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Interaction of allosteric ligands with $GABA_A$ receptors containing one, two, or three different subunits

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Abstract

The presence of allosteric binding sites on recombinant $GABA_A$ receptors formed after transfection of human embryonic kidney (HEK) 293 cells with α_1-, β_3-, or γ_2-subunits, or with various combinations of these subunits, was systematically investigated. From all possible subunit combinations, high affinity [^{3}H]muscimol binding sites were induced in cells transfected with $\alpha_1\beta_3$- or $\alpha_1\beta_3\gamma_2$-subunits only. $GABA_A$ receptor associated [^{3}H]flunitrazepam binding sites were induced in cells after transfection with $\alpha_1\gamma_2$- or $\alpha_1\beta_3\gamma_2$-subunits, and [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) binding sites were found in cells transfected with β_3-, $\beta_3\gamma_2$-, $\alpha_1\beta_3$-, or $\alpha_1\beta_3\gamma_2$-subunits. Binding of [^{35}S]TBPS could be inhibited by pentobarbital, etazolate, (+)-etomidate, alphaxalone, propofol, chlormethiazole, and 4′-chlorodiazepam (Ro 5-4864) with a potency which differed in cells transfected with β_3-, $\beta_3\gamma_2$-, $\alpha_1\beta_3$-, or $\alpha_1\beta_3\gamma_2$-subunits. Results obtained indicate that receptors with different subunit composition actually can be formed in HEK cells and exhibit distinct pharmacological properties.

Keywords: $GABA_A$ receptor, recombinant; [^{3}H]Flunitrazepam; [^{3}H]Muscimol; [^{35}S]TBPS ([^{35}S]*t*-butylbicyclophosphorothionate); Allosteric binding site

1. Introduction

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system, mediates fast synaptic inhibition by opening a chloride ion channel intrinsic to the $GABA_A$ receptor (Silvilotti and Nistri, 1991). This receptor is a hetero-oligomeric membrane glycoprotein and is the site of action of a variety of pharmacologically and clinically important drugs. Thus, it has been demonstrated that benzodiazepines, barbiturates, steroids, anesthetics and convulsants modulate GABA-induced chloride ion flux by interacting with separate and distinct allosteric binding sites on $GABA_A$ receptors (Sieghart, 1995).

Recently, 6α-, 4β-, 4γ-, 1δ-, and 2ρ-subunits of vertebrate $GABA_A$ receptors have been cloned and sequenced, and it is assumed that five subunits have to assemble to form native receptors (Nayeem et al., 1994). Studies with recombinant receptors have demonstrated that an α-, a β-, and a γ-subunit have to be present simultaneously in a cell in order to produce receptors exhibiting a pharmacology resembling that of $GABA_A$ receptors found in the brain (Sieghart, 1995). Other studies, however, have indicated that at least some of the allosteric binding sites of $GABA_A$ receptors are formed on receptors containing only one or two different subunits (Blair et al., 1988; Pritchett et al., 1988).

So far only three binding sites present on $GABA_A$ receptors can be directly investigated by binding studies: the GABA-, the benzodiazepine- and the picrotoxinin/*t*-butylbicyclophosphorothionate- (TBPS) binding site. Due to the lack of suitable radioactively labeled ligands, the binding of compounds to other sites on $GABA_A$ receptors can be investigated only indirectly by their allosteric interaction with these three binding sites (Sieghart, 1995).

In order to investigate the subunit requirements for the formation of the different binding sites on $GABA_A$ receptors, in this study the induction of [^{3}H]muscimol, [^{3}H]flunitrazepam, or [^{35}S]TBPS binding sites in human embryonic kidney (HEK) 293 cells transfected with one, two, or three different subunits was systematically examined. In addition, the allosteric modulation of [^{35}S]TBPS binding to these cells was investigated and compared. For these experiments, α_1-, β_3-, or γ_2-subunits of $GABA_A$ receptors were used, because these subunits are frequently

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colocalized in the same brain region and neurons (Persohn et al., 1992; Wisden et al., 1992), and $GABA_A$ receptors consisting of various combinations of these subunits might therefore actually occur in the brain. Although receptors consisting of $\alpha_1\beta_2\gamma_2$-subunits seem to be more abundant, the actual presence of α_1-, β_3-, and γ_2-subunits in the same $GABA_A$ receptors recently has been demonstrated (Pollard et al., 1991; Jechlinger et al., in preparation). Results obtained indicate that recombinant $GABA_A$ receptors with different subunit composition exhibit distinct pharmacological properties.

2. Materials and methods

2.1. Materials

[^{35}S]TBPS (specific activity 61.2 Ci/mmol) and [^{3}H]muscimol (specific activity 17.1 Ci/mmol) were purchased from DuPont-New England Nuclear, Dreieich, Germany, and [^{3}H]flunitrazepam (specific activity 85 Ci/mmol) was purchased from Amersham, Buckinghamshire, UK. Other compounds were obtained from the following sources: diazepam and Ro5-4864 (4′-chlorodiazepam) from Hoffmann La Roche, Basle, Switzerland; IPTBO (4-(isopropyl)-1-phospho-2,6,7-trioxabicyclo-(2,2,2)octane-1-oxide) from J.S. Collins, City of London Polytechnic, London; TBPS (*t*-butylbicyclophosphorothionate) from R.F. Squires, Orangeburg, NY, USA; alphaxalone from Glaxo Group Res., Middlesex, UK; propofol from ICI-Pharma, Milan, Italy; chlormethiazole from Astra Arcus, Södertälje, Sweden; etazolate from E.R. Squibb, Princeton, NJ, USA; (+)-etomidate from Janssen Pharmaceuticals, Belgium.

2.2. Culturing and transfecting of human embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells (American Type of Culture Collection, Rockville, MD, USA, CRL 1573) were maintained in Dulbecco's modified Eagle medium (GIBCO-BRL., Grand Island, NY, USA) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin G, and 100 μg/ml streptomycin in 75-cm^2 Petri dishes by using standard cell culture techniques.

cDNAs for the α_1-, β_3- and γ_2-subunits of $GABA_A$ receptors were cloned as described previously (Slany et al., 1995a). Using the calcium phosphate precipitation method (Chen and Okayama, 1988), 3×10^6 HEK 293 cells were transfected either with 20 μg cDNA encoding for the rat α_1-, β_3- or γ_2-subunit of $GABA_A$ receptors subcloned individually into pCDM8 expression vectors, or with a total of 24 μg of a 2 : 1 : 1 mixture of these cDNAs. When cells were transfected with two different subunits, a total of 20 μg and a cDNA ratio of 1 : 1 was used. The medium was changed 20 h after transfection.

Cells were harvested 96 h after transfection by scraping into phosphate-buffered saline. After centrifugation at $12\,000 \times g$ for 10 min the cell pellets were homogenized in 50 mM Tris-citrate buffer, pH 7.4 by using an Ultraturax, followed by three centrifugation ($200\,000 \times g$ for 20 min)-resuspension cycles, and were then used for ligand binding or were stored at −20°C.

2.3. Radioligand binding studies

Similar results were obtained when freshly prepared or frozen membranes were used for binding studies. Frozen membranes from cerebellum (Slany et al., 1995a) or from HEK cells were thawed, centrifuged and resuspended in 50 mM Tris-citrate buffer, pH 7.4, at a protein concentration of about 1 mg/ml as measured by the BCA-protein assay kit of Pierce Chem. Co. with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 150 mM NaCl and 20 nM [^{3}H]flunitrazepam or 20 nM [^{3}H]muscimol in the absence or presence of 10 μM diazepam or 10 μM GABA, for 90 min at 4°C, respectively (Sieghart and Schuster, 1984; Drexler and Sieghart, 1984). For [^{35}S]TBPS binding, membranes were incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 200 mM NaBr and various concentrations (2–200 nM) of [^{35}S]TBPS in the absence or presence of 10 μM IPTBO for 180 min at room temperature (Drexler and Sieghart, 1984). The stock solutions for saturation experiments were obtained by diluting the original [^{35}S]TBPS solution (61.2 Ci/mmol) by various concentrations of cold TBPS. At 200 nM the specific activity of the stock solution of [^{35}S]TBPS was 6.1 Ci/mmol.

Membranes were then filtered through Whatman GF/B filters. When [^{3}H]flunitrazepam or [^{3}H]muscimol binding was investigated, the filters were rinsed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer. When [^{35}S]TBPS binding was investigated, the filters were rinsed three times with 3.5 ml of this buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml Hydrofluor (National Diagnostics, NJ, USA) scintillation fluid. Non-specific binding determined in the presence of 10 μM diazepam, 10 μM GABA or 10 μM IPTBO was subtracted from total [^{3}H]-flunitrazepam, [^{3}H]muscimol or [^{35}S]TBPS binding, respectively, to result in specific binding.

3. Results

Membranes from HEK 293 cells transfected with α_1-, β_3-, or γ_2-subunits of $GABA_A$ receptors, or with various combinations of these subunits, were incubated with either 20 nM [^{3}H]muscimol, 20 nM [^{3}H]flunitrazepam, or with

200 nM [^{35}S]TBPS, under conditions where the actual expression of the respective subunit proteins has been demonstrated by Western blot analysis using subunit specific antibodies (Slany et al., 1995a). For these experiments relatively high concentrations of radioligands were used in order to increase the chances for a detection of low affinity binding sites possibly formed in HEK cells transfected with some of the subunit combinations. Specific binding of radioligands was determined and compared with that obtained with membranes from non-transfected HEK cells. As shown in Fig. 1, among all combinations including homo-oligomeric sets, only in membranes from cells transfected with $\alpha_1\beta_3$- or $\alpha_1\beta_3\gamma_2$-subunits of $GABA_A$ receptors a significant increase in specific

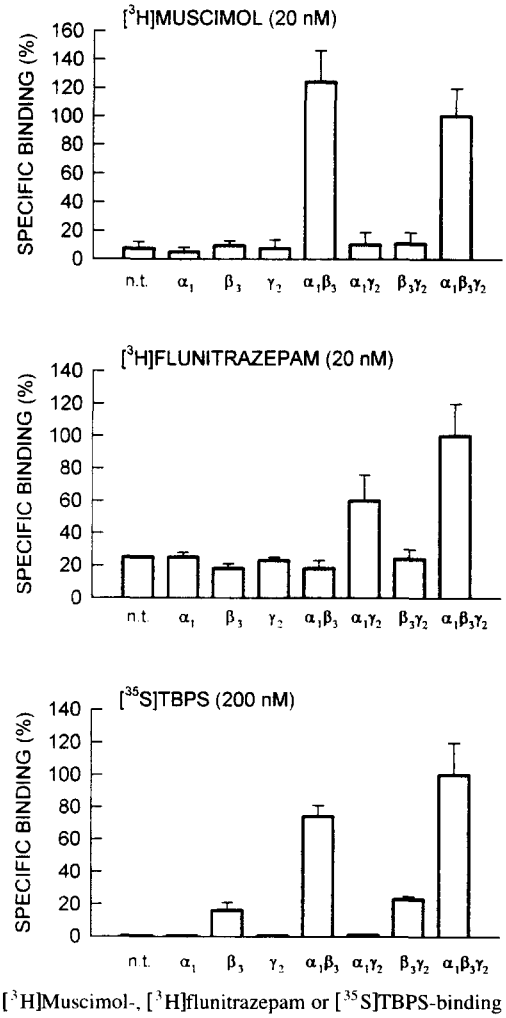

Fig. 1. [^{3}H]Muscimol-, [^{3}H]flunitrazepam or [^{35}S]TBPS-binding to membranes from HEK cells transfected with α_1-, β_3-, or γ_2-subunits, or with various combinations of these subunits. Membranes from non-transfected HEK 293 cells (n.t.) or from cells transfected with $GABA_A$ receptor subunits as indicated, were incubated with 20 nM [^{3}H]muscimol-, 20 nM [^{3}H]flunitrazepam or 200 nM [^{35}S]TBPS in the absence or presence of 10 μM GABA, 10 μM diazepam or 100 μM IPTBO, respectively. Specific radioactivity bound to membranes was then determined as described in Materials and methods. Data are mean values ± S.D. from three to six different experiments performed in duplicate, and are presented as percentage of the specific binding observed in $\alpha_1\beta_3\gamma_2$-transfected cells.

Table 1
Scatchard analysis of [^{35}S]TBPS binding to membranes from HEK cells transfected with various combinations of α_1-, β_3-, or γ_2-subunits

Subunit combination	K_D (nM)	B_{max} (fmol/mg protein)	n
$\alpha_1\beta_3\gamma_2$	15.1 ± 4.5	3488 ± 694	4
$\alpha_1\beta_3$	8.8 ± 3.3	2571 ± 271	3
$\beta_3\gamma_2$	27.4 ± 4.9	648 ± 85	3
β_3	38.9 ± 13.4	553 ± 178	3

Membranes from HEK cells transfected with the $GABA_A$ receptor subunit combinations as indicated, were incubated with various concentrations of [^{35}S]TBPS in the absence or presence of 10 μM IPTBO. Membranes were filtered through Whatman GF/B filters and specific radioactivity bound to membranes was subjected to Scatchard analysis. Data are means ± S.D. from n separate experiments performed in duplicate.

[^{3}H]muscimol binding was observed. As demonstrated previously, a relatively high endogenous [^{3}H]flunitrazepam binding, unrelated to $GABA_A$ receptors, was present in non-transfected HEK cells (Fuchs et al., 1995), and the formation of additional, $GABA_A$ receptor associated specific [^{3}H]flunitrazepam binding sites could be induced by transfection of HEK cells with $\alpha_1\gamma_2$- or $\alpha_1\beta_3\gamma_2$-subunits only (Slany et al., 1995b). Specific [^{35}S]TBPS binding sites, however, were found in HEK cells transfected with β_3- (Slany et al., 1995a), as well as in those transfected with $\alpha_1\beta_3$-, $\beta_3\gamma_2$-, or $\alpha_1\beta_3\gamma_2$-subunits (Fig. 1).

Since most of the subunit combinations which can be investigated by binding studies exhibited [^{35}S]TBPS binding sites, and since all the other allosteric binding sites so far identified on $GABA_A$ receptors can be investigated by their interaction with these sites (Sieghart, 1995), in all subsequent experiments only [^{35}S]TBPS binding was studied. Scatchard analysis of equilibrium binding data obtained from incubating membranes of $\alpha_1\beta_3\gamma_2$-, $\alpha_1\beta_3$-, $\beta_3\gamma_2$-, or β_3-transfected cells with various [^{35}S]TBPS concentrations, indicated the existence of apparently single types of binding sites in each of the membrane preparations investigated. As shown in Table 1, cells transfected with $\alpha_1\beta_3\gamma_2$-subunits exhibited an affinity for this compound (K_D of 15.1 ± 4.5 nM, mean ± S.D., $n = 4$) which was similar to that observed in cerebellar membranes (K_D of 20.7 ± 8.7 nM, mean ± S.D., $n = 3$). Whereas the affinity of [^{35}S]TBPS for cells transfected with $\alpha_1\beta_3\gamma_2$-subunits was not significantly different from that of $\alpha_1\beta_3$-transfected cells (K_D of 8.8 ± 3.3 nM, mean ± S.D., $n = 3$; Student's t-test, $P = 0.14$), it was significantly higher than that for cells transfected with $\beta_3\gamma_2$- (K_D of 27.4 ± 4.9 nM, mean ± S.D., $n = 3$; Student's t-test, $P = 0.03$) or β_3-subunits (K_D of 38.9 ± 13.4 nM, mean ± S.D., $n = 3$; Student's t-test, $P = 0.04$). Similarly, the total number of [^{35}S]TBPS binding sites induced in HEK cells transfected with $\alpha_1\beta_3$-subunits, due to variations in the transfection efficiency, was not significantly different from that of $\alpha_1\beta_3\gamma_2$-transfected cells. In contrast, the number of bind-

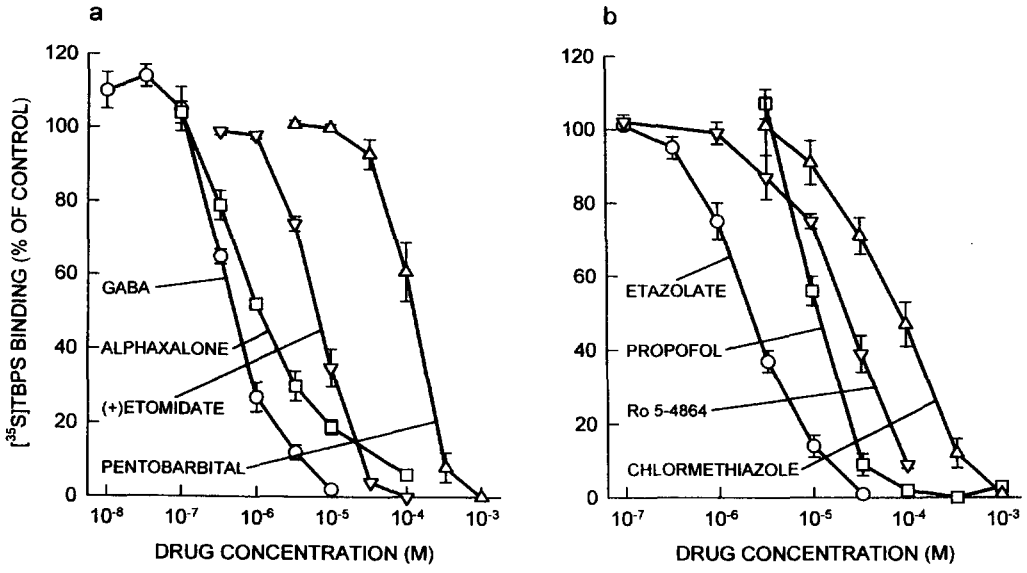

Fig. 2. Inhibition of specific [^{35}S]TBPS binding to membranes from HEK cells transfected with $\alpha_1 \beta_3 \gamma_2$-subunits by various compounds. Membranes from HEK 293 cells transfected with $\alpha_1 \beta_3 \gamma_2$ subunits of $GABA_A$ receptors were incubated with 2 nM [^{35}S]TBPS in the absence or presence of 10 μM IPTBO or various concentrations of drugs as indicated. Membranes were then filtered through Whatman GF/B filters and radioactivity specifically bound to membranes was determined as described in Materials and methods. Data shown represent mean values ± S.D. from three separate experiments performed in duplicate.

ing sites induced in membranes from cells transfected with $\beta_3 \gamma_2$-, or β_3-subunits, was significantly smaller than that found in cells transfected with $\alpha_1 \beta_3 \gamma_2$- or $\alpha_1 \beta_3$-subunits (Table 1). These results are in agreement with previous results from electrophysiological studies which led to the conclusion that the assembly of receptors consisting of $\alpha\beta$- and $\alpha\beta\gamma$-subunits is much more efficient than that of receptors consisting of single subunits or of other subunit combinations (Verdoorn et al., 1990; Sieghart, 1995).

In other experiments the potency of various allosteric modulators of $GABA_A$ receptors for inhibition of [^{35}S]TBPS binding (Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Gee, 1988; Cross et al., 1989; Concas et al., 1991) was investigated in recombinant $\alpha_1 \beta_3 \gamma_2$-receptors. As shown in Fig. 2a, GABA, alphaxalone, (+)-etomidate, and pentobarbital, dose dependently inhibited specific binding of 2 nM [^{35}S]TBPS to recombinant $\alpha_1 \beta_3 \gamma_2$-receptors. Similar inhibition curves were obtained for etazolate, propofol, Ro 5-4864 and chlormethiazole (Fig. 2b). The slight stimulation of [^{35}S]TBPS binding at low concentrations of some of these compounds has been observed previously in brain membranes, and has been interpreted as a $GABA_A$ receptor agonists induced enhancement of the slow time course of association of [^{35}S]TBPS with its binding site (Maksay and Simonyi, 1986).

As shown in Table 2, the potency of all compounds investigated for inhibition of [^{35}S]TBPS binding was comparable in recombinant $\alpha_1 \beta_3 \gamma_2$-receptors and in cerebellar membranes. In addition, the potency of these compounds

Table 2
Potencies of GABA and allosteric $GABA_A$ receptor ligands for the inhibition of [^{35}S]TBPS binding to membranes from HEK cells transfected with $\alpha_1 \beta_3 \gamma_2$ subunits, or to cerebellar membranes. Comparison with potencies of these compounds for stimulation of [^{3}H]flunitrazepam binding

Compounds	[^{35}S]TBPS binding (IC_{50}, μM)		[^{3}H]Flunitrazepam binding (EC_{50}, μM)	
	$\alpha_1 \beta_3 \gamma_2$	Cerebellum	$\alpha_1 \beta_3 \gamma_2$	Cerebellum
GABA	0.5 ± 0.1 (4)	0.24 ± 0.07 (4)	0.5 ± 0.2 (4)	0.43 ± 0.2 (4)
Alphaxalone	1.1 ± 0.12 (3)	1.7 ± 0.5 (4)	0.8 ± 0.5 (4)	0.77 ± 0.3 (7)
Pentobarbital	113 ± 29 (3)	100 ± 22 (4)	65.5 ± 21 (3)	125 ± 34 (5)
(+)-Etomidate	6.5 ± 0.7 (3)	4.6 ± 0.3 (4)	4.5 ± 0.4 (3)	7.0 ± 2.9 (3)
Etazolate	2.4 ± 0.2 (3)	1.6 ± 0.4 (3)	1.0 ± 0.3 (4)	1.2 ± 0.9 (5)
Propofol	10.8 ± 1.9 (3)	16 ± 7 (3)	7.2 ± 1.3 (3)	16.3 ± 3.7 (3)
Chlormethiazole	88.3 ± 25.2 (3)	53 ± 7 (3)	–	–
Ro 5-4864	20.9 ± 1.6 (3)	19 ± 5 (4)	–	–

Membranes from HEK cells transfected with $\alpha_1 \beta_3 \gamma_2$-subunits of $GABA_A$ receptors or membranes from rat cerebellum were incubated with 2 nM [^{35}S]TBPS in the absence or presence of 10 μM IPTBO or various concentrations of compounds to be investigated. Data are means ± S.D. with the number of experiments performed in duplicate in parentheses. Data for [^{3}H]flunitrazepam binding are taken from Slany et al. (1995b).

for stimulation of [^{3}H]flunitrazepam binding to recombinant $\alpha_1\beta_3\gamma_2$-receptors (Slany et al., 1995b) was similar to that for inhibition of [^{35}S]TBPS binding to this receptor, and was comparable with that observed in cerebellar membranes (Table 2).

In previous studies, some of the allosteric modulators of $GABA_A$ receptors have been demonstrated to accelerate the dissociation of [^{35}S]TBPS from its binding sites on brain membranes (Maksay and Ticku, 1985; Maksay and Simonyi, 1986). In order to further investigate the similarity between [^{35}S]TBPS binding sites in brain membranes and in membranes from HEK cells transfected with $\alpha_1\beta_3\gamma_2$-subunits, the latter membranes were incubated with 2 nM [^{35}S]TBPS until binding equilibrium was reached and then maximally inhibiting concentrations of the compounds to be investigated were added (Maksay and Ticku, 1985; Maksay and Simonyi, 1986). As shown in Fig. 3, dissociation initiated by a completely displacing concentration of TBPS or IPTBO, similar to that of picrotoxin (experiments not shown) was slow and monophasic. This was in contrast to the rapid biphasic dissociation observed when completely displacing concentrations of alphaxalone, etazolate, propofol, chlormethiazole, Ro 5-4864 or pentobarbital were added (Fig. 3).

Recently, the presence of allosteric binding sites for alphaxalone, pentobarbital, (+)-etomidate, etazolate, propofol, chlormethiazole, and Ro5-4864 have been demonstrated in homo-oligomeric $GABA_A$ receptors consisting of β_3-subunits (Slany et al., 1995a). In the present study, therefore, the inhibition of [^{35}S]TBPS binding by GABA and these allosteric $GABA_A$ receptor ligands was investigated in recombinant receptors consisting of $\alpha_1\beta_3$- or $\beta_3\gamma_2$-subunits, and was compared with that of receptors consisting of $\alpha_1\beta_3\gamma_2$- or β_3-subunits. As shown in Table 3, all compounds investigated exhibited a similar potency for the inhibition of [^{35}S]TBPS binding to receptors consisting of $\alpha_1\beta_3\gamma_2$- or $\alpha_1\beta_3$-subunits. In addition, the potency of etazolate and chlormethiazole for inhibition of [^{35}S]TBPS binding to receptors consisting of $\beta_3\gamma_2$- or

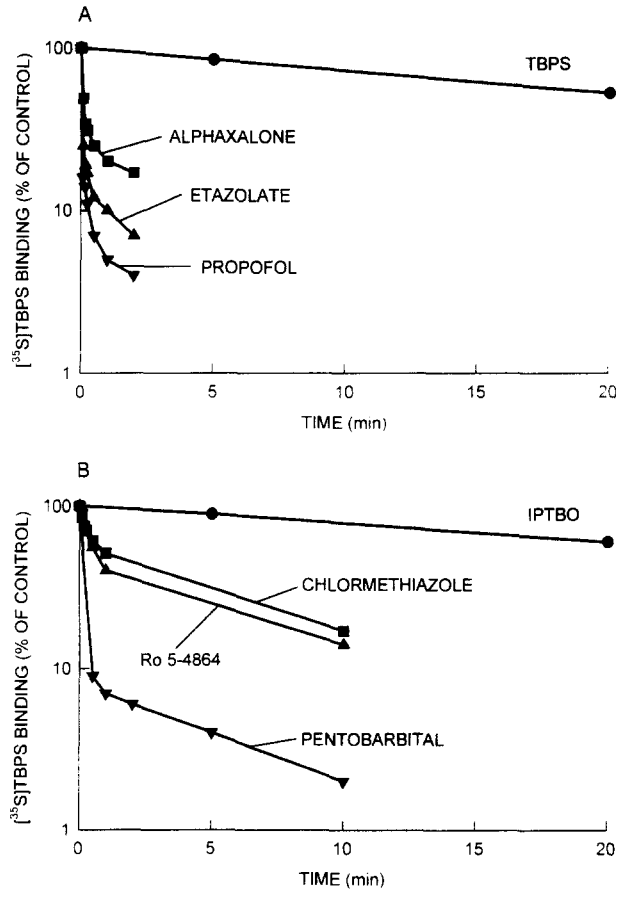

Fig. 3. Displacement of [^{35}S]TBPS from its binding sites on membranes from HEK cells transfected with $\alpha_1\beta_3\gamma_2$ subunits by various $GABA_A$ receptor ligands. Membranes from HEK cells transfected with cDNAs encoding for $\alpha_1\beta_3\gamma_2$ subunits were incubated with 2 nM [^{35}S]TBPS until equilibrium. Then 2 μM TBPS, 100 μM alphaxalone, 100 μM etazolate, 333 μM propofol, 10 μM IPTBO, 1 mM chlormethiazole, 100 μM Ro 5-4864, or 1 mM pentobarbital were added and radioactivity remaining bound to membranes was determined after rapid filtration through Whatman GF/B filters at various timepoints after the addition of the dissociating compounds. Results are from a single experiment performed in duplicate. The experiment was performed twice with similar results.

Table 3
Potencies of GABA and allosteric $GABA_A$ receptor ligands for the inhibition of [^{35}S]TBPS binding to membranes from HEK cells transfected with various combinations of α_1-, β_3-, or γ_2-subunits

Compounds	IC_{50} (μM)			
	$\alpha_1\beta_3\gamma_2$	$\alpha_1\beta_3$	$\beta_3\gamma_2$	β_3
GABA	0.5 ± 0.1 (4)	0.49 ± 0.07 (4)	– (4)	– (3)
Alphaxalone	1.1 ± 0.12 (3)	1.1 ± 0.2 (3)	54.5 ± 1.1 (3)	11.7 ± 9.0 (3)
Pentobarbital	113 ± 29 (3)	123 ± 17 (3)	42.8 ± 10 (3)	19.5 ± 4.1 (3)
(+)-Etomidate	6.5 ± 0.7 (3)	5.8 ± 1.0 (3)	1.8 ± 0.4 (3)	1.6 ± 0.5 (3)
Etazolate	2.4 ± 0.2 (3)	2.2 ± 0.1 (3)	3.4 ± 0.4 (3)	3.8 ± 0.3 (3)
Propofol	10.8 ± 1.9 (3)	11.9 ± 1.2 (3)	19.3 ± 1.2 (3)	20.2 ± 0.9 (3)
Chlormethiazole	88.3 ± 25.2 (3)	99 ± 10 (3)	97.6 ± 9.0 (3)	84 ± 20 (4)
Ro 5-4864	20.9 ± 1.6 (3)	20 ± 2.6 (3)	8.0 ± 1.3 (3)	8.8 ± 3.2 (4)

Membranes from HEK cells transfected with the subunit combinations indicated were incubated with 2 nM [^{35}S]TBPS in the absence or presence of 10 μM IPTBO or various concentrations of compounds to be investigated. Membranes were then filtered through Whatman GF/B filters and radioactivity specifically bound to membranes was determined. Data are means ± S.D. with the number of experiments performed in duplicate in parentheses. Data for receptors consisting of β_3-subunits are from Slany et al. (1995a).

β_3-subunits was comparable with that for $\alpha_1\beta_3\gamma_2$- or $\alpha_1\beta_3$-receptors. The potency of other compounds for inhibition of [^{35}S]TBPS binding to $\beta_3\gamma_2$- or β_3-receptors, however, was different from that for $\alpha_1\beta_3\gamma_2$- or $\alpha_1\beta_3$-receptors. For instance, as with homo-oligomeric β_3-receptors (Slany et al., 1995a), GABA was not able to inhibit [^{35}S]TBPS binding to receptors consisting of $\beta_3\gamma_2$-subunits. In addition, in receptors consisting of $\beta_3\gamma_2$- or β_3-subunits, alphaxalone and propofol exhibited a lower, and pentobarbital, (+)-etomidate, and Ro 5-4864 a higher potency for inhibition of [^{35}S]TBPS binding than in those consisting of $\alpha_1\beta_3$- or $\alpha_1\beta_3\gamma_2$-subunits (Table 3). Whereas the potency of (+)-etomidate, etazolate, propofol, chlormethiazole, and Ro 5-4864 was similar for receptors consisting of $\beta_3\gamma_2$- or β_3-subunits, alphaxalone and pentobarbital exhibited a higher potency for inhibition of [^{35}S]TBPS binding to receptors consisting of β_3- than for those consisting of $\beta_3\gamma_2$-subunits (Table 3).

4. Discussion

In the present study membranes from HEK cells transfected with α_1-, β_3-, or γ_2-subunits of $GABA_A$ receptors, or with various combinations of these subunits, were systematically investigated for the presence of binding sites for various $GABA_A$ receptor ligands. In agreement with previous results (Pritchett et al., 1988; Pregenzer et al., 1993), high affinity binding sites for [^{3}H]muscimol were identified on membranes from cells transfected with $\alpha_1\beta_3$- or $\alpha_1\beta_3\gamma_2$-subunits. No such binding sites, however, could be identified in HEK cells transfected with single subunits, under conditions where the actual expression of these subunits has been demonstrated (Ewert et al., 1990; Slany et al., 1995a), or in cells transfected with $\alpha_1\gamma_2$- or $\beta_3\gamma_2$-subunits, under conditions where these cells exhibited high affinity [^{3}H]flunitrazepam or [^{35}S]TBPS binding, respectively. These results support previous conclusions (Pregenzer et al., 1993; Sieghart, 1995) that the high affinity [^{3}H]muscimol binding site is localized at the interface of the α- and β-subunits of $GABA_A$ receptors. Since GABA has been demonstrated to open chloride ion channels in all homo- or hetero-oligomeric $GABA_A$ receptors investigated (Sieghart, 1995), these results additionally support the conclusion that high affinity [^{3}H]muscimol binding sites seem to have structural requirements different from those of low affinity GABA binding sites involved in opening of $GABA_A$ receptor associated chloride ion channels.

Specific high affinity [^{3}H]flunitrazepam binding sites have been shown previously to be localized on recombinant $\alpha_1\beta_3\gamma_2$- or $\alpha_1\gamma_2$-receptors only (Wong et al., 1992; Slany et al., 1995b), suggesting that these binding sites are localized at the interface of α- and γ-subunits. In contrast, high affinity [^{35}S]TBPS binding sites in the present study have been identified on all $GABA_A$ receptors containing β_3-subunits. These results suggest that a β-subunit is necessary for the formation of the [^{35}S]TBPS binding site.

This conclusion is supported by the observation that a point mutation (alanine to serine) localized at the cytoplasmic inner end of the channel lining domaine of an insect $GABA_A$ receptor (ffrench-Constant et al., 1993), not only caused a resistance of the insect to the picrotoxin-TBPS binding site ligand dieldrin, but also greatly reduced the binding of a structural analogue of TBPS to these receptors (Cole et al., 1995). Although this *Rdl* insect clone exhibits a rather low overall homology to subunits of vertebrate $GABA_A$ receptors, the homology of the channel lining domaine of these proteins is significant (ffrench-Constant et al., 1993). Interestingly, from all subunits of the vertebrate $GABA_A$ receptors, only the β-subunits, similar to the wild type of the insect receptors (ffrench-Constant et al., 1993), contain an alanine at the corresponding amino acid position. From the present observation that only receptors containing β_3-subunits exhibited [^{35}S]TBPS binding, it is tempting to speculate that the [^{35}S]TBPS binding to the rat $GABA_A$ receptors, in analogy to that of the insect receptors, might be dependent on the presence of this alanine within the chloride ion channel.

The slow time course of interaction of [^{35}S]TBPS with $GABA_A$ receptors and its acceleration by GABA and $GABA_A$ receptor agonists under conditions where these compounds opened chloride ion channels (Squires et al., 1983; Maksay and Ticku, 1985; Maksay and Simonyi, 1986) further support the conclusion that [^{35}S]TBPS might block $GABA_A$ receptors by binding to a site within the chloride ion channel of these receptors. The slightly higher affinity of [^{35}S]TBPS for receptors consisting of $\alpha_1\beta_3\gamma_2$- or $\alpha_1\beta_3$- over those consisting of $\beta_3\gamma_2$- or β_3-subunits, might indicate that this binding site is slightly better accomodated in $\alpha_1\beta_3\gamma_2$- or $\alpha_1\beta_3$- than in $\beta_3\gamma_2$- or homo-oligomeric β_3-receptors.

[^{35}S]TBPS binding to membranes from $\alpha_1\beta_3\gamma_2$-transfected HEK cells not only could be inhibited by GABA (Pregenzer et al., 1993; Korpi and Lüddens, 1993), and neuroactive steroids (Korpi and Lüddens, 1993; Hawkinson et al., 1994), but also by pentobarbital, etazolate, (+)-etomidate, propofol, chlormethiazole, and Ro 5-4864 with potencies closely similar to those observed in cerebellar membranes. Several lines of evidence support the conclusion that these compounds allosterically interact with [^{35}S]TBPS binding sites of $GABA_A$ receptors. Thus, in contrast to TBPS or IPTBO, these compounds accelerated the dissociation of [^{35}S]TBPS from its binding site on $\alpha_1\beta_3\gamma_2$-receptors. In addition, their similar potency for inhibition of [^{35}S]TBPS and for stimulation of [^{3}H]flunitrazepam binding support the hypothesis that both effects were caused by the same conformational change induced in $GABA_A$ receptors on interaction of these compounds with their respective allosteric binding site. And finally, most of the compounds which inhibited [^{35}S]TBPS binding in the present study, are able to enhance GABA-

induced chloride ion flux (Sieghart, 1995). Since binding of these compounds within the channel would inhibit rather than enhance chloride ion flux, their binding sites probably are localized on the outside of the channel.

The potency of alphaxalone, pentobarbital, etazolate, (+)-etomidate, propofol, chlormethiazole, and Ro 5-4864 for inhibition of [^{35}S]TBPS binding varied with the composition of receptors induced in HEK cells. Whereas chlormethiazole and etazolate exhibited a similar potency for inhibition of [^{35}S]TBPS binding in all receptors containing β_3-subunits, other compounds, such as pentobarbital, (+)-etomidate, and Ro 5-4864, or alphaxalone and propofol exhibited the highest potency in homo-oligomeric β_3-receptors or in receptors consisting of $\alpha_1\beta_3$- or $\alpha_1\beta_3\gamma_2$-subunits, respectively. From these data, however, no firm conclusion on the subunit localization of the various allosteric binding sites can be made, because the difference in potency observed simply might reflect a differential ability of individual receptors to accomodate the various binding sites. In addition, other experiments have demonstrated that barbiturates are able to potentiate the GABA-induced chloride ion flux not only in homo-oligomeric $GABA_A$ receptors consisting of β_1- (Sanna et al., 1995), but also in those consisting of α_1- or γ_2-subunits (Blair et al., 1988; Shivers et al., 1989). GABA-induced chloride ion flux in receptors consisting of $\alpha_1\gamma_2$-subunits has been demonstrated to be stimulated by barbiturates and inhalation anesthetics and to be inhibited by Ro 5-4864 (Puia et al., 1989; Harrison et al., 1993). And finally, [^{3}H]flunitrazepam binding to receptors consisting of $\alpha_1\gamma_2$-subunits has been shown to be inhibited by pentobarbital (Harris et al., 1995; Slany et al., 1995b), as well as by alphaxalone, (+)-etomidate, etazolate, propofol, and chlormethiazole (Slany et al., 1995b). These results seem to indicate that binding sites for all these allosteric ligands can also be formed on $GABA_A$ receptors in the absence of β-subunits. These sites, thus, might be formed by amino acid sequences highly conserved in different $GABA_A$ receptor subunits.

Further experiments will have to be performed to support this conclusion and to identify the exact amino acid sequence of the GABA-binding site as well as that of binding sites for the other allosteric ligands of $GABA_A$ receptors.

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