Biochemical Pharmacology, Vol. 41, No. 8, pp. 1141–1144, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

ENANTIOSELECTIVITY AT THE PHYSIOLOGICALLY ACTIVE $GABA_A$ RECEPTOR

JULIANNA KARDOS,* ILONA KOVÁCS, EDIT SIMON-TROMPLER and FERENC HAJÓS†
Central Research Institute for Chemistry, The Hungarian Academy of Sciences, P.O. Box 17, H-1525 and †Department of Anatomy, School of Veterinary Medicine, Budapest, Hungary

(*Received* 16 *April* 1990; *accepted* 14 *November* 1990)

Abstract—A subfraction of cortical tissue from rat brain, containing membrane vesicles was prepared freshly with added protease inhibitors and antioxidant. The preparation was used to measure stimulation of transmembrane $^{36}Cl^-$ flux and inhibition of bicuculline-sensitive [^{3}H]muscimol binding by (+)-(*S*) and (−)-(*R*) enantiomers of dihydromuscimol at 30° in physiological salt solution. Displacement of bound [^{3}H]muscimol and stimulation of $^{36}Cl^-$ flux appeared in the 0.1–10 μM concentration range of the enantiomers, channel gating, however, required rather high concentrations. Degrees of enantioselectivity for channel gating, desensitization of and binding to $GABA_A$ receptors were estimated by the concentration ratios of dihydromuscimol enantiomers, [(−)-(*R*)]/[(+)-(*S*)], at the same level of response or displacement. Different enantioselectivities were observed for channel gating (6 ± 3), receptor binding (3 ± 2) and desensitization (no selectivity). The low and concentration-dependent enantioselectivities found for channel gating and receptor binding can be explained by desensitization and heterogeneity of $GABA_A$ receptors.

The degrees of ligand enantioselectivity for GABA binding sites, expressed as the ratio of IC_{50} values obtained from displacement of [^{3}H]GABA,‡ [^{3}H]THIP and [^{3}H]P4S bound to specific sites on isolated and purified rat brain synaptosomal membranes by DHM enantiomers, are far higher than one would expect on the basis of the inhibitory effect of DHM enantiomers on the firing of cat spinal interneurons [1]. Results obtained with other chiral analogues of muscimol and GABA having the same or no affinity for the GABA transport system have also suggested that $GABA_A$ receptors in the physiological state exhibit a lower degree of enantioselecitvity (for a review see Ref. 2). The implications of these findings can be rather fundamental as they make binding methods for studying physiological $GABA_A$ receptor questionable.

An understanding of this discrepancy may require taking into account the experimental differences between binding and functional studies of the $GABA_A$ receptor, such as differences in the integrity of the membrane-bound GABA receptor and the use of incubation media. In the present study, therefore, we applied a new experimental approach in which both the binding and the functional tests of the $GABA_A$ receptor, i.e. inhibition of bicuculline-sensitive [^{3}H]muscimol binding and stimulation of the transmembrane $^{36}Cl^-$ flux by DHM enantiomers were performed on native membrane vesicles, freshly prepared with added protease inhibitors and antioxidant in physiological media. A preliminary report on this subject has been published recently [3].

MATERIALS AND METHODS

Membrane vesicles. Preparation of the membrane vesicle fraction was carried out according to the method of Cash and Subbarao [4] with minor modifications. Three sliced cerebral cortices from male Wistar rats, 6 weeks old were suspended in 60 mL of HEPES buffered (pH 7.5) 0.32 M sucrose solution containing antipain, pepstatin A, leupeptin (5 μg/mL each), aprotinin (10 μg/mL) and phenylmethylsulphonyl fluoride (1 mM) as well as butylated hydroxytoluene (20 μM). The mixture was homogenized with Ultra-Turrax (setting 4) for 2 × 10 sec. Thereafter the short procedure (omitting the Ficoll gradient separation, [4]) followed. Final pellets were resuspended in physiological salt solution and adjusted to 20 mg of protein/mL. The protein concentration was assayed by the Folin reagent method [5]. Applying standard electron microscopy [6] we have observed that the preparation contains numerous membrane vesicles of different sizes and shapes. In addition, a few intact synaptosomes and free mitochondria are also present in the preparation.

$^{35}Cl^-$ *Influx measurements.* We used the procedure previously described [7] with slight modifications as follows. Each sample was preincubated at 30° (pH 7.5) for 15 min. A 0.100 mL suspension was then mixed with 0.300 mL prewarmed physiological salt solution containing $^{36}Cl^-$ (2.5 μCi/mL, ICN) in the presence or absence of DHM enantiomers (kindly provided by Prof. Krogsgaard-Larsen, Royal Danish School of Pharmacy, Copenhagen, Denmark) or GABA (Sigma Chemical Co., St Louis, MO,

* To whom correspondence should be addressed.

‡ Abbreviations: GABA, 4-aminobutyric acid; (+)-(*S*)-DHM, (+)-(*S*)-dihydromuscimol; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol); P4S, piperidine-4-sulphonic acid; bicuculline, (−)-(1*S*,9*R*)-bicuculline methiodide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulphonic acid; physiological salt solution, 145 mM NaCl, 5.0 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5; IC_{50}, concentration for half-inhibition of binding.

U.S.A.). After 7 sec, $^{36}Cl^-$ influx was terminated by the addition of 0.5 mL quench solution (ice-cold physiological salt solution containing picrotoxin and furosemide, 3 mM each). The mixture was filtered through Whatman GF/B filters. The vesicles on the filters were washed with 2 × 2 mL quench solution and the radioactivity on the filters was counted for 10 min. Specific influx was determined by subtracting unspecific uptake (without any drug added) from the total uptake. Five hundred counts of the total of 1500 counts were specific with 1 mM GABA and 7 sec assay time. Under this condition, the specific chloride influx shared 13% of the inter-nal volume [4] of the vesicles.

[^{3}H]*Muscimol binding*. Binding of [^{3}H]muscimol to membrane vesicles was determined by incubation of membrane vesicles (2 mg of protein) in 1 mL of physiological salt solution containing [^{3}H]muscimol (8 Ci/mmol, Amersham) in the presence or absence of the DHM enantiomers. After 10 min of incubation at 30°, the binding process was terminated by centrifugation for 5 min at 10,000 rpm. The supernatant was aspirated and the pellets were rapidly rinsed twice with 1 mL of ice-cold physiological salt solution. Pellets were removed with 2 × 0.3 mL warm physiological solution and their radioactivity was counted. Specific binding was determined by subtracting non-specific binding (determined in the presence of 2 mM bicuculline, Sigma Chemical Co.) from the total binding. Typically 40% of the counts were found to be bicuculline-sensitive.

Enantiomeric purity and stability. Enantiomeric purity of DHM enantiomers was established by i.r. spectroscopy as described in Ref. 1 and found to be >98% for both enantiomers (Prof. Krogsgaard-Larsen, personal communication). Aqueous stock solutions of the enantiomers were stored at −20°. In control experiments, vesicles were disrupted and their membranes were isolated and purified [6]. The enantioselectivity of DHM isomers as inhibitors of [^{3}H]muscimol binding to sites present on disrupted and purified membranes was determined in different media and at different temperatures and compared to the enantioselectivity of [^{3}H]muscimol binding sites present on native vesicles. The enantioselectivity at the $GABA_A$ sites on disrupted membranes was not changed by substituting Tris–HCl buffer for physiological salt solution and decreasing the temperature from 30° to 4°. Thus, the extent of racemization is either the same or not significant under these different assay-conditions. When enantioselectivities at the $GABA_A$ sites present on disrupted membranes and native vesicles were compared, the latter was found to be less. The difference in enantioselectivities suggests that the low degree of enantioselectivity can not be due to enantiomeric impurity of DHM stereoisomers.

RESULTS

$^{36}Cl^-$ *Influx experiments*

The specific agonist-stimulated uptake of $^{36}Cl^-$ by native membrane vesicles in physiological salt solution was followed over its whole range of concentration-dependence. With all three agonists tested the dose–response curves reached the same plateau. Figure 1 shows the specific $^{36}Cl^-$ uptake induced by (+)-(*S*)-DHM, GABA and (−)-(*R*)-DHM. The effect of GABA was completely inhibited by the $GABA_A$ receptor antagonist bicuculline (2 mM). The unspecific chloride influx (with no agonist added) was not affected by the presence of bicuculline indicating that this value reflects spontaneous uptake and contains no contribution from chloride influx mediated by endogenous GABA. Examination of the dependence of $^{36}Cl^-$ influx on drug concentration revealed the following characteristics. (1) (+)-(*S*)-DHM was the more potent stereoisomer. (2) GABA was more potent than (−)-(*R*)-DHM. (3) Dose–response curves for (+)-(*S*)-DHM and GABA were parallel, while that for (−)-(*R*)-DHM was shallower. Hence, differences in the potencies of DHM enantiomers increased with concentration. Degrees of enantioselectivity were estimated on the basis of the ratio of the concentration of the enantiomers, [(−)-(*R*)-DHM]/[(+)-(*S*)-DHM]. Degrees of enantioselectivity were 3, 6 and 9 (i.e. 6 ± 3) at 25%, 50% and 75% stimulation of transmembrane $^{36}Cl^-$ flux, respectively.

Desensitization of $GABA_A$ receptor with varying concentrations of DHM enantiomers (Table 1) was quantified using a GABA test (assay time: 3 sec, [GABA] = 1 mM) after 7 sec of preincubation with various concentrations of the stereoisomers. The values obtained without stereoisomers in the preincubation are independent of the time of preincubation and represent zero preincubation $^{36}Cl^-$ influx (M_0). The $^{36}Cl^-$ influx measured after preincubation with DHM enantiomers (M_{DHM}) was smaller than M_0; the decrease of $^{36}Cl^-$ influx was expressed as $(M_{DHM}/M_0) \times 100$ [%]. Only small and insignificant differences in the potencies of DHM enantiomers to desensitize $GABA_A$ receptor were observed suggesting that desensitization does not exhibit enantioselectivity.

[^{3}H]*Muscimol binding experiments*

Binding of [^{3}H]muscimol to $GABA_A$ sites present on native membrane vesicles in physiological salt solution was characterized by a dissociation constant in the 0.1 μM concentration range ($K_D = 0.15$ μM, $B_{max} = 1.9$ pmol/mg protein, $r = 0.77$). Hence, displacement studies were performed with 80 nM [^{3}H]muscimol. Inhibition of bicuculline-sensitive binding of [^{3}H]muscimol to $GABA_A$ sites present on membrane vesicles by DHM enantiomers exhibited the following characteristics (Fig. 2). (1) (+)-(*S*)-DHM was the more potent enantiomer. (2) Displacement curves were not parallel; the differences in the potencies of DHM enantiomers increased with concentration. Degrees of enantioselectivity were 1, 2 and 5 (i.e. 3 ± 2) at 25%, 50% and 75% inhibition of [^{3}H]muscimol binding, respectively. (3) Enantioselectivity of binding was less than the enantioselectivity observed in $^{36}Cl^-$ flux measurements. (4) Both, the displacement of [^{3}H]muscimol binding and the stimulation of $^{36}Cl^-$ influx occurred in the 0.1–10 μM range of concentration of the enantiomers, however, significant responses required five-fold higher concentrations.

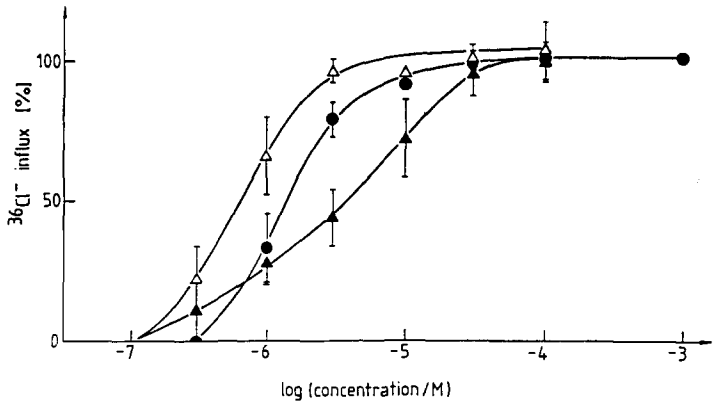

Fig. 1. Differences in the potency of dihydromuscimol enantiomers and GABA to stimulate $^{36}Cl^-$ influx into membrane vesicles. Symbols: △, (+)-(S)-DHM; ●, GABA; ▲, (−)-(R)-DHM. Experiments were performed as described in Materials and Methods. Bars indicate mean ± SEM from three experiments performed in triplicate.

Table 1. Desensitization of $GABA_A$ receptor with varying concentrations of dihydromuscimol enantiomers

Concentration [μM]	$(M_{DHM}/M_o) \times 100$ [%] (+)-(S)-DHM	(−)-(R)-DHM
0.01	100 ± 11	100 ± 10*
0.05	85 ± 12	87 ± 13*
0.50	72 ± 11	94 ± 9†
1.00	83 ± 11	84 ± 3*
2.00	53 ± 9	57 ± 2*
10.0	57 ± 7	64 ± 4*
100	50 ± 3	46 ± 2*

Membrane vesicles were preincubated for 7 sec with buffer. In a subsequent assay, the GABA-stimulated $^{36}Cl^-$ influx was measured (M_o = 100%). Assay conditions were as follows: [GABA] = 1 mM, t = 3 sec. Preincubation with DHM stereoisomers (M_{DHM}) decreased the value of M_o, expressed as $(M_{DHM}/M_o) \times 100$ [%].

Data are mean ± SEM values from three experiments performed in triplicates. Student's t-test indicated: *not significant (i.e. $P > 0.05$) and †$P < 0.05$.

DISCUSSION

Comparison of the results obtained with different assays of $GABA_A$ receptor activity, i.e. stimulation of transmembrane $^{36}Cl^-$ flux into cortical membrane vesicles (this work) and into cerebellar granule cells [8] as well as reduction of the firing rate of the cat spinal interneurons [1] revealed identical rank orders of agonist-potencies, namely (+)-(S)-DHM > GABA > (−)-(R)-DHM.

In $^{36}Cl^-$ flux measurements the degree of enantioselectivity varied from 3 to 9 with increasing $^{36}Cl^-$ influx (0.5–5 μM range of concentration of the enantiomers) because of non-parallel slopes of the concentration–effect curves of the enantiomers. Concentration-dependence of enantioselectivity could be interpreted in terms of high-affinity uptake processes (uptake of the stereoisomers or inhibition of the uptake of endogenous GABA by DHM enantiomers) exhibiting enantioselectivity opposite to channel gating [1, 2]. These uptake processes would result in a decrease of enantioselectivity with increasing concentration of the enantiomers. (We cannot rule out the possibility, however, that a low-affinity and high-capacity uptake could account to

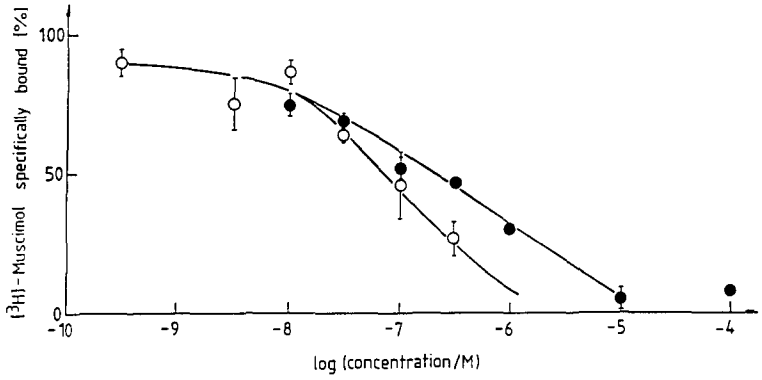

Fig. 2. Differences in the potency of dihydromuscimol enantiomers to inhibit bicuculline-sensitive binding of [^{3}H]muscimol to sites present on membrane vesicles. Symbols: ○, (+)-(S)-DHM; ●, (−)-(R)-DHM. Experiments were performed as described in Materials and Methods. Bars indicate mean ± SEM from three experiments performed in triplicate.

some extent for the phenomenon.) Differences in the wideness of the ranges of effective concentrations of the enantiomers may arise from the heterogeneity [4] of $GABA_A$ receptors. Preliminary fast kinetic experiments show that the time courses of $^{36}Cl^-$ influx mediated by (+)-(*S*)-DHM and (−)-(*R*)-DHM are different. It is the (−)-(*R*)-DHM enantiomer which can differentiate between a faster and a more slowly desensitizing $GABA_A$ receptor.

A significant stimulation of $^{36}Cl^-$ influx by DHM enantiomers occurred at about five-fold higher concentration than the inhibition of [^{3}H]muscimol binding. Self-triggered enhancements in the binding affinity of cholinergic agonists were observed and coupled to desensitization of cholinergic receptors [9, 10]. In general, thermodynamics predicts an increased affinity of the receptor for agonists in the agonist-mediated desensitized conformation. Following this reasoning our results suggest that desensitization of the physiologically active $GABA_A$ receptor during the binding assay may induce a five-fold increase in receptor affinity.

In conclusion, the degree of enantioselectivity in $^{36}Cl^-$ flux measurements is not smaller than that found in the [^{3}H]muscimol binding experiments. The physiologically active $GABA_A$ receptors exhibit a rather small degree of enantioselectivity in binding experiments, most probably because these measurements reflect $GABA_A$ receptors in the desensitized state.

REFERENCES

1. Krogsgaard-Larsen P, Nielsen L, Falch E and Curtis DR, GABA agonists. Resolution, absolute stereochemistry and enantioselectivity of (*S*)-(+)- and (*R*)-(−)-dihydromuscimol. *J Med Chem* **28**: 1612–1617, 1985.
2. Krogsgaard-Larsen P, Lenique P and Jacobsen P, GABAergic drugs: configurational and conformational aspects. In: *Handbook of Stereoisomers: Drugs in Psychopharmacology* (Ed. Smith DF), pp. 369–399. CRC Press, Boca Raton, 1984.
3. Kardos J, Kovacs I and Simonyi M, Enantioselectivity in the potency of agonists at the functional $GABA_A$ receptor. *Soc Neurosci Abstr* **15**: 997, 1989.
4. Cash DJ and Subbarao K, Desensitization of γ-aminobutyric acid receptor from rat brain: two distinguishable receptors on the same membrane. *Biochemistry* **26**: 7556–7562, 1987.
5. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
6. Kardos J, Hajós F and Simonyi M, Differential localization of GABA-dependent and GABA-independent benzodiazepine binding sites within synapses of rat cerebral cortex. *Neurosci Lett* **48**: 355–359, 1984.
7. Kardos J, Kovács I, Hajós F, Kálmán M and Simonyi M, Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. *Neurosci Lett* **103**: 139–144, 1989.
8. Kardos J and Guidotti A, Desensitization of GABA-stimulated $^{36}Cl^-$ influx in cerebellar granule cells. In: *Chloride Channels and their Modulation by Neurotransmitters and Drugs* (Eds. Biggio G and Costa E), pp. 161–173. Raven Press, New York, 1988.
9. Changeux J-P, Benedetti L, Bourgeois J-P, Brisson A, Cartaud J, Devaux P, Grunhagen H, Moreau M, Popot J-L, Sobel A and Weber M, Some structural properties of the cholinergic receptor protein in its membrane environment relevant to its function as a pharmacological receptor. *Cold Spring Harbor Symp Quant Biol* **40**: 211–230, 1976.
10. Taylor P and Sine SM, Ligand occupation and the functional states of the nicotinic-cholinergic receptor. *Trends Pharmacol Sci* **3**: 197–200, 1982.