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Binding characteristics of γ-hydroxybutyric acid as a weak but selective $GABA_B$ receptor agonist

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Abstract

The aim of this study was to reexamine the concept that γ-hydroxybutyric acid (GHB) is a weak but selective agonist at γ-aminobutyric $acid_B$ ($GABA_B$) receptors, using binding experiments with several radioligands. K_i values of GHB were similar (≈ 100 μM) in three agonist radioligand assays for $GABA_B$ receptors, [^{3}H]baclofen (β-*para*-chlorophenyl-γ-aminobutyric acid), [^{3}H]CGP 27492 (3-aminopropyl-phosphinic acid) and [^{3}H]GABA, in the presence of the $GABA_A$ receptor agonist isoguvacine with rat cortical, cerebellar and hippocampal membranes. In competition experiments between GHB and the $GABA_B$ receptor antagonist, [^{3}H]CGP 54626 (3-*N* [1-{(*S*)-3,4-dichlorophenyl}-ethylamino]-2-(*S*)-hydroxypropyl cyclo-hexylmethyl phosphinic acid), the IC_{50} values were significantly increased with 300 μM of 5′-guanyl-imidodiphosphate (Gpp(NH)p), which suggested that guanine nucleotide binding proteins (G-proteins) modulate GHB binding on $GABA_B$ receptors. The inhibition by GHB of [^{3}H]CGP 27492 binding in cortical membranes was not altered in the presence of 0.3 or 3 mM of the two GHB dehydrogenase inhibitors, valproate and ethosuximide. Thus, GHB is not reconverted into GABA by GHB dehydrogenase. Taken together, the results of this study demonstrated that GHB is an endogenous weak but selective agonist at $GABA_B$ receptors.

Keywords: $GABA_B$ receptor; GHB (γ-hydroxybutyric acid); G-protein-coupled receptor; GTP shift; Absence seizure

1. Introduction

γ-Hydroxybutyric acid (GHB) is a structural analogue of γ-aminobutyric acid (GABA) occurring in the mammalian brain and is formed primarily following GABA metabolism through GABA transaminase (EC 2.6.1.19) and specific succinic semialdehyde reductase (EC 1.1.1.2) (for a recent review see Cash, 1994). The main characteristics of brain GHB, conditions of synthesis, release and turnover, presence of active transport and of high- (nanomolar) and low-affinity (micromolar) binding sites (Benavides et al., 1982), as well as the conditions for degradation, and the fact that this compound is concentrated in the synaptosomal fraction, correspond to the criteria generally accepted for defining a neurotransmitter (Cash, 1994; Vayer et al., 1987). Guanine nucleotide binding proteins (G-proteins) are coupled to the GHB binding sites, modifying the high-affinity GHB binding (Ratomponirina et al., 1995). These results lend support to the hypothesis that central GHB binding sites belong to the family of G-protein-linked receptors, a pertussis-sensitive (G_i or G_o family) G-protein being involved in the coupling to the cellular response. However, to date no neuronal pathways that use this neurotransmitter have been demonstrated and any effects that GHB might have on ion fluxes or on signals involved in cellular transduction remain to be demonstrated (Cash, 1994).

In rodents and monkeys GHB induces rhythmic spike-and-wave discharges and behavioural changes resembling those seen in human generalized non-convulsive epilepsy (absence seizures) (Bearden et al., 1980; Godschalk et al., 1976, 1977; Marescaux et al., 1992a,b; Snead, 1988). The development of absence seizures requires high doses (3.5 mmol/kg) of GHB. This effect is reversed by the centrally active $GABA_B$ receptor antagonist, CGP 35348 (3-

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aminopropane-diethoxymethylphosphinic acid), since micromolar concentrations of the antagonist completely inhibit the effects induced by millimolar concentrations of GHB (Marescaux et al., 1992a,b; Snead, 1992a,b). Conversely, the prototypic $GABA_B$ receptor agonist, *R*-(−)-baclofen, potentiates the total duration of spike-and-wave discharges induced by GHB (Snead, 1992a,b).

High doses of GHB markedly increase rat striatal dopamine synthesis early after administration and later reduce it. During this later phase, GHB caused a marked accumulation of dopamine and its deaminated metabolites (Waldmeier, 1991). This sequence of effects closely resembles that caused by *R*-(−)-baclofen. The $GABA_B$ receptor antagonist, CGP 35348, did not alter dopamine synthesis on its own, but antagonized the increase elicited by GHB and *R*-(−)-baclofen, respectively. In addition, like *R*-(−)-baclofen, GHB dose dependently decreases cerebellar cGMP levels and these effects are antagonized by CGP 35348. Taken together, these results indicate that GHB behaves as an agonist at $GABA_B$ receptors (Waldmeier, 1991). Thus, these results suggest that the generation of absence seizures and the increase of rat striatal dopamine synthesis observed after administration of 3.5–5 mmol/kg GHB could directly or indirectly be mediated through $GABA_B$ receptors. Engberg and Nissbrandt (1993), Xie and Smart (1992a,b) and Williams et al. (1995) have observed neurophysiological similarities between the potent and selective $GABA_B$ receptor agonist, *R*-(−)-baclofen, and GHB with regard to their activation of pre- and postsynaptic $GABA_B$ receptors in thalamocortical, hippocampal (CA1) and substantia nigra neurons and have thus confirmed by using functional assays that GHB is a $GABA_B$ receptor agonist. We have found by using the agonist radioligands, [^{3}H]baclofen and [^{3}H]CGP 27492 (Bernasconi et al., 1992), that GHB but not its biologically inactive cyclic derivative γ-butyrolactone, had a selective, although weak affinity for $GABA_B$ receptors (IC_{50} = 150 μM). The average GHB brain levels are in the order of 2–4 nmol/g (≈ 2–4 μM) (Bernasconi et al., 1992; Vayer et al., 1988) and are lower than the concentrations necessary to stimulate $GABA_B$ receptors. After injections of 3.5 mmol/kg of GHB, a dose sufficient to induce spike-and-wave discharges or to enhance striatal dopamine levels, brain concentrations reach levels high enough (240 ± 31 nmol/g, ≈ 240 μM) (Snead, 1991) to stimulate $GABA_B$ receptors.

However, according to Banerjee and Snead (1995) several pieces of evidence mitigate against the hypothesis that GHB is a simple $GABA_B$ receptor agonist. For example, there is a report by Snead (1992a, 1995) that in the presence of the $GABA_A$ receptor agonist, isoguvacine, GHB does not compete for [^{3}H]GABA binding.

The present experiments were designed to further characterize the interactions between GHB and $GABA_B$ receptors by using the agonist radioligands, [^{3}H]GABA and [^{3}H]CGP 27492, and the antagonist radioligand, [^{3}H]CGP 54626. We also investigated whether GHB binding to $GABA_B$ receptors was dependent on the coupling between G-proteins and $GABA_B$ receptors in a manner similar to *R*-(−)-baclofen. We also examined the hypothesis proposed by Cash (1994) that GHB may exert its $GABA_B$ receptor-like effects, at least in part, via transformation through GHB dehydrogenase (EC 1.1.1.19) (Kaufman and Nelson, 1983; Vayer et al., 1985a,b) to a pool of GABA which is formed in a compartment which directly activates $GABA_B$ receptors rather than by a direct effect of GHB on these receptors. The results of the present study indicate that the binding characteristics of GHB to $GABA_B$ receptors are similar to those of *R*-(−)-baclofen.

2. Materials and methods

2.1. Drugs

Drugs for this study were obtained as follows: the $GABA_B$ receptor agonists radioligands, [^{3}H]CGP 27492 (3-aminopropyl-phosphinic acid) (15.0 Ci/mmol) and [^{3}H]GABA (57 Ci/mmol), from Ciba (Horsham, UK) and Amersham (Amersham, UK), respectively; the $GABA_B$ receptor antagonist radioligand, [^{3}H]CGP 54626 (3-*N* [1-{(*S*)-3,4-dichlorophenyl}-ethylamino]-2-(*S*)-hydroxypropyl cyclo-hexylmethyl phosphinic acid) (60 Ci/mol), from Anawa (Wangen, Switzerland); the cold $GABA_B$ receptor agonists, *R*-(−)-baclofen (β-*para*-chlorophenyl-γ-aminobutyric acid), CGP 27492 and GHB, from Ciba (Basel, Switzerland); the $GABA_A$ receptor agonist, isoguvacine, from Research Biochemicals International (Natick, MA, USA); Gpp(NH)p (5′-guanyl-imidodiphosphate) from Boehringer-Mannheim (Mannheim, Germany); ethylene imine polymer (PEI) from Fluka (Buchs, Switzerland); valproate sodium and ethosuximide from Ciba Basel. The term baclofen indicates the racemic mixture (±)-baclofen as compared to *R*-(−)-baclofen which characterizes the stereoisomer. The purity of radioligands was determined by high-pressure liquid chromatography. All other reagents were obtained from commercial sources and were of the highest available purity.

2.2. Animals

To avoid the influence of cyclic changes in female sex hormones on $GABA_B$ receptors (Al-Dahan et al., 1994) and to keep the parameters consistent with those of our previous experiments, adult (12–16 weeks) non-epileptic male Wistar rats from the breeding colony at the INSERM U.398 in Strasbourg were chosen at random. They were housed in groups of 4–6 per cage at constant temperature (22 ± 2°C), humidity (60 ± 5%), and normal light/dark cycle (12/12 h); they received food and water ad libitum. All animals were drug-naive.

2.3. Membrane preparation

Ten rats, not anaesthetized, of about 200–250 g body weight were decapitated, the brains removed, the cerebral cortices, cerebella and hippocampi dissected and homogenized in 10 vols. of ice-cold 0.32 M sucrose containing $MgCl_2$ (1 mM) and K_2HPO_4 (1 mM), with a glass/Teflon homogenizer. Crude membrane fractions were then prepared according to the method of Bittiger et al. (1990).

2.4. Binding asssays

$GABA_B$ receptor assays using the agonist radioligand, [^{3}H]CGP 27492, were performed as described by Bittiger et al. (1990) in a total volume of 2 ml Krebs-Henseleit buffer with 2 nM radioligand and non-specific binding was determined in the presence of 10 μM *R*-(−)-baclofen. The incubation time was 40 min at 20°C. The $GABA_B$ receptor assay, using the antagonist radioligand, [^{3}H]CGP 54626, was performed according to Bittiger et al. (1992) in 1 ml Krebs-Henseleit buffer pH 7.40 containing 1 nM [^{3}H]CGP 54626. Non-specific binding was assessed in the presence of 10 μM CGP 54626. Specific binding in these two receptor assays was more than 80%. For studying the binding of [^{3}H]GABA to $GABA_B$ sites we used the method of Hill and Bowery (1981). Membranes were suspended in a total volume of 1 ml Tris-HCl buffer (50 mM, pH 7.40) containing 2.5 mM $CaCl_2$, isoguvacine hydrobromide (40 μM) and 10 nM [^{3}H]GABA. The IC_{50} of the $GABA_A$ receptor agonist, isoguvacine, for the inhibition of [^{3}H]baclofen binding is > 700 μM (Hill and Bowery, 1981). Under the binding conditions described by Hill and Bowery (1981) [^{3}H]GABA labels selectively $GABA_B$ receptors. Non-specific binding was determined in the presence of 100 μM *R*-(−)-baclofen and represents 40–50% of the total binding. The incubation time was 10 min at 20°C.

2.5. Data analysis

The calculations of IC_{50} values and Hill coefficients of GHB for the inhibition of [^{3}H]CGP 27492, [^{3}H]GABA and [^{3}H]CGP 54626 binding were performed by non-linear (sigmoidal) fitting using a commercially available PC software program (GraphPad Prism 2.0, GraphPad Software, San Diego, CA, USA). K_i values were calculated as approximations from the IC_{50} values by means of the Cheng-Prusoff equation (Cheng and Prusoff, 1973). IC_{50} values were obtained from binding assays with 8–12 different concentrations of GHB and performed in triplicate. The highest concentration of GHB used in the various displacement experiments was 30 mM, so that the IC_{50} values obtained with [^{3}H]CGP 54626 in the presence of Gpp(NH)p were calculated by extrapolation. Data analysis was performed for each separate displacement curve, and IC_{50} values are given as means of at least three experiments. The significance of differences in the IC_{50} values obtained from different radioligands was evaluated using the unpaired Student's *t*-test. A P value < 0.05 was considered to be significant.

3. Results

3.1. Competition of [^{3}H]CGP 27492, [^{3}H]GABA and [^{3}H]CGP 54626 binding by GHB in cortical, hippocampal and cerebellar membranes of rats

Displacement curves of GHB in $GABA_B$ receptor agonist radioligand assays with [^{3}H]CGP 27492 and [^{3}H]GABA had Hill coefficients of 0.85 ± 0.02 and 0.81 ± 0.07, respectively (Fig. 1a and Table 1). Similar results were obtained for hippocampal and cerebellar membranes. For comparison the displacement curves of *R*-(−)-baclo-

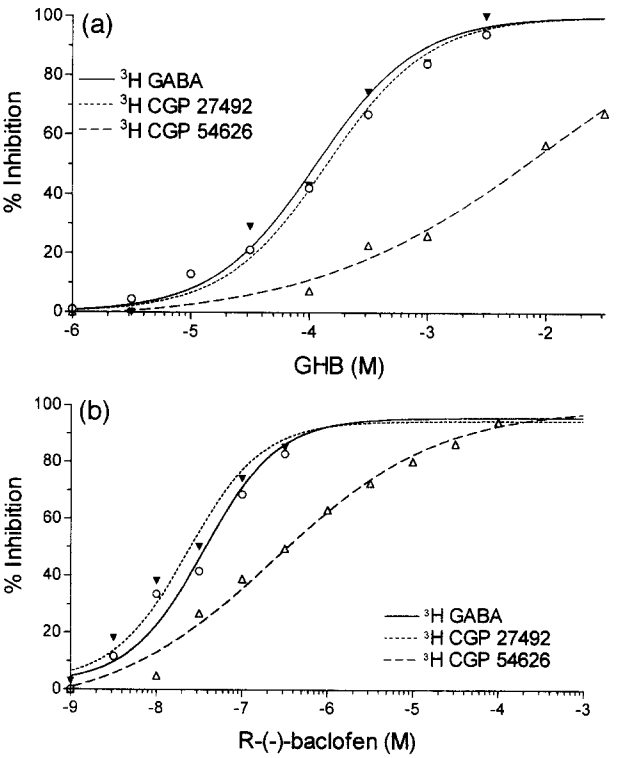

Fig. 1. Comparison of the inhibition by GHB (a) and *R*-(−)-baclofen (b) of [^{3}H]GABA binding in the presence of isoguvacine, [^{3}H]CGP 27492 and [^{3}H]CGP 54626 specificically bound to frozen and thawed crude membranes from rat cerebral cortex incubated in Krebs-Henseleit solutions. Frozen and thawed membranes were washed four times in Krebs-Henseleit solution before resuspension for assay. Binding conditions specific for each radioligand are described in Section 2. Varying concentrations of unlabelled GHB or *R*-(−)-baclofen (abscissae: log molar) were added simultaneously with each radioligand and incubated at 20°C. The data shown in this figure are taken from a single experiment performed in triplicate, which was repeated at least three times. Standard errors of the means were less than than 5% and have been omitted for clarity. Similar results were observed for membranes prepared from hippocampus and cerebellum. The IC_{50} and K_i values as well as Hill coefficients of GHB and *R*-(−)-baclofen are shown in Table 2Table 3.

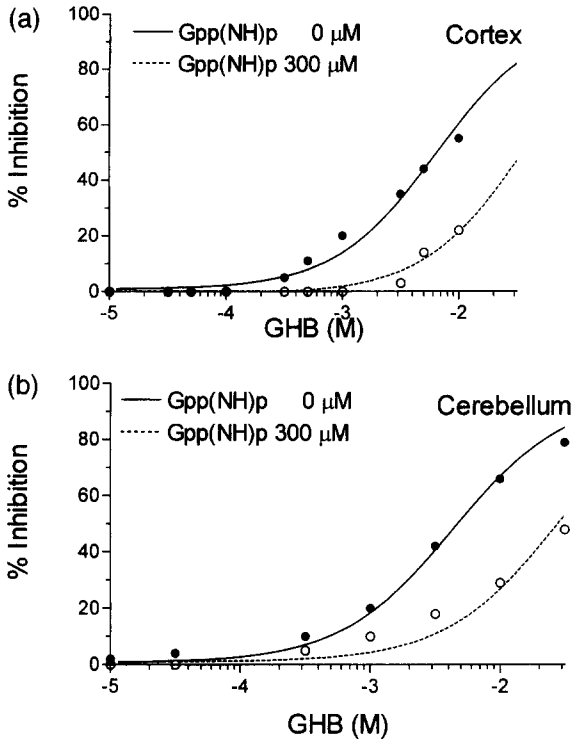

Fig. 2. Displacement of the antagonist radioligand for $GABA_B$ receptors, [^{3}H]CGP 54626, by GHB in the absence (————) or presence (---) of 300 μM of the GTP analogue, Gpp(NH)p, in membranes prepared from cerebral cortex (a) and from cerebellum (b). 1 nM [^{3}H]CGP 54626 was incubated with unlabelled GHB in concentrations ranging from 10 μM to 30 mM. The data shown in this figure are from a single representative experiment performed in triplicate, which was repeated twice. S.E.M. were less than 5% and were not plotted for the sake of clarity. Similar results were observed for hippocampal membranes. The IC_{50} values are given in Table 2.

fen in [^{3}H]GABA, [^{3}H]CGP 27492 and [^{3}H]CGP 54626 are shown in Fig. 1b. The IC_{50} and K_i values of GHB in agonist and antagonist radioligand assays are shown in Tables 1 and 2 and compared with the values previously obtained with *R*-(−)-baclofen (Table 3) (Bernasconi et al., 1992). The IC_{50} values for the inhibition by GHB of antagonist [^{3}H]CGP 54626 specific binding in cortex, hippocampus and cerebellum were significantly higher than those observed with the three agonist radioligands. The complete inhibition was not reached at the highest concentration of GHB (30 mM) used in the displacement experiments with [^{3}H]CGP 54626 so that the IC_{50} values obtained were calculated by extrapolation (Figs. 1 and 2,

Table 1
Inhibition of agonist radioligand binding by GHB

Radioligand	IC_{50} (μM)	n_H
[^{3}H]GABA	93 ± 18	0.81 ± 0.07
[^{3}H]CGP 27492	93 ± 2	0.85 ± 0.02

IC_{50} values and Hill coefficients were calculated using the GraphPad Prism 2.0 program. Data are means ± S.E.M. of three experiments performed in triplicate.

Table 2
Inhibition of [^{3}H]CGP 54626 antagonist binding by GHB

Gpp(NH)p	IC_{50} (mM)		
	Cortex	Hippocampus	Cerebellum
0 μM	5.5 ± 0.8	3.9 ± 0.5	5.9 ± 2.4
300 μM	38 ± 13 [a]	28 ± 7 [a]	18 ± 6

IC_{50} values were calculated by non-linear regression fitting of the experimental data shown in Fig. 2a and 2b using the GraphPad Prism 2.0 program. Values are the means ± S.E.M. of three experiments performed in triplicate. [a] $P < 0.05$ for the IC_{50} in the absence of Gpp(NH)p compared to the IC_{50} in the presence of Gpp(NH)p (unpaired Student's *t*-test).

Table 2). Hill coefficients could not be calculated accurately and are not given.

3.2. Inhibition by GHB of antagonist [^{3}H]CGP 54626 specific binding to brain membranes in the absence and in the presence of Gpp(NH)p

Agonist binding to $GABA_B$ receptors is inhibited by GTP and its metabolically stable analogue, Gpp(NH)p, through interaction with a G-protein (Hill et al., 1984; Mathivet et al., 1994, 1996a). Gpp(NH)p decreased the affinity of agonists to $GABA_B$ receptors by transforming the high-affinity conformation into a conformation with lower affinity (Hill et al., 1984). The effects of 300 μM Gpp(NH)p on the inhibition by GHB of [^{3}H]CGP 54626 specific binding in cortical, hippocampal and cerebellar membranes are shown in Table 2 and in Fig. 2a,b. The concentration of 300 μM Gpp(NH)p is sufficient to transform all high-affinity conformations into low-affinity ones (Mathivet et al., 1994, 1996a). The addition of Gpp(NH)p

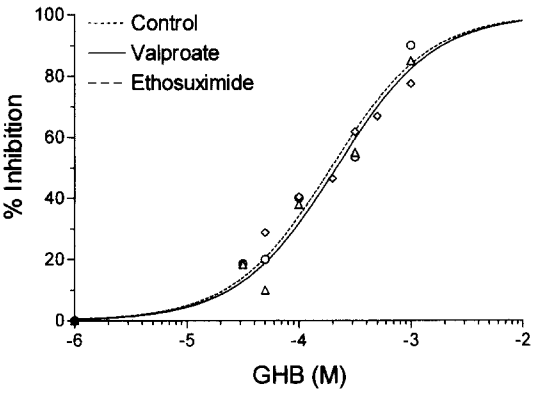

Fig. 3. Inhibition by GHB of [^{3}H]CGP 27492 specific binding to $GABA_B$ receptors in cortical membranes of Wistar rats in the absence and presence of 3 mM of the two GHB dehydrogenase inhibitors, valproate and ethosuximide. Membranes were incubated in the presence of 2 nM [^{3}H]CGP 27492 with increasing concentrations of GHB (from 10 μM to 30 mM). S.E.M. were less than 5% and are not plotted for the sake of clarity. The displacement curves shown in this figure are from a representative experiment performed in triplicate, which was repeated twice. The IC_{50} values and the number of binding sites labelled by the radioligand were similar in the absence or presence of valproate and ethosuximide.

Table 3
Comparative analysis of the interactions of γ-hydroxybutyric acid (GHB), GABA and *R*-(−)-baclofen with $GABA_B$ receptors

Radioligand	Membrane		GHB K_i (μM)	GABA K_i (nM)	(−)-Baclofen K_i (nM)	References
[^{3}H]GABA	Cortex	Wistar rat	79		32	Present data
[^{3}H]Baclofen	Cortex	Wistar rat	125		12	Bernasconi et al. (1986)
	Primary cultures of mouse cerebellar granule cells		398		79	Ito et al. (1995)
	Cortex	Mouse	630	125 [a]	250 [a]	Ishige et al. (1993) [a] Ishige et al. (1996)
[^{3}H]CGP 27492	Cortex	Wistar rat	80	250 [b]	13 [b]	Present data Bittiger et al. (1992) [b]
	Cerebellum	Wistar rat	100			Bernasconi et al. (1992)
	Thalamus	Wistar rat	126			Bernasconi et al. (1992)
[^{3}H]CGP 54626	Cortex	Wistar rat	3300	200 [b]	200 [b]	Present data Bittiger et al. (1992) [b]
	Cerebellum	Wistar rat	3500			Present data
	Hippocampus	Wistar rat	2300			Present data

IC_{50} values of $GABA_B$ receptor agonists for the inhibition of [^{3}H]GABA, [^{3}H]baclofen and [^{3}H]CGP 27492 binding were calculated by non-linear fitting using GraphPad Prism 2.0. K_i were calculated by means of the Cheng-Prusoff equation. IC_{50} values were calculated using 8–12 different concentrations run in triplicate. Data were analysed for each separate displacement curve, and K_i are the means of at least three experiments.

to the assay shifted the competition curves to the right and the IC_{50} values were significantly increased in the cortex and hippocampus (Table 2).

3.3. Lack of effect of valproate and ethosuximide on the inhibition by GHB of [^{3}H]CGP 27492 specifically bound to cortical membranes

Under physiological conditions GHB can be reconverted to GABA in vitro and in vivo via GHB dehydrogenase, an enzyme that is completely blocked by 3 mM valproate or ethosuximide (Kaufman and Nelson, 1991). The K_i values of GHB dehydrogenase for the two anti-absence drugs have been reported to be between 60 and 80 μM (Kaufman and Nelson, 1983; Vayer et al., 1985a,b), 3 mM concentrations of valproate and ethosuximide correspond approximately to $30–50 \times K_i$. Valproate and ethosuximide have no affinity for $GABA_B$ receptors (results not shown) and as shown in Fig. 3 they had no effect at 0.3 and 3 mM on the displacement of [^{3}H]CGP 27492 by GHB from cortical membranes. The IC_{50} values and the number of binding sites occupied by GHB were similar in the presence or absence of valproate or ethosuximide. Thus, under the conditions used in the $GABA_B$ receptor assay, GHB is not converted into GABA.

4. Discussion

The main conclusions of this investigation, which confirm results of our previous experiments (Bernasconi et al., 1992), are: (i) GHB inhibits the binding of the $GABA_B$ receptor agonists, [^{3}H]CGP 27492, [^{3}H]GABA and [^{3}H]*R*-(−)-baclofen ($K_i \approx 100$ μM), as well as of the $GABA_B$ receptor antagonist, [^{3}H]CGP 54626 ($K_i \approx 3$ mM), to cerebrocortical, cerebellar and hippocampal membranes. (ii) Interactions with 11 other receptors (including $GABA_A$ and central benzodiazepine receptors) were absent at a concentration of 100 μM (0% inhibition) (Bernasconi et al., 1992). γ-Butyrolactone, the biochemically inactive cyclic analogue of GHB, did not interact at a concentration of 100 μM with the 12 receptors, including $GABA_B$ (Bernasconi et al., 1992). Thus, the interactions of GHB with $GABA_B$ receptors appear to be selective. (iii) The inhibition by GHB of the antagonist radioligand [^{3}H]CGP 54626 binding to $GABA_B$ receptors was decreased by the GTP analogue, Gpp(NH)p, indicating that the GHB-sensitive $GABA_B$ receptors are associated with G-proteins. (iv) Valproate (3 mM) and ethosuximide (3 mM) block completely the non-specific semialdehyde reductase (= GHB dehydrogenase) and have no effect on $GABA_B$ binding and on the displacement of [^{3}H]CGP 27492 by GHB from cortical membranes. Thus, under the conditions used in the receptor assay the results observed are not due to reconversion of GHB into GABA.

4.1. GHB as a $GABA_B$ receptor agonist

The data from this and previous studies (Bernasconi et al., 1992) have characterized GHB as an agonist at $GABA_B$ receptors. The K_i values of GHB are similar in the three agonist radioligand assays, but the K_i measured in the [^{3}H]CGP 54626 antagonist assay are much higher than with $GABA_B$ receptor agonists (Tables 2 and 3). The cause for these differences may be the different binding states of $GABA_B$ receptors. Scatchard analysis of saturable binding of [^{3}H]CGP 54626 to cortical membranes uncovered the presence of two affinity states of $GABA_B$ receptors (Mathivet et al., 1994) and showed that the antagonist radioligand labels 2–3 times more receptor sites

than do agonist radioligands (Bittiger et al., 1992). Curves for the inhibition of the specifical binding of [^{3}H]CGP 54626 by agonists suggest that the antagonist radioligand labels high- and low-affinity states of the receptor (Bittiger et al., 1992). $GABA_B$ receptor antagonists like CGP 35348 and CGP 36742 have similar IC_{50} values in the [^{3}H]CGP 27492 agonist assay and in the antagonist [^{3}H]CGP 54626 $GABA_B$ receptor assay (30–35 μM). On the contrary, the IC_{50} values of $GABA_B$ receptor agonists are higher in the antagonist [^{3}H]CGP 54626 assay than in agonist radioligand assays: GABA (309 vs. 16 nM), *R*-(−)-baclofen (350 vs. 32 nM) and CGP 27492 (34 vs. 4.1 nM) (Bittiger et al., 1992). The values for cortex, hippocampus and cerebellum with GHB (5500 vs. 93 μM, Table 3) indicate that GHB is an agonist at $GABA_B$ receptors. This notion is supported by the observation that, in the presence of Gpp(NH)p, a significant shift of the IC_{50} values for the inhibition by GHB of [^{3}H]CGP 54626 specific binding to lower affinity values was observed for cortex, cerebellum and hippocampus. These results show that GHB shares the same binding characteristics as GABA, CGP 27492 and *R*-(−)-baclofen and is a weak but selective agonist at $GABA_B$ receptors. The results also indicate that central GHB-sensitive $GABA_B$ receptors are of the G-protein-linked receptor type. The K_i values of GHB observed with the agonist radioligands are similar in the three brain structures investigated and the higher K_i values obtained with [^{3}H]CGP 54626 are identical for cortex, cerebellum and hippocampus. There is no regional selectivity of the K_i values of GHB.

Snead (1992a, 1995) has reported that, in the presence of the $GABA_A$ receptor agonist isoguvacine, 200 μM of GHB did not displace specifically bound [^{3}H]GABA from $GABA_B$ receptors localized in synaptosomal membranes prepared from cerebral cortex. We have no explanation for the discrepancy between the results of Snead and those from the present study. On the other hand, our results obtained with [^{3}H]GABA in the presence of isoguvacine are highly consistent with those obtained with the agonist radioligands, [^{3}H]CGP 27492 and [^{3}H]baclofen, and with the antagonist, [^{3}H]CGP 54626, and confirmed that GHB binds to $GABA_B$ receptors.

Recently, Ito et al. (1995) and Ishige et al. (1996) have confirmed that GHB inhibits the binding of [^{3}H]baclofen. They used cerebellar granule cells and mouse cerebellar membranes. [^{3}H]Baclofen binding in the cells was markedly reduced by 1 mM GHB (about 75% inhibition). The IC_{50} values reported by Ito et al. (1995) and by Ishige et al. (1996) for mouse cerebellar membranes were 500 μM and 796 ± 10 μM, respectively. The IC_{50} values reported by Ishige et al. (1993) for GABA and *R*-(−)-baclofen in the [^{3}H]baclofen assay were 159 nM and 316 nM, respectively, as compared to 16 and 32 nM observed by Bittiger et al. (1992). Thus, the IC_{50} values observed by Ishige et al. in the [^{3}H]baclofen assay for $GABA_B$ receptor agonists are always higher than those obtained with the binding methods used in the present study, but the ratio of IC_{50} values obtained with the two methods remains constant and was 8.5 for GHB, 9.9 for GABA and 9.8 for *R*-(−)-baclofen. The higher values observed by these authors compared to those of the present study could be the result of differences in animal species used (mouse versus rat) or in membrane preparation.

Benavides et al. (1982) reported that, like GABA and the $GABA_A$ receptor agonist, isoguvacine, 10 μM (±)-baclofen does not displace [^{3}H]GHB from high-affinity GHB binding sites in crude membranes prepared from cerebral hemispheres. The binding techniques used by Benavides et al. (1982) are completely different from those utilized with $GABA_B$ receptors. The optimum pH for GHB binding is 5.5 compared to 7.5 for $GABA_B$ binding and the incubation temperatures in the GHB and $GABA_B$ binding assays were 0°C and 20°C, respectively. The fact that GHB displaces agonist and antagonist radioligands bound specifically to $GABA_B$ receptors does not necessarily imply that baclofen is an agonist or an antagonist at GHB binding sites and GHB binding sites, and $GABA_B$ receptors may be two different entities (see Section 4.3).

4.2. GHB is not reconverted into GABA during the receptor assay

GHB metabolism has been well characterized in the mammalian central nervous system. GHB is formed from GABA via transamination to succinic semialdehyde, a reaction catalyzed by GABA transaminase. An $NADP^+$-dependent aldehyde reductase (specific succinic semialdehyde reductase) reduces succinic semialdehyde to GHB (Vayer et al., 1985a, 1987). Valproate is not a substrate of specific succinic semialdehyde reductase (Rumigny et al., 1980). Several pieces of evidence indicate that GHB is converted back to succinic semialdehyde by GHB dehydrogenase, a cytosolic $NADP^+$-dependent oxidoreductase, a non-specific enzyme inhibited by valproate and ethosuximide (Kaufman and Nelson, 1983; Vayer et al., 1985a). The kinetic characteristics of pure GHB dehydrogenase coupled to purified GABA transaminase are consistent with the rapid transformation of micromolar amounts of GHB present in vitro (Vayer et al., 1985a,b) and in vivo (Kaufman and Nelson, 1991). Based on the interconversion between GHB and succinic semialdehyde, Cash (1994) has proposed that at least some GHB is reconverted to GABA in neuronally active GABA pools, which in turn are capable of interacting with both $GABA_A$ and $GABA_B$ receptors. As the two GABA receptors display different anatomical distributions (Bowery et al., 1987), distinct pools of GHB-derived GABA are required to activate each one.

We used the strong inhibition of GHB dehydrogenase by valproate and ethosuximide (Kaufman and Nelson, 1983; Vayer et al., 1985b) to test the hypothesis proposed by Cash (1994). At the concentrations of valproate and

ethosuximide used in the binding experiments (Fig. 3) GHB dehydrogenase is completely inhibited (valproate and ethosuximide concentrations equal about $30–50 \times K_i$ for GHB dehydrogenase). The results of those experiments demonstrate that the affinity of GHB for $GABA_B$ receptors is not due to GHB-derived GABA acting selectively at $GABA_B$ receptors and strengthen the concept that GHB is a direct agonist of $GABA_B$ receptors. It must be stressed that under other in vitro conditions and in vivo the hypothesis of the interconversion of GHB and succinic semialdehyde may still be valid.

4.3. Relation between GHB and $GABA_B$ receptors

GHB binds to $GABA_B$ receptors but $GABA_B$ receptor agonists and antagonists do not displace [^{3}H]GHB from its binding sites, suggesting that GHB binding sites and $GABA_B$ receptors are probably two different entities. This hypothesis is supported by observations reported by Snead (1994) showing that GHB binding sites and $GABA_B$ receptors have a different anatomical distribution in rat brain, suggesting a lack of correlation between GHB binding sites and $GABA_B$ receptors and that direct coupling of these receptors does not seem to be involved in the inhibition of $GABA_B$ binding by GHB. For example, there was greater intensity of [^{3}H]GHB binding in hippocampus versus greater intensity of [^{3}H]$GABA_B$ binding in the thalamus. One of the highest concentrations of $GABA_B$ sites occurs in the molecular layer of the cerebellum (Bowery et al., 1987) where GHB binding sites are almost absent (Benavides et al., 1982; Hechler et al., 1992; Snead, 1994). However, there was high intensity and co-localization of both [^{3}H]$GABA_B$ and [^{3}H]GHB binding sites in laminae I–III of cortex. The ontogeny of [^{3}H]GHB binding sites was also distinctly different from that of [^{3}H]$GABA_B$ receptors (Snead, 1994).

Even so, recent data suggest functional interactions between both receptors. Mathivet et al. (1996b) have shown that another GHB receptor agonist, (−)-HA 966, displaced [^{3}H]CGP 27492 (IC_{50} = 300 μM) and [^{3}H]GHB (IC_{50} = 150 nM) from their binding sites and like GHB (IC_{50} for GHB and $GABA_B$ binding sites are 95 nM and 100 μM, respectively), (−)-HA 966 induces absence seizures in non-epileptic Wistar rats and exacerbates absences in a strain of Wistar rats selected in our laboratory and called 'Genetic Absence Epilepsy Rats from Strasbourg' (GAERS). The generation of spike-and-wave discharges as well as the increase of dopamine synthesis induced by (−)-HA 966 were antagonized by $GABA_B$ receptor antagonists (Mathivet et al., 1996b; Waldmeier, 1991). Both specific GHB and $GABA_B$ receptor antagonists block the occurrence of spike-and-wave discharges in experimental absence models (Maitre et al., 1990; Snead, 1992a,b). Thus, both binding sites appear to be involved in the pathogenesis of absence seizures and in the alteration of impulse flow in dopaminergic neurons. It is conceivable that GHB as well as $GABA_B$ receptor activity are required for the expression of GHB-induced absence seizures and increase of rat striatal dopamine synthesis.

Taken together, these data suggest a complicated interplay between GHB binding sites and $GABA_B$ receptors.

4.4. Physiological significance of the stimulation of $GABA_B$ receptors by GHB

Based on purely quantitative criteria, the normal measured endogenous level of GHB (2–4 nmol/g) (Bernasconi et al., 1992) could only be expected to produce meaningful neuropharmacological effects by interacting with its high-affinity binding sites (K_d = 95 nM; Benavides et al., 1982). Although physiological effects of endogeneously released GHB have not been demonstrated, systemic administration to animals of non-anaesthetic high doses of GHB (3.5 mmol/kg) induces various neuropharmacological effects. The most striking effects include the modulation of dopaminergic activity (Waldmeier, 1991) and the induction of absence-like seizures (Bernasconi et al., 1992; Snead, 1988). According to Snead (1991), the mean cortical GHB concentration at the onset of absence seizures after injection of the threshold dose of 3.5 mmol/kg i.p. GHB is 240 ± 31 nmol/g (= 240 μM), i.e., is sufficient to stimulate $GABA_B$ receptors (K_i of GHB for $GABA_B$ receptors = 87 μM). High, non-anaesthetic doses of GHB could stimulate $GABA_B$ receptors, and the generation of absence-like seizures in the GHB model of epilepsy or the increase in dopamine levels might be due to the $GABA_B$ receptor agonist-like properties of GHB (Bernasconi et al., 1992).

4.5. Conclusions

The results of this study have indicated that the binding characteristics of GHB are those of a $GABA_B$ receptor agonist and support the finding of our first study (Bernasconi et al., 1992). Two recent reports by Ito et al. (1995) and Ishige et al. (1996) are also consistent with our findings. The data presented in the present communication suggest that GHB-sensitive $GABA_B$ receptors are linked to a G-protein. Recently, we have shown (Bernasconi et al., 1995) that, like *R*-(−)-baclofen, GHB had no effect on basal GABA and glutamate release, but it dose-dependently decreased, electrically evoked release of [^{3}H]GABA and of endogenous glutamate from cortical slices. $GABA_B$ receptor antagonists inhibited the effects of GHB on the release of the amino-acid neurotransmitters. These results are in keeping with those of our first study showing a dose- and time-dependent decrease of cGMP induced either by *R*-(−)-baclofen or by GHB and antagonized by CGP 35348 (Bernasconi et al., 1992). Thus, in a functional assay and in the measurement of a second messenger, GHB shares the same $GABA_B$ receptor agonist-like effects as *R*-(−)-baclofen.

In addition, Xie and Smart (1992a,b) have shown that GHB could activate presynaptic as well as postsynaptic $GABA_B$ receptors. Stimulation of postsynaptic $GABA_B$ receptors by GHB has also been reported by Engberg and Nissbrandt (1993) and by Williams et al. (1995). The binding results of the present study do not allow us to differentiate between pre- and postsynaptic $GABA_B$ receptors.

New displacement experiments have shown that GHB can be used as a tool to differentiate between GHB-sensitive and GHB-non-sensitive $GABA_B$ receptor subtypes (Mathivet et al., to be published).

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