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The Effects of Acute Administration of Diazepam on the Binding of [^{3}H]-Diazepam and [^{3}H]-GABA to Rat Cortical Membranes

JOSEPH COUPET, CHARLES E. RAUH, ARNOLD S. LIPPA AND BERNARD BEER

Department of Central Nervous System Research
Medical Research Division of American Cyanamid Company
Lederle Laboratories, Pearl River, NY 10965

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COUPET, J., C. E. RAUH, A. S. LIPPA AND B. BEER. *The effects of acute administration of diazepam on the binding of [^{3}H]-diazepam and [^{3}H]-GABA to rat cortical membranes.* PHARMAC. BIOCHEM. BEHAV. 15(6) 965–968, 1981.—Specific [^{3}H]-diazepam binding and [^{3}H]-GABA binding were measured in cortical membranes of untreated rats and rats which had been administered unlabeled diazepam (5.0 mg/kg, IP) thirty minutes prior to sacrifice. Washed and unwashed membranes from control animals showed identical levels of [^{3}H]-diazepam binding. Unwashed membranes of diazepam-treated animals showed consistently and significantly lower binding of [^{3}H]-diazepam than membranes derived from control animals and treated similarly. [^{3}H]-GABA was almost non-existent in unwashed membranes of either group of animals. The binding capability of membranes of treated animals for [^{3}H]-diazepam returned to control levels upon washing with buffer prior to the binding assay. The specific binding of [^{3}H]-GABA in membranes derived from either group of animals also improved after the buffer washes. However, no difference could be detected in [^{3}H]-GABA binding between control and diazepam-treated animals. The failure of diazepam to modulate [^{3}H]-GABA binding in unwashed membranes and the participation of an endogenous inhibitory material repressing [^{3}H]-GABA binding are discussed.

Benzodiazepine receptors [^{3}H]-GABA Endogenous inhibitor GABA receptors Ligands Conflict/punishment [^{3}H]-diazepam

A LARGE body of evidence has implicated GABA in mediating some of the therapeutic effects of the benzodiazepines in the mammalian central nervous system [3, 4, 6, 16, 17]. Moreover, numerous studies have shown that GABA and GABA agonists increase the affinity of the benzodiazepine receptor for [^{3}H]-diazepam [2, 7, 15]. Such compelling evidence in support of a GABA hypothesis for the actions of the benzodiazepines has prompted many investigators to explore whether these drugs can modulate GABA receptor binding. While some workers observed an enhancement [5,10] others failed to detect any pertubation of GABA receptor binding affinity by pharmacologically active benzodiazepines [1, 11, 13, 14]. One group of investigators [5] has demonstrated that incubation of synaptic membranes with low concentrations of benzodiazepines revealed a high affinity [^{3}H]-GABA binding site which could also be seen after freezing then thawing and followed by detergent treatment and extensive washing to remove a proposed inhibitor protein.

In this study, we have investigated whether *in vivo* treatment of rats with a pharmacologically active dose of diazepam could also reveal this high affinity site for [^{3}H]-GABA binding in unwashed membranes *in vitro*.

METHOD

Tissue Preparation

Groups of six male Wistar rats weighing 150–175 g were injected intraperitoneally (IP) with 5.0 mg/kg of diazepam, a dose which has previously shown a significant increase in punished responses in rats [8]. Control animals received equivolume of vehicular solution. Thirty minutes after treatment, all animals were sacrificed and their brains removed and placed on crushed ice. Frontal cortices were removed and pooled in pairs of rats. These tissues were homogenized gently in 20 volumes of ice cold 0.32 M sucrose in a Potter Elvejhem glass homogenizer with a teflon pestle and centrifuged twice at 1,000 × G for 10 minutes. The pellets were discarded and supernatant from each pool was divided into four equal parts and recentrifuged at 30,000 × G for 20 minutes to produce crude P_2, synaptosome-rich fractions. From each pool, two fractions were labeled unwashed preparations and two other fractions were lysed in 40 volumes of ice cold deionized water, incubated at 37°C for 20 minutes and twice washed with 80 volumes of 50 mM Tris·HCl (pH 7.4 or 50 mM Tris·citrate (pH 7.1) buffer. These latter fractions were labeled washed preparations. Final resuspension

TABLE 1

SPECIFIC BINDING OF [^{3}H]-DIAZEPAM IN WASHED AND UNWASHED RAT CORTICAL MEMBRANES

Drug Treatment	Unwashed Membranes (pmole bound/mg protein) ±S.E.M.	Washed Membranes (pmole bound/mg protein) ±S.E.M.
Vehicle Control	0.209 ± 0.012	0.203 ± 0.0016
Diazepam (5mg/kg, IP)	0.165 ± 0.001*	0.190 ± 0.008

*Significantly lower than vehicle control (untreated) at $p<0.05$.
[^{3}H]-diazepam was assayed at 1.5 nM.
The values are expressed in pmole bound/mg protein ± standard error of the mean (S.E.M.) of three separate determinations.

TABLE 2

SPECIFIC BINDING OF [^{3}H]-GABA IN WASHED AND UNWASHED RAT CORTICAL MEMBRANES

Drug Treatment	Unwashed Membranes pmole bound/mg protein ±S.E.M.	Washed Membranes pmole bound/mg protein ±S.E.M.
Vehicle Control	Undetectable*	0.161 ± 0.023
Diazepam (5.0 mg/kg, IP)	Undetectable*	0.148 ± 0.031

[^{3}H]-GABA was assayed at 8 nM.
*No difference existed between the amount of CPM found in total binding and binding determined in the presence of 1.0 mM of GABA.
The values are expressed in pmole bound/mg protein ± standard error of the mean (S.E.M.) of three separate determinations.

was made in the appropriate buffer to give a protein concentration of 1.0 mg per ml of suspension. Protein was determined by the method of Lowry [9] using bovine serum albumin as standard.

[^{3}H]-*Diazepam Binding*

[^{3}H]-diazepam binding was performed as described previously [8] in all fractions (unwashed and washed) for both control and treated animals, using 300 μl of tissue suspension in 50 mM Tris·HCl (pH 7.4). Incubation was carried out for 20 minutes at 0°C and terminated by rapid filtration on glass fiber filters, Whatman GF/C. The filter discs were rinsed twice with 5.0 ml of cold buffer and the radioactivity was determined by liquid scintillation spectrometry in a Beckman Scintillation Counter. All binding was expressed as specific binding and was calculated by subtracting non specific binding, determined in the presence of 3 μM unlabeled diazepam, from total binding.

[^{3}H]-*GABA Binding*

Sodium (Na+) independent [^{3}H]-GABA binding was determined in 50 mM Tris-citrate (pH 7.1) buffer in all fractions including diazepam treated and control animals, by a filtration method. Briefly, 100 μl of tissue suspension in 50 mM Tris·citrate buffer (pH 7.1) was incubated in a final volume of 2.0 ml with 8 nM of [^{3}H]-GABA (S.A., 43 Ci/mole, New England Nuclear Corp., Boston, MA) in the absence or in the presence of 1.0 mM of unlabeled GABA. Incubation was carried out for 30 minutes at 0°C and terminated by rapid filtration of GF/C filter discs under reduced pressure. The filters were rinsed twice with 5.0 ml of ice cold buffer and the radioactivity determined by liquid scintillation spectrometry. All binding was expressed as specific binding and was calculated by subtracting non specific binding, determined in the presence of unlabeled GABA, from total binding.

RESULTS

Unwashed membranes of diazepam treated animals showed significantly lower specific [^{3}H]-diazepam binding than unwashed membranes derived from control animals (Table 1). This is highly indicative of receptor occupancy by diazepam on the membranes of treated animals. On the other hand, unwashed membranes derived either from control or diazepam treated animals showed no appreciable binding of [^{3}H]-GABA (Table 2). This is suggestive of the presence of an inhibitor of GABA binding in these preparations, as has been previously shown by other investigators [13,14]. However, when these membranes were twice washed with buffer in the absence of detergent before assaying for diazepam binding, quite a different effect was observed. [^{3}H]-diazepam binding in washed membranes of the treated animals were not significantly different from the level observed in un-

washed membranes of control animals (Table 1). In addition, no significant difference could be detected in the binding of [^{3}H]-diazepam between the washed membranes of either group of animals (Table 1). It was highly likely that the mild washing procedure had removed the diazepam that had previously occupied the diazepam receptors in the course of the *in vivo* treatment with this agent. Furthermore, these findings question the existence of a putative endogenous ligand of small molecular size as claimed by Skolnick, *et al.* [15], since low molecular size endogenous substances would have been removed by the washing procedure. Therefore, different level of binding of [^{3}H]-diazepam would have been observed in unwashed and washed membranes of control animals.

Mild washing improved the binding of [^{3}H]-GABA in membranes of both control and treated animals (Table 2). However, there was no significant difference in [^{3}H]-GABA binding between washed membranes derived from either group of animals. This finding suggests that mild washing had partially removed an endogenous inhibitor of [^{3}H]-GABA binding. However, questions should be raised as to the validity of the report [5] claiming that addition of low concentration of exogenous diazepam to unwashed membranal preparations can substitute for freezing then thawing followed by detergent treatment and extensive washing to remove an inhibitor of [^{3}H]-GABA binding.

DISCUSSION

Evidence is presented in these studies for a correlation between diazepam receptor occupancy achieved by *in vivo* treatment and diminished binding of [^{3}H]-diazepam, in unwashed membranes from animals so treated. We have also been able to demonstrate that mild washing of membranes from treated animals could restore their binding capacity for [^{3}H]-diazepam. As other investigators [12] have already demonstrated, high specific binding of [^{3}H]-GABA (8 nM) to unwashed membranes was undetectable in unwashed membranes. *In vivo* treatment with diazepam to achieve receptor occupancy, did not reverse nor prevent the inhibitory material from interfering with [^{3}H]-GABA binding. While, mild washing partially removed this inhibitory material and facilitated [^{3}H]-GABA binding.

The present findings do not corroborate those of Guidotti, *et al.* [5] who have shown that low concentrations of exogenously applied benzodiazepines revealed a high affinity [^{3}H]-GABA binding site in unwashed membranes. These authors stated that the effect of low concentrations of benzodiazepine was similar in degree and could substitute for the harsh treatment of freezing then thawing, followed by detergent treatment and extensive washing. The present data indicate that mild washings with buffer without prior freezing then thawing and detergent treatment could restore to some degree the binding capacity of these membranes for [^{3}H]-GABA. Although no attempt was made to identify whether a high or a low affinity site was revealed by these mild washings, the 2 to 3 differential ratios in counts between total binding and non-specific binding would tend to suggest that the high affinity binding site may not have been fully revealed by such mild treatment. Moreover, it is unlikely that such mild treatment could have removed a large protein as "GABA modulin." It is to be speculated that the treatment described in these studies may have removed endogenous GABA, thereby facilitating [^{3}H]-GABA binding in washed membranes. Napias *et al.* [12] have previously identified an inhibitory material to [^{3}H]-GABA binding in unwashed membranes as GABA on the basis of its size, chemical properties and its sensitivity to destruction by GABAase.

In conclusion, like other reporters, [1, 11, 13, 14] we have failed to modulate [^{3}H]-GABA binding by diazepam, despite the fact that GABA modulates [^{3}H]-diazepam binding [14,15]. Although our methodology differs from that of these earlier investigators, the *in vivo* treatment with diazepam (5 mg/kg, IP) did allow diazepam to occupy the benzodiazepine receptor sites since a reproducible 20% statistically significant decrease in [^{3}H]-diazepam binding *in vitro* was seen in the unwashed membranes of the treated animals. To ascertain that it was diazepam that inhibited [^{3}H]-diazepam binding in unwashed membranes of drug-treated animals, we were able to remove the inhibition by mild washings and restore [^{3}H]-diazepam binding.

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REFERENCES

1. Andrews, P. R. and G. A. R. Johnston. GABA agonists and antagonists. *Biochem. Pharmac.* **28:** 2697–2702, 1979.
2. Briley, M. S. and S. Z. Langer. Influence of GABA receptor agonists and antagonists on the binding of [^{3}H]-diazepam to the benzodiazepine receptor. *Eur. J. Pharmac.* **52:** 129–132, 1978.
3. Costa, E. and A. Guidotti. Molecular mechanisms in the receptor action of benzodiazepines. *A. Rev. Pharmac. Toxicol.* **19:** 531–545, 1979.
4. Costa, E., A. Guidotti, C. Mao and A. Suria. New concepts on the mechanism of action of benzodiazepines. *Life Sci.* **17:** 167–186, 1975.
5. Guidotti, A., G. Toffano and E. Costa. An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain. *Nature* **275:** 553–555, 1978.
6. Haefely, W., P. Pole, R. Schaffner, H. H. Keeler, L. Pieri and H. Mohler. Facilitation of GABA-ergic transmission by drugs. In: *GABA-Neurotransmitters*, edited by P. Krogagaard-Larsen, J. Scheel-Kruger and H. Kofod. Copenhagen: Munksgaard, 1979, pp. 357–375.
7. Karobath, M. and G. Sperk. Stimulation of benzodiazepine receptor binding by gamma-aminobutyric acid. *Proc. natn. Acad. Sci. U.S.A.* **76:** 1004–1006, 1979.
8. Lippa, A. S., C. A. Klepner, L. Yunger, M. C. Sano, W. V. Smith and B. Beer. Relationship between benzodiazepine receptors and experimental anxiety in rats. *Pharmac. Biochem. Behav.* **9:** 853–856, 1978.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193:** 265–275, 1951.
10. Meiners, B. A. and A. I. Salama. Enhancement of [^{3}H]-GABA binding by the novel anxiolytic ICI 136,753 and benzodiazepines. *Soc. Neurosci. Abstr.* **6:** 189, 1980.
11. Mohler, H. and T. Okada. Properties of γ-aminobutyric acid receptor binding with (+)[^{3}H] bicuculine methiodide in rat cerebellum. *Molec. Pharmac.* **14:** 256–265, 1978.
12. Napias, C., M. O. Bergman, P. C. Van Ness, D. V. Greenler and R. W. Olsen. GABA binding in mammalian brain: Inhibition by endogenous GABA. *Life Sci.* **27:** 1001–1011, 1980.

13. Olsen, R. W. Biochemistry of GABA receptor proteins in mammalian brain. In: *Psychopharmacology and Biochemistry of Neurotransmitter Receptors*, edited by H. I. Yamamura, R. W. Olsen and E. Usdin. New York: Elsevier, 1980, pp. 537–550.
14. Olsen, R. W., M. K. Ticku, P. C. Van Ness and D. Greenlee. Effects of drugs on γ-aminobutyric acid receptors, uptake, release and synthesis *in vitro*. *Brain Res.* **139:** 277–294, 1978.
15. Skolnick, P., P. J. Marangos, P. Syapin, F. K. Goodwin and S. M. Paul. CNS benzodiazepine receptors: Physiological studies and putative endogenous ligands. *Pharmac. Biochem. Behav.* **10:** 815–823, 1979.
16. Tallman, J. F., J. W. Thomas and D. W. Gallager. GABA-ergic modulation of benzodiazepine binding sensitivity. *Nature* **274:** 383–385, 1978.
17. Tsuchiya, T. and H. Fukushima. Effects of benzodiazepine and pentobarbitone on the GABA-ergic recurrent inhibition of hippocampal neurons. *Eur. J. Pharmac.* **48:** 421–424, 1978.
18. Waddington, J. C. and A. Longden. Rotational behaviour and cGMP responses following manipulation of nigral mechanisms with chlordiazepoxide. *Naunyn-Schmiedebergs Arch. Pharmac.* **300:** 233–237, 1977.