Biochemical Pharmacology, Vol. 28, pp. 1123–1128.

0006-2952/79/0401-1123 $02.00/0

CHARACTERIZATION OF [^{3}H]MUSCIMOL BINDING TO MOUSE BRAIN MEMBRANES

YNG-JIIN WANG, PAUL SALVATERRA and EUGENE ROBERTS *
Division of Neurosciences, City of Hope National Medical Center, Duarte, CA 91010, U.S.A.

(*Received* 3 *May* 1978; *accepted* 25 *August* 1978)

Abstract—The binding of [^{3}H]muscimol {[methylene-^{3}H(N)]-3-hydroxy-5-aminoethyl isoxazole} to a membrane fraction from mouse brain was studied as a possible model for the GABA recognition site of the γ-aminobutyric acid (GABA)–anionophore receptor complex. Kinetic studies showed two distinct association and dissociation rate constants, 2.3×10^{-4} and 1.4×10^{-5} sec^{-1} nM^{-1} for association and 1.2×10^{-2} and 1.0×10^{-3} sec^{-1} for dissociation. Equilibrium analysis (Scatchard plot) of binding data also indicated two types of sites with K_D equal to 9×10^{-9} and 70×10^{-9} M. Detergents (0.1% Triton X-100, 0.1% Lubrol PX or 5% Tween 20) had no effect on the binding of [^{3}H]muscimol or other conformationally restricted agonists such as imidazoleacetic acid, isoguvacine, THIP (4,5,6,7-tetrahydroisoxazole [5,4-c] pyridine-2-ol), and (±)-isonipecotic acid. In contrast to this, the detergents potentiated the ability of the less conformationally restricted agonists such as GABA and β-alanine in displacing [^{3}H]muscimol. Binding of bicuculline was reduced by all of the detergent treatments.

Muscimol {[methylene-^{3}H(N)]-3-hydroxy-5-aminoethyl isoxazole}, a γ-aminobutyric acid (GABA) analogue with restricted conformation, is a powerful GABA-mimetic substance [1–5]. This neuroactive substance, which occurs naturally in the mushroom *Amanita muscaria*, is the most potent specific GABA agonist known to date. In animals and humans, muscimol seems to exert its major neuropharmacologic action by central GABAergic activity. In humans it is hallucinogenic. *In vitro* studies employing membrane preparations from calf or rat brain have shown that binding of muscimol probably takes place to the GABA recognition site of the GABA receptor complex [6–8]. Quantitative electrophysiological studies have shown that muscimol acts on GABA receptors of the slowly adapting neurons of the crayfish stretch receptor [9] and mammalian central nervous system [10]. In all quantitative studies to date, muscimol has proven to be more potent than GABA itself in activating GABA receptors. This paper presents our results on the biochemical and pharmacological characterization of muscimol receptors in mouse brain.

MATERIALS AND METHODS

The brains (minus the stems) of fifty Swiss mice were homogenized in 200 ml of 0.25 M sucrose solution at 4°. The homogenate was centrifuged at 1,050 *g* avg. for 10 min in a Beckman type 30 rotor and the supernatant fraction (S_1) was collected. The pellet obtained after centrifuging the S_1 fraction at 17,000 *g* for 15 min was resuspended in 125 ml of 5 mM Tris buffer, pH 8.1, containing 50 μM $CaCl_2$ by passing three times through a 14 gauge hypodermic needle connected to a 50 ml Luer-lock syringe. This suspension was kept at 4° for 10 min and then centrifuged at 70,000 *g* for 10 min. The pellet (P_2) was frozen and thawed, suspended in 50 ml of ice-cold distilled water, and centrifuged at 70,000 *g* for 10 min. This freeze–thaw–centrifugation procedure was repeated once and the final pellet was suspended in 25 ml of distilled water to give a protein concentration of 10–11 mg/ml and stored frozen.

For binding studies, the suspension was thawed, diluted in 7 vol. of ice-cold distilled water and centrifuged at 70,000 *g* for 20 min. The final pellet was resuspended in distilled water and immediately assayed. Usually 0.5 to 0.6 mg protein of the above P_2 preparation was incubated in 0.05 M Tris–HCl buffer (pH 7.3) with various concentrations of [^{3}H]muscimol in the absence (specific binding) or presence (nonspecific binding control) of 10^{-3} M muscimol in a final volume of 1 ml. After incubation at 4° for 30 min, the mixture was centrifuged at 20,000 *g* in a Sorvall centrifuge using the SM-24 rotor. The pellet was superfically rinsed two times with 3-ml aliquots of distilled water, solubilized in 0.5 ml Soluene at 37°, transferred to a scintillation vial containing 10 ml of acidified liquid scintillation mixture (PPO+ 4.95 g, POPOP‡ 0.5 g, and 0.59 ml glacial acetic acid in 1 liter toluene), and counted in a Packard Tri-Carb (model 3320) scintillation spectrometer. For kinetic studies a filtration assay was used. The mixture of membranes and radioactive muscimol was filtered through a GF/B glass filter (2.5 cm disc) and washed twice very quickly with 0.05 M Tris, pH 7.3. The filtration and washing took less than 15 sec and the GF/B filters retained no specific [^{3}H]muscimol when tissue was omitted from the assay mixture or the samples were preincubated at 90° for 15 min before assay.

To determine the chemical nature of the membrane-bound radioactivity, the following experiment was done. An aliquot (0.6 mg) of mouse P_2 was prepared for binding as described above. After washing the final pellet with distilled water, all radioactivity was quantitatively extracted by incubation at 0° for 30 min with 50% ethanol. After centrifugation, the extract was lyophilized, resuspended in 10 μl of 10^{-3} M unlabeled muscimol and analyzed by silica gel G thin-layer chromatography. The chromatogram was developed with

* To whom reprint requests should be sent.
+ 2,5-Diphenyloxazole.
‡ 1,4-Bis-[2-(4-methyl-5-phenyloxazolyl)-thiophenel.

isopropanol–methyl ethyl ketone–ammonium hydroxide (12:3:5, v/v) and spots were visualized with ultraviolet light. Nearly all (90–95 per cent) of the radioactivity co-migrated with authentic muscimol, indicating little if any metabolic breakdown of this compound under the conditions of our binding assay.

[^{3}H]muscimol (12 Ci/m-mole) was obtained from the New England Nuclear Corp. (Boston, MA). Triton X-100 was purchased from the Packard Co. (Downers Grove, Il), and Tween 20 from the J. T. Baker Co. (Phillipsburg, NJ). Lubrol PX and digitonin were obtained from the Sigma Chemical Co. (St. Louis, MO). Isoguvacine, and THIP (4,5,6,7-tetrahydroisoxazole [5,4-c] pyridine-3-ol) were gifts from Dr. Paul Krogsgaard-Larsen, 3-aminopropanesulfonic acid was a gift from Dr. R. Olsen, and muscimol from Dr. E. Costa and the Ciba-Geigy Corp. (Ardsley, NY). Imidazoleacetic acid was purchased from CalBiochem (San Diego, CA). The method of Lowry *et al.* [11] was used for protein assay.

RESULTS

Dependence of [^{3}H]*muscimol binding on receptor and ligand concentrations.* Figure 1 shows the equilibrium binding curve of [^{3}H]muscimol. Nonspecific binding increased linearly up to 400 nM muscimol, whereas specific binding showed a typical saturation pattern. At a low concentration of muscimol (1 nM), the signal/noise ratio was high, approximately 12/1, a value much higher than the approximately 3/1 reported for the binding of GABA [12]. At higher muscimol concentrations, nonspecific binding became predominant and the signal/noise ratio became less than 1 for muscimol concentrations above 270 nM. A Scatchard plot (Fig. 2) of muscimol binding suggested two possible mechanisms: (1) one homogeneous binding site with a K_D about 10 nM, $B_{max} = 2.1$ pmoles/mg; or (2) two different binding sites with $K_D = 9$ nM, $B_{max} = 2.1$ pmoles/mg and $K_D = 70$ nM, $B_{max} = 0.7$ pmoles/mg respectively. Statistical analysis of the Scatchard plot did not rule out either case, and measurements of binding which would be sufficiently accurate to reach a decision were not possible at high concentrations of muscimol because of the low signal/noise ratio. The slope (n) of the Hill plot for [^{3}H]muscimol binding was calculated to be 0.93, indicating that there was no positive or negative cooperativity in the binding (Fig. 3).

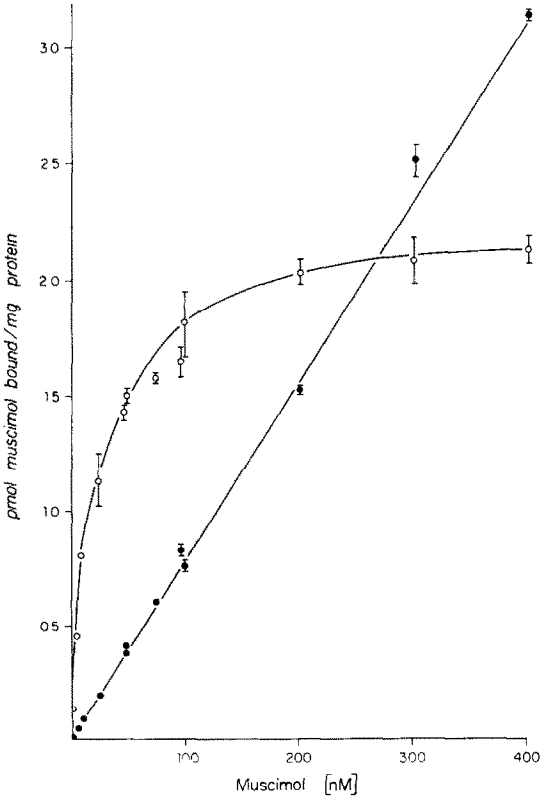

Fig. 1. Specific and nonspecific binding of [^{3}H]muscimol to mouse P_2 fraction. The P_2 preparation (0.6 mg/ml) was incubated in 50 mM Tris, pH 7.3, containing various concentrations of [^{3}H]muscimol. Incubation was carried out as described in Materials and Methods. Nonspecific binding represents the binding of [^{3}H]muscimol in the presence of 1 mM non-radioactive muscimol (●). Specific binding was calculated by subtracting the nonspecific binding from that found when only the [^{3}H]muscimol was present in the incubation mixture (○).

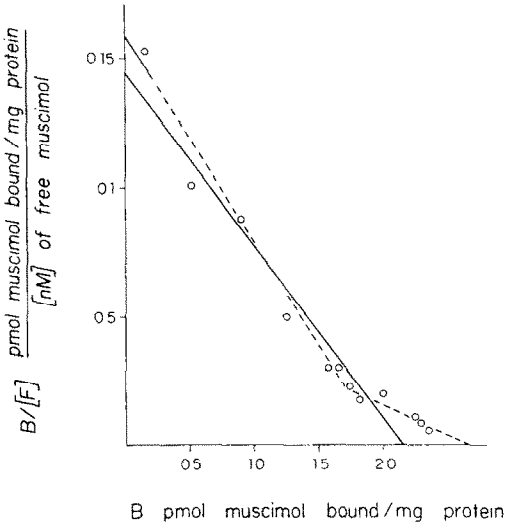

Fig. 2. Scatchard plot of [^{3}H]muscimol binding. Data are from the experiments of Fig. 1. Points were fitted by least squares linear regression. With $P < 0.01$, two possible cases were proposed: (1) one binding site (———) with $K_D = 10$ nM, $B_{max} = 2.1$ pmoles/mg; and (2) two binding sites (– – – – –) with $K_D^1 = 9$ nM, $B_{max}^1 = 2.1$ pmoles/mg and $K_D^2 = 70$ nM, $B_{max}^2 = 0.7$ pmole/mg respectively. Data from five experiments were used.

Figure 4 shows that [^{3}H]muscimol binding was linear with protein concentrations up to 1 mg/ml.

Kinetics of muscimol binding. Dynamic features of ligand–receptor interaction can only be understood after the derivation of kinetic constants. In the case of neurotransmission, the association and dissociation of transmitter from receptor may be related closely to the opening and closing, respectively, of ion channels. Moreover, it is necessary to know the relationship of the structures of various agonists and antagonists to their kinetic behavior, since one of our aims is to devise ligands that associate with receptors extremely rapidly and dissociate only very slowly. The availability of a high affinity, slowly dissociating ligand for GABA

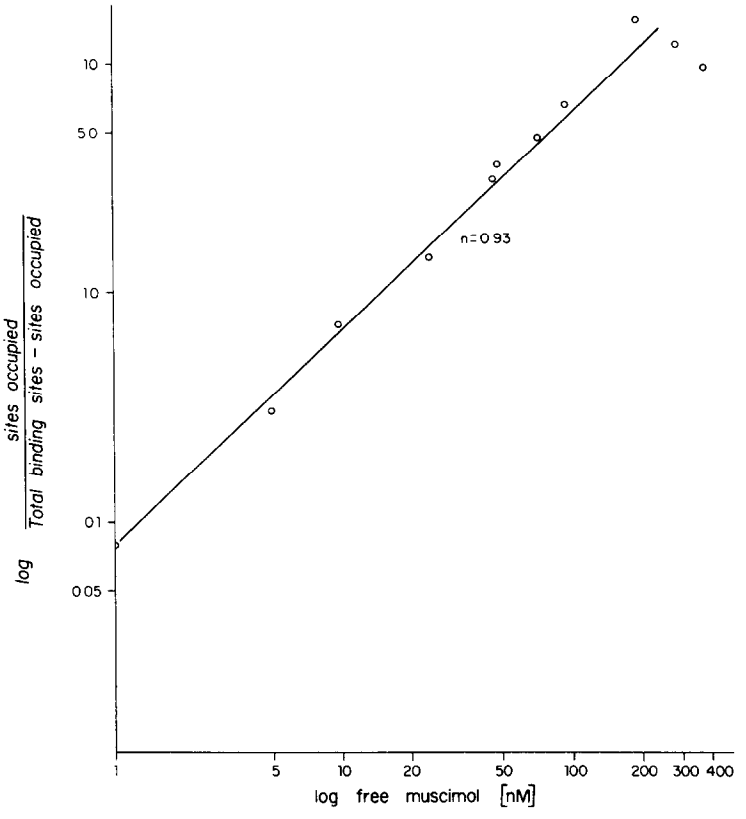

Fig. 3. Hill plot of [^{3}H]muscimol binding. See legend for Figs. 1 and 2.

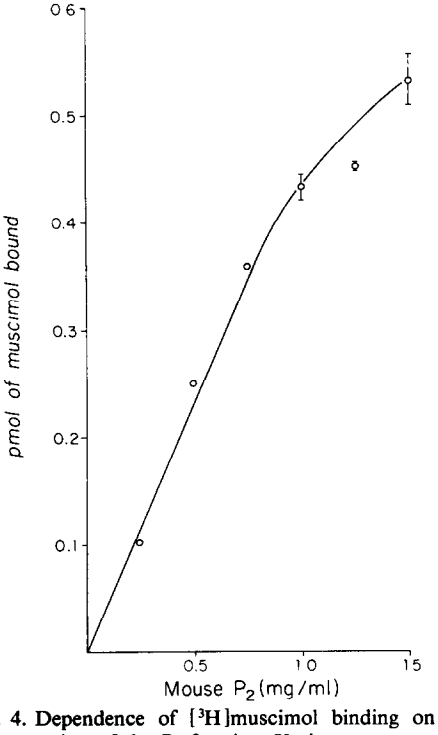

Fig. 4. Dependence of [^{3}H]muscimol binding on protein concentration of the P_2 fraction. Various concentrations of mouse P_2 were incubated with 5 nM of [^{3}H]muscimol in 50 nM Tris, pH 7.3 Only the specific binding is shown. Assays were carried out in duplicate as described in Materials and Methods. Protein determinations were done before and after the binding assay.

receptor recognition sites would be an invaluable aid in attempts to isolate these receptors.

According to Enna and Snyder [12], Na^+-independent binding of [^{3}H]GABA to its receptor showed very rapid association and dissociation, making it impossible to measure the kinetic constants. Taking advantage of the stronger binding of [^{3}H]muscimol and using a very rapid filtration assay with 10-sec resolution, we have successfully determined the association and dissociation rates of this GABA agonist with the binding sites on the membrane particles.

Two association rates differing by a factor of 16 were derived from the data shown in Fig. 5a. The rates are characterized by two constants, 2.3×10^{-4} sec^{-1} nM^{-1} and 1.4×10^{-5} sec^{-1} nM^{-1}. These values were reproducible on replication with less than 10 per cent error. The dissociation of [^{3}H]muscimol from its binding sites was also characterized by two different rates, 0.012 sec^{-1} and 0.001 sec^{-1} (Fig. 5b), corresponding to half-lives of 58 sec and 16.7 min respectively. Approximately 20–30 per cent of the total binding sites could not be monitored with the assay system employed.

Effects of several GABA agonists and antagonists on muscimol binding. Sixteen substances structurally related to GABA were tested for their ability to inhibit [^{3}H]muscimol binding (Table 1). The relative potencies were as follows: muscimol > 3-aminopropanesulfonate > GABA > isoguvacine > THIP > isonipecotic acid > imidazoleacetic acid > bicuculline = β-alanine. β-4-Chlorophenyl GABA (Lioresal), picrotoxin, and δ-aminolevulinic acid required more than 1 mM to produce an inhibition of 50 per cent (IC_{50}). Figure 6 shows

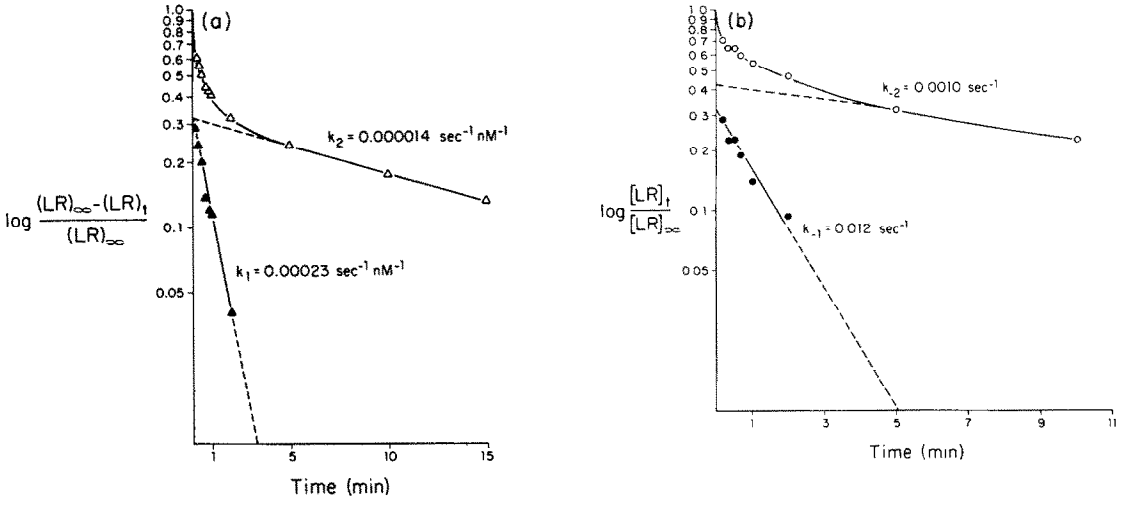

Fig. 5. Kinetic studies of [^{3}H]muscimol binding to the P_2 fraction. (a) Association. One ml of incubation mixture contained 0.7 mg of P_2 protein and 84 nM muscimol in 0.05 M Tris, pH 7.3. After incubation at 4° for the time indicated, free and bound [^{3}H]muscimol were separated by filtration (see Materials and Methods). $(LR)_t$ represents the amount of [^{3}H]muscimol–receptor complex at time t. $(LR)_\infty$ represents the total [^{3}H]muscimol binding sites. With the concentration of [^{3}H]muscimol (84 nM) we used in this study $(LR)_\infty = B_{max}^1 + B_{max}^2 = 2.8$ pmoles/mg. (b) Dissociation. The same mixture as above was incubated for 30 min, then 10 ml of 10^{-2} M muscimol was added, incubation was continued for various times at 4°, and filtration was as described in Materials and Methods.

typical binding curves (solid lines) of [^{3}H]muscimol in the absence and presence of several concentrations of non-radioactive muscimol, 3-aminopropanesulfonate, GABA, β-alanine, and bicuculline.

Effects of detergents on muscimol binding. Triton X-100, which solubilizes acetylcholine receptors, fails to extract GABA receptors from rat brain membranes [13, 14]. In an initial attempt to isolate the GABA receptor complex from mouse brain, we have studied the effect of several detergents on muscimol binding. Treatment of the P_2 fraction with Tween 20 (5%) did not decrease muscimol binding but actually increased it approximately 1.4-fold (Table 2). Scatchard analysis of [^{3}H]muscimol binding on Tween 20-

Table 1. The IC_{50} values of various agonists and antagonists on [^{3}H]muscimol binding*

Ligand	Mouse P_2 (μM)
Muscimol	0.02
3-Aminopropanesulfonate	0.10
GABA	0.20
Isoguvacine	0.25
THIP†	0.63
Imidazoleacetic acid	1.6
Isonipecotic acid	2.5
Bicuculline	13
β-Alanine	14
Others‡	>1000

* About 0.6 mg/ml of mouse P_2 was incubated with various concentrations of different agonists and antagonists in the presence of 8.4 nM [^{3}H]muscimol. [^{3}H]muscimol binding in the absence of any other ligand gave a value of about 6000 cpm. Nonspecific binding was 500 cpm. The IC_{50} was obtained from 50 per cent of specific binding (for typical case, see Fig. 6).

† 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol.

‡ Picrotoxin, glycine, L-carnosine, δ-aminolevulinic acid, β-4-chlorophenyl GABA, nicotinic acid and isonicotinic acid.

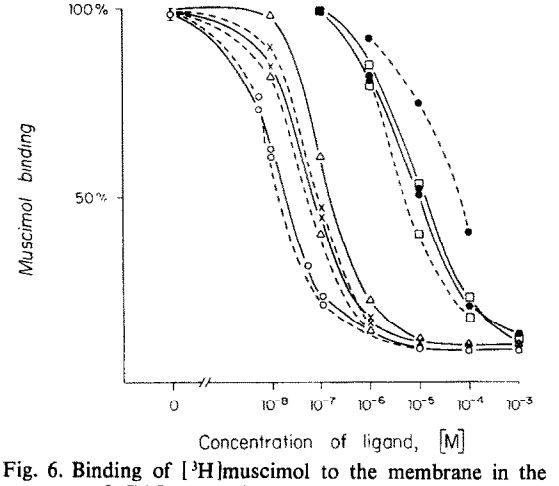

Fig. 6. Binding of [^{3}H]muscimol to the membrane in the presence of GABA agonists and antagonists. One ml of 0.6 mg/ml of mouse P_2 was incubated with various concentrations of muscimol (○), 3-aminopropanesulfonate (×), GABA (△), β-alanine (□) and bicuculline (●) in the presence of 8.4 nM [^{3}H]muscimol. The binding of [^{3}H]muscimol in the absence of other ligands is expressed as 100 per cent binding (~6000 cpm). Nonspecific binding was about 10 per cent (~ 500 cpm). In some cases, the same amount of the P_2 fraction was treated with Lubrol PX (90.1%) at 37° for 30 min. The membrane was spun down, resuspended and incubated with 8.4 nM [^{3}H]muscimol in the presence of muscimol (○), aminopropanesulfonate (×), GABA (△), β-alanine (□) and bicuculline (●). Solid lines represent the binding of Lubrol PX-treated mouse P_2.

Table 2. Effect of detergent treatment on muscimol binding*

Detergent	Concn (w/v)	[^{3}H]muscimol binding (% of control ±S.D.)	Protein amount (% of control)
Triton X-100	0.01%	112 ± 6	84
	0.05	111 ± 6	54
	0.1	116 ± 4	53
	0.5	80 ± 7	44
	1.0	71 ± 2	42
	2.5	59 ± 1	37
	5.0	48 ± 25	37
Digitonin	0.002	102 ± 3	†
	0.02	105 ± 5	†
	0.05	107 ± 5	†
	0.1	105 ± 10	†
	0.2	106 ± 7	†
	0.5	70 ± 3	†
	1.0	84 ± 7	†
Tween 20	0.01	104 ± 2	93
	0.05	109 ± 3	84
	0.1	111 ± 9	84
	0.5	126 ± 7	74
	1.0	127 ± 1	84
	2.5	140 ± 1	84
	5.0	142 ± 1	93
Lubrol PX	0.01	110 ± 1	79
	0.05	99 ± 3	60
	0.1	96 ± 4	67
	0.5	69 ± 7	58
	1.0	70 ± 3	57
	2.5	70 ± 2	58
	5.0	76 ± 2	65

* One ml P_2 (0.6 to 0.7 mg/ml of protein) was incubated in 0.05 M Tris, pH 7.3, containing various amounts of detergent, as indicated in the table. After incubation at 37° for 30 min, the suspension was spun down at 20,000 *g* in the Sorvall centrifuge. The pellet was resuspended and incubated in 0.05 M Tris, pH 7.3, containing 8.4 nM [^{3}H]muscimol. Incubation and processing of this sample were then carried out as indicated in Materials and Methods. Data were obtained from two experiments, each run in duplicate. Binding of muscimol to mouse P_2 without any detergent treatment gives ~5500 cpm (100 per cent). This is used as control to all of the detergent-treated membranes.

† Detergent strongly interferes with protein determination.

treated mouse P_2 yields a K_D of 5 nM and a B_{max} of 3.2 pmoles/mg. Triton X-100, Lubrol PX, and digitonin up to 0.1% did not effectively remove muscimol bound to the particulate fraction. Treatment of membranes with more than 0.5 per cent of the latter three detergents caused a decrease in muscimol binding.

Treatment of mouse P_2 fraction with 0.1% Lubrol PX changed the potency of some agonists and antagonists in competing with muscimol binding. After 0.1% Lubrol PX treatment, the IC_{50} value for bicuculline increased 3-fold (Fig. 6), those for GABA and β-alanine decreased 2-fold, while the efficacy of muscimol, 3-aminopropanesulfonate, isoguvacine, THIP and imidazoleacetic acid remained the same.

DISCUSSION

From our kinetic study of [^{3}H]muscimol binding with GABA receptors, it is reasonable to assume that two different binding sites were observed in the equilibrium analysis with $K_D = 9$ nM, $B_{max} = 2.1$ pmoles/mg and $K_D = 70$ nM, $B_{max} = 0.7$ pmole/mg, respectively. The binding sites with lower affinity represent 25 per cent of the total binding sites. This is comparable to the number of the binding sites seen for the slower associating and dissociating component. Since $t_{\frac{1}{2}}$ was about 60 sec for the faster dissociating component, this would give a 25 per cent loss of total binding if the membranes stay on the GF/B filter disc for 15 sec during the assay. This may be the reason that we were not able to detect 30 per cent of the total binding. Assigning this 30 per cent to the faster dissociating and associating components, we calculate that 60–70 per cent of total binding sites were of the latter class. This number is in the range of the 75 per cent obtained from the Scatchard plot for higher affinity sites. Since the Hill constant ($n = 0.93$) indicates that there was no cooperative interaction between these two different binding sites, we can assume two heterogeneous sites with receptor ligand

binding expressed by the following equations:

$$[\text{muscimol}] + [\text{receptor}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{complex}]_1 \quad (1)$$

$$[\text{muscimol}] + [\text{receptor}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [\text{complex}]_2 \quad (2)$$

With $k_1 = 0.00023\ \text{sec}^{-1}\ \text{nm}^{-1}$, $k_{-1} = 0.012\ \text{sec}^{-1}$, $k_2 = 0.000014\ \text{sec}^{-1}\ \text{nM}^{-1}$ and $k_{-2} = 0.0010\ \text{sec}^{-1}$, the values of 52 and 72 nM were obtained for K_D^1 and K_D^2 respectively. The two apparent sites for muscimol binding may be at subsynaptic and extrasynaptic membrane loci. Such sites appear to have been distinguished in a recent study of GABA-induced membrane current noise in the crayfish muscle fibers [15].

In contrast to our results on the effects of detergents, Beaumont *et al.* [6] reported that the binding of muscimol rat brain P_2 increased at least 4-fold when membranes were treated with 0.05% Triton X-100, whereas we observed little if any effect. This apparent discrepancy may be attributed to the presence of an endogenous inhibitor of GABA binding in brain P_2 which can be removed by repeated freeze–thaw cycles or alternatively by Triton X-100 washing [16, 17]. Our mouse P_2 was subjected to several freeze–thaw cycles and prewashed immediately before the binding assay and hence should not contain any significant amount of endogenous inhibitor [16].

Our studies of [^{3}H]muscimol binding to detergent-treated membranes also show that the GABA recognition site of the GABA receptor is resistant to extraction with non-ionic detergents in concentrations up to 0.1%. At the latter concentration, Triton X-100 and Lubrol PX extracted about half of the protein from membrane. An especially interesting feature of the effect of Tween 20 was that it enhanced the total muscimol binding 1.4-fold. This may be attributed to the exposure of buried receptor by the action of Tween 20.

Treatment of membranes with 0.1% Lubrol PX did not decrease binding of [^{3}H]muscimol. However, the ability of GABA and β-alanine to compete with [^{3}H]muscimol binding was increased. On the other hand, the potency of bicuculline was decreased. Agonists with a more restricted conformation than GABA such as imidazoleacetic acid, THIP, isoguvacine or isonipecotic acid did not change significantly in their potencies. Treatment of the membrane preparations with 0.1% Triton X-100 gave similar results. Thus, non-ionic detergents tend to increase the binding of agonists with flexible conformations, such as GABA and β-alanine, and to decrease that of a purported atangonist, i.e. bicuculline. The mechanism of this action is currently under investigation.

The [^{3}H]muscimol binding sites we have studied are most likely enriched in synaptic regions since our P_2 preparation is enriched in synaptic plasma membrane and exhibits a 10-fold higher specific muscimol binding when compared to a mouse brain myelin fraction containing little if any synaptic plasma membrane. Moreover, the binding of [^{3}H]muscimol in rats has been demonstrated to be brain specific since no specific binding could be detected in other tissues, including heart, stomach, intestine, diaphragm, liver and kidney [6].

In summary, our results support the hypothesis that [^{3}H]muscimol may be used as a GABA receptor ligand in mouse brain by confirming and extending the observations of others using calf or rat brain [6–8]. We have also presented a kinetic analysis of [^{3}H]muscimol binding which should be useful in further pharmacological characterization of the GABA recognition site of the GABA receptor complex.

Acknowledgements—This work was supported in part by Grants NS-1615 and NS-12116 from the National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health. We thank Phyllis Degener for her skillful technical assistance.

REFERENCES

1. C. H. Eugster, in *Ethnopharmacologic Search for Psychoactive Drugs* (Eds D. H. Efron, B. Holmstedt and N. S. Kline) pp. 416–439. U.S. Dept. of Health, Education and Welfare, Washington, D.C. (1967).
2. G. A. R. Johnston, D. R. Curtis, W. C. deGroat and A. W. Duggan, *Biochem. Pharmac.* **17**, 2488 (1968).
3. G. F. R. Müller and C. H. Eugster, *Helv. chim. Acta* **48**, 910 (1965).
4. A. Scotti de Carolis, F. Lipparini and V. G. Longo, *Psychopharmacologia* **15**, 186 (1969).
5. W. Theobald, O. Büch, H. A. Kunz, P. Krupp, E. G. Stenger and H. Heimann, *Arzneimittel-Forsch.* **18**, 311 (1968).
6. K. Beaumont, W. S. Chilton, H. I. Yamamura and S. J. Enna, *Brain Res.* **148**, 153 (1978).
7. S. R. Snodgrass, *Nature, Lond.* **273**, 392 (1978).
8. M. J. Leach and J. A. Wilson, *Eur. J. Pharmac.* **48**, 329 (1978).
9. N. Hori, K. Ikeda and E. Roberts, *Brain Res.* **141**, 364 (1978).
10. P. Krogsgaard-Larsen, G. A. R. Johnston, D. R. Curtis, C. J. A. Game and R. M. McCulloch, *J. Neurochem.* **25**, 803 (1975).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. S. J. Enna and S. H. Snyder, *Brain Res.* **100**, 81 (1975).
13. D. T. Wong and J. S. Horng, *Life Sci.* **20**, 445 (1977).
14. S. J. Enna and S. H. Snyder, *Molec. Pharmac.* **13**, 442 (1977).
15. J. Dudel, W. Finger and H. Stettmeier, *Neurosci. Lett.* **6**, 203 (1977).
16. D. V. Greenlee, P. C. Van Ness and R. W. Olsen, *Life Sci.*, in press.
17. A. Guidotti, G. Toffano, M. Baraldi, J. P. Schwartz and E. Costa, *GABA Neurotransmitters*, Alfred Benzon Symp. #12. Munksgaard Press, Copenhagen, in press.