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Benzodiazepine Binding Sites in Mice Forebrain and Kidneys: Evidence for Similar Regulation by GABA Agonists

LEMBIT K. RÄGO, RAUL A. K. KIIVET,[1] JAANUS E. HARRO AND LEMBIT H. ALLIKMETS

Department of Pharmacology, Tartu State University, 18 Ulikooli Street, 202400, Tartu, Estonia, USSR

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RÄGO, L. K., R. A. K. KIIVET, J. E. HARRO AND L. H. ALLIKMETS. *Benzodiazepine binding sites in mice forebrain and kidneys: Evidence for similar regulation by GABA agonists.* PHARMACOL BIOCHEM BEHAV **24**(1) 1–3, 1986.—Benzodiazepine binding sites in mice forebrain and kidney homogenate were labelled either in vivo (SC injection) or in vitro by ^{3}H-flunitrazepam. The binding in both cases was carried out in vitro in the presence or absence of 10 μM unlabelled flunitrazepam during 60 min at 0°C. When benzodiazepine binding sites were labelled in vivo pretreatment of animals with muscimol (0.75 and 1.5 mg/kg, IP) and fenibut (25 and 100 mg/kg, IP) in a dose-dependent manner facilitated ^{3}H-flunitrazepam binding both in mice forebrain and kidneys. In vitro labelling of benzodiazepine binding sites demonstrated similar to in vivo labelling in forebrain but not in kidneys. The peripheral benzodiazepine binding sites in kidneys are therefore modulated similarly to benzodiazepine binding sites in forebrain by GABA-ergic drugs.

Benzodiazepine binding sites Forebrain Kidneys Modulation GABA agonists

SPECIFIC binding sites for the benzodiazepines exist in both the brain and kidney [3,12]. The pharmacology of the brain and kidney sites is quite distinct. The clinically inactive benzodiazepine Ro 5-4864 is a very potent inhibitor of ^{3}H-diazepam binding sites in kidney and quite inactive in brain. Clonazepam, vice versa, is highly potent in brain and inactive in kidney [3, 11, 14]. Several works have suggested that a significant proportion of brain diazepam and flunitrazepam binding represents labelling of peripheral rather than central sites. It has been claimed that peripheral type benzodiazepine binding sites is not modulated by GABA [9]. Although the physiological role of these peripheral-type binding sites is unknown, recent evidence demonstrating the involvement of these sites in the modulation of phospholipid methylation and prolactin release supports the idea of possible receptor function [6,13]. In an effort to learn more about GABAergic modulation of central and peripheral benzodiazepine binding sites the effect of administration of GABA receptor agonists muscimol and fenibut (β-phenyl-γ-aminobutyric acid) on benzodiazepine binding sites in mice forebrain and kidneys was studied. Muscimol is a very potent and relatively selective $GABA_A$ receptor agonist while fenibut is a weak $GABA_B$ receptor agonist not possessing $GABA_A$ receptoral activity in vitro [10]. In this report we present evidence indicating that both $GABA_A$ and $GABA_B$ receptor agonists can similarly regulate benzodiazepine binding sites in mice forebrain and kidneys.

[1]Requests for reprints should be addressed to R. A. K. Kiivet.

METHOD

The experiments were performed on male albino mice weighing 25–30 g (Rappolovo Farm, Leningrad). The mice were maintained on food and water ad lib at 20±1°C on a reversed lighting cycle with lights off from 0800 to 2000 hr. The animals were killed by decapitation, forebrains and kidneys rapidly dissected on ice, carefully weighed and stored at −20°C until binding assayed. In both binding experiments muscimol (Research Biochemicals Inc., Wayland, U.S.A.) and fenibut (NPO Biokhimreaktiv, Olaine, USSR) were dissolved in saline and injected in a volume of 10 ml/kg IP 60 min prior to the sacrifice.

Experiment 1

^{3}H-Flunitrazepam (50 μCi/kg, 84 Ci/mmol, Radiochem Center Amersham, U.K.) was injected SC into nape region 30 min before sacrifice. Individual mice forebrains and kidneys were homogenized in 50 mM Tris-HCl buffer (pH=7.3) by glass-teflon Potter-S homogenizer (1000 rpm, 8 passings). Tissue samples (25 mg w/w per 500 μl) were incubated in the presence or absence of 10 μM unlabelled flunitrazepam (Hoffmann-La Roche, Switzerland) for 60 min on ice. The incubation was followed by centrifugation at 10,000 rpm for 10 min in Microfuge 12 (Beckman). After centrifugation the supernatants were carefully removed, resulting pellets washed once with 1 ml ice-cold buffer and resuspended in

TABLE 1

EXPERIMENT I: THE EFFECT OF ADMINISTRATION OF MUSCIMOL (0.75 AND 1.5 mg/kg) AND FENIBUT (25 AND 100 mg/kg) ON BENZODIAZEPINE BINDING SITES IN MICE FOREBRAIN AND KIDNEYS

Treatment, mg/kg		Number of animals	Cpm specifically bound per 100 mg tissue: Forebrain	(%)	Kidneys	(%)
Saline		6	1342 ± 89	(100)	2654 ± 112	(100)
Muscimol	0.75	4	1512 ± 189	(113)	3391 ± 234*	(128)
	1.5	5	2174 ± 214†	(162)	5467 ± 281†	(206)
Fenibut	25	5	2127 ± 158†	(158)	4589 ± 311†	(173)
	100	5	2973 ± 306†	(222)	8221 ± 608†	(310)

*$p<0.05$; †$p<0.01$ vs. saline.

Benzodiazepine binding sites were labeled by in vivo administration of ^{3}H-flunitrazepam (50 μCi/kg, SC).

TABLE 2

EXPERIMENT II: THE EFFECT OF ADMINISTRATION OF MUSCIMOL (0.75 AND 1.5 mg/kg) AND FENIBUT (25 AND 100 mg/kg) ON BENZODIAZEPINE BINDING SITES IN MICE FOREBRAIN AND KIDNEYS

Treatment, mg/kg		Number of animals	Cpm specifically bound per 100 mg tissue: Forebrain	(%)	Kidneys	(%)
Saline		7	1529 ± 92	(100)	2898 ± 131	(100)
Muscimol	0.75	4	1758 ± 128	(115)	2930 ± 87	(101)
	1.5	6	2629 ± 102*	(172)	3275 ± 249	(113)
Fenibut	25	5	2110 ± 121*	(138)	2985 ± 161	(103)
	100	6	2889 ± 141*	(189)	3101 ± 187	(107)

*$p<0.01$ vs. saline.

Benzodiazepine binding sites were labeled in vitro by ^{3}H-flunitrazepam (0.08 nM).

500 μl distilled water. This suspension was immersed into dioxane scintillation cocktail (counting efficiency between 40–43%). Nonspecific binding was generally about 45 percent of total binding.

Experiment II

All the procedures were the same as described in experiment I with the exception that ^{3}H-flunitrazepam (0.08 nM) was portioned out into test tubes in a volume of 5 μl. The concentration of radiolabelled ligand in this experiment was chosen to give similar amount of cpm as in experiment I. Nonspecific binding in the presence of 10 μM flunitrazepam was also about 45 percent of total binding.

Student's *t*-test was used for statistical analyses of the results. The term "significant" is used in the text to mean a significant difference of at least $p<0.05$.

RESULTS

Experiment I

In this experiment benzodiazepine binding sites in mice forebrain and kidney homogenates were labelled by in vivo administration of ^{3}H-flunitrazepam to the animals. The binding studies were carried out in vitro as indicated in the Method section. The density of benzodiazepine binding sites in kidneys was about two times higher than that in forebrain (Table 1). In our studies both muscimol, a classical $GABA_A$ agonist, and fenibut, a $GABA_B$ receptor agonist stimulated in a dose-dependent manner significantly (with the exception of 0.75 mg/kg of muscimol in the forebrain) ^{3}H-flunitrazepam binding to benzodiazepine binding sites in mice forebrain and kidneys. It was found that muscimol and fenibut caused more marked changes in benzodiazepine binding sites in kidneys, i.e., the elevation percent of specific ^{3}H-flunitrazepam binding in kidneys was higher than in forebrain from 15 to 88 percent (Table 2).

Experiment II

In contrast to experiment I benzodiazepine binding sites in mice forebrain and kidney homogenates were labelled in vitro by ^{3}H-flunitrazepam. In this experiment specific ^{3}H-

flunitrazepam binding in mice kidneys was also about two times higher than that in forebrain (Table 2). The changes in benzodiazepine binding sites in forebrain caused by muscimol and fenibut were very similar to those in experiment I. The administration of both GABAergic compounds to mice resulted in elevation of benzodiazepine binding in mice forebrain. No significant modulation of benzodiazepine binding by pretreatment with muscimol and fenibut was observed in mice kidneys. Therefore the main difference of experiments I and II was the lack of modulation of benzodiazepine binding sites in kidneys by GABAergic drugs in experiment II.

DISCUSSION

The fact that muscimol, a $GABA_A$ receptor agonist, is able to modulate benzodiazepine binding is well known [7,15]. In this context our results concerning the augmentation of benzodiazepine binding in mice forebrain by muscimol are in agreement with the numerous data in literature. In contrast to muscimol, fenibut is a $GABA_B$ receptor agonist devoid of $GABA_A$ receptoral activity [10]. It has been claimed that $GABA_B$ receptors are not linked to benzodiazepine receptors [2,4]. Indeed, fenibut is not able to modulate benzodiazepine binding in vitro in traditional benzodiazepine binding studies (our unpublished data). Nevertheless according to the present results, fenibut effectively potentiates ^{3}H-flunitrazepam binding similarly to muscimol in "ex vivo" studies. Pretreatment of rats "in vivo" with baclofen, a structurally closely related compound to fenibut and a specific $GABA_B$ receptor agonist is also able to increase ^{3}H-diazepam binding in cerebellar cortex [5]. In our previous studies it has been shown that Ro 15-1788, a benzodiazepine antagonist, is able to modify behavioural effects of fenibut in rats similarly to the behavioural effects of muscimol [1]. Recently it has been shown that a subpopulation of benzodiazepine receptors (detergent sensitive one) is stimulated by Ca^{2+} ions [8]. As $GABA_B$ receptors are Ca^{2+} dependent and they have been assumed to be connected with Ca^{2+} transport processes [2], a possibility exists that modulation of benzodiazepine binding by $GABA_B$ agonists is realized through Ca^{2+} sensitive mechanisms.

In our study the extremely low concentration of radiolabelled ligand used must be taken into consideration. It is obvious that in our experiment only a very small part of benzodiazepine binding sites existing are labelled and the importance of this fact for current study is difficult to estimate. The pharmacokinetic interaction between GABAergic drugs studied and ^{3}H-flunitrazepam can be a possible reason for altered benzodiazepine binding in experiments where ^{3}H-flunitrazepam was administered in vivo to mice. However this possibility is almost excluded when benzodiazepine binding sites are labelled in vitro. Our comparative experiments with in vivo and in vitro use of radiolabelled ligand demonstrate clearly that pharmacokinetics is not the reason to be considered. It seems that changes in central but not in peripheral benzodiazepine binding sites can be registered with equal success no matter how the binding sites are labelled, in vivo or in vitro. Apparently in vivo labelling of benzodiazepine binding sites in kidneys is able to fix and to preserve changes caused in vivo.

In conclusion we have to assume that the real nature of GABA receptor agonists and peripheral benzodiazepine binding sites interaction remains to be elucidated. Nevertheless the present study confirms the possibility of modulation of peripheral benzodiazepine binding sites similarly to that of central ones by GABA receptor agonists. Whether the regulation of benzodiazepine binding sites by GABA is of central or peripheral origin remains to be studied.

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REFERENCES

1. Allikmets, L. H. and L. K. Rägo. The action of benzodiazepine antagonist Ro 15-1788 on the effects of GABA-ergic drugs. *Naunyn Schmiedebergs Arch Pharmacol* **324:** 235–237, 1983.
2. Bowery, N. G., D. R. Hill and A. L. Hudson. Characteristics of $GABA_B$ receptor binding sites in rat whole brain synaptic membranes. *Br J Pharmacol* **78:** 191–206, 1983.
3. Braestrup, C. and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high affinity ^{3}H-diazepam binding. *Proc Natl Acad Sci USA* **74:** 3805–3809, 1977.
4. Doble, A. and M. J. Turnbull. Lack of effect of benzodiazepines on bicuculline-insensitive GABA-receptors in the field stimulated guinea-pig vas deferens preparation. *J Pharm Pharmacol* **33:** 267–268, 1981.
5. Gallager, D. W., J. W. Thomas and J. F. Tallman. Effect of GABA-ergic drugs on benzodiazepine binding site sensitivity in rat cerebral cortex. *Biochem Pharmacol* **27:** 2745–2749, 1978.
6. Grandison, L. Characterization of benzodiazepine binding and action on rat pituitary cells. *Soc Neurosci Abstr* **7:** 503, 1981.
7. Krogsgaard-Larsen, P. and J. Arnt. Pharmacological studies of interaction between benzodiazepines and GABA receptors. *Brain Res Bull* **5:** 867–872, 1980.
8. Lo Mathew, M. S. and S. Snyder. Two distinct solubilised benzodiazepine receptors: differential modulation by ions. *Life Sci* **3:** 2270–2279, 1983.
9. Patel, J. and P. J. Marangos. Differential effects of GABA on peripheral and central type benzodiazepine binding sites in brain. *Neurosci Lett* **30:** 157–160, 1982.
10. Rägo, L. K., A. M. Nurk, A. Ya. Korneyev and L. H. Allikmets. Fenibut binds to bicuculline-insensitive GABA receptors in rat brain. *Bull Exp Med Biol* **11:** 58–59, 1982 (in Russian).
11. Regan, J. W., W. R. Roeske, J. B. Malick, S. H. Yamamura and H. I. Yamamura. γ-Aminobutyric acid enhancement of CL 218,872 affinity and evidence of benzodiazepine receptor heterogenity. *Mol Pharmacol* **20:** 477–483, 1981.
12. Regan, J. W., H. I. Yamamura, S. Yamada and W. R. Roeske. High affinity renal ^{3}H-flunitrazepam binding: characterization, localization and alteration in hypertension. *Life Sci* **28:** 991–997, 1981.
13. Strittmatter, W. J., W. J. Hirata, J. Axelrod, P. Mallorga, J. F. Tallman and R. C. Henneberry. Benzodiazepine and β-adrenergic receptor ligands independently stimulate phospholipid methylation. *Nature* **282:** 857–859, 1979.
14. Syapin, P. J. and P. Skolnick. Characterization of benzodiazepine binding sites in cultured cells of neural origin. *J Neurochem* **32:** 1047–1051, 1979.
15. Tallman, J. F., J. W. Thomas and D. W. Gallager. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* **274:** 383–385, 1978.