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# PURINE INHIBITION OF [$^3$H]-γ-AMINOBUTYRIC ACID RECEPTOR BINDING TO RAT BRAIN MEMBRANES

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**Abstract**—Several purines, including inosine and hypoxanthine, inhibit the binding of [$^3$H]-γ-aminobutyric acid (GABA) and [$^3$H]diazepam to freeze–thawed and extensively washed rat brain membranes. While purines have been reported to inhibit diazepam binding competitively, their interactions with GABA receptors in both mitochondrial and mitochondrial plus microsomal fractions are noncompetitive. The possibility that purines may bind at one site and affect the GABA receptor–ionophore–benzodiazepine complex is discussed.

Although the molecular mechanisms involved in benzodiazepine actions are unknown, several lines of neurophysiological [1–3] and biochemical [4–6] evidence suggest that an intimate relationship exists between the benzodiazepines and the gamma-aminobutyric acid (GABA) receptor–ionophore system. It is also widely accepted that benzodiazepines bind to specific sites in the mammalian central nervous system (CNS) [7–11].

Recently, a great deal of interest has been focused on the identification of endogenous ligands for the benzodiazepine receptor sites. The purines, inosine and hypoxanthine, have been isolated from brain extracts and, based on their abilities to inhibit benzodiazepine binding, it has been speculated that they may be endogenous ligands for benzodiazepine receptors [12–14]. In addition to inosine and hypoxanthine, several other purines, including xanthine derivatives, also inhibit benzodiazepine binding [14–15]. Furthermore, multiple sites of action of purines and cross-desensitization between benzodiazepines and purines have been demonstrated [16].

In view of the known interactions between benzodiazepines and the GABA receptor system, we investigated the effects of purines on the binding of [$^3$H]GABA to its receptor sites.

## METHODS

Male Sprague–Dawley rats (125–200 g) were used. [$^3$H]GABA (65–66 Ci/mmole) was purchased from Amersham/Searle (Arlington Heights, IL) and [$^3$H]diazepam (64 Ci/mmole) was purchased from New England Nuclear (Boston, MA). Muscimol was purchased from Research Organics (Cleveland, OH), and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

*Tissue preparation.* The rats were decapitated, and whole brains, excluding the brain stem caudal to the cerebellum, were removed and used for tissue preparation as described previously [17–19]. Briefly, brains (or cerebella) were homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1000 *g* for 10 min. The supernatant fraction was collected and centrifuged at 100,000 *g* for 45 min to collect the crude mitochondrial plus microsomal ($P_2 + P_3$) fractions. Crude mitochondrial ($P_2$) fraction was obtained according to published procedures [20]. The pellet ($P_2$ or $P_2 + P_3$) was subjected to two osmotic shock treatments, two freeze–thaw cycles and four buffer washes (0.05 M Tris-citrate, pH 7.1) prior to the binding studies, as described elsewhere [17–19]. The freeze–thawing and excessive washing of the tissue preparation is necessary to remove endogenous inhibitors of GABA receptor binding [5, 18, 19]. Routinely, $P_2 + P_3$ or $P_2$ rat brain membranes were used in the present study.

*Binding studies.* The binding of [$^3$H]GABA to $P_2$ or $P_2 + P_3$ membranes was measured by a modification of the centrifugation assay of Enna and Snyder [21] as described previously [17–19]. Aliquots of homogenate (~1 mg protein ) were incubated with various concentrations of [$^3$H]GABA, without or with excess nonradioactive GABA (0.1 mM), and other ligands for 10 min at 0° in scintillation Biovials. Assays were done in triplicate, with background values obtained in the presence of 0.1 mM nonradioactive GABA. Following incubation, the vials were centrifuged at 48,000 *g* for 10 min. The supernatant fraction was discarded, the pellets were rapidly rinsed and solubilized, and the radioactivity in the pellet was counted in 3 ml of toulene containing 5 g/l of 2,5-diphenyloxazole. The efficiency of counting was 40 ± 1 per cent. Specific binding usually represented 74 ± 6 per cent of the total radioactivity in the pellet. Protein was estimated by the method of Lowry *et al.* [22].

[$^3$H]Diazepam binding to extensively washed $P_2 + P_3$ membranes (see above) was measured by a centrifugation assay [23, 24]. Aliquots of membranes in 0.05 M Tris-maleate (pH 7.4) were incubated in triplicate with 1 nM [$^3$H]diazepam (64 Ci/mmole), with or without other ligands, for 30 min at 0–4°. Following incubation, the vials were centrifuged and processed for solubilization and scintillation counting as described for [$^3$H]GABA binding. Background radioactivity obtained in the presence of 50 μM flurazepam was subtracted from the total radioactivity in the pellet. Under these conditions, the benzodiazepine binding is specific, saturable and has all the characteristics of benzodiazepine receptors [23, 24].

## RESULTS

As seen in Table 1, various purines inhibited the binding of [$^3$H]GABA to rat brain $P_2 + P_3$ membranes. Inosine and hypoxanthine, which were postulated to be the endogenous ligands for benzodiazepine sites [12–14], inhibited GABA binding in a dose-related manner. [$^3$H]GABA binding to rat brain $P_2 + P_3$ membranes was also inhibited by adenosine and its chloro-derivative but not by cyclic AMP or ATP. Xanthine derivatives, such as theophylline, also potently inhibited specifically bound GABA.

Table 2 shows that inosine and its deoxy-derivative inhibit [$^3$H]GABA binding in $P_2$ (whole brain) and $P_2 + P_3$ fractions of whole brain and cerebellum. Diazepam and flurazepam (up to 25 $\mu$M) did not affect the binding of [$^3$H]GABA to extensively washed $P_2$ or $P_2 + P_3$ membranes as used in this study (data not shown). To further characterize the interaction between purines and GABA, we analyzed the inhibition of [$^3$H]GABA binding by double reciprocal analysis. Figure 1 shows that purines appear to inhibit [$^3$H]GABA binding noncompetitively in both $P_2$ (whole brain) and $P_2 + P_3$ (cerebellum) membranes. Under identical conditions of tissue preparation (see Methods), flurazepam (up to 25 $\mu$M) did not alter the kinetics of [$^3$H]GABA binding (data not shown).

Table 1. Inhibition of specific [$^3$H]GABA binding to rat brain $P_2 + P_3$ membranes by various purines*

| Ligand | Percent inhibition of specific [$^3$H]GABA binding | | |
|---|---|---|---|
| | 200 $\mu$M | 500 $\mu$M | 1 mM |
| Inosine | 13 ± 4 | 28 ± 11 | 41 ± 5 |
| 2′-Deoxyinosine | 18 ± 3 | 36 ± 5 | 65 ± 3 |
| Hypoxanthine | 17 ± 5 | 34 ± 14 | — |
| Theophylline | 30 ± 9 | 54 ± 12 | — |
| Adenosine | 20 ± 6 | 38 ± 10 | — |
| Chloradenosine | 33 ± 8 | 58 ± 9 | — |
| Thymine | 0 | 0 | 3 |
| Cyclic AMP | 0 | 0 | 2 |
| ATP | 0 | 0 | 5 |

* [$^3$H] GABA binding was measured as described in Methods. The purine derivatives were added to the incubation mixture to give the indicated final concentration. The data are means ± S.D. of three to nine determinations, each done in triplicate; (—) denotes not tested . $P_2$ is the crude mitochondrial fraction; $P_3$ is the crude microsomal fraction.

Since purines inhibit [$^3$H]diazepam binding [12–14], and benzodiazepine receptors interact with GABA systems [1–6], we looked for the presence of [$^3$H]diazepam binding sites in our freeze-thawed and extensively washed preparation. Table 3 shows

Table 2. Inhibition of specific [$^3$H]GABA binding to $P_2$ and $P_2 + P_3$ membranes*

| | Per cent inhibition of specific [$^3$H]GABA binding | | |
|---|---|---|---|
| | $P_2$ (whole brain) | $P_2 + P_3$ (whole brain) | $P_2 + P_3$ (cerebellum) |
| Inosine (1 mM) | 51 ± 6 (3) | 41 ± 5 (6) | 48 ± 8 (2) |
| 2′-Deoxyinosine (1 mM) | 68 ± 4 (4) | 65 ± 3 (6) | 63 ± 7 (2) |

* [$^3$H]GABA binding was measured as described in Methods. Results are the means ± S.D. of the number of experiments given in parentheses. $P_2$ is the crude mitochondrial fraction; $P_3$ is the crude microsomal fraction.

Table 3. Effects of GABA receptor ligands and purines on [$^3$H]diazepam binding to rat brain $P_2 + P_3$ membranes*

| Treatment | Specific [$^3$H]diazepam bound (fmoles/mg protein) | Per cent of control |
|---|---|---|
| Control | 121 ± 7 | 100 |
| $+10^{-5}$ M GABA | 198 ± 7 | 164 |
| $+10^{-5}$ M Muscimol | 215 ± 5 | 176 |
| $+10^{-5}$ M Muscimol + $10^{-4}$ (+)-bicuculline | 128 ± 7 | 106 |
| $+2 \times 10^{-4}$ M Inosine | 99 ± 7 | 82 |
| $+5 \times 10^{-4}$ M Inosine | 90 ± 5 | 74 |
| $+2 \times 10^{-4}$ M 2′-Deoxyinosine | 100 ± 4 | 83 |

* [$^3$H]Diazepam binding to freeze–thawed and extensively washed rat brain $P_2 + P_3$ membranes was measured by a centrifugation assay as described in Methods. Vials containing aliquots of homogenate, 1 nM [$^3$H]diazepam, and the given concentrations of the ligands were incubated for 30 min at 0–4° prior to centrifugation. Results are means ± S.D. of three experiments, each done in triplicate. $P_2$ is the crude mitochondrial fraction; $P_3$ is the crude microsomal fraction.

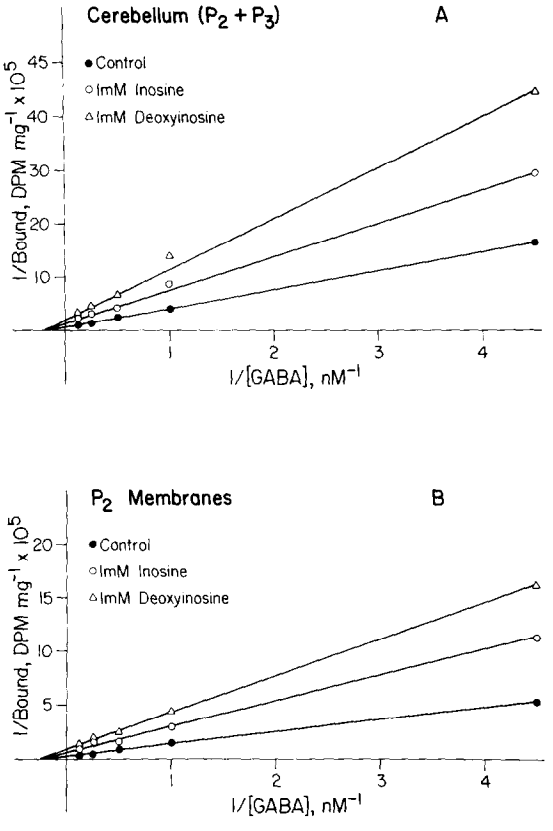


Fig. 1. Double reciprocal plots of specific [$^3$H]GABA binding to $P_2 + P_3$ cerebellar membranes (panel A) and $P_2$ whole brain membranes (panel B) in the absence (●) and in the presence of 1 mM inosine (○) and 1 mM 2′-deoxyinosine (△). Results are the mean values of triplicates and typical of three to four experiments. $P_2$ is the crude mitochondrial fraction; $P_3$ is the microsomal fraction.

that extensively washed $P_2 + P_3$ membranes which bind GABA with receptor-like properties [17–19, 25] also contain [$^3$H]diazepam binding sites. As reported by others for fresh $P_2$ membranes [7–10], the [$^3$H]diazepam binding in freeze–thawed and extensively washed $P_2 + P_3$ (whole brain) membranes was increased by GABA agonists, such as muscimol, and this enhancement was prevented by the GABA synaptic antagonist bicuculline (Table 3). These results agree with published reports [4, 6]. Furthermore, [$^3$H]diazepam binding was inhibited in these membranes by inosine and 2′-deoxyinosine (Table 3).

## DISCUSSION

Several lines of evidence suggest a possible interaction between benzodiazepine binding sites and the GABA receptor–ionophore system [1–6]. Recent studies have indicated that inosine and hypoxanthine may be endogenous ligands for the benzodiazepine receptor [12–14]. Furthermore, a selectivity of purines for benzodiazepine binding sites has also been reported [14]. Asano and Spector [14] reported that inosine and hypoxanthine selectively inhibited diazepam binding, but not opiate, muscarinic, β-adrenergic or GABA binding. In contrast to this study, we found that a variety of purines, including inosine and hypoxanthine, inhibited GABA binding to its receptor-like sites.

The binding of GABA to freeze–thawed and extensively washed $P_2$ and $P_2 + P_3$ membranes, as measured in this study, meets the necessary criteria for GABA receptor-like properties [17–19, 25]. In contrast to fresh membranes where benzodiazepines have been reported to alter the kinetics of GABA binding [5], such interaction has not been observed in the freeze–thawed and Triton-treated or extensively washed membranes [5, 25]. In the present study, flurazepam and diazepam did not affect the kinetics of GABA binding to freeze–thawed and extensively washed $P_2$ or $P_2 + P_3$ membranes. However, the $P_2 + P_3$ membranes in which we observed an inhibition of GABA binding by purines (Table 1) contained [$^3$H]diazepam binding sites with the expected properties (Table 3). GABA and muscimol enhanced the binding of [$^3$H]diazepam in these membranes, and this enhancement was prevented by the concurrent presence of the GABA synaptic antagonist (+)-bicuculline. These results are in agreement with other published studies [4, 6] and suggest a link between the benzodiazepine binding site and GABA receptors. [$^3$H]Diazepam binding to $P_2 + P_3$ rat brain membranes was inhibited by inosine and deoxyinosine (Table 3). These findings suggest that, in freeze–thawed and extensively washed membranes, an interaction between GABA receptors and benzodiazepine binding sites occurs (Table 3); we did not observe an interaction in an opposite direction (i.e. benzodiazepine site → GABA receptors). This lack of interaction may be due to the absence of endogenous proteins [5] in these membranes or the absence of some, as yet unknown, coupling mechanism. While the interaction of purines with benzodiazepine binding sites is competitive [12–14], their interaction with GABA receptors in both $P_2$ and $P_2 + P_3$ membranes appears to be noncompetitive (Fig. 1). Furthermore, purines also inhibit the binding of the GABA synaptic antagonist [$^3$H] dihydropicrotoxinin (DHP) to rat brain $P_2 + P_3$ membranes [26,*]. Several lines of evidence suggest that DHP binds at a site distinct from the GABA receptor sites, and that DHP sites are associated with the GABA receptor-linked chloride ionophore [25, 27, 28]. Thus, purines inhibit the binding of three ligands (benzodiazepines, GABA and DHP) which appear to bind at three different sites; nonetheless, they are associated with the GABA receptor–ionophore–benzodiazepine complex.

GABA is known to bind to two distinct sites [5, 17–19, 25], and multiple sites may also exist for benzodiazepines [29, 30] and purines [16]. In view of all of these findings, the relationship of purines to benzodiazepines and the GABA receptor–ionophore system must be investigated further before any conclusions about their role can be drawn.

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* M. K. Ticku, unpublished observations.

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