ELSEVIER

European Journal of Pharmacology 360 (1998) 99–104

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[^{3}H]*t*-butylbicycloorthobenzoate binding to recombinant $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptor

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Received 2 September 1998; accepted 4 September 1998

Abstract

The interaction of several selected compounds with the binding of the cage convulsant *t*-[^{3}H]butylbicycloorthobenzoate ([^{3}H]TBOB) to membranes isolated from human embryonic kidney (HEK) 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subtype of $GABA_A$ receptors was studied. Scatchard analysis of binding data revealed the existence of a single type of binding site for [^{3}H]TBOB with a K_d of 47.06 ± 4.06 nM and a B_{max} value of 6.72 ± 0.52 pmol/mg protein. GABA, thiopental, TBOB, picrotoxin and the neurosteroid dehydroepiandrosterone sulfate displaced concentration—dependently the binding of [^{3}H]TBOB to this recombinant receptor. Dehydroepiandrosterone sulfate reversed the 5 μM GABA-induced inhibition of specific [^{3}H]TBOB binding. It is concluded that membranes isolated from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits exhibit specific high-affinity [^{3}H]TBOB binding. The potency of drugs to inhibit [^{3}H]TBOB binding mainly corresponded to that observed for the inhibition of the binding of cage convulsants to the native receptors or to transiently transfected HEK 293 cells.

Keywords: [^{3}H]TBOB (*t*-[^{3}H]butylbicycloorthobenzoate) binding; $GABA_A$ receptor, recombinant; GABA (γ-aminobutyric acid); Thiopental; Picrotoxin; Dehydroepiandrosterone sulfate

1. Introduction

$GABA_A$ receptor is a hetero-oligomeric membrane protein known to consist of various subunits whose regional distribution and prevalence in the brain may vary considerably (Wisden et al., 1992; Laurie et al., 1992). So far, a total of 6α-, 3β-, 3γ-, 1δ-, and 2ρ-subunits of the $GABA_A$ receptor have been cloned and sequenced from mammalian brain (reviewed by: Wisden and Seeburg, 1992; Sieghart, 1995). Whereas α-, β-, γ- and δ- subunits appear in the rat brain, most probably forming a pentameric structure (Nayeem et al., 1994), ρ subunits recently detected in the avian and rat brain (Albrecht et al., 1997; Boue-Grabot et al., 1998) may constitute homomeric $GABA_C$ rather than heteromeric $GABA_A$ receptors (Enz and Cutting, 1998). For some subunits, e.g., γ_2, also the spliced forms (termed γ_{2s} and γ_{2l}) have been identified (Whiting et al., 1990). The functional and pharmacological properties of $GABA_A$ receptors, including the potency and efficacy of the neurotransmitter itself, depend on their subunit composition (Bureau and Olsen, 1993; Korpi and Lüddens, 1993; Dučić et al., 1995; Korpi et al., 1996). Unlike the studies on the native receptors obtained from brain tissue, the experiments on the recombinant $GABA_A$ receptors make it possible to study the pharmacological properties of well-defined receptor forms and to elucidate the importance of each particular subunit for receptor pharmacology and function. Particularly attractive models represent the stable cell lines expressing different subtypes of $GABA_A$ receptors. Recently Besnard et al. (1997) described the development of three clonal mammalian cell lines in which relatively high levels of $GABA_A$ receptor subtypes were expressed.

In the present study we describe the interaction of several selected compounds with the binding of the cage convulsant [^{3}H]TBOB (*t*-[^{3}H]butylbicycloorthobenzoate) to one of these cell lines expressing the $\alpha_1\beta_2\gamma_{2s}$ subtype of $GABA_A$ receptor. Besides GABA, we used picrotoxin and barbiturate, drugs known to bind in the chloride channel region (Olsen, 1981), i.e., at the same site as the convulsant TBOB (Lawrence et al., 1985). Further, since steroids

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00661-X

are also known to allosterically modulate the $GABA_A$ receptor-associated Cl^- channel (Gee et al., 1988), we used one neurosteroid, dehydroepiandrosterone sulfate, which has been described as being inactive at the [^{3}H]TBOB binding site (Majewska et al., 1990a).

2. Materials and methods

2.1. Materials

Compounds were obtained from the following sources: GABA and picrotoxin from Sigma (St. Louis, MO, USA), dehydroepiandrosterone sulfate (dehydroisoandrosterone 3-sulfate, sodium salt hydrate) from Aldrich (Milwaukee, WI) and thiopental from Pliva (Zagreb, Croatia).

[^{3}H]TBOB (*t*-[^{3}H]butylbicycloorthobenzoate, specific activity 24 Ci/mmol) was purchased from Amersham. Unlabeled TBOB was a gift from Prof. H.I. Yamamura. TBOB was dissolved at a concentration of 1 mM in dimethyl sulfoxide. All other drugs were dissolved in distilled water. Culture medium, antibiotics and fetal bovine serum were purchased from Sigma.

2.2. Culturing of HEK 293 cells

Cells of the human embryonic kidney (HEK) 293 cell line expressing the $\alpha_1\beta_2\gamma_{2s}$ subtype of $GABA_A$ receptor (Besnard et al., 1997) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin in 250 ml tissue culture flasks according to standard cell culture techniques.

2.3. Preparation of the membranes

Membranes from stably transfected HEK 293 cells were prepared mainly as described by Fuchs et al. (1995). The cells were washed, harvested by scraping into phosphate-buffered saline and centrifuged at $12\,000 \times g$ for 12 min. The cell pellet was homogenised in 50 mM Tris–citrate buffer, pH 7.4, and centrifuged at $200\,000 \times g$ for 20 min. The pellet was resuspended, centrifuged at $200\,000 \times g$ for 20 min two more times, resuspended again and stored at −20°C. On the day of assay, the suspension was centrifuged once more at $200\,000 \times g$ for 20 min and used for binding studies.

2.4. [^{3}H]TBOB binding assay

2.4.1. Inhibition studies

Aliquots of the cell membrane preparation (~90 μg protein) were incubated in 50 mM Tris–citrate buffer (pH = 7.4) containing 200 mM NaCl at 25°C for 90 min with 8 nM [^{3}H]TBOB, in the presence or absence of various drugs (50 μl). The total assay volume was 0.5 ml. Non-specific [^{3}H]TBOB binding, defined in the presence of 100 μM picrotoxin, was < 23% of the total binding.

2.4.2. Saturation studies

Assay conditions were in general as described above. Data for Scatchard plots were obtained by adding varying concentrations of nonradioactive TBOB to a fixed concentration (8 nM) of [^{3}H]TBOB so that 10 final concentrations (8–200 nM) were achieved.

2.5. Protein determination

Protein concentration was determined in 10 μl of membrane suspension according to Lowry et al. (1951), using bovine serum albumin as standard.

2.6. Data analysis

The binding data were analysed by using a computer-based equilibrium binding data analysis (EBDA) program (McPherson, 1983). EBDA calculates the apparent dissociation constant (K_d) and the maximum density of binding

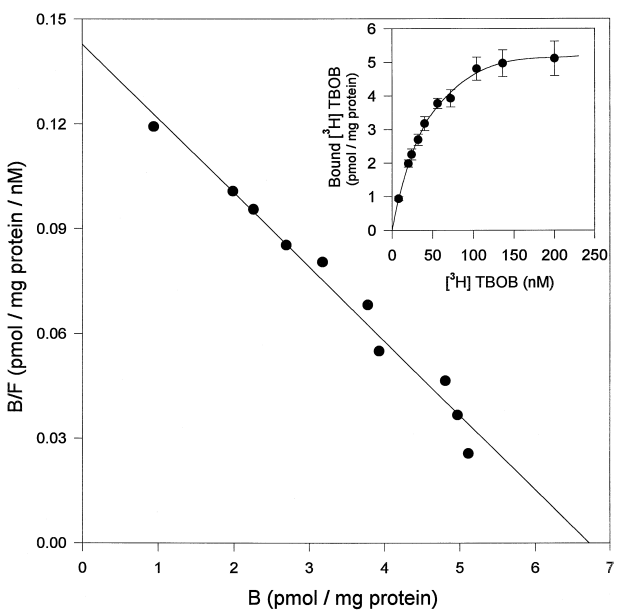

Fig. 1. Scatchard analysis of [^{3}H]TBOB binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits of $GABA_A$ receptors. Membranes were incubated with increasing concentrations of non-radioactive TBOB and a fixed concentration (8 nM) of [^{3}H]TBOB so that 10 final concentrations (8–200 nM) were achieved. Radioactivity bound to membranes was determined after rapid filtration on Whatman GF/C filters. Binding in the presence of picrotoxin (100 μM) was subtracted from total [^{3}H]TBOB binding to give specific [^{3}H]TBOB binding. Data were subjected to Scatchard analysis. Each data point is the mean of four independent experiments, each performed in duplicate. Inset: saturation isotherms. Bars represent S.E.M. K_d for [^{3}H]TBOB was 47.06 ± 4.06 nM and B_{max} was 6.72 ± 0.52 pmol/mg protein.

sites by Scatchard transformation of the saturation binding data. Inhibition data were fit to the sigmoidal equation:

$$Y = A + (B - A)/(1 + \exp((X - C) * D))$$

(Hawkinson et al., 1996) by using SigmaPlot, where Y is the percent specific bound, A is the bottom plateau, B is the top plateau, X is log concentration, C is the log of the IC_{50}, and D is the Hill coefficient.

3. Results

As shown in Fig. 1, [^{3}H]TBOB bound to membranes of HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits of the $GABA_A$ receptor in a saturable manner. Scatchard analysis of equilibrium binding data revealed the existence of a single type of binding site for [^{3}H]TBOB with a K_d of 47.06 ± 4.06 nM and a B_{max} value of 6.72 ± 0.52 pmol/mg protein.

[^{3}H]TBOB binding sites present in HEK cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits of the $GABA_A$ receptor were further studied by determining the inhibitory potencies of several compounds known to bind or allosterically modulate the convulsant binding site. Fig. 2 shows the displacement of [^{3}H]TBOB binding to this membrane preparation by TBOB (1 nM–5 μM), picrotoxin (1 nM–100 μM), GABA (10 nM–100 μM) and thiopental (1 μM–1 mM). As expected, TBOB was the most potent inhibitor of [^{3}H]TBOB binding. Picrotoxin and GABA were less potent, but their IC_{50} values were still in the nanomolar range. Evaluation of the binding data by the curve-fitting program revealed that the slope factors for all three drugs were near unity. Thiopental and dehydroepiandrosterone sulfate inhibited the binding of [^{3}H]TBOB in a concentration-dependent manner (Figs. 2 and 3). The inhibitory potency of these drugs was similar, i.e., the IC_{50} values of both drugs were in the micromolar range, although the slope factor for picrotoxin was significantly higher and that for GABA was significantly lower than unity. All IC_{50} values and slope factors are given in Table 1. When 5 μM GABA was included in the [^{3}H]TBOB assay, dehydroepiandrosterone sulfate reversed the 5 μM GABA-induced inhibition of specific [^{3}H]TBOB binding with an EC_{50} value of 99.52 ± 23.73 μM. The inhibition produced by 0.5–2 mM dehydroepiandrosterone sulfate in

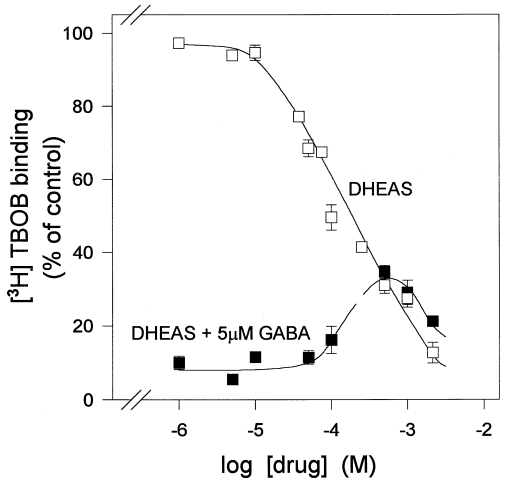

Fig. 3. Inhibition of specific [^{3}H]TBOB binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$-subunits of $GABA_A$ receptors by dehydroepiandrosterone sulfate (DHEAS) in the absence or presence of GABA (5 μM). The conditions of incubation and the description correspond to those described in the legend of Fig. 2.

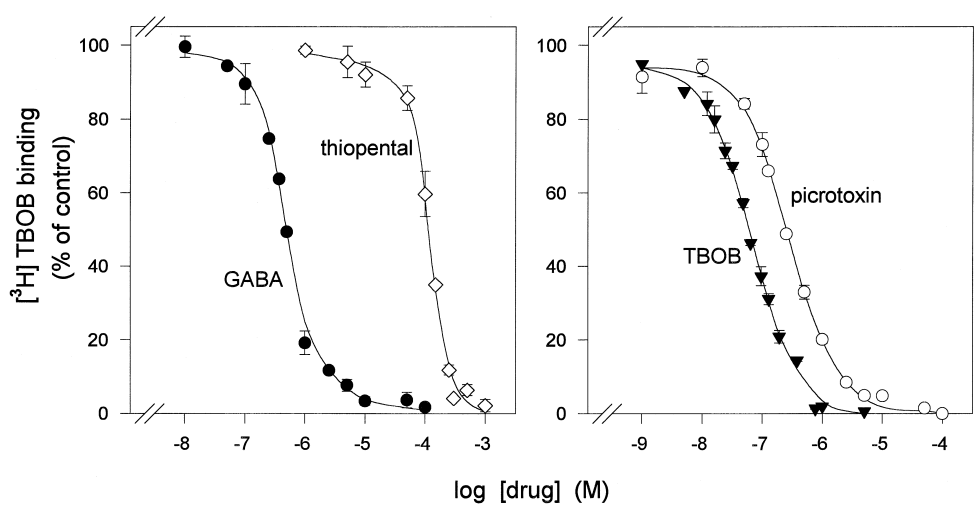

Fig. 2. Inhibition of specific [^{3}H]TBOB binding to membranes from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$-subunits of $GABA_A$ receptors by various compounds. Membranes from HEK cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$-subunits of rat $GABA_A$ receptors (~ 90 μg protein) were incubated with 8 nM [^{3}H]TBOB in the absence or presence of 100 μM picrotoxin or various concentrations of drugs as indicated. Inhibition data were fit to the sigmoidal equation, as described in Section 2. Data points represent means ± S.E.M. of at least three individual experiments performed in duplicate, each using different membranes preparation. IC_{50} values of these curves are listed in Table 1.

Table 1
The potencies of selected compounds for inhibiting [^{3}H]TBOB binding to the recombinant $\alpha_1\beta_2\gamma_{2s}$ form of the rat $GABA_A$ receptor

Drugs	IC_{50} (nM)	Slope factor	(n)
TBOB	62.98 ± 0.79	1.09 ± 0.04	(4)
Picrotoxin	251.37 ± 21.06	0.95 ± 0.06	(5)
GABA	361.04 ± 52.60	1.10 ± 0.03	(4)
Thiopental	130403 ± 22853	2.05 ± 0.30	(3)
Dehydroepiandrosterone sulfate	109090 ± 7211	0.71 ± 0.07	(5)

Aliquots of the cell membrane preparation (~90 μg protein) were incubated in 50 mM Tris–citrate buffer in a final volume of 0.5 ml at 25°C for 90 min with 8 nM [^{3}H]TBOB and different concentrations of drugs. Non-specific binding was defined in the presence of 100 μM picrotoxin.
Data are means ± S.E.M. of (n) separate experiments performed in duplicate.

the absence of GABA equalled approximately that obtained with the same concentration of steroid in the presence of GABA. The maximum inhibition (I_{max}) obtained with dehydroepiandrosterone sulfate (2 mM) was 89.48 ± 12.07% of specific [^{3}H]TBOB binding (Fig. 3).

4. Discussion

The present study demonstrates that membranes isolated from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits of the rat $GABA_A$ receptor exhibit specific high-affinity [^{3}H]TBOB binding. [^{3}H]TBOB bound to a single population of binding sites. GABA, thiopental, TBOB, picrotoxin and the neurosteroid dehydroepiandrosterone sulfate displaced concentration—dependently the binding of [^{3}H]TBOB to this recombinant receptor.

Studies using [^{3}H]TBOB to label a chloride ionophore associated binding site within the $GABA_A$ receptor complex are less common than those using *t*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS), but since both ligands are supposed to label the same population of binding sites (Lawrence et al., 1985; Van Rijn et al., 1995) and have the analogous kinetics (Maksay and Van Rijn, 1993), studies using the two radioligands may well be compared. To our knowledge, until now [^{3}H]TBOB has not been used to label the binding site for convulsants in the recombinant $GABA_A$ receptor. In different studies performed with the brain tissue membranes or sections, the K_d values for [^{3}H]TBOB (Lawrence et al., 1985; Van Rijn et al., 1990; Sakurai et al., 1994; Kume et al., 1996) mainly correspond to those observed for [^{35}S]TBPS (Ito et al., 1989; Holland et al., 1990; Lloyd et al., 1990; Slany et al., 1995; Sousa and Ticku, 1997). The K_d value for [^{3}H]TBOB obtained in our study was similar to that observed for the binding of [^{35}S]TBPS to different subtypes of $GABA_A$ receptors transiently expressed in HEK 293 or in SF-9 insect cells (Korpi and Lüddens, 1993; Im et al., 1994; Ebert et al., 1996).

The total number of binding sites for cage convulsants varies considerably from one preparation to the other. The highest number (15.0 pmol/mg protein) was obtained for the $\alpha_1\beta_1$ combination of subunits stably expressed in HEK 293 cells (Drewe et al., 1995). In HEK cells transiently transfected with different $GABA_A$ receptor subunits, B_{max} values from 3.1 to 3.5 pmol/mg protein were obtained (Lüddens et al., 1994; Slany et al., 1995; Zezula et al., 1996). The total number of [^{3}H]TBOB binding sites obtained in our study exceeded this number, which suggests that the cell line stably expressing the $\alpha_1\beta_2\gamma_{2s}$ form of the $GABA_A$ receptor (Besnard et al., 1997) is very suitable for pharmacological studies. The binding of [^{3}H]TBOB could not be detected in non-transfected HEK 293 cells.

Although many reports have described the modulation of convulsant binding by GABA and its analogs, different results have been obtained depending on the brain region under study and on the $GABA_A$ receptor subtype (Korpi and Lüddens, 1993; Im et al., 1994; Lüddens and Korpi, 1995). While Korpi and Lüddens (1993) found that the regional sensitivity of [^{35}S]TBPS binding to GABA depended on the $GABA_A$ receptor α subunit, Zezula et al. (1996) pointed out the importance of the β subunit for [^{35}S]TBPS binding to membranes from HEK 293 cells transfected with α_1, β_3, or γ_2 subunits of $GABA_A$ receptors. At low levels GABA allosterically increases, while at higher levels decreases, [^{35}S]TBPS binding to different $GABA_A$ receptor subtypes transiently or stably expressed in the HEK 293 cell line (Korpi and Lüddens, 1993; Lüddens and Korpi, 1995; Drewe et al., 1995) or in SF-9 cells infected with baculovirus carrying cDNAs for $GABA_A$ receptors (Im et al., 1994). However, in the report of Pregenzer et al. (1993), GABA inhibited [^{35}S]TBPS binding to the $\alpha_1\beta_2\gamma_2$ subtype of $GABA_A$ receptor expressed in insect SF-9 cells without the early stimulatory phase. The potencies of GABA, picrotoxin and TBOB as displacers of [^{3}H]TBOB binding to the recombinant $\alpha_1\beta_2\gamma_{2s}$ form of the rat $GABA_A$ receptor, observed in this study, are very similar to the potencies of the same drugs as displacers of [^{35}S]TBPS binding to rat brain membranes (Squires et al., 1983). Besides, the inhibitory potency of GABA mainly corresponded to that obtained for the inhibition of [^{35}S]TBPS binding to cerebellar membranes and to the $\alpha_1\beta_3\gamma_2$, $\alpha_1\beta_3$ or to the $\alpha_4\beta_3\gamma_2$ subtype of $GABA_A$ receptor (Zezula et al., 1996; Ebert et al., 1996). GABA was a less potent inhibitor of [^{3}H]TBOB binding to a crude membrane fraction from the rat brain (Van Rijn et al., 1990). The displacement potency of picrotoxin observed in our study was similar to that observed for the inhibition of [^{3}H]TBOB binding to synaptoneurosomes, to rat forebrain membranes (Van Rijn et al., 1990) and brain sections (Sakurai et al., 1994). In general, the literature data describing the modulation of [^{3}H]TBOB/[^{35}S]TBPS binding by GABA vary to a greater extent than the data for the inhibition of the binding of the same radioligands by picrotoxin, although some results (Van Rijn et al., 1990),

which are opposite to other results (Sakurai et al., 1994), suggest that picrotoxin and TBOB do not bind to identical sites in a simple competitive manner.

As shown in Section 3, the affinity of neurosteroid for the chloride channel region resembled the affinity of thiopental, although the slope factors were different. Some previous studies failed to demonstrate the effect of dehydroepiandrosterone sulfate on [^{3}H]TBOB/[^{35}S]TBPS binding to brain homogenates (Majewska and Schwartz, 1987; Majewska et al., 1990a). However, dehydroepiandrosterone sulfate inhibited slightly the binding of [^{3}H]muscimol and [^{3}H]flunitrazepam (Demirgören et al., 1991). Sousa and Ticku (1997) have demonstrated that dehydroepiandrosterone sulfate competitively inhibits the binding of [^{35}S]TBPS to rat brain membranes. Hence, the authors concluded that the picrotoxin/TBPS site on the $GABA_A$ receptor complex is the site of action for dehydroepiandrosterone sulfate. Our results, showing that dehydroepiandrosterone sulfate was able to abolish the inhibitory effect of 5 μM GABA on [^{3}H]TBOB binding, are in good agreement with the data showing that dehydroepiandrosterone sulfate is a $GABA_A$ receptor antagonist, because it inhibited GABA-induced [^{36}Cl]-influx in cultured cortical neurons (Sousa and Ticku, 1997). Some other authors have also demonstrated that dehydroepiandrosterone sulfate inhibits electrophysiological responses to GABA (Majewska et al., 1990a,b; Spivak, 1994).

In summary, the present study demonstrates that membranes isolated from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2s}$ subunits exhibit specific high-affinity [^{3}H]TBOB binding. Dehydroepiandrosterone sulfate, GABA, and several compounds known to modulate $GABA_A$ receptors inhibited [^{3}H]TBOB binding with a potency similar to that observed for the inhibition of the binding of cage convulsants to the native receptors or transiently transfected HEK 293 cells.

Acknowledgements

This study was supported by the Croatian Ministry of Science and Technology. Unlabelled TBOB was generously provided by Prof. Dr. Henry I. Yamamura. The skilful technical assistance of Mrs. Zlatica Tonšetić is gratefully acknowledged.

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