Exp Ther* **235:** 619–628, 1985.
9. Pabreza LA, Dhawan S and Kellar KJ, [^{3}H]cytisine binding to nicotinic cholinergic receptors in brain. *Mol Pharmacol* **39:** 9–12, 1990.
10. Hall M, Zerbe L, Leonard S and Freedman R, Characterization of [^{3}H]cytisine binding to human brain membrane preparations. *Brain Res* **600:** 127–133, 1993.
11. Borea PA, Varani K, Guerra L, Gilli P and Gilli G, Binding thermodynamics of A_1 adenosine receptor ligands. *Mol Neuropharmacol* **2:** 273–281, 1992.
12. Weiland GA, Minneman KP and Molinoff PB, Fundamental difference between the molecular interactions of agonists and antagonists with the β-adrenergic receptor. *Nature* **281:** 114–117, 1979.
13. Borea PA, Dalpiaz A, Varani K, Guerra L and Gilli G, Binding thermodynamics of adenosine A_{2a} receptor ligands. *Biochem Pharmacol* **49:** 461–469, 1995.
14. Maksay G, Thermodynamics of γ-aminobutyric acid A type receptor binding differentiate agonists from antagonists. *Mol Pharmacol* **46:** 386–390, 1994.
15. Borea PA, Dalpiaz A, Gessi S and Gilli G, Thermodynamics of 5-HT_3 receptor binding discriminates agonistic from antagonistic behaviour. *Eur J Pharmacol* **298:** 329–334, 1996a.
16. Maksay G, Distinct thermodynamic parameters of serotonin 5-HT_3 agonists and antagonists to displace [^{3}H]granisetron binding. *J Neurochem* **67:** 407–412, 1996.
17. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. *Anal Biochem* **72:** 248, 1976.
18. Munson PJ and Rodbard D, LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal Biochem* **107:** 220–239, 1980.
19. Cheng YC and Prusoff WH, Relationships between the inhibitory constant (K_i) and the concentration of inhibitory which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
20. Osborne JP, Palumbo JC, Brewer HB and Edlhoch H, The thermodynamics of the self-association of the reduced and carboxymethylated form of Apo A-II from the human high density lipoprotein complex. *Biochem* **15:** 317–320, 1976.
21. Krug RR, Hunter WG and Grieger RA, Statistical interpretation of enthalpy-entropy compensation. *Nature* **261:** 566–567, 1976.
22. Kauzmann W, Some factors in the interpretation of protein denaturation. *Adv Prot Chem* **14:** 1–63, 1959.
23. Waelbroeck M, Van Obberghen E and De Meyts P, Thermodynamics of the interaction of insuline with its receptor. *J Biol Chem* **254:** 7736–7740, 1979.
24. Sturtevant JM, Heat capacity and entropy changes in processes involving proteins. *Proc Natl Acad Sci USA* **74:** 2236–2240, 1977.
25. Hinz H-J, Thermodynamics of protein-ligand interactions: calorimetric approaches. *Ann Rev Biophys Bioeng* **12:** 285–317, 1983.
26. Spolar RS, Livingstone JR and Record MT, Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry* **31:** 3947–3955, 1992.
27. Spolar RS and Record MT, Coupling of local folding to site-specific binding of proteins to DNA. *Science* **263:** 777–784, 1994.
28. Kilpatrick GJ, El Tayar N, Van de Waterbeemd H, Jenner P, Testa B and Marsden CD, The thermodynamics of agonist and antagonist binding to dopamine D_2 receptors. *Mol Pharmacol* **30:** 226–234, 1986.
29. Dalpiaz A, Borea PA, Gessi S and Gilli G, Binding thermodynamics of 5-HT_{1A} receptor ligands. *Eur J Pharmacol* **312:** 107–114, 1996.
30. Kochman RL and Hirsch JD, Thermodynamic changes asociated with benzodiazepine and alkyl β-carboline-3-carboxylate binding to rat brain homogenates. *Mol Pharmacol* **22:** 335–341, 1982.
31. Borea PA, Dalpiaz A, Varani K, Gessi S and Gilli G, Binding thermodynamics at A_1 and A_{2a} adenosine receptors. *Life Sci* **59:** 1373–1388, 1996b.
32. Ruiz-Gómez A, García-Calvo M, Vázquez J, Marvizón JCG, Valdivieso F and Mayor F, Thermodynamics of agonist and antagonist interaction with the strychnine-sensitive glycine receptor. *J Neurochem* **52:** 1775–1780, 1989.
33. Gilli P, Ferretti V, Gilli G and Borea PA, Enthalpy-entropy compensation in drug-receptor binding. *J Phys Chem* **98:** 1515–1518, 1994.
34. Lumry R and Rajender S, Enthalpy-entropy compensation phe-

nomena in water solutions of proteins and small molecules: A ubiquitous property of water. *Biopol* **9:** 1125–1227, 1970.
35. Tomlinson E, Enthalpy-entropy compensation analysis of pharmaceutical, biochemical and biological systems. *Int J Pharmaceutics* **13:** 115–144, 1983.
36. Grunwald E and Steel C, Solvent reorganization and thermodynamic enthalpy-entropy compensation. *J Am Chem Soc* **117:** 5687–5692, 1995.
37. Colombo MF, Rau DC and Parsegian VA, Protein solvation in allosteric regulation: A water effect on hemoglobin. *Science* **256:** 655–659, 1992.
38. Testa B, Jenner P, Kilpatrick GJ, El Tayar N, Van de Waterbeemd H and Marsden CD, Do thermodynamic studies provide information on both the binding to and the activation of dopaminergic and other receptors? *Biochem Pharmacol* **36:** 4041–4046, 1987.