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Inhibition of γ-aminobutyric acid stimulated [^{3}H]diazepam binding by benzodiazepine receptor ligands

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The recent demonstration of specific high affinity receptor sites for the benzodiazepines in brain tissue [1, 2] has provided insights concerning the mechanism of action of these drugs. Furthermore, the high correlation between receptor binding affinity and the pharmacologic potency of the benzodiazepines [3] indicates that this receptor is involved in mediating the action of these drugs and provides a convenient means for screening new potential anxiolytic and anticonvulsant drugs. Recent studies have focused on the isolation and characterization of endogenous ligands [4] for the benzodiazepine receptor. Using inhibition of specific [^{3}H]diazepam binding to assay fractionated tissue extracts, a growing list of putative endogenous ligands for the benzodiazepine receptor has emerged which includes the purines [5–7], nicotinamide [8], γ-aminobutyric acid (GABA) modulin [9], β-carboline ethyl esters [10], and various other unidentified fractions [11, 12]. The purines and nicotinamide are the only putative ligand candidates which also display benzodiazepine-like neurophysiologic [8, 13] and pharmacologic [8, 14] effects, but their relatively low receptor binding potencies have raised questions concerning their physiologic relevance as ligands or modulators of the receptor *in vivo*.

A large body of evidence indicates that the benzodiazepines exert at least some of their effects via the inhibitory neurotransmitter GABA. The benzodiazepines potentiate GABA-mediated processes [15] and antagonize convulsions caused by GABA depletion [16]. *In vitro* studies have also shown that GABA stimulates [^{3}H]diazepam binding to extensively washed synaptosomal membranes [17, 18]. The neurophysiological and biochemical evidence, therefore, clearly indicates that the benzodiazepine and GABA receptors are tightly coupled, although the biochemical basis of this coupling remains largely unknown.

We now report that the benzodiazepines and several putative endogenous ligand candidates inhibit GABA-stimulated [^{3}H]diazepam binding at concentrations 2- to 5-fold lower than those required to inhibit basal, non-GABA-stimulated binding.

Synaptosomal membranes were prepared from fresh rat forebrain tissue (male Sprague–Dawley, 100–125 g) according to the procedure described previously [4]. The P_2 pellet was washed four times in 50 original tissue volumes of 50 mM Tris–Cl buffer, pH 7.3 (assay buffer). The final membrane suspension was frozen for at least 16 hr at −20°. Immediately before use, the membrane suspension was thawed, centrifuged at 30,000 *g* for 30 min and resuspended in 50 volumes of assay buffer. Protein determinations were performed as described by Lowry *et al.* [19].

The [^{3}H]diazepam receptor binding assay was performed as described previously [3, 6] with minor modifications. Each assay contained 0.1 to 0.2 mg of the extensively washed membrane protein, [^{3}H]diazepam (New England Nuclear Corp., Boston, MA, 80 Ci/mmole), and the indicated additions. The total assay volume was 0.5 ml and the final buffer concentration 50 mM (assay buffer). The incubations were terminated by vacuum filtration using Whatman GF-B filters and four 3 ml washes with ice-cold assay buffer. The filters were counted by liquid scintillation counting in Aquasol. Under these conditions approximately 5000 dpm per assay was obtained at 1.25 nM [^{3}H]diazepam. Non-specific binding was determined by the incorporation of 3 μM unlabeled diazepam (Hoffmann-LaRoche, Nutley, NJ) and routinely represented 5 per cent of the total bind-

ing. Incorporation of 1 μM GABA routinely increased the binding to 8000 dpm with negligible effects on non-specific binding. The IC_{50} values for inhibition of GABA-enhanced binding were estimated by reading the ligand concentration inhibiting basal or GABA-stimulated binding by 50 per cent directly from a semilog plot.

Table 1 summarizes the effects of all the compounds tested on GABA-stimulated and basal [^{3}H]diazepam binding. The benzodiazepines, purines, methylxanthines, and nicotinamide were all more potent as inhibitors of GABA-stimulated, than of basal, binding. The inosine derivative 7-methyl inosine which has been shown previously to be virtually without activity as an inhibitor of basal [^{3}H]diazepam binding [6] was also inactive as an inhibitor of GABA-stimulated binding. The rank order of potencies of the benzodiazepine receptor ligands tested as inhibitors of basal [^{3}H]diazepam binding was very similar to their relative potencies as inhibitors of GABA-stimulated binding.

Reciprocal Lineweaver–Burk analysis of bound [^{3}H]diazepam versus the concentration of [^{3}H]diazepam, in the presence and absence of 1 μM GABA, revealed that GABA increased the apparent affinity of the benzodiazepine receptor for diazepam, a finding that is consistent with previous reports [17]. K_D values of 5.0 nM were routinely obtained for [^{3}H]diazepam binding in the absence of added GABA and 3 nM in the presence of 1 μM GABA. Incorporation of IC_{50} concentrations of inosine (Fig. 1) to the GABA-stimulated curve yielded a K_D value (absissa intersect) intermediate between the basal and stimulated values, indicating that the inhibition of GABA-enhanced [^{3}H]diazepam binding by inosine was competitive. Similar results were obtained with unlabeled diazepam and with 2-deoxyinosine.

The benzodiazepines diazepam, clonazepam, and chlordiazepoxide have all been shown to inhibit GABA-stimulated [^{3}H]diazepam binding at concentrations lower than those required to inhibit basal binding. The inhibition was competitive and was also exhibited by putative endogenous benzodiazepine receptor ligands such as the purines and nicotinamide. It is especially interesting that chlordiazepoxide was greater than 7-fold more potent as an inhibitor of GABA stimulated binding compared to clonazepam and diazepam, which were 2- to 3-fold more potent. Chlordiazepoxide was, therefore, only 20-fold less potent than diazepam as an inhibitor of GABA-enhanced diazepam binding, whereas it was 60-fold less potent as an inhibitor of basal binding. The closer relative potencies of diazepam and chlordiazepoxide as inhibitors of GABA-stimulated diazepam binding are more consistent with their relative clinical potencies since the average daily therapeutic dose of chlordiazepoxide is only approximately 5-fold higher than that of diazepam [3].

The data suggests that modulation of the GABA-enhanced state of the benzodiazepine receptor may be more relevant to the actions of these agents than modulation of the artifically created unstimulated receptor (in the absence of GABA). The high levels of GABA present in brain suggest that the benzodiazepine receptor is in the stimulated state *in vivo*. Furthermore, the greater potency of the purines as inhibitors of GABA-stimulated diazepam binding increases the likelihood that these compounds are physiologically relevant since purine concentrations that would have significant effects on GABA-stimulated binding *in vitro* have been reported to exist in brain [20] (P. J. Marangos, E. Trams, R. L. Clark-Rosenberg, S. M. Paul and P. S. Kolnick, unpublished results). The benzodiazepine RO-3663, a convulsant, also inhibits GABA-stimulated [^{3}H]diazepam binding at concentrations that do not affect basal binding [21]. It, therefore, appears that both benzodiazepine receptor ligands with activating effects, i.e. RO-3663 and the methylxanthines (caffeine and theophylline), and those with tranquilizing anticonvulsant effects (diazepam and inosine), are able to inhibit GABA-stimulated diazepam binding with a higher potency than basal binding.

Table 1. Inhibition of GABA-stimulated and basal [^{3}H]diazepam binding by various benzodiazepine receptor ligands*

Compound	K_i GABA-stimulated diazepam binding (1.0 μM GABA)	K_i basal diazepam binding
	(nM)	
Diazepam	2.4	5.2
Chlordiazepoxide	49	375
Clonazepam	0.289	1.0
	(μM)	
Inosine	310	720
Hypoxanthine	384	780
Guanosine	316	704
Adenosine	804	1920
7-Methyl inosine	>2000	>5000
2-Deoxyinosine	109	264
2-Deoxyguanosine	114	256
Caffeine	129	284
Theophylline	216	472
Nicotinamide	1115	3600

* The compounds indicated were tested for their inhibition of basal and GABA-stimulated [^{3}H]diazepam binding as described in the text. The concentration of [^{3}H]diazepam was 1.25 nM in all experiments. The K_i values were calculated using the formula

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}.$$

The K_D values used were 5.0 nM (basal binding) and 3.0 nM for 1.0 μM GABA. The values reported are means of three to five separate IC_{50} determinations. Each determination was done using eight concentrations of the competing unlabeled ligand.

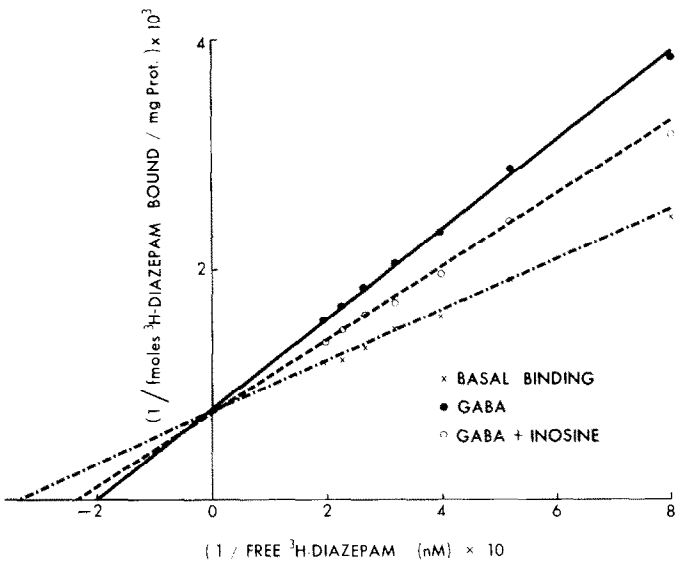

Fig. 1. Lineweaver–Burk analysis of the inhibition of GABA-stimulated [^{3}H]diazepam binding by inosine. Increasing amounts of [^{3}H]diazepam (1.25 to 10.1 nM) were incubated with synaptosomal membranes in the presence and absence of GABA (1 μM) and with GABA + 150 μM inosine. The observed K_D values for [^{3}H]diazepam binding were 5.0 nM in the absence of GABA and 3.0 nM in the presence of GABA.

Although the neurophysiologic and pharmacologic consequences of the benzodiazepine–GABA synergism have been amply observed, the molecular mechanism of this phenomenon remains unknown. The existence of the GABA agonists THIP (4,5,6,7-tetrahydroisoxazolo) (4,5-C) pyridin (3-OL) and isoquavicine which inhibit GABA binding but do not enhance diazepam binding [22–24] suggest that a novel state of the GABA receptor may be associated with the benzodiazepine receptor. The observed greater potency of benzodiazepines as inhibitors of GABA-enhanced [^{3}H]diazepam binding relative to basal binding is, however, consistent with a two-state model of the benzodiazepine receptor, as has been postulated previously [15, 17]. It is not clear at present whether the GABA-stimulated receptor is a separate entity or simply a GABA-induced conformer of the binding site observed in the absence of GABA. Further characterization of this complex system is required to differentiate between these hypotheses.

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Inhibition of rat heart superoxidase dismutase activity by diethyldithiocarbamate and its effect on mitochondrial function

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Superoxidase dismutase (E.C. 1.15.1.1) (SOD)* is an ubiquitous enzyme that catalyzes the dismutation of superoxide anions $(O_2^-)^+$ to hydrogen peroxide and molecular oxygen [1]. SOD is thought to play an important role in protecting the cells against O_2^- radical toxicity [2]. Maximal SOD activity has been observed in liver, adrenal and kidney tissues. Heart muscle contains a considerable amount of SOD [3], but little research has been done to ascertain its role in cardiac metabolism. Heikkila *et al.* [4] have reported that when diethyldithiocarbamate $(DDC)^+$, a powerful copper chelating agent, was injected intraperitoneally in mice, SOD activity in the brain, liver and blood was markedly lowered. SOD inhibition was also observed after incubation of DDC either with solutions of purified bovine SOD [5] or with mouse tissue homogenates that contained endogeneous SOD [4]. More recently, DDC has been found to potentiate oxygen toxicity and the *in vitro* effects of hemolytic agents [6] or the lethal effects of ozone and paraquat [7]. The present study was undertaken to investigate the effect of the administration *in vivo* of DDC on rat cardiac SOD activity. Since cardiac mitochondria are able to generate O_2^- radicals [8], we have also studied the effects of cardiac SOD inhibition on mitochondrial function.

Materials and Methods

Male Sprague–Dawley rats weighing 200–300 g were injected intraperitoneally (1.2 g/kg wt) with sodium DDC (Sigma Chemical Company, St. Louis, MO) in 0.9 per cent (w/v) NaCl solution. Control animals received the same volume of the saline vehicle (usually 0.5 ml). After the rats were killed by cervical dislocation, their hearts were rapidly removed and immersed in a Krebs-Henseleit oxygenated buffer [9] at 2–4°. In order to remove blood contamination, the hearts were perfused for 15 min by the non-recirculating Langendorff technique [10] using an oxygenated buffer containing 11 mM glucose as substrate [11]. At the end of each perfusion, the hearts were removed and homogenized in 180 mM KCl, 10 mM EDTA and 0.5 per cent (w/v) bovine serum albumin, pH 7.2, using an Ultra-Turrax homogenizer [12]. The homogenates were centrifuged at 1000 *g* for 5 min. The supernatants were filtered and recentrifuged at 10,000 *g* for 10 min. The supernatants were collected and recentrifuged at 40,000 *g* for 60 min to prepare cytosolic fractions. The above resulting crude mitochondrial pellets were resuspended in a medium containing 180 mM KCl and 0.5 per cent (w/v) bovine serum albumin, pH 7.2 (medium-1) and centrifuged for 10 min at 5000 *g*. The mitochondrial pellets were washed again in medium-1, and after centrifugation at 5000 *g* for 10 min, the final pellets were collected and resuspended in the medium-1. Mitochondrial oxygen consumption (QO_2, *n* atoms oxygen uptake in presence of ADP/mg mitochondrial protein/min), the respiratory control index (RCI) and the ADP/O ratio were calculated from the decrease of the O_2-partial pressure measured by a Clark electrode in a closed water jacketed at 25° (Gilson Instruments, France). The assay medium consisted of 3 mM substrate (glutamate, succinate, pyruvate or α-ketoglutarate), 250 mM sucrose, 0.5 mM EDTA, 3 mM KH_2PO_4 at pH 7.4. ADP (250 μM) was added to the incubation mixture to initiate mitochondrial respiration. Superoxide dismutase activity was measured by the method of Nishikimi *et al.* [13] which utilizes the inhibitory action of SOD on the rate of reduction of nitroblue tetrazolium $(NBT)^+$ by O_2^- mediated by phenazine methosulfate $(PMS)^+$. The assay medium consisted of 19 mM sodium pyrophosphate buffer, pH 8.3, containing 33 μM of NBT and 86 μM NADH. After mixing briefly, 50 μl of mitochondrial or cytosolic sample was added, and after addition of 69 μM of PMS, the reduction rate of NBT was monitored at 550 nm for 2 min. For all the samples the inhibition of the reaction was kept close to 50 per cent. Malondialdehyde $(MDA)^+$ content was evaluated in mitochondrial and cytosolic fractions using thiobarbituric acid as reagent [14]. The proteins were measured by the method of Bradford [15] using serum albumin as a standard.

Results

Table 1 reports the SOD activity measured in mitochondria and cytosol prepared from perfused rat hearts isolated 2 hr after intraperitoneal injection with DDC.

Both mitochondrial and cytosolic SOD activity were significantly depressed by DDC. Two hours after injection, cardiac SOD inhibition reached its highest value and remained constant for another 2 hr (data not shown). The amounts of cardiac MDA produced in the animals treated with DDC are reported in Table 2. The content of mitochondrial MDA was greatly increased in the treated ani-

* *Abbreviations used:* SOD, superoxide dismutase; O_2^-, superoxide anions; DDC, diethyldithio carbamate; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; MDA, malondialdehyde.