Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 1004–1006, February 1979
Physiological Sciences

Stimulation of benzodiazepine receptor binding by γ-aminobutyric acid

(receptor interactions/diazepam/muscimol/brain membranes)

MANFRED KAROBATH* AND GÜNTHER SPERK†

*Department of Experimental Psychiatry, Psychiatrische Universitätsklinik, University of Vienna, A-1090, Wien, Austria; and †Institute of Biochemical Pharmacology, University of Vienna, A-1090, Wien, Austria

Communicated by Seymour S. Kety, November 27, 1978

ABSTRACT **The effect of the neurotransmitter γ-aminobutyric acid (GABA) on high-affinity binding of benzodiazepines to brain membranes has been investigated. GABA stimulated [^{3}H]diazepam binding by more than 100% when extensively washed membranes from brain tissue were used. This GABA-stimulated benzodiazepine binding occurred in all brain regions examined. The stimulation was specific for GABA agonists. It was inhibited by the GABA receptor blocker bicuculline methiodide. A large number of compounds structurally closely related to GABA but without direct effect on the GABA receptor failed to enhance [^{3}H]diazepam binding. The stimulation of benzodiazepine binding was caused by an increase in affinity; the number of binding sites remained unchanged. Half-maximal activation of [^{3}H]diazepam binding occurred in the presence of 300 nM muscimol or 900 nM GABA. β-Guanidinopropionic acid and imidazoleacetic acid were much weaker activators. It is suggested that the described stimulation of benzodiazepine high-affinity binding is mediated by a receptor for GABA. This site of GABA action exhibits different properties when compared to GABA receptors, as characterized by high-affinity binding of GABA agonists.**

Evidence from several laboratories, based on electrophysiological and pharmacological studies, suggests that benzodiazepines exert their action in the central nervous system by indirect potentiation of γ-aminobutyric acid (GABA) neurons (1–3). By use of radiolabeled diazepam or flunitrazepam, specific high-affinity binding sites for benzodiazepines have been demonstrated and characterized in brain (4–7). The potency of various benzodiazepine derivatives in displacing [^{3}H]diazepam or [^{3}H]flunitrazepam from their binding sites correlates well with their potency in pharmacological tests in animals and with their clinical efficacy in man (4–7). It has therefore been suggested that the benzodiazepine high-affinity binding site represents the pharmacological benzodiazepine receptor in the brain.

In addition to benzodiazepines, a great number of compounds of pharmacological interest have been tested for possible interference with [^{3}H]diazepam binding sites. All of these, including GABA and its agonists and antagonists, were reported to be ineffective (4–7). When we reexamined the effects of GABA and GABA-receptor agonists, we found that GABA and muscimol slightly enhanced [^{3}H]diazepam binding although, with the relatively crude membrane preparations that were used originally, this enhancement never exceeded 15%. However, after extensive washing of the membranes, including freezing and thawing, a marked stimulation of benzodiazepine binding by GABA and its agonists could consistently be measured. In the present communication we describe the properties and regional distribution of the GABA-stimulated benzodiazepine binding.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

METHODS

Membrane Preparation. Fresh brain tissue of several species was homogenized in 10 vol of ice-cold 0.32 M sucrose in a Potter–Elvehjem homogenizer fitted to a clearance of 0.25 mm. The homogenate was centrifuged at 1000 × *g* for 10 min at 3°C and the resulting supernatant was recentrifuged at 20,000 × *g* for 10 min. The pellet from the second centrifugation was suspended and rehomogenized with an Ultra-Turrax homogenizer in 40 vol of 5 mM Tris·HCl buffer (pH 7.4). After freezing and thawing, these membranes were washed at least five times by rehomogenization in the same amount of 50 mM Tris·HCl buffer (pH 7.4) and recentrifugation.

Binding Assays. [^{3}H]Diazepam binding was determined as described (8). The standard incubation medium (final volume 0.2 ml) contained: membranes (about 250 μg of protein), 0.8 nM [^{3}H]diazepam [[N-*methyl*-^{3}H]diazepam, 39.1 Ci (1 Ci = 3.7×10^{10} becquerels)/mmol; New England Nuclear] in 150 mM Tris·HCl buffer (pH 7.4). Compounds to be tested were added in the stated concentrations. In some experiments, 0.4 nM [^{3}H]flunitrazepam ([N-*methyl*-^{3}H]flunitrazepam, 87.4 Ci/mmol; New England Nuclear) was used as labeled ligand. At the end of the incubation period (0°C, 60 min) 5 ml of ice-cold buffer (50 mM Tris, pH 7.4) was added immediately and membranes with bound [^{3}H]diazepam or [^{3}H]flunitrazepam were trapped on Whatman GF/B glass fiber filters. The filters were washed instantly with an additional 5 ml of ice-cold buffer, and total binding was estimated by liquid scintillation counting. Unspecific binding was determined in the same incubation mixture in the presence of excess (3 μM) unlabeled diazepam and substracted from total binding to yield specific binding. The same values for unspecific binding were obtained in the presence of 1 μM unlabeled flunitrazepam.

RESULTS

In extensively washed membranes, prepared from rat brain cortex, [^{3}H]diazepam binding was enhanced by more than 100% in the presence of GABA and GABA-agonists. This effect was inhibited by bicuculline methiodide, a known GABA-receptor blocker (Table 1). It was not altered by the addition of the following cations: NaCl, 100 mM; KCl, 100 mM; $CaCl_2$, 10 mM. Analogous results were obtained in experiments in which [^{3}H]diazepam was replaced by [^{3}H]flunitrazepam as the labeled ligand. Various amino acids and drugs structurally related to GABA but without direct pharmacological action on the GABA receptor failed to stimulate [^{3}H]diazepam binding (Table 1 legend). For example, compounds that differ in the chain length (β-alanine, 5-aminovaleric acid) or with the amino group altered (γ-hydroxybutyric acid) compared to GABA, as well as the GABA-containing dipeptide homocarnosine, did not

Abbreviation: GABA, γ-aminobutyric acid.

Table 1. Stimulation of [^{3}H]diazepam binding by GABA-ergic compounds and its inhibition by bicuculline methiodide (BCM)

Agonist	Specific binding, % of control	
	No BCM	With BCM (30 μM)
None	100.0 ± 0.4	82.7 ± 1.3
GABA		
3 μM	172.3 ± 4.4	107.2 ± 2.6
10 μM	210.3 ± 0.8	115.3 ± 2.1
Muscimol		
3 μM	192.5 ± 3.3	119.2 ± 0.2
10 μM	224.8 ± 3.5	138.5 ± 2.3
4-*trans*-Aminocrotonic acid		
3 μM	163.0 ± 0.8	102.1 ± 2.6
10 μM	198.2 ± 2.5	ND
DL-4-Amino-2-hydroxybutyric acid		
20 μM	151.9 ± 4.8	98.3 ± 1.0
β-Guanidinopropionic acid		
3 μM	122.0 ± 3.6	92.1 ± 3.0
10 μM	137.6 ± 3.2	ND
Imidazoleacetic acid		
3 μM	110.4 ± 3.2	88.0 ± 1.7
10 μM	119.2 ± 1.9	ND

[^{3}H]Diazepam binding was performed with rat brain cortex membranes in the presence of 0.8 nM [^{3}H]diazepam. Essentially the same results were obtained when [^{3}H]flunitrazepam was used as labeled ligand or when bicuculline was used instead of bicuculline methiodide as GABA receptor blocker. The following compounds structurally related to GABA were examined at a concentration of 10 μM and found to be inactive (<5% stimulation) in stimulating specific [^{3}H]-diazepam binding: β-alanine, glycine, 5-aminovaleric acid, glutamic acid, taurine, baclofen (Lioresal), histidine, homocarnosine, 2-aminobutyric acid, oxamic acid, 4-amino-5-hexen-1-oic acid (γ-vinylic GABA), DL-4-aminohex-5-yonic acid (γ-acetylenic GABA), δ-aminolevulinic acid, γ-hydroxybutyric acid, (−)nipecotic acid, guvacine, and gabaculine. Data are shown as mean ± SEM. ND, not determined.

alter [^{3}H]diazepam binding. Amino acids with a hyperpolarizing (glycine, β-alanine, taurine) or depolarizing (glutamic acid) effect on neurons were also inactive. Compounds that inhibit GABA uptake [guvacine, (−)nipecotic acid] or inhibitors of GABA degradation (oxamic acid, γ-vinylic GABA, γ-acetylenic GABA, gabaculine) also failed to enhance benzodiazepine binding.

The stimulation of [^{3}H]diazepam binding by GABA and the GABA-agonist muscimol was sigmoid, with half-maximal activation at 900 nM for GABA and 300 nM for muscimol (Fig. 1). 4-*trans*-Aminocrotonic acid and 4-amino-2-hydroxybutyric acid were also potent activators of benzodiazepine binding. Imidazoleacetic acid, which has about the same potency as GABA in displacing [^{3}H]muscimol binding (9, 10), was less than 1/100th as potent as GABA in stimulating [^{3}H]diazepam binding.

A kinetic analysis of ^{3}H-diazepam binding indicated that the affinity of benzodiazepine binding sites was increased when a maximally stimulating concentration of muscimol (30 μM) was present, but the number of binding sites remained unchanged (Fig. 2). In unstimulated rat brain cortex membranes the apparent K_d for [^{3}H]diazepam was 8.83 ± 1.02 nM (mean ± SEM; n = 4). In the presence of 30 μM muscimol this K_d value was decreased to 3.71 ± 0.59 nM. When unwashed membranes were analyzed, a similar low K_d value (3.82 nM) was observed, which is in agreement with values previously reported (4, 5). GABA-stimulated [^{3}H]diazepam binding of similar magnitude could be observed in membranes prepared

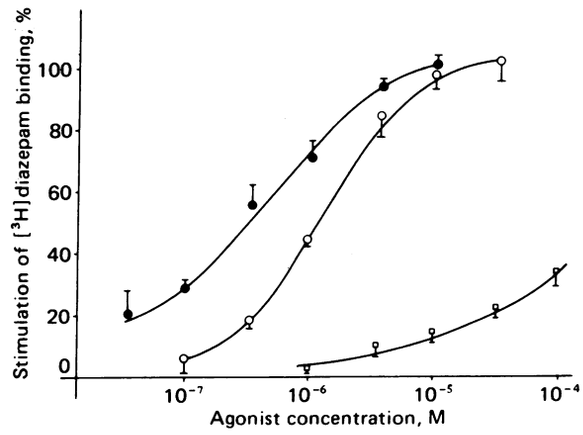

FIG. 1. Stimulation of specific [^{3}H]diazepam binding by GABA, muscimol, and imidazoleacetic acid. Binding experiments were performed with previously frozen rat brain cortex membrane preparations that were washed five times. The concentration of [^{3}H]diazepam was 0.8 nM. Increasing concentrations of GABA (○), muscimol (●), or imidazoleacetic acid (□) were included in the binding assay. [^{3}H]Diazepam binding in the presence of a saturating concentration of GABA (30 μM) was stimulated 81–122% over basal binding. This stimulation was taken as 100% and the results are expressed as percentage of maximal GABA-stimulated [^{3}H]diazepam binding. Results are mean ± SEM; n = 3.

from mouse, rat, guinea pig, beef, and human brain (data not shown). The regional distribution of basal and GABA-stimulated [^{3}H]diazepam binding was studied in membranes prepared from six brain regions, and stimulation by GABA was measurable in all regions. The distribution is shown in Table 2.

DISCUSSION

Investigations by several research groups suggest that one of the mechanisms by which benzodiazepines may produce their specific pharmacological effects is stimulation of the GABA system in the brain (1–3). The present results provide evidence that the reverse seems also to be possible and GABA may in-

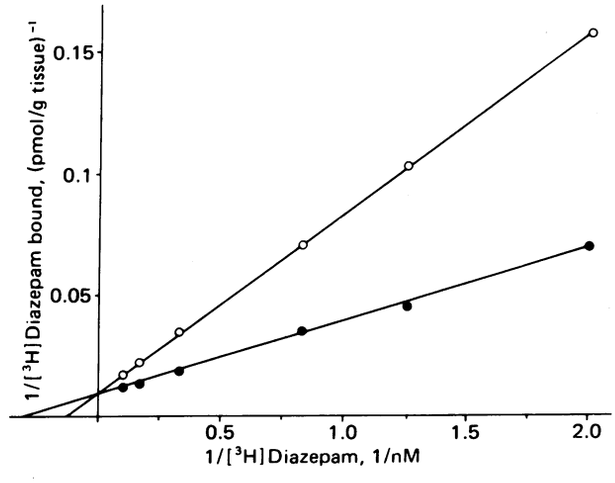

FIG. 2. Double reciprocal analysis of specific [^{3}H]diazepam binding with or without muscimol. [^{3}H]Diazepam (2–40 nM) was incubated in triplicate in the presence (●) or absence (○) of 30 μM muscimol. Kinetic analysis of specific binding was performed according to Lineweaver and Burk (11). This experiment was replicated three times and the K_d values were 8.83 ± 1.02 nM for unstimulated membranes and 3.71 ± 0.59 nM for muscimol-stimulated membranes (mean ± SEM; n = 4).

Table 2. Regional distribution of basal and GABA-stimulated [^{3}H]diazepam binding

Region	Specific binding, pmol/g tissue	
	Basal	With muscimol (10 μM)
Brain cortex	6.47 ± 0.15	14.26 ± 0.27
Olfactory bulb	5.99 ± 0.03	10.03 ± 0.08
Striatum	3.35 ± 0.03	6.36 ± 0.09
Cerebellum	3.40 ± 0.05	5.55 ± 0.12
Medulla oblongata	3.55 ± 0.08	5.30 ± 0.07
Spinal cord	3.08 ± 0.10	4.35 ± 0.01

All membranes were prepared from rat brain. They were thawed, frozen, and washed four times with 40 vol/g of tissue. Specific [^{3}H]-diazepam binding in the presence of 0.8 nM [^{3}H]diazepam was determined. Data are shown as mean ± SEM (n = 3).

fluence the characteristics of the benzodiazepine receptor, thus pointing to an interdependence of these two systems.

During the preparation of this manuscript similar findings were reported by Tallman *et al.* (12). These authors observed a stimulation of [^{3}H]diazepam binding by 25 or 33% in the presence of 10 μM GABA or muscimol, respectively. We believe that the quantitatively much greater effects of GABA and muscimol that we report here are due to the more stringent washing procedure during the preparation of the membranes. We therefore were able to detect also stimulation by compounds that have a weaker affinity to the GABA receptor. A decrease in affinity of [^{3}H]diazepam binding was observed after the washing procedure and later was reversed by the addition of GABA or GABA agonists. We do not know whether, during the washing procedure, contaminating GABA or another factor that influences benzodiazepine binding is removed from the membranes.

The stimulation of benzodiazepine binding appears to be specific for GABA agonists because structural analogues of GABA that do not act on the GABA receptor have no effect. Bicuculline methiodide, a GABA receptor-blocker, antagonizes GABA and GABA-agonist-stimulated benzodiazepine binding. These findings suggest that the stimulation of benzodiazepine binding is the consequence of the interaction of GABA with a GABA receptor. A close functional and spatial interaction of GABA and benzodiazepine receptors in the membrane therefore appears to be likely. However, several differences exist between the properties of GABA-stimulated benzodiazepine binding and of GABA agonist binding (9, 10, 13). Higher concentrations of GABA or muscimol are required for half-maximal stimulation of benzodiazepine binding than for half-maximal saturation of [^{3}H]muscimol (9, 10) or Na^+-independent [^{3}H]GABA binding (13). Furthermore, imidazoleacetic acid and β-guanidinopropionic acid, both potent in their interaction with [^{3}H]muscimol or [^{3}H]GABA binding (9, 10, 13), are much weaker in stimulating benzodiazepine binding. Therefore, the GABA receptor site that is responsible for the stimulation of the benzodiazepine receptor and the GABA receptor characterized by ^{3}H-labeled agonist binding appear to represent anatomically or functionally different sites of action.

This notion of a possible heterogeneity of GABA receptors is supported by large differences in the regional distribution of basal and GABA-stimulated [^{3}H]diazepam binding and of [^{3}H]muscimol binding (ref. 10; unpublished observation). For instance, in the cerebellum a low density of basal and GABA-stimulated [^{3}H]diazepam binding is accompanied by the highest density of [^{3}H]muscimol binding sites. Thus, in this brain region (as well as in some other brain regions) there may be GABA receptors that are not associated with benzodiazepine receptors. This suggestion is in accordance with the late evolutionary appearance of benzodiazepine receptors (14), whereas GABA has been shown to be a neurotransmitter in phylogenetically old species (15).

Apart from the functional implications of an interaction of benzodiazepines and GABA at the membrane level, the present observations represent an *in vitro* model system in which an effect of GABA mimetic compounds may be examined and distinguished from the effect of GABA receptor blockers. More work is needed in order to establish the specificity of this test system. If found to be specific for compounds acting selectively on the GABA receptor, this experimental model may permit the detection of new GABA agonists and antagonists by a simple *in vitro* assay.

We thank Prof. O. Hornykiewicz for his help during the preparation of this manuscript. We also thank E. Schlögl and M. Lippitsch for excellent technical assistance and J. Reisenhofer for typing this manuscript. DL-4-Aminohex-5-ynoic acid and 4-amino-5-hexen-1-oic acid were obtained from Centre de Recherche Merrell International, Strasbourg, France. This project was supported by Fonds zur Förderung der wissenschaftlichen Forschung in Österreich.

1. Costa, E., Giudotti, A., Mao, C. C. & Suria, A. (1975) *Life Sci.* **17,** 167–186.
2. Haefely, W., Kulcsar, A., Möhler, H., Pieri, L., Polc, P. & Schaffner, R. (1975) *Adv. Biochem. Psychopharmacol.* **14,** 131–152.
3. MacDonald, R. & Barker, J. L. (1978) *Nature (London)* **271,** 563–564.
4. Squires, R. F. & Braestrup, C. (1977) *Nature (London)* **266,** 732–734.
5. Möhler, H. & Okada, T. (1977) *Science* **198,** 849–851.
6. Speth, R. C., Wastek, G. J., Johnson, P. C. & Yamamura, H. I. (1978) *Life Sci.* **22,** 859–866.
7. Braestrup, C. & Squires, R. F. (1978) *Eur. J. Pharmacol.* **48,** 263–270.
8. Karobath, M., Sperk, G. & Schönbeck, G. (1978) *Eur. J. Pharmacol.* **49,** 323–326.
9. Leach, M. J. & Wilson, J. A. (1978) *Eur. J. Pharmacol.* **48,** 329–330.
10. Beaumont, K., Chilton, W. S., Yamamura, H. I. & Enna, S. J. (1978) *Brain Res.* **148,** 153–162.
11. Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56,** 658–666.
12. Tallman, J. F., Thomas, J. W. & Gallager, D. W. (1978) *Nature (London)* **274,** 383–385.
13. Enna, S. J. & Snyder, S. H. (1975) *Brain Res.* **100,** 81–97.
14. Nielsen, M., Braestrup, C. & Squires, R. F. (1978) *Brain Res.* **141,** 342–346.
15. Gerschenfeld, H. M. (1973) *Physiol. Rev.* **53,** 1–119.