DIAZEPAM BIPHASICALLY MODULATES $[^3H]$TBOB BINDING TO THE CONVULSANT SITE OF THE GABA$_A$ RECEPTOR COMPLEX

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ABSTRACT

Interactions of GABA, bicuculline methochloride and diazepam with $[^3H]$TBOB binding to rat brain membranes were evaluated in vitro. GABA displaced $[^3H]$TBOB binding with an IC$_{50}$ of 4 µM and a slope factor near unity. The competitive GABA antagonist bicuculline methochloride shifted the displacement curve of GABA parallelly to the right, indicating that the interaction of GABA with $[^3H]$TBOB binding is of an allosteric nature.

In the presence of GABA, diazepam displaced the binding of $[^3H]$TBOB according to a two-site model: a high affinity site with an IC$_{50}$ of about 50 nM and a lower affinity site with an IC$_{50}$ of about 30 µM. Bicuculline methochloride abolished the nanomolar displacement by diazepam and increased the micromolar IC$_{50}$ value. These results indicate that the interaction of the high affinity diazepam site with the $[^3H]$TBOB binding site is totally GABA dependent and that the low affinity effect of diazepam on $[^3H]$TBOB binding is at least partially GABA dependent.

It is likely that the low affinity potency of diazepam to displace $[^3H]$TBOB binding has physiological relevance.
INTRODUCTION

The GABA$_A$ receptor complex is a heterogeneous oligomeric protein (1). It comprises the Cl' channel as well as binding sites for a variety of compounds, e.g. binding sites for GABA (gamma-aminobutyric acid), benzodiazepines, barbiturates and convulsants (2). All the receptor sites on the complex are allosterically coupled, resulting in a network of interactions ultimately controlling the ion channel.

Several interactions have been described: Benzodiazepine agonists allosterically enhance the affinity of GABA for its low affinity receptor site (3,4,5,6). GABA allosterically displaces TBPS ([35S]t-Butylbicyclophosphorothionate) binding to the convulsant sites (7,8). Based on these findings we may expect a benzodiazepine effect on the binding of ligands to the convulsant site: a potentiation of the displacement by GABA should be found. Yet, conflicting data have been published on the effects of benzodiazepines on convulsant binding. Benzodiazepine agonists either increased (9,10) or decreased (11,12) convulsant binding depending on the amount of GABA present (13). The enhancement has been attributed to non-equilibrium conditions of TBPS binding (14,15). The displacement of convulsant binding requires micromolar concentrations of benzodiazepines, in contrast to the nanomolar affinities of the benzodiazepines for their specific binding sites (16,17,18,19).

We conducted a detailed study on the interactions between GABA, bicuculline methochloride and diazepam on the binding of the convulsant site ligand [3H]-t-butylbicycloorthobenzoate: [3H]TBOB (12,20). As will be seen, [3H]TBOB labels a site linked to the GABA$_A$ receptor complex. We show that diazepam, in the presence of GABA, displaces [3H]TBOB according to a two sites model.

MATERIALS AND METHODS

Preparation of the membranes.
Female Wistar rats were used, they were bred in our laboratory, their weight was 200 ± 20 g. Forebrains were homogenized in 9 volumes 0.32 M sucrose at
0 °C with a Teflon-glass homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min at 4 °C. The supernatant was decanted and centrifuged at 48,000 x g for 30 min at 4 °C. The pellets were washed two times by suspension in 50 mM sodium-potassium-phosphate buffer, pH 7.4, containing 500 mM NaCl (assay buffer) and centrifugation at 48000 x g for 10 min at 4 °C. The pellets were stored at -20 °C until assay. Before assay, the pellets were washed once.

**Chemicals.**

GABA and Bicuculline methochloride were obtained from Sigma Chemical Company. Diazepam was a generous gift of Dr. K. Dingelhoff, Roche Nederland B.V. \[^3\text{H}\]TBOB was obtained from Amersham. The specific activity was 30 Ci/mmol. Unlabelled TBOB was a generous gift of Dr. C.J. Palmer, University of California, USA.

**Assays.**

Membrane pellets were homogenized in assay buffer: the tissue concentration in incubation medium was 12.5 mg/ml. Into glass tubes we pipetted consecutively: 25 µl of \[^3\text{H}\]TBOB and either drugs or buffer in volumes of 25 µl. The final concentration of \[^3\text{H}\]TBOB in the incubation mixture was 8 nM. The incubation was started by adding 200 µl of tissue homogenate. Incubations were performed at 25 °C, lasted 90 min and were terminated by adding 3 ml ice cold buffer to the incubation tubes and rapid filtration of the mixture through Whatman GF/B filters. The filters were washed two times with 3 ml ice cold assay buffer. Radioactivity retained in the filters was counted by liquid scintillation spectrometry. Specific \[^3\text{H}\]TBOB binding was defined as total binding minus the remaining binding in the presence of 4 µM TBOB or of 100 µM picrotoxin. Specific binding was 70-75 % of total binding at 8 nM \[^3\text{H}\]TBOB.

**Data analysis.**

Models were fitted to the data using the nonlinear regression program INPLOT to the function of a two sites model:

\[
B_{\text{drug}} - B_{\min} + (B_{\max} - B_{\min}) \left( \frac{F_1}{1 + \left( \frac{IC_{50}(1) \text{[drug]}}{\text{[drug]}} \right)^{H_1}} \right) + \frac{1 - F_1}{1 + \left( \frac{IC_{50}(2) \text{[drug]}}{\text{[drug]}} \right)^{H_2}}
\]
where [drug] is the concentration of the test drug in moles/l. $B_{\text{drug}}$ is the % binding in the presence of the drug of investigation and $B_{\text{min}}$ is the minimum binding in % of control binding. Control binding is defined as $[^{3}\text{H}]$TBOB binding in the presence of 10 µM bicuculline methochloride. $B_{\text{drug}}$ and $B_{\text{min}}$ were determined experimentally. $F_1$ is the fraction of binding with an IC$_{50}(1)$, being 0 for a one site model. $(1-F_1)$ is the fraction of binding with an IC$_{50}(2)$. IC$_{50}$'s are the concentrations of the drug that gives half maximum inhibition or enhancement expressed as moles/l, and $H$'s are the slope factors being positive in case of an enhancement and negative in case of an inhibition. The parameters that are estimated by non-linear regression are: $B_{\text{max}}$, IC$_{50}(1)$, IC$_{50}(2)$, $F_1$, $H_1$ and $H_2$.

Statistical analysis.

F-tests were used to analyze whether a two site model fits the data better than a one site model. Parameters estimates were tested for differences with ANOVA followed by Scheffé's post hoc analysis.

RESULTS

The effect of GABA on $[^{3}\text{H}]$TBOB binding.

The equation given in Materials and Methods was fitted to the data, according to a one site model: i.e. $F_1=0$. Results are summarized in table 1 and in fig. 1.

1) GABA displaced all specifically bound $[^{3}\text{H}]$TBOB: The nonspecific binding obtained by inhibition of $[^{3}\text{H}]$TBOB with GABA resembled the nonspecific binding determined by inhibition with unlabelled TBOB (4 µM) or picrotoxin (100 µM) and was about 30 % of total binding.

2) GABA displaced $[^{3}\text{H}]$TBOB binding with an IC$_{50}$ of 5.2 µM (value not corrected for endogenous GABA).

3) The concentration of endogenous GABA in our samples, determined by aminoacid analysis (21) was $2.43 \pm 0.13$ µM (mean ± SEM, n=16).

4) Control binding was defined as $[^{3}\text{H}]$TBOB binding in the presence of 10 µM bicuculline methochloride. The binding of $[^{3}\text{H}]$TBOB in the presence of endogenous GABA was $63 \pm 2$ % of control binding (mean ± SEM, n=4).
The effect of GABA on the binding of $[^3\text{H}]$TBBOB in the absence and in the presence of bicuculline methochloride (BMC).

<table>
<thead>
<tr>
<th>Drugs present</th>
<th>IC50 GABA (µM)</th>
<th>Slope factor (H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>endogenous GABA</td>
<td>5.2 ± 0.1</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>+ 10 µM BMC</td>
<td>44 ± 2</td>
<td>1.12 ± 0.05</td>
</tr>
</tbody>
</table>

Parameters estimates obtained by fitting the formula given in Materials and Methods to the data. Without addition of bicuculline methochloride: mean ± SEM of n=4 in triplicate. In the presence of bicuculline methochloride: cumulated data points of 2 experiments: fit result ± SE of fit. Data are not corrected for endogenous GABA. When adjusted for the endogenous GABA content, as depicted in fig.1, the IC50 of GABA was 4.0 ± 0.2 µM.

Figure 1: The effect of GABA on $[^3\text{H}]$TBBOB binding in the presence of 2.4 µM endogenous GABA (circles) (data points: mean ± SD, n = 4 in triplicate) and in the presence of bicuculline methochloride (10 µM, triangles, cumulated data of n = 2 in triplicate). Endogenous GABA inhibits $[^3\text{H}]$TBBOB to 63 % of control binding (i.e. the binding in the presence of 10 µM bicuculline methochloride). GABA displaces $[^3\text{H}]$TBBOB according to a one site model. The estimated B_max value was not significantly different from 100 %. Bicuculline methochloride shifts the displacement curve of GABA to the right.
TABLE 2

The effect of bicuculline methochloride (BMC) on the binding of [³H]TBOB in the absence and in the presence of GABA or of diazepam (Dia).

<table>
<thead>
<tr>
<th>Drugs present</th>
<th>Bmin (%)</th>
<th>EC50 BMC (µM)</th>
<th>Bmax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA endo</td>
<td>63.9 ± 2.1*</td>
<td>0.52 ± 0.08*</td>
<td>101.5 ± 0.8</td>
</tr>
<tr>
<td>GABA endo + GABA 8</td>
<td>19.9 ± 1.7</td>
<td>2.30 ± 0.21</td>
<td>106.2 ± 3.8</td>
</tr>
<tr>
<td>GABA endo + Dia 50</td>
<td>23.4 ± 3.4</td>
<td>1.77 ± 0.26</td>
<td>94.0 ± 2.3*</td>
</tr>
</tbody>
</table>

Parameters estimates obtained by fitting the formula given in Materials and Methods to the data. Mean ± SEM of n = 4 in triplicate. Per column an * indicates significant differences, ANOVA, F(2,11) ≥ 12.6 followed by Scheffé post hoc analysis, p<0.05. Slope factors were not different from unity.

Figure 2: The effect of bicuculline methochloride on [³H]TBOB binding in the presence of endogenous GABA (circles), of (endogenous + 8 µM) GABA (squares), or of (endogenous GABA + 50 µM diazepam) (stars) (data points: mean ± SD, n = 4 in triplicate). Bicuculline methochloride reverses the inhibitory effect of GABA and that of diazepam.
5) After correction for endogenous GABA, GABA was found to displace \[^3H\]TBOB binding with an IC\(_{50}\) of 4.0 \(\mu\)M. The estimated value for B\(_{\text{max}}\) was 108 ± 8 %. This was not significantly different from 100 %.

6) In the presence of 10 \(\mu\)M bicuculline methochloride the displacement curve of \[^3H\]TBOB by GABA was shifted parallelly to the right.

The effect of bicuculline methochloride on \[^3H\]TBOB binding.
The equation was fitted to the data, according to a one site model: i.e. F1=0.
Results are summarized in table 2 and in figure 2.

1) Bicuculline methochloride reversed the inhibitory effect of endogenous GABA with an EC\(_{50}\) of 0.5 \(\mu\)M (table 2).

2) In the presence of (endogenous + 8 \(\mu\)M) GABA the binding is 20 % of control. Bicuculline methochloride reversed this effect completely with an EC\(_{50}\) of 2.3 \(\mu\)M.

3) In the presence of endogenous GABA and 50 \(\mu\)M diazepam the binding is 23 % of control. The nonspecific binding was not influenced by the addition of 50 \(\mu\)M diazepam. The displacing effect of endogenous GABA and diazepam is reversed by bicuculline methochloride with an EC\(_{50}\) of 1.8 \(\mu\)M, to a maximum of 94 % of control.

The effect of diazepam on \[^3H\]TBOB binding.
The equation was fitted to the data, results are listed in table 3 and fig. 3.

1) See fig. 3, upper line. In the presence 10 \(\mu\)M bicuculline methochloride, diazepam displaced \[^3H\]TBOB to a minimum of 72 ± 4 % at 10\(^{-4}\) M (n=4). In a one site model an extrapolated IC\(_{50}\) of 215 \(\mu\)M was found. In order avoid extrapolation, we determined the IC\(_{10}\)'s as well: In the presence of bicuculline methochloride a 10 % displacement was achieved at 24 \(\mu\)M. The highest obtainable concentration diazepam was 10\(^{-4}\) M.

2) See fig. 3, lower two lines. Diazepam displaced \[^3H\]TBOB completely in the presence GABA. This displacement could best be described by a two-sites model (F-tests, p<0.0001).

2.1) \[^3H\]TBOB binding was 63 % of control in the presence of 2.4 \(\mu\)M endogenous GABA. Diazepam displaced \[^3H\]TBOB binding with the following
TABLE 3

The effect of diazepam on the binding of \([^3H]TBOB\) in the absence and the presence of bicuculline methochloride or of GABA.

<table>
<thead>
<tr>
<th>Drug present (µM)</th>
<th>Bₘₐₓ (%)</th>
<th>B(1) (%)</th>
<th>IC₅₀(1) (µM)</th>
<th>B(2) (%)</th>
<th>IC₅₀(2) (µM)</th>
<th>IC₁₀(2) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BicMCl 10</td>
<td>100</td>
<td>0</td>
<td>65±18*</td>
<td>100</td>
<td>215±31*</td>
<td>24.5±3.5*</td>
</tr>
<tr>
<td>GABA endo</td>
<td>62.6±2.2*</td>
<td>10.6±1.9*</td>
<td>8.1±1.4*</td>
<td>52.1±1.7*</td>
<td>43.7±4.5*</td>
<td>10.4±1.0*</td>
</tr>
<tr>
<td>GABA endo+4</td>
<td>35.1±2.1±</td>
<td>8.1±1.4*</td>
<td>44±18*</td>
<td>26.9±1.80</td>
<td>20.9±1.18</td>
<td>12.9±1.3*</td>
</tr>
</tbody>
</table>

Parameters estimates obtained by fitting the formula given in Materials and Methods to the data. B(1) is the binding with IC₅₀(1) (FₓxBₘₐₓ), B(2) is the binding with IC₅₀(2) ((1-Fₓ)xBₘₐₓ). Mean ± SEM, n=4-6 in triplicate. Per column different symbols indicate significant differences: ANOVA, F(2,15) ≥ 11.2, followed by Scheffé post hoc analysis, p<0.05. Slope factors were not different from unity.

Figure 3: The effect of diazepam on \([^3H]TBOB\) binding in the presence of 10 µM bicuculline methochloride (upper line, triangles), of endogenous GABA (middle line, circles) or of (endogenous + 4 µM) GABA (lower line, squares) (data points: mean ± SD, n = 4-6 in triplicate). In the presence of bicuculline methochloride, diazepam displaces \([^3H]TBOB\) according to a one site model, in the presence of GABA, diazepam displaces \([^3H]TBOB\) according to a two site model.
parameters: 10% of the binding had an IC$_{50}$ of 65 nM, while the remaining 53% had an IC$_{50}$ of 44 µM. A 10% of control displacement of the lowest affinity site was achieved at 10 µM diazepam.

2.2) $[^3]H$TBOB binding was 35% of control in the presence of 6.4 µM GABA. Diazepam displaced this binding as follows: 8% had an IC$_{50}$ of 44 nM, while the remaining 27% had an IC$_{50}$ of 21 µM. A 10% of control displacement of the lowest affinity site was achieved at 13 µM.

**DISCUSSION**

The present study provides additional evidence that $[^3]H$TBOB, like $[^{35}]S$TBPS, specifically labels a site associated with the GABA$_A$-receptor complex (22,23) since i) GABA completely allosterically displaces $[^3]H$TBOB and ii) the displacing effect was reversed by the competitive GABA$_A$ antagonist bicuculline methochloride (24).

The micromolar IC$_{50}$ value of GABA indicates that the GABA site that is allosterically coupled to the convulsant site, is the low affinity one, as was reported (8). Bicuculline methochloride shifts the GABA displacement curve parallelly to the right. Such a right shift by antagonists is usually observed in functional dose-response studies (25). Therefore, the $[^3]H$TBOB receptor binding assay can be interpreted in terms of a physiological dose-response experiment. Indeed it has been shown that the inhibition of the binding of the cage convulsant $[^{35}]S$TBPS correlates with a physiological parameter namely the enhancement of Cl⁻ uptake (26).

Diazepam displaces $[^3]H$TBOB as well. In the presence of micromolar concentrations of GABA, the data can be described with a two-sites model with nM and µM affinities. The nM affinity site is not demonstrable when the GABA low affinity site is occupied by the antagonist bicuculline methochloride. This observation confirms that nM concentrations of diazepam displace $[^3]H$TBOB indirectly via potentiation of the displacing effect of GABA (3,4). Using in vitro receptor binding studies with $[^3]H$ muscimol or $[^3]H$ diazepam, it
is well documented that μM concentrations of GABA enhances the nM affinity of diazepam and vice versa (6,17). We show that the [³H]TBOB binding assay can be used to demonstrate the interaction between the low affinity site of GABA and the high affinity site of diazepam as well. Moreover, parameter estimates describing the allosteric interaction between the GABA low affinity site and the diazepam high affinity site obtained by [³H]TBOB binding (table 3) are in agreement with those obtained by [³H]muscimol binding or [³H]diazepam binding (6,17). [³H]TBOB binding revealed an additional interaction between the GABA₀ receptor complex and diazepam. Diazepam displaces [³H]TBOB binding with a potency in the micromolar range as well. The displacement of [³H]TBOB by μM concentrations of diazepam is found in the presence of GABA as well as in the presence of bicuculline methochloride, but the parameters estimates differ: the IC₅₀ in the presence of GABA is lower than the IC₅₀ in the presence of bicuculline (table 3). Moreover bicuculline reverses not only the inhibitory effect of the nM concentrations of diazepam but at least part of the effect of μM concentrations diazepam as well (fig. 2, table 2). We conclude therefore that GABA modulates the μM potency of diazepam to displace [³H]TBOB.

The low affinity potency of diazepam can be explained in different ways. It may represent an interaction with a specific binding site on the GABA₀ receptor complex or it may reflect an allosteric negative cooperation between two interdependent receptor sites on the GABA₀ complex (25). Another possibility is an interaction with a membrane component resulting in a secondary interference with the GABA₀ complex. The presence of specific μM affinity sites for benzodiazepines have been described (27,28). Delorenzo (29) reported a micromolar benzodiazepine site coupled to Ca²⁺ channels. Since the binding of [³H]TBOB is restricted to GABA₀ receptors, the μM benzodiazepine effect described here is not the result of an interaction with the site described by Delorenzo. A μM effect of benzodiazepines related to the convulsant site on the GABA₀ receptor is reported by Gee (28). Micromolar concentrations of the
convulsant benzodiazepine Ro 5-4864 modulates the convulsant site in a GABA dependent manner (28,30,31). No specific Ro 5-4864 binding sites on the GABA<sub>A</sub> complex are characterized yet. The interaction of micromolar concentrations of Ro 5-4864 with the GABA receptor complex is believed to be responsible for the proconvulsive action of the compound (32). This proconvulsive action can be blocked by diazepam but not by flumazenil (32), a benzodiazepine antagonist with nM affinity (33). Preliminary results with [<sup>3</sup>H]TBOB binding show that flumazenil (1 μM) is able to eliminate the inhibitory effect of nM concentrations of diazepam but not its micromolar effects. Therefore diazepam and Ro 5-4864 might interact with the same low affinity site for benzodiazepines on the GABA complex, with opposite effects.

The question remains what is the clinical pharmacological relevance of the low affinity benzodiazepine influence? The ability of nanomolar concentrations of diazepam to enhance the affinity of GABA is generally held to be responsible for the majority of its clinical effects (3,4,34). The slopes of functional dose-response curves of benzodiazepines for overall CNS depression are less than unity and the EC<sub>50</sub> are in the order of μM (3,35). Fitting our data to the equation of a one site model yields a slope factor of 0.6 and an IC<sub>50</sub> value of 15 μM. The performance of a two site model was significantly better and therefore we hypothesize that the slight in vivo slopes camouflage a two site model. Recent in vivo data for midazolam support this hypothesis: a two phasic dose-response curve on partial convulsive epilepsy was reported (36). Flumazenil did not antagonize the anticonvulsant effect of the high doses midazolam (36), being in agreement with our preliminary in vitro data. Therefore it is likely that the low affinity benzodiazepine site found in vitro has physiological relevance.

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REFERENCES


