Characterization of [³H]-imidazenil binding to rat brain membranes

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1 The binding of [³H]-imidazenil, an imidazobenzodiazepine carboxamide, to rat cerebellar membranes was characterized at different temperatures.

2 Specific binding was linear with tissue concentrations and reached maximum after 90, 30 and 5 min incubation at 0, 21 and 37°C, respectively. The binding was of high affinity, specific and saturable; non linear regression and Scatchard analysis of the data was compatible with the presence of a single population of receptor sites with $B_{\max}$ of 0.74 ± 0.020 , 0.90 ± 0.011 and 1.0 ± 0.036 pmol mg⁻¹ protein at 0, 21 and 27°C, respectively. Binding affinity decreased with increasing temperature: K_d were 0.29 ± 0.051 nM (0°C), 1.0 ± 0.080 nM (21°C) and 2.4 ± 0.38 nM (37°C).

3 At all tested temperatures, [³H]-imidazenil binding was reversible and the K_d calculated from the dissociation and association rate constants approximated the equilibrium K_d .

4 In the presence of γ -aminobutyric acid (GABA), K_d increased 4 fold at 0°C, whereas $B_{\max}$ increased, albeit slightly, at all temperatures.

5 Benzodiazepines (BZDs), imidazopyridines and methyl- β -carboline-3-carboxylate (β CCM) were effective inhibitors of [³H]-imidazenil binding. Conversely, GABA_A antagonists, barbiturates, picrotoxin and peripheral BZD receptor ligands were devoid of any activity.

6 Comparing [³H]-imidazenil to [³H]-flumazenil binding in various brain areas, similar densities of recognition sites as well as like regional differences in the distribution of binding sites for both radioligands were observed (cortex = striatum > cerebellum > spinal cord).

7 The present results indicate that [³H]-imidazenil specifically binds to the BZD sites of GABA_A receptors. Furthermore, the effects of GABA and temperature differentiate imidazenil from classical BZDs. It is suggested that the characteristics of imidazenil binding may be relevant to the *in vivo* pharmacology of the drug.

Keywords: GABA_A receptor complex; benzodiazepine; partial agonist; [³H]-imidazenil binding; thermodynamic analysis; kinetic analysis

Introduction

Benzodiazepines (BZDs) are in widespread use as anxiolytics, anticonvulsants, hypnotics and myorelaxants (Greenblatt *et al.*, 1983). These neuropharmacological actions are presumably mediated by an enhancement of GABAergic synaptic transmission at GABA_A receptors (Costa *et al.*, 1975; Haefely *et al.*, 1975). Specific binding sites for BZDs have been reported on brain membranes, in close association with GABA_A receptors (Olsen, 1982). GABA and BZD binding sites are reciprocally regulated, since GABA or muscimol stimulate binding of [³H]-flunitrazepam to brain membranes (Olsen, 1982) and BZDs increase binding of GABA at GABA_A receptors (Matsumoto & Fukuta, 1982). Furthermore, the clinical potency of BZDs is positively correlated with their affinity for these [³H]-flunitrazepam binding sites. Therefore, it is assumed that the pharmacological and clinical effects of BZDs are exerted via a positive allosteric modulation of GABA action following a specific interaction with these binding sites named central BZD receptors (Haefely *et al.*, 1985). Conversely, unrelated to GABA_A receptor-associated BZD binding sites are peripheral BZD receptors, which are localized on the outer mitochondrial membranes of many tissues including the brain (Verma & Snyder, 1989).

A whole family of GABA_A receptor subtypes is now known following the successful cloning and expression of the receptor subunits (Seeburg *et al.*, 1990). The exact interplay of BZD with these receptor subtypes is currently being investigated. Recently, based on their distinctive actions at

different recombinant GABA_A receptors, a classification of BZDs into three categories has been proposed: (i) full allosteric modulators (FAMs) that act with high potency and efficacy at many GABA_A receptors; (ii) selective allosteric modulators (SAMs) that act with high potency and high efficacy at selective GABA_A receptors; and (iii) partial allosteric modulators (PAMs) that act with high potency but low efficacy at many GABA_A receptors (Puia *et al.*, 1991; Ducic *et al.*, 1993). The *in vivo* pharmacological activity of PAMs appears different from that of high efficacy allosteric modulators (i.e. SAMs and FAMs). In particular, the separation of the desirable (i.e. anxiolytic) and undesirable (i.e. sedation and ataxia) effects of some BZD receptor ligands has been attributed to their partial allosteric modulation and explained in terms of different receptor reserves of neural systems involved in the various effects of BZDs (Haefely *et al.*, 1990).

Imidazenil (6-(2-bromophenyl)-8-fluoro-4H-imidazo [1,5-a]-benzodiazepine-3-carboxamide), an imidazobenzodiazepine carboxamide has been recently characterized as a representative of the PAM class (Giusti *et al.*, 1993). In *in vivo* tests, imidazenil showed anticonflict and anticonvulsant properties. Furthermore, it was virtually devoid of side effects such as ataxia, sedation, potentiation of ethanol and barbiturates at doses considerably higher than those producing the anticonflict and anticonvulsant effects. In binding experiments, imidazenil displaced [³H]-flumazenil binding both *in vivo* and in crude synaptic membranes. On the other hand, it failed to displace a number of other radioligands from their binding sites, thus indicating no interaction with peripheral BZD,

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GABA_B, dopamine, 5-hydroxytryptamine (5-HT₁ and 5-HT₂), glutamate, opiate, cholecystokinin (CCK) and β -adrenoceptors.

To investigate further the binding properties of imidazenil, [³H]-imidazenil with high specific activity was synthesized and its interactions with BZD receptor sites on washed rat cerebellar membranes were studied. Moreover, the effects of GABA and temperature on [³H]-imidazenil binding were evaluated, since the affinity of many ³H-BZDs for their receptors is strongly affected by both factors (Speth *et al.*, 1978; Quast *et al.*, 1982; Earle *et al.*, 1987). Finally, binding parameters were measured in different rat brain areas and compared with those obtained from the binding of [³H]-flumazenil, which is a selective, high affinity and competitive ligand at central BZD receptors (Hunkeler *et al.*, 1981). We are now reporting that the high affinity, saturable and specific binding of [³H]-imidazenil to rat cerebellar membranes is associated with the BZD receptor site on the GABA_A receptor macromolecular complex at which it exhibits a peculiar profile with respect to known positive allosteric modulators.

Methods

Membrane preparation

Male SD/Rij rats (FRAR, S. Pietro al Natisone, UD, Italy) weighing 200–250 g were used. Different brain areas were rapidly dissected on ice and homogenized in 20 ml of ice-cold 0.32 M sucrose, pH 7.4, in a glass homogenizer with a Teflon pestle (10 up and down strokes).

The homogenate was centrifuged at 1,000 g at 4°C for 10 min, the P₁ pellet was discarded and the supernatant was collected and recentrifuged at 20,000 g at 4°C for 20 min. The resulting crude mitochondrial pellet (P₂) was resuspended in 20 ml of ice-cold distilled water and homogenized. The homogenate was centrifuged at 8,000 g at 4°C for 20 min, the supernatant was collected and recentrifuged at 48,000 g at 4°C for 20 min and the final crude microsomal pellet (P₃) was frozen for at least 24 h.

The pellet was resuspended in 10 ml of 50 mM Tris-HCl pH 7.4, centrifuged at 48,000 g at 4°C for 20 min and then resuspended in 10 volumes of Tris-HCl, pH 7.4 + NaCl 120 mM + KCl 5 mM (incubation medium) for standard binding assay.

Radioligand binding assay

Aliquots of membrane suspensions (100 μ l, or 0.15 mg of protein) were added to incubation medium containing different concentrations of [³H]-imidazenil (specific activity 20.65 Ci mmol⁻¹) or [³H]-flumazenil (specific activity 72.4 Ci mmol⁻¹) in a final volume of 1 ml.

Incubations were carried out in triplicate and non specific binding measured in the presence of 1 μ M flumazenil, dissolved in 1% dimethylsulphoxide (DMSO). In saturation experiments, reactions were carried out for 90 min at 0°C, 30 min at 21°C and 5 min at 37°C for [³H]-imidazenil and for 60 min at 21°C for [³H]-flumazenil. Reactions were stopped by the addition of 5 ml ice-cold Tris-HCl followed by rapid filtration through Whatman GF/C glass fibre filters (Whatman Inc., Clifton, NJ) and two additional washes.

The radioactivity trapped on the filters was counted after the addition of 8 ml of Filter Count (Packard), by liquid scintillation spectrometry. Saturation binding isotherm were analysed either according to Scatchard (1949) or by a non linear regression computer programme (Sacchi-Landriani *et al.*, 1983).

Kinetic experiments

Kinetic studies of [³H]-imidazenil were performed at 0, 21 and 37°C. In association studies, membranes were incubated

with [³H]-imidazenil (1 nM) in the presence or absence of 1 μ M flumazenil to define specific binding. Samples were filtered at different times as described above. Because less than 10% of the radioligand added was bound to the tissue homogenate, pseudo-first-order kinetics could be assumed. The association rate constants K_1 were determined from the slope of the pseudo-first-order plot, $\ln B_0/(B_0 - B_t)$, where B_0 is the equilibrium binding in association experiments and B_t is the amount of specific binding at time t (Weiland & Molinoff, 1981). The slope of the plot is the observed rate constant (K_{obs}) for the pseudo-first-order reaction. The association rate constant K_1 was calculated from $K_1 = (K_{obs} - K_{-1})/[^3H\text{-imidazenil}]$. K_{-1} is the dissociation rate constant and [³H]-imidazenil is the used concentration of radioligand. Dissociation studies were performed by incubating membranes with [³H]-imidazenil (1 nM) to equilibrium and then adding excess cold flumazenil (10 μ M) to prevent rebinding of dissociated [³H]-imidazenil. The remaining binding of [³H]-imidazenil was measured at different time intervals. Dissociation rate constant (K_{-1}) followed first-order kinetics and was calculated from the slope of the first order plot, $\ln B_0/B_t$, where B_0 and B_t are binding at equilibrium and time t , respectively and t is the time after the addition of flumazenil. The slope of the first-order plot is the dissociation constant $-K_{-1}$ (Weiland & Molinoff, 1981). The K_d for the kinetic binding experiments was determined from the quotient of K_{-1}/K_1 .

Effect of ATP on association kinetics

The association of [³H]-imidazenil (1 nM) was measured as a function of time at 21°C in the absence or presence of 5 mM ATP. The rate constant K_{obs} was calculated as described above.

Thermodynamics

Thermodynamic analysis was performed according to Weiland & Molinoff (1981). The K_d values were plotted on a semilogarithmic scale versus T^{-1} (Van't Hoff plot). The change in enthalpy (ΔH) can be calculated from the slope of the plot, $-\Delta H/R$, where R is the gas constant (1.987 cal degree⁻¹ mol⁻¹). The free energy of binding (ΔG), is related to the equilibrium dissociation constant K_d by the following formula: $\Delta G = -RT \ln K_A$, where R is the gas constant, T is the absolute temperature and K_A is $1/K_d$. The variations in free energy and enthalpy are connected to the changes in entropy (ΔS) by the Gibbs equation: $\Delta G = \Delta H - T\Delta S$.

Effect of GABA on the specific binding of [³H]-imidazenil

GABA (100 μ M) was added to washed, frozen-thawed membranes in the presence of different concentrations of [³H]-imidazenil (0.1–90 nM) at 0, 21 and 37°C. Incubations were carried out as described above. Specific binding was defined in the presence of 1 μ M flumazenil.

Inhibition of [³H]-imidazenil binding by various drugs

Increasing concentrations (0.01 nM–10 μ M) of drugs were dissolved in DMSO (1%) and added to 1 nM [³H]-imidazenil. All subsequent steps were performed as above. IC₅₀ was defined as the concentration of drug that elicited half-maximal inhibition of specific binding. The inhibition constant (K_i) was calculated with the Cheng and Prusoff equation, $K_i = IC_{50}/(1 + L/K_d)$ where L is the concentration of free labelled ligand and K_d the equilibrium dissociation constant of the labelled ligand (Cheng & Prusoff, 1973).

Drugs

Imidazenil and [³H]-imidazenil were synthesized at Hoffmann-La Roche (Nutley, NJ, U.S.A.). The radiochemical

purity of $[^3\text{H}]$ -imidazenil was initially found to be 99% when determined by high pressure liquid chromatography on a ODS-10 column (250 by 4 mm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using the following mobile phase: 60% acetonitrile-0.1% trifluoroacetic acid. $[^3\text{H}]$ -flumazenil was obtained from New England Nuclear (Boston, MA, U.S.A.).

Diazepam, bretazenil, flumazenil, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]benzodiazepine-3-carboxylate (Ro 15-4513) and clonazepam were a gift from Hoffman-La Roche (Nutley, NJ, U.S.A.). Zolpidem and alpidem were generously supplied from Synthelabo (Paris, France). Triazolam was obtained from Upjohn Co. (Kalamazoo, MI, U.S.A.). Thiopentone, bicuculline, pentylene-tetrazole (PTZ), GABA and ATP were purchased from Sigma (St. Louis, MO, U.S.A.). 1-(2-Chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline carboxamide (PK 11195) and methyl- β -carboline-3-carboxylate (βCCM) were obtained from RBI (Natick, MA, U.S.A.).

Results

Radioligand binding assay

The specific binding of $[^3\text{H}]$ -imidazenil increased linearly with the concentration of rat cerebellar membranes up to 0.25 mg ml^{-1} (data not shown). A tissue concentration of 0.15 mg ml^{-1} was subsequently used for routine binding studies. The binding of $[^3\text{H}]$ -imidazenil to rat cerebellar membranes, evaluated as a function of radioactive ligand concentration, indicated the occurrence of high-affinity, specific and saturable binding sites at 0, 21 and 37°C (Figure 1). Non linear regression and Scatchard analysis of the data were compatible with the presence of a single population of receptor sites at all tested temperatures. The maximal density of binding sites (B_{max}) was 0.74 ± 0.02 , 0.90 ± 0.01 and $1.01 \pm 0.04 \text{ pmol mg}^{-1}$ protein at 0, 21 and 37°C , respectively. The equilibrium dissociation constants (K_d) were $0.29 \pm 0.052 \text{ nM}$ (0°C), $1.0 \pm 0.084 \text{ nM}$ (21°C) and $2.4 \pm 0.38 \text{ nM}$ (37°C).

Kinetic experiments

Kinetics of $[^3\text{H}]$ -imidazenil binding was performed at the same temperatures and indicated that binding was rapid and fully reversible. In association studies, the reaction reached equilibrium after 90 min at 0°C , 25 min at 21°C and less than 5 min at 37°C . No further binding was observed up to 24 h (0°C) (data not shown), 4 h (21°C) and 1 h (37°C) (Figure 2). At 37°C , the maximum specific binding attained with 1 nM $[^3\text{H}]$ -imidazenil was $0.45 \pm 0.0073 \text{ pmol mg}^{-1}$ protein, whereas it was 0.77 ± 0.083 and 0.77 ± 0.0051 at 0 and 21°C , respectively (Figure 2). The association rate constants (K_1) were calculated, as described in Methods, at all temperatures with the exception of 37°C , where the equilibrium state was reached too fast to allow the calculation of a reliable value of K_1 (Table 1). The addition of 5 mM ATP to the incubation medium did not modify the association rate constant at 21°C . In fact, K_{obs} values were 0.097 ± 0.015 and $0.098 \pm 0.017 \text{ min}^{-1}$ in the absence or presence of ATP, respectively. Maximum specific binding was 0.77 ± 0.021 ($- \text{ATP}$) and 0.72 ± 0.023 ($+ \text{ATP}$) pmol mg^{-1} protein. In dissociation studies, $[^3\text{H}]$ -imidazenil binding appeared fully reversible at all tested temperatures (Figure 3). The reaction was very slow at 0°C , but it proceeded faster with increasing temperatures. For instance, at 37°C , $[^3\text{H}]$ -imidazenil was completely displaced from its binding sites in less than 5 min (Figure 3). The calculated dissociation rate constants (K_{-1}) are shown in Table 1. The K_d values calculated according to the formula ($K_d = K_{-1}/K_1$) approximated the dissociation constants measured in equilibrium binding studies, when determined (Table 1).

Thermodynamics

Thermodynamic analysis of receptor-ligand interaction showed that binding of $[^3\text{H}]$ -imidazenil to its recognition sites was associated with a decrease in enthalpy and only a slight decrease in entropy. ΔH was $-9.12 \text{ kcal mol}^{-1}$ at all the temperatures; ΔG were -11.91 , -12.08 and $-12.23 \text{ kcal mol}^{-1}$ at 0, 21 and 37°C , respectively; ΔS were $+10.22$,

Figure 1 Binding of $[^3\text{H}]$ -imidazenil ($0.1\text{--}90 \text{ nM}$) to rat cerebellar membranes was performed under standard conditions at 0°C (○), 21°C (●) and 37°C (▽), as described in Methods. Results are presented as the mean values $\pm$ s.e.mean from at least three observations. Inset shows Scatchard plot for specific $[^3\text{H}]$ -imidazenil binding at the same temperatures. The mean values $\pm$ s.e.mean of the K_d and B_{max} are given in the text.

Figure 2 Association kinetics of $[^3\text{H}]$ -imidazenil (1.0 nM) binding were measured as a function of time at 0°C (○), 21°C (●) and 37°C (▽). Specific binding was determined in the presence of $1 \mu\text{M}$ flumazenil. Inset shows pseudo-first-order kinetic plot of specific $[^3\text{H}]$ -imidazenil binding at all tested temperatures. Association rate constants are shown in Table 1.

Table 1 $[^3\text{H}]$ -imidazenil kinetic parameters on rat cerebellar membranes

Temperature ($^\circ\text{C}$)	K_1 ($\text{min}^{-1} \text{ nM}^{-1}$)	K_{-1} (min^{-1})	K_d (nM)
0	0.023 ± 0.0011	0.0041 ± 0.0030	0.18 ± 0.0020
21	0.032 ± 0.015	0.063 ± 0.0056	1.99 ± 0.012
37	ND ^a	0.70 ± 0.047	ND

Assays of $[^3\text{H}]$ -imidazenil binding were performed as described in Methods. Each kinetic value is the mean $\pm$ s.e.mean from three independent determinations. ^aND, not determined.

+10.07 and +10.03 kcal mol⁻¹ T⁻¹ at 0, 21 and 37°C, respectively. In addition, when the dependence upon temperature of the K_d of $[^3\text{H}]$ -imidazenil binding was studied using a Van't Hoff plot, the plotted data were fitted by a straight line ($r = 0.984$). K_d were: 0.29 ± 0.051 nM (0°C); 0.83 ± 0.10 nM (12°C); 1.0 ± 0.080 nM (21°C); 1.9 ± 0.25 (30°C); and 2.4 ± 0.38 (37°C).

Effect of GABA on the specific binding of $[^3\text{H}]$ -imidazenil

The addition of GABA (100 μM) to the incubation medium slightly increased the B_{max} values of $[^3\text{H}]$ -imidazenil binding to washed cerebellar membranes. Similar values of K_d were obtained at 21° and 37°C, in the absence or presence of GABA. In contrast, at 0°C, a fourfold increase in K_d could be observed (Table 2).

Inhibition of $[^3\text{H}]$ -imidazenil binding by various drugs

Specificity of imidazenil binding to central BZD receptors was studied in displacement experiments by use of several substances interacting with distinct sites on the GABA_A receptor complex. $[^3\text{H}]$ -imidazenil binding to rat cerebellar membranes was effectively inhibited by BZDs (bretazenil, clonazepam, diazepam, flumazenil, imidazenil, Ro 15-4513 and triazolam), imidazopyridines (alpidem and zolpidem) and a β -carboline (βCCM). Among these drugs, bretazenil, imidazenil and βCCM were the most effective displacers (Table 3). Triazolam and flumazenil were effective inhibitors at nanomolar concentrations, followed by Ro 15-4513 and clonazepam. Diazepam, alpidem and zolpidem were less

effective as displacing agents, whereas bicuculline, thiopentone, PTZ and PK 11195 were completely inactive (Table 3).

Regional distribution of $[^3\text{H}]$ -imidazenil binding sites

Marked regional variations in $[^3\text{H}]$ -imidazenil binding parameters were measured in different rat brain areas. The greatest density of binding sites was found in rat cerebral cortex and striatum; the cerebellum exhibited a smaller number of binding sites and the spinal cord displayed the least amount of binding (Table 4). A similar number as well as regional distribution of $[^3\text{H}]$ -flumazenil binding sites was observed (Table 4).

Discussion

Our data showed that the binding of $[^3\text{H}]$ -imidazenil to rat cerebellar membranes met most conventional criteria defining a specific receptor-ligand interaction. It was of high affinity, saturable, reversible and specific between 0 and 37°C. Moreover, binding analysis indicated the presence of a single population of receptor sites. We measured a temperature-dependent change in the binding affinity whereas the maximal number of recognition sites was unchanged. The same pattern of K_d changes was observed using a kinetic analysis of $[^3\text{H}]$ -imidazenil binding. These results are consistent with previous findings, that indicate a temperature dependence for *in vitro* ^3H -BZD binding (Speth *et al.*, 1978; Quast *et al.*, 1982; Möhler *et al.*, 1981).

In association studies, the $[^3\text{H}]$ -imidazenil maximum

Figure 3 Dissociation kinetics of $[^3\text{H}]$ -imidazenil binding. $[^3\text{H}]$ -imidazenil was allowed to equilibrate for 90 min at 0°C (○), 30 min at 21°C (●) and 5 min at 37°C (▽). After equilibrium binding, excess cold flumazenil (10 μM) was added for the indicated time and residual binding was measured. Inset shows a semilogarithmic plot of B_t/B_0 as a function of time. Dissociation rate constants are shown in Table 1.

Table 3 Inhibition of $[^3\text{H}]$ -imidazenil binding on rat cerebellar membranes

Displacers	K_i (nM)
Bretazenil	0.03 ± 0.0051
Imidazenil	0.24 ± 0.032
βCCM	0.45 ± 0.042
Triazolam	0.98 ± 0.14
Flumazenil	1.40 ± 0.23
Ro 15-4513	3.6 ± 0.42
Clonazepam	4.5 ± 0.43
Diazepam	20 ± 1.8
Zolpidem	33 ± 2.9
Alpidem	39 ± 2.7
Bicuculline	$>30,000$
Thiopentone	$>30,000$
PTZ	$>30,000$
PK 11195	$>30,000$

Displacement of $[^3\text{H}]$ -imidazenil (1 nM) was performed as described in Methods at 21°C. Data are presented as mean values $\pm$ s.e. mean from three observations. Each curve consisted of at least nine concentrations, each performed in triplicate.

Table 2 Effect of GABA on $[^3\text{H}]$ -imidazenil binding to rat cerebellar membranes

Temperature (°C)	- GABA		+ GABA	
	B_{max} (pmol mg ⁻¹ protein)	K_d (nM)	B_{max} (pmol mg ⁻¹ protein)	K_d (nM)
0	0.74 ± 0.020	0.29 ± 0.051	0.9 ± 0.026	1.2 ± 0.15
21	0.90 ± 0.011	1.0 ± 0.080	1.2 ± 0.055	1.4 ± 0.15
37	1.0 ± 0.036	2.4 ± 0.38	1.2 ± 0.035	2.3 ± 0.13

Binding of $[^3\text{H}]$ -imidazenil (0.1–90 nM) was performed under standard conditions, at the indicated temperatures, in the presence or absence of GABA (100 μM). Results are presented as mean values $\pm$ s.e. mean from three independent experiments, each performed in triplicate.

Table 4 Regional distribution of specific $[^3\text{H}]$ -imidazenil and $[^3\text{H}]$ -flumazenil binding in rat CNS

	$[^3\text{H}]$ -imidazenil		$[^3\text{H}]$ -flumazenil	
	B_{max} (pmol mg^{-1} protein)	K_d (nM)	B_{max} (pmol mg^{-1} protein)	K_d (nM)
Cerebellum	0.90 ± 0.01	1.04 ± 0.08	0.88 ± 0.12	1.3 ± 0.18
Cortex	1.4 ± 0.14	0.89 ± 0.012	1.2 ± 0.014	1.3 ± 0.023
Striatum	1.2 ± 0.0012	1.5 ± 0.056	0.98 ± 0.052	1.6 ± 0.13
Spinal cord	0.24 ± 0.011	0.90 ± 0.014	0.22 ± 0.023	3.8 ± 0.12

Washed brain membranes from various CNS regions were prepared as described in Methods and incubated with different concentrations of $[^3\text{H}]$ -imidazenil for 90 min at 21°C or $[^3\text{H}]$ -flumazenil for 60 min at 21°C . Results are presented as mean values $\pm$ s.e.mean from three independent experiments.

specific binding at 37°C was nearly a half of that measured both at 0 and 21°C . This observation is apparently in contrast with the results of saturation experiments. An explanation for this discrepancy could be that a conformational rearrangement of the GABA_A receptor complex takes place at high temperature. The β -subunits of the GABA_A receptors contain sites for intracellular phosphorylation and this process may be crucial in the regulation of GABA_A receptors (Zorumski *et al.*, 1991). Therefore, a temperature-dependent high level of GABA_A receptor phosphorylation could account for the reduced number of $[^3\text{H}]$ -imidazenil binding sites measured at 37°C . Should this be the case, the addition of ATP, at 21°C , to stimulate a receptor kinase that phosphorylates ligand-receptor complexes (Sibley *et al.*, 1987), would have reduced the amount of $[^3\text{H}]$ -imidazenil binding to the same value observed at 37°C . The number of binding sites, however, was not decreased in the presence of ATP. It is possible that at 37°C , when incubation is protracted beyond equilibrium, some proteolysis of receptors occurs. Because of the inadequacy of the binding approach, an answer to this question can be obtained only by using transfected cells containing cDNA encoding for the different GABA_A receptor subunits.

Thermodynamic studies showed that $[^3\text{H}]$ -imidazenil binding was enthalpy and entropy driven. K_d and temperature were related by a linear Van't Hoff plot. Similar results have been reported for $[^3\text{H}]$ -flumazenil binding (Möhler *et al.*, 1981; Kopp *et al.*, 1990). Conversely, a break in linearity at about 16°C has been observed for $[^3\text{H}]$ -flunitrazepam (Speth *et al.*, 1978), $[^3\text{H}]$ -clonazepam (Möhler *et al.*, 1981) and $[^3\text{H}]$ -diazepam (Braestrup *et al.*, 1977) binding. This phenomenon has been described in the context of a two-step interaction model of receptor binding (Weiland *et al.*, 1979; Möhler *et al.*, 1981). Accordingly, all BZD ligands bind to their receptors with similar thermodynamic components at low temperature, but only BZD ligands provided with intrinsic activity are able to induce a conformational change of the receptors above 16°C .

The interaction of BZDs with their recognition site at the GABA_A receptor is allosterically modulated by the presence of GABA (GABA shift) (Möhler *et al.*, 1981; Braestrup *et al.*, 1982; Earle *et al.*, 1987). The binding of SAMs and FAMs is markedly enhanced by GABA, whereas that of PAMs or ligands with no intrinsic activity is largely unaffected (Giusti *et al.*, 1993). Unlike positive allosteric modulators, the potency of βCCM (a negative allosteric modulator) in displacing $[^3\text{H}]$ -flumazenil is decreased in the presence of GABA (Möhler *et al.*, 1981). A negative (at 0°C) or no shift (21 and 37°C) in the K_d of $[^3\text{H}]$ -imidazenil binding was measured in the presence of GABA. On the contrary, in a previous paper (Giusti *et al.*, 1993) we showed that GABA increased 1.7 fold the potency of imidazenil in displacing $[^3\text{H}]$ -flumazenil binding and the effect was less than that obtained with diazepam. Differences in temperature and binding methodology could explain such discrepancies. It should be considered, however, that only few partial agonists

have been studied so far to infer a general behaviour for this class of drugs in the GABA shift.

In displacement experiments, only BZD ligands (BZDs, imidazopyridines and βCCM) were shown to be active inhibitors of $[^3\text{H}]$ -imidazenil binding. Compounds acting at other sites on the GABA_A receptor complex showed no significant inhibition of $[^3\text{H}]$ -imidazenil binding. The experiments were performed at 21°C . Then, similar to what has been reported in saturation studies, a leftward shift in K_i could be expected if binding is measured at lower temperatures. However, the rank order of potency of the various compounds should be maintained (Speth *et al.*, 1978).

In regional studies $[^3\text{H}]$ -imidazenil marked the same number of binding sites as $[^3\text{H}]$ -flumazenil, in agreement with data reported for positive allosteric modulators such as clonazepam (Möhler *et al.*, 1981) and zolpidem (Arbilla *et al.*, 1986), which selectively bind to the central type of the BZD receptors.

Altogether, the present results show that imidazenil selectively binds to the BZD site located on the supramolecular GABA_A receptor complex. Moreover, GABA- and temperature-induced effects on K_d indicate that imidazenil interacts with this receptor in a manner similar to flumazenil. Imidazenil, therefore, should be classified as a BZD ligand with no intrinsic activity. Findings that this agent failed to modify $[^3\text{S}]\text{-TBPS}$ binding and muscimol-stimulated $^{36}\text{Cl}^-$ uptake (Serra *et al.*, 1994) seem consistent with this suggestion. However, a clear PAM profile for imidazenil has emerged from both *in vitro* and *in vivo* tests (Giusti *et al.*, 1993). In these studies imidazenil was compared to several BZDs, including clonazepam. Clonazepam is the only commercially available BZD ligand showing features of a PAM; in fact, its intrinsic efficacy is lower than that of diazepam (Bonetti *et al.*, 1987). Nevertheless, imidazenil and clonazepam exhibit different pharmacological profiles in rats. Imidazenil is more potent but less effective than clonazepam against bicuculline- and PTZ-induced seizures (Giusti *et al.*, 1993). Clonazepam, but not imidazenil, was found to potentiate the action of ethanol and thiopentone and to produce sedation and ataxia at anxiolytic doses (Giusti *et al.*, 1993). Moreover, tolerance to the anticonvulsant effects of clonazepam has been reported (Gent *et al.*, 1985; Young *et al.*, 1987), while preliminary evidence indicates low tolerance liability for imidazenil (Auta *et al.*, 1994). Furthermore, binding studies showed that, unlike imidazenil, clonazepam interacts with BZD receptors in a manner that is closer to that of FAMs and SAMs (Möhler *et al.*, 1981). It is suggested that the peculiar nature of imidazenil interaction with the BZD- GABA_A supramolecular receptor complex could account for the production of beneficial effects (i.e. anxiolytic and anticonvulsant) with less unwanted outcomes (i.e. sedation, ataxia and tolerance) after imidazenil administration. The study of the thermodynamic interactions at BZD- GABA_A receptors may then allow the identification of active BZD ligands with minimal side effects.

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