Acknowledgements—I thank Dr. J. D. Gardner for his support of this study and also J. Dyer and W. Thompson for their technical assistance.

Departments of Pharmacology and Surgery
Uniformed Services University of the Health Sciences, and
The National Institute of Health
Bethesda, MD 20014, *U.S.A.*

SHMUEL BATZRI

REFERENCES

1. M. I. Grossman, in *Handbook of Physiology, Alimentary Canal* (Ed. C. F. Code) Section 6. Vol. 2, p. 835. American Physiological Society, Washington, DC (1967).
2. E. D. Jacobson and W. J. Thompson in *Advances in Cyclic Neucleotide Research* (Eds. P. Greengard and G. A. Robinson), Vol. 7, p. 199. Raven Press, New York (1976).
3. S. Batzri and J. D. Gardner, *Biochim. biophys. Acta* **541**, 181 (1978).
4. S. Batzri and J. D. Gardner, *Molec. Pharmac.* **16**, 406 (1979).
5. S. Batzri and J. Dyer, *Biochim. biophys. Acta*, in press.
6. A. H. Soll and A. Wollin, *Am. J. Physiol.* **237**, E444 (1979).
7. P. Scholes, A. Cooper, D. Jones, J. Major, M. Walters and C. Wilde, *Agents Actions* **6**, 677 (1976).
8. A. H. Soll, *Am. J. Physiol.* **238**, G366 (1980).
9. A. Sonnenberg, W. Hunziker, H. R. Koelz, J. A. Fisher and A. L. Blum, *Acta physiol. scand.* (Special Suppl.) 307 (1978).
10. A. Sonnenberg, T. Berglindh, M. J. M. Lewin, J. A. Fischer, G. Sachs and A. L. Blum, in *Hormone Receptors in Digestion and Nutrition* (Eds. G. Rosselin, P. Fromageot and S. Bonfils) p. 337. Elsevier/North Holland Biomedical Press, Amsterdam (1979).
11. C. V. Perrier and M. Griessen, *Eur. J. clin. Invest.* **6**, 113 (1976).
12. S. Batzri and J. D. Gardner, *Biochim. biophys. Acta* **508**, 328 (1978).
13. M. Osband and R. McCaffrey, *J. biol. Chem.* **254**, 9970 (1979).
14. P. DeMeyts, J. Roth, D. M. Neville, Jr., J. R. Gavin, III and M. A. Lesniak, *Biochem. biophys. Res. Commun.* **55**, 154 (1973).
15. M. A. Beaven, Z. Horakova and H. Keiser, *Eur. J. Pharmac.* **29**, 138 (1974).
16. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1948).
17. E. D. Brown and G. D. Aurbach, *J. biol. Chem.* **251**, 1232 (1976).
18. T. Berglindh, H. F. Helander and K. J. Obrink, *Acta physiol. scand.* **97**, 401 (1976).
19. G. J. Durant, C. R. Ganellin and M. E. Parson, *J. med. Chem.* **18**, 905 (1975).
20. R. Levi, C. R. Ganellin, G. Allan and H. J. Willens, *Eur. J. Pharmac.* **34**, 237 (1975).
21. T. Berglindh and G. Sachs, in *Hormone Receptors in Digestion and Nutrition* (Eds. G. Rosselin, P. Fromageot and S. Bonfils), p. 373. Elsevier/North-Holland Biomedical Press, Amsterdam (1979).
22. J. W. Black, W. A. M. Duncan, C. R. Furant, C. R. Ganellin and M. E. Parson, *Nature Lond.* **236**, 385 (1972).

---

* Send reprint requests and correspondence to: Shmuel Batzri, Ph.D., Uniformed Services University of the Health Sciences, Room A3017, 4301 Jones Bridge Road, Bethesda, MD 20014, U.S.A.

Biochemical Pharmacology, Vol. 30, No. 21, pp. 3016–3019, 1981.
Printed in Great Britain.

0006-2952/81/213016-04 $02.00/0

Inhibition of benzodiazepine receptor binding by several tryptophan and indole derivatives

(*Received* 26 *March* 1981; *accepted* 14 *May* 1981)

Today, increasing evidence exists that the pharmacological activities of tranquilisers of the benzodiazepine group are mediated via benzodiazepine specific, high-affinity binding sites. These "benzodiazepine receptors" are stereospecific and are only present in the central nervous system, but not in peripheral organs of many vertebrate species, with a pronounced regional distribution [1–4]. However, the physiological function of the benzodiazepine receptor is still unclear mainly because it is still not known which endogenously occurring substance represents the native ligand of this receptor. Inosine, hypoxanthine and nicotinamide have had most attention as possible candidates for the unknown endogeous ligand [5], though their affinities for the benzodiazepine receptor are very low (Table 1). Much higher affinities have been found for some β-carboline derivatives [6–9] whereof norharmane-3-carboxylic acid ethylester is the most potent [6]. However, this compound has not yet been found *in vivo* [6]. At the present state, harmane (1-methyl-β-carboline) is the most potent endogenously occurring [10–12] inhibitor of benzodiazepine receptor binding [7]. Therefore, harmane or another related β-carboline derivative are the most attractive candidates for the unknown endogenous ligand of the benzodiazepine receptor.

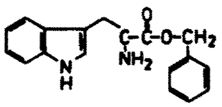
While the complete biosynthetic pathway of β-carbolines in animals or man is not known it is quite clear that these

Table 1. Inhibition of benzodiazepine receptor binding by several tryptophan and indole derivatives

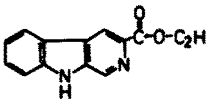
Inhibitor	IC$_{50}$	[mM]
L-Tryptophan	2.8	± 0.7
D-Tryptophan	4.1	± 1.1
L-Tryptophanamid	5.8	± 0.4
5-Hydroxy-L-tryptophan	2.5	± 0.2
L-Tryptophan methylester	1.1	± 0.1
L-Tryptophan ethylester	0.3	± 0.02
D/L-Tryptophan butylester	0.2	± 0.01
L-Tryptophan benzylester	0.06	± 0.007
D/L-5-Hydroxytryptophan ethylester	0.6	± 0.01
Tryptophanylglycine	1.2	± 0.2
Tryptophanylalanine	2.5	± 0.2
Tryptophanylphenylalanine	4.5	± 0.5
Glycyltryptophan	4.0	± 0.4
Tryptophol	1.9	± 0.3
5-Hydroxytryptophol	2.0	± 0.2
5-Methoxytryptophol	1.7	± 0.3
Tryptamine	1.8	± 0.6
5-Hydroxytryptamine	6.8	± 1.5
Melatonin	0.7	± 0.03
6-Methoxyharmalane	0.15	± 0.02
Indole	1.8	± 0.2
5-Hydroxyindole	1.9	± 0.1
Indole-2-carboxylic acid	2.8	± 0.5
Indole-2-carboxylic acid ethylester	2.2	± 0.5
3-Indolylacetone	1.5	± 0.1
Indole-3-acetaldehyde	1.5	± 0.3
Indole-3-acetic acid	10.2	± 0.02
Indole-3-acetic acid ethylester	0.5	± 0.02
Norharmane	0.008	± 0.001
Norharmane-3-carboxylic acid	0.03*	
Norharmane-3-carboxylic acid ethylester	0.000007*	
Harmane	0.007	± 0.001
Harmane-3-carboxylic acid ethylester	0.005*	
Harmine (7-methoxyharmane)	0.13	± 0.01
6-Methoxyharmane	0.01	± 0.001
2-Methylharmine	0.04	± 0.004
Harminic acid	0.75	± 0.03
Harminic acid diethylester	0.029	± 0.001
Inosine	1.8	± 0.2
Hypoxanthine	2.3	± 0.1
Nicotinamide	4.6	± 1.1

IC$_{50}$ Values, the concentrations of the inhibitors which inhibit specific [^{3}H]flunitrazepam binding by 50% were determined by log-probit analysis each performed for 5 or 6 concentrations of the inhibitors. The data are means of 4–6 individual determinations.

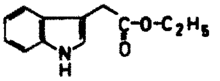
* Data from reference [6].



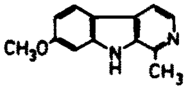
Tryptophan benzyl ester



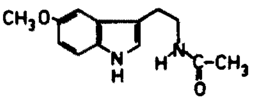
Norharmane 3-carboxylic acid ethyl ester



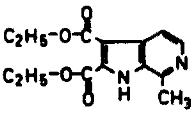
Indole-3-acetic acid ethyl ester



7-Methoxy harmane (Harmine)



Melatonine



Harminic acid diethyl ester

Fig. 1. Structural formulas of some of the compounds investigated.

compounds are condensation products of tryptophan, 5-hydroxytryptophan, tryptamine, or 5-hydroxytryptamine with C_1-fragments (norharmanes) or C_2-fragments (harmanes). Out of the 20 naturally occurring amino acids L-tryptophan had clearly the highest affinity for the benzodiazepine receptor [13] with an IC_{50} value similar to inosine, hypoxanthine, or nicotinamide (Table 1). This observation could be an indication that the cyclisation of tryptophan or tryptamine derivatives leading to the β-carboline structure is not mandatory to obtain derivatives with high affinity for the benzodiazepine receptor. Accordingly, we investigated the interaction of a large variety of tryptophan and indole derivatives with the benzodiazepine receptor in order to find structure activity relationships between indole derivatives and benzodiazepine receptor binding.

Materials and methods

[^{3}H]Flunitrazepan (sp. act. 87 Ci/mmole) was obtained from New England Nuclear (Dreieichenhain, West Germany). All indole derivatives were obtained from Sigma (Munich, West Germany), Serva (Heidelberg, West Germany), or Fluka (Neu-Ulm, West Germany). All other chemicals were obtained from commercial suppliers.

Synthesis of harminic acid diethyl ester (8-methyl-1-H-pyrrolo-2,3-C-pyridine-2,3-dicarboxylic acid diethyl ester): Harminic acid (220 mg, 1 mmole) was refluxed with thionyl chloride (2 ml) and one drop of *N,N*-dimethyl formamide for 90 min. Unreacted thionyl chloride was removed *in vacuo*. The dark oily residue was treated with dry ethanol (5 ml) under reflux for 30 min.

The reaction product was partitioned between 5% sodium carbonate solution and ether, the organic phase washed neutral, dried over anhydrous magnesium sulphate, and brought to dryness leaving a yellow oil This was chromatographed over silica gel with chloroform–methanol (19:1, v/v), yielding harminic acid diethyl ester (190 mg, 69%) after removal of the solvent as a nearly colourless oil which was pure according to t.l.c. and n.m.r. ($CDCl_3$) 1.25 (3H, t, $CH_2—C\underline{H}_3$), 1.43 (3H, t, $CH_2—C\underline{H}_3$), 2.73 (3H, s, $C\underline{H}_3$ at C-8), 4.34 (2H, q, $C\underline{H}_2—CH_3$), 4.47 (2H, q, $C\underline{H}_2—CH_3$), 7.81 (1H, d, H-5), 8.18 (1H, d, H-6), 9.23 (1H, s, $N\underline{H}$); FD-mass spectrum, *m/e* 276 ($M^{+\cdot}$, 100%). Anal. calcd. for $C_{14}H_{16}O_4N_2$ (276.29): C, 60.86; H, 5.84; N, 10.14. Found: C, 60.8; H, 5.7; N, 10.3.

Harminic acid diethyl ester could be recrystallised from carbon tetrachloride/hexane and was then obtained as an off-white microcrystalline powder, m.p. 112–114°.

Benzodiazepine receptor binding studies were performed as previously described using a centrifugation technique and [^{3}H]flunitrazepam as ligand [14]. One millilitre aliquots of a whole rat brain homogenate in 0.067 M phosphate buffer pH 7.4 (about 1 mg protein/ml) were incubated in triplicate together with about 0.1 nM [^{3}H]flunitrazepam and different concentrations of the displacers for 30 min at 4°. Unspecific binding was obtained from experiments performed in the presence of unlabelled diazepam 10 μM. Under these conditions, specific [^{3}H]flunitrazepam binding displays all the characteristics of brain specific benzodiazepine receptor binding [14].

Results and discussion

In agreement with the observations of Squires *et al.* [13] L- and D-tryptophan inhibit benzodiazepine receptor binding in a concentration dependent but not stereospecific fashion with IC_{50} values in the low mM range (Table 1). Compared to L-tryptophan, only slightly different IC_{50} values were found in the case of tryptophanamide, L-5-hydroxy-tryptophan, and L-tryptophan methyl ester. However, L-tryptophan ethyl and butyl ester are one order of magnitude more active than L-tryptophan itself. The affinity is further increased by one order of magnitude in the case of the L-tryptophan benzyl ester, whose IC_{50} is as low as 60 μmole/l (Table 1). A comparable effect of the ethyl ester formation was found for 5-hydroxytryptophan. Several tryptophan containing dipeptides inhibit benzodiazepine receptor binding with affinities similar to that of tryptophan itself (Table 1).

Some tryptophol derivatives and tryptamine interact with the benzodiazepine receptor with affinities similar to L-tryptophan while 5-hydroxytryptamine is less active (Table 1). Melatonin (5-methoxy-*N*-acetyl-tryptamine) is about one order of magnitude more potent than 5-hydroxytryptamine. But more important, the cyclic derivative of melatonin, 6-methoxyharmalane, is even more potent with an IC_{50} of about 150 μmole/l (Table 1).

In respect to the binding of trytophan derivatives to the benzodiazepine receptor, the mandatory part of the molecule seems to be the indole moiety as indicated by the IC_{50} values of indole, 5-hydroxyindole, indoleacetone, and indoleacetaldehyde which are similar to that of tryprophan (Table 1). While the ethyl ester formation has no effect on the affinity of indole-2-carboxylic acid it increases the affinity of indole-3-acetic acid by more than one order of magnitude (Table 1).

As already mentioned, the most pronounced effect of the ethyl ester formation has been reported for the tricyclic (β-carboline) derivative norharmane-3-carboxylic acid, where the ethylester has a more than three orders of magnitude higher affinity than the free acid or than norharmance itself (Table 1). This effect is not seen in the case of harmane where harmane-3-carboxylic acid ethylester is only as potent as harmane itself in inhibiting specific [^{3}H]flunitrazepam binding (Table 1).

Harmine (7-methoxyharmane) is considerably less potent than harmane itself while the isomeric 6-methoxyharmane is nearly as potent as harmane (Table 1). A similar although not so pronounced effect of the methoxy group in the 6-instead of the 7-position can be seen in the case of harmaline (7-methoxy-harmalan; IC_{50} = 400 μmole/l) [7] and of 6-methoxy-harmalan (IC_{50} = 150 μmol/l) (Table 1). Harminic acid, a metabolite of harmaline [15], is only five times less potent than harmine thought the structure of the molecule is distinctly changed by opening the benzene ring of the indole moiety. Again the esterification of the free carboxyl acid is followed by a 20 fold increase of the potency. It is noteworthy that harminic acid diethyl ester is only four times less active than the most potent endogenous β-carboline derivative, harmane, thought the tricyclic structure of the β-carbolines is no longer completely intact.

The data reported clearly indicate that a large variety of tryptophan and indole derivatives interact with the benzodiazepine receptor with affinities similar or even higher than that of L-tryptophan. However, in all cases lower affinities that that of harmane were found. Thus, we do not think that one of the compounds is directly related to the endogenous ligand of the benzodiazepine receptor although a few of them are present in mammals, e.g. 5-hydroxyindole, indole-3-carboxylic acid and possibly 6-methoxy-harmalan [16, 17]. However, two important conclusions can be drawn from our data in respect to β-carboline derivatives as possible endogenous ligands of the benzodiazepine receptor. (1) In several cases, the ethyl ester formation of an indole acetic or propionic acid derivative led to a pronounced increase of the affinity for the benzodiazepine receptor which raises serious doubts in accepting this structure–activity parameter *per se* as a specific indication for its biological significance. (2) Some tryptophan derivatives which do not have the β-carboline structure have also a much higher affinity than tryptophan itself (e.g. L-tryptophan benzyl ester). This supports our previous assumption that the endogenous ligand of the benzodiazepine receptor must not have in any case the tricyclic β-carboline structure. Therefore, we suggest to extend the search for the still unknown endogenous ligand of the benzodiazepine receptor to all indole derivatives and related compounds found in the body.

Acknowledgements—This study was supported by a grant of the Deutsche Forschungsgemeinschaft to W.E.M. The technical assistance of Ingeborg Zube is gratefully acknowledged.

Pharmakologisches Institut der Universität Mainz
Obere Zahlbacher Straße 67
D-6500 Mainz
West Germany

KLAUS J. FEHSKE
WALTER E. MÜLLER*
KARL L. PLATT
ANGELIKA E. STILLBAUER

REFERENCES

1. C. Braestrup and R. F. Squires, *Br. J. Psychiat.* **133**, 249 (1978).
2. H. Möhler and T. Okada, *Br. J. Psychiat.* **133**, 261 (1978).
3. J. F. Tallman, S. M. Paul, P. Skolnik and D. W. Gallager, *Science* **207**, 274 (1980).
4. W. E. Müller, *Pharmacology* **22**, 153 (1981).
5. P. J. Marangos, S. M. Paul, F. K. Goodwin and P. Solnick, *Life Sci.* **25**, 1093 (1979).
6. C. Braestrup, M. Nielsen and C. E. Olsen, *Proc. Natn. Acad. Sci. U.S.A.* **77**, 2288 (1980).
7. H. Rommelspacher, C. Nanz, H. O. Borbe, K. J. Fehske, W. E. Müller and U. Wollert, *Naunyn-Schmiedebergs Arch. Pharmacol.* **314**, 97 (1980).
8. H. Rommelspacher, Ch. Nanz, H. O. Borbe, K. J. Fehske, W. E. Müller and U. Wollert, *Eur. J. Pharmac.* **70**, 409 (1981).
9. W. E. Müller, K. J. Fehske, H. O. Borbe, U. Wollert, H. Rommelspacher and Ch. Nanz, *Pharm. Biochem. Behav.* **14**, 693 (1981).
10. H. Rommelspacher, S. Strauss and J. Lindemann, *FEBS Lett.* **109**, 209 (1980).
11. D. W. Shoemaker, J. T. Cummins, T. G. Bidder, H. G. Boettger and M. Evans, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmacol.* **310**, 227 (1980).
12. T. G. Bidder, D. W. Shoemaker, H. G. Boettger, M. Evans and J. T. Cummins, *Life Sci.* **25**, 157 (1979).
13. R. F. Squires, D. I. Benson, C. Braestrup, J. Coupet, C. A. Klepner, V. Myers and B. Beer, *Pharm. Biochem. Behav.* **10**, 825 (1979).
14. H. W. Damm. W. E. Müller, U. Schläfer and U. Wollert, *Res. Comm. chem. Path. Pharmac.* **22**, 597 (1978).
15. F. Flury, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **64**, 105 (1911).
16. M. D. Armstrong, N. F. Shaw, J. Gortakowski and H. Singer, *J. biol. Chem.* **234**, 17 (1958).
17. W. McIssaac, *Postgrad. Med.* **30**, 111 (1961).

---

* To whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 30, No. 21, pp. 3019–3022, 1981.
Printed in Great Britain.

0006–2952/81/213019–04 $02.00/0

Regulation of cerebellar L-[^{3}H]glutamate binding: influence of guanine nucleotides and Na$^+$ ions

(*Received* 10 *February* 1981; *accepted* 29 *May* 1981)

Purine nucleotides serve important functions in many biological processes. For example, the cyclic hydrolytic products of ATP and GTP (cylcic AMP and cyclic GMP, respectively) regulate cell growth and development [1], mediate hormonal [2], and certain neurotransmitter [3] responses, and, in addition purines themselves may have transmitter roles in the periphery [4] and central nervous system [5].

The receptor binding of many hormones or transmitters which effect changes in adenylate cyclase activity, is sensitive to guanine nucleotides: GTP and GDP have been demonstrated to produce selective decreases in the affinities of epinephrine [6], dopamine [7], serotonin [8] and opiates [9] for their receptors by an allosteric regulatory mechanism. It has been proposed that sensitivity to guanine nucleotides is a property of only these receptors that are linked to adenylate cyclase [10]. However, recent evidence indicates that ligand binding to the D2 dopamine receptor may also be regulated by these nucleotides [11].

We have reported previously that the binding of L-glutamate to its postsynaptic receptor on rat cerebellar membranes is influenced by an as yet unidentified endogenous inhibitor [12] and that guanine nucleotides, in contrast to adenine derivatives, produce substantial inhibition of specific binding [13].

This study was carried out in order to investigate the mechanisms underlying the inhibitory actions of guanine nucleotides and sodium ions on glutamate binding.

Materials and methods

Preparation of synaptic membranes. Female Wistar rats (250–300 g) were killed by decapitation and cerebella were homogenised in 20 vol. (w/v) 0.32 M sucrose in a Teflon–glass homogeniser (0.25 mm clearance). Synaptic membranes were then prepared as described previously [14]. The membrane pellet was suspended in glass-distilled water and subjected to a 30 min preincubation at 37°, followed by two or three washing stages with 50 vol. water, and recentrifugation of the membranes (50,000 *g* for 10 min).

The final pellet was dispersed in 50 mM Tris–citrate buffer (pH 7.1) or in buffer with added nucleotide (as the sodium salts) or sodium chloride, and used immediately for the binding assay.

During some of these experiments, aliquots of homogenates and supernatants were taken during various stages of membrane preparation, and were assayed for the presence of L-glutamate by a microenzymic fluorometric method [15]. No free glutamate was detectable in the final membrane preparation as compared with 250 nmole/g tis-