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Rat β3 Subunits Expressed in Human Embryonic Kidney 293 Cells Form High Affinity [^{35}S]*t*-Butylbicyclophosphorothionate Binding Sites Modulated by Several Allosteric Ligands of γ-Aminobutyric Acid Type A Receptors

ASTRID SLANY, JÜRGEN ZEZULA, VERENA TRETTER, and WERNER SIEGHART

Department of Biochemical Psychiatry, University Clinic for Psychiatry, Vienna, Austria

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SUMMARY

Human embryonic kidney 293 cells transiently transfected with β3 subunits of γ-aminobutyric acid type A receptors from the rat exhibited a specific high affinity binding for [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) that could be inhibited by pentobarbital, etazolate, (+)-etomidate, alphaxalone, propofol, chlormethiazole, and Ro 5–4864. The potency of these compounds for inhibition of [^{35}S]TBPS binding was similar in membranes from β3 subunit-transfected human embryonic kidney 293 cells and in cerebellar membranes. In contrast to maximally inhibiting concentrations of unlabeled TBPS or picrotoxin, which caused a monophasic and rather slow dissociation of [^{35}S]TBPS, maximally inhibiting concentrations of pentobarbital, etazolate, alphaxalone, propofol, chlormethiazole, and Ro 5–4864 accelerated the dissociation of [^{35}S]TBPS from β3 subunit-containing membranes. Immunoaffinity chromatography and Western blot analysis with subunit-specific antibodies indicated that other endogenous subunits possibly present in these cells were not associated with β3 subunits. These results appear to indicate that most of the allosteric binding sites present on γ-aminobutyric acid type A receptors can be formed by the β subunit of these receptors. Homo-oligomeric β3 receptors therefore are an excellent model system for the structural investigation of γ-aminobutyric acid type A receptors.

GABA$_A$ receptors are ligand-gated chloride ion channels and the site of action of a variety of pharmacologically and clinically important drugs. 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 (1). So far, six α, four β, four γ, one δ, and two ρ 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 (1, 2). Transfection studies have demonstrated that an α, a β, and a γ subunit have to be present simultaneously in a cell to produce receptors exhibiting pharmacology resembling that of GABA$_A$ receptors found in the brain (1, 3).

Other studies, however, have indicated that on transfection of *Xenopus* oocytes (4, 5), human embryonic kidney cells (6, 7), or insect cell lines (8) with a single type of GABA$_A$ receptor subunit, GABA-gated chloride ion channels are formed that could be blocked by bicuculline and picrotoxin and potentiated by pentobarbital. Currents activated by 10 μM GABA, however, were 1 order of magnitude smaller than those activated in cells transfected with most dual- or triple-subunit combinations (7), indicating either an infrequent channel opening or a low efficiency of expression and assembly of single subunits. Thus, it appears that single subunits of the GABA$_A$ receptor are capable of forming gated receptors that exhibit at least some pharmacological properties of GABA$_A$ receptors found in the brain.

In the present study, we demonstrated that HEK transfected with β3 subunits of the rat GABA$_A$ receptors exhibit high affinity [^{35}S]TBPS binding and that this binding could be inhibited by various allosteric modulators of GABA$_A$ receptors. Results indicate that most of the allosteric binding sites present on GABA$_A$ receptors can be formed by the β subunit of these receptors.

Experimental Procedures

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]fluni-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; HEK, human embryonic kidney 293 cells; IPTBO, (4-(isopropyl)-1-phospho-2,6,7-trioxabicyclo(2,2,2)octane-1-oxide; Ro 5–4864, 4′-chlorodiazepam.

trazepam (specific activity, 85 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Other compounds were obtained from the following sources: diazepam and Ro 5–4864, Hoffmann La Roche (Basel, Switzerland); IPTBO, J. S. Collins (City of London Polytechnic, London, UK); TBPS, R. F. Squires (Orangeburg, NY); alphaxalone, Glaxo Group Research (Middlesex, UK); propofol, ICI-Pharma (Milan, Italy); chlormethiazole, Astra Arcus (Södertälje, Sweden); etazolate, E. R. Squibb (Princeton, NJ); and (+)-etomidate, Janssen Pharmaceuticals (Beerse, Belgium).

Cloning of α1, β3, and γ2 subunits of $GABA_A$ receptors. A rat brain cDNA library was constructed in λZAP (Stratagene, La Jolla, CA) from (poly)A^+ mRNA isolated from the brains of 8–10-day-old rats as detailed in the protocol from Stratagene. The α1, β3, and γ2 subunits of $GABA_A$ receptors were cloned from this cDNA library, and their sequence proved to be identical with the respective sequence published previously (9–11). Cloned cDNAs encoding for the α1, β3, or γ2 subunits of $GABA_A$ receptors plus varying amounts of 5′ (4–400 bp) and 3′ (0–150 bp) untranslated regions were subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA) by using standard rDNA procedures. Each plasmid was purified after growth from a single bacterial colony.

Culturing and transfecting of HEK. HEK (CRL 1573, American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 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.

With the calcium phosphate precipitation method (12), 3×10^6 HEK were transfected either with 20 μg cDNA encoding for the rat α1, β3, or γ2 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. The medium was changed 20 hr after transfection.

Isolation of cerebellar membranes or of membranes from HEK. Adult rats were killed by decapitation, and the cerebellum was rapidly dissected and homogenized in 0.32 M sucrose and 10 mM phosphate buffer, pH 7.4. The suspension was centrifuged at 50,000 × *g* for 20 min. The pellet was resuspended in 50 mM Tris/citrate buffer, pH 7.4, and the membranes were recentrifuged at 50,000 × *g* for 20 min. Membranes were resuspended in 50 mM Tris/citrate buffer, pH 7.4, and stored frozen overnight at −20°. Samples then were thawed, and membranes were centrifuged and washed three times as described. Membrane suspensions were stored frozen until use. Before use, the frozen suspension was thawed, and membranes were centrifuged and resuspended in the same volume of 50 mM Tris/citrate buffer, pH 7.4.

Nontransfected HEK or cells 96 hr after transfection with plasmids encoding for $GABA_A$ receptor subunits were washed twice and then harvested by scraping into phosphate-buffered saline. After centrifugation at 12,000 × *g* for 10 min, the cell pellets were homogenized in 50 mM Tris/citrate buffer, pH 7.4, with the use of an Ultraturax, followed by three centrifugation-resuspension cycles (200,000 × *g* for 20 min), and then used for ligand binding or stored at −20°.

Immunoaffinity chromatography and Western blot analysis. Membranes from HEK transfected with α1, β3, or γ2 subunits of $GABA_A$ receptors, or with a mixture of these subunits, were extracted with a buffer containing 0.5% desoxycholate, 150 mM NaCl, 10 mM Tris chloride, pH 7.8, 0.5 mg/ml phosphatidylcholine, and several protease inhibitors (0.1 mg/ml bacitracine, 0.5 mM benzamidine, and 0.3 mM phenylmethylsulfonylfluoride). The clear extracts were then chromatographed on an immunoaffinity column containing either anti-peptide α1(1–9), anti-peptide β3(345–408), or anti-peptide γ2(316–352) antibodies (13). The anti-peptide α1(1–9) antibodies selectively recognize α1 subunits of the rat $GABA_A$ receptors (13). To identify human α1 subunits possibly expressed in HEK, the monoclonal antibody bd 24 (14) was used in some Western blot experiments. The anti-peptide β3(345–408) antibody has been raised by immunizing rabbits with a maltose-binding protein-β3(345–408)-fusion protein and was purified by affinity chromatography on a glutathion-*S*-transferase-β3(345–408)-fusion protein. This antibody is able to selectively recognize the β3 subunit of rat and human $GABA_A$ receptors, as is described.[1] Similarly, the anti-peptide γ2(316–352) antibodies are able to selectively recognize the rat and human γ2 subunits (13). The immunoaffinity columns were washed with 10 volumes of a buffer consisting of 0.5% Triton X-100, 600 mM NaCl, 50 mM Tris chloride, pH 8.0, 1 mM EDTA, and protease inhibitors as mentioned and then with 10 volumes of the same buffer containing 0.2% Triton X-100 and 150 mM NaCl.

Proteins bound to the immunoaffinity columns were then eluted from the columns with a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 100 mM glycine/HCl, pH 2.45. The column eluate was neutralized, and proteins were precipitated (15) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis as described previously (13). $GABA_A$ receptor subunits were detected by using digoxigenin-labeled anti-peptide α1(1–9), anti-peptide β3(345–408), or anti-peptide γ2(316–352) antibodies (13). Antibodies bound to membranes were detected using the anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim, Mannheim, Germany) and the Western exposure chemiluminescence detection system (Clontech, Palo Alto, CA). For identification of monoclonal antibody bd 24, alkaline phosphatase-conjugated goat anti-mouse IgG (Accurate Chemical and Scientific Corp., Westbury, NY) and the same chemiluminescence detection system were used.

Radioligand binding studies. For binding studies, both freshly prepared and frozen membranes from untransfected cells or from cells transfected with plasmids encoding for $GABA_A$ receptor subunits were used, with similar results. Frozen membranes from cerebellum or HEK were thawed, centrifuged, and resuspended in 50 mM Tris/citrate buffer, pH 7.4, at a protein concentration of ~1 mg/ml as measured with the BCA protein assay kit (Pierce Chemical) with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated for 90 min at 4° in a total of 1 ml of a solution containing 50 mM Tris/citrate buffer, pH 7.4, 150 mM NaCl, and 5 nM [^{3}H]flunitrazepam or 20 nM [^{3}H]muscimol in the absence or presence of 10 μM diazepam or 10 μM GABA, respectively (16, 17). For [^{35}S]-TBPS binding, membranes were incubated for 180 min at room temperature in a total of 1 ml of a solution containing 50 mM Tris/citrate buffer, pH 7.4, 200 mM NaBr, and various concentrations of [^{35}S]TBPS in the absence or presence of 10 μM IPTBO (17).

Membranes were then filtered through Whatman GF/B filters, and the filters were rinsed twice with either 5 ml of ice-cold 50 mM Tris/citrate buffer (for [^{3}H]flunitrazepam or [^{3}H]muscimol binding) or rinsed three times with 3.5 ml of ice-cold 50 mM Tris/citrate buffer (for [^{35}S]TBPS binding). Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml Hydrofluor (National Diagnostics, Somerville, NJ) scintillation fluid. Nonspecific 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.

Results

Membranes from HEK were transfected with cDNAs encoding for the α1, β3, or γ2 subunit of the $GABA_A$ receptor to investigate whether $GABA_A$ receptors assembled from a single type of subunit exhibit high affinity [^{3}H]muscimol, [^{3}H]flunitrazepam, or [^{35}S]TBPS binding sites. As shown in Table 1, no [^{3}H]muscimol or [^{3}H]flunitrazepam binding sites

[1] K. Fuchs, S. Karall, B. Haver, and W. Sieghart. Evidence for a co-existence of β1 and β3 subunits in the same γ-aminobutyric $acid_A$ receptor complex. Manuscript in preparation.

TABLE 1

Specific binding of [^{3}H]muscimol, [^{3}H]flunitrazepam, or [^{35}S]TBPS induced in membranes from HEK transfected with various subunits of $GABA_A$ receptors

Membranes from HEK transfected with cDNAs encoding for α1, β3, or γ2 subunits of $GABA_A$ receptors or with a mixture of these cDNAs were incubated with 20 nM [^{3}H]muscimol, 5 nM [^{3}H]flunitrazepam, or 5 nM [^{35}S]TBPS in the absence or presence of 10 μM GABA, 10 μM diazepam, or 10 μM IPTBO, respectively. Membranes were then filtered through Whatman GF/B filters, and specific radioactivity bound to membranes was determined as described in Experimental procedures. Values are given as mean ± SD from three separate experiments performed in duplicate.

Transfected subunits	[^{3}H]Muscimol binding	[^{3}H]Flunitrazepam binding	[^{35}S]TBPS binding
		fmol/mg protein	
α1			
β3			68± 13
γ2			
α1β3γ2	480±169	578±80	850±167

were formed in membranes from cells transfected with either of these subunits. Interestingly, however, the formation of high affinity [^{35}S]TBPS binding sites could be observed in membranes from cells transfected with β3 subunits but not in those from cells transfected with α1 or γ2 subunits (Table 1). In control experiments, it was demonstrated that membranes from HEK cotransfected with α1, β3, and γ2 subunits of the $GABA_A$ receptor exhibited specific high affinity [^{3}H]muscimol, [^{3}H]flunitrazepam, and [^{35}S]TBPS binding sites (Table 1).

[^{35}S]TBPS binding to membranes from β3-transfected HEK, in accord with that to cerebellar membranes (17, 18), in three different experiments was absolutely dependent on the presence of chloride or bromide ions in the incubation solution (experiments not shown). Scatchard analysis of equilibrium binding data obtained from membranes of β3 subunit-transfected cells revealed the existence of a single type of binding site for [^{35}S]TBPS (Fig. 1), which exhibited an apparent K_D of 39.6 ± 12.1 nM (mean ± SD, n = 3). The affinity of [^{35}S]TBPS for these binding sites was thus similar to that for membranes of HEK cotransfected with α1β3γ2 subunits (K_D, 15.1 ± 2.6 nM [mean ± SD]; n = 3) or for cerebellar membranes (K_D, 20.7 ± 8.7 nM; n = 3). However, the total number of [^{35}S]TBPS binding sites induced in HEK through transfection with β3 subunits (B_{max}, 563 ± 170 fmol/mg protein [mean ± SD]; n = 3) was much smaller than that induced in HEK transfected with α1β3γ2 subunits (B_{max}, 3488 ± 491 fmol/mg protein; n = 3), supporting previous

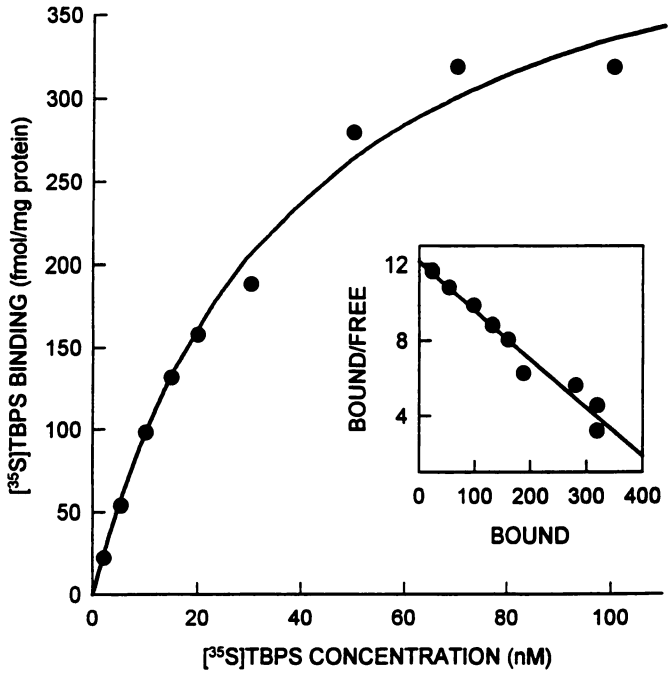

Fig. 1. [^{35}S]TBPS binding to membranes from HEK transfected with β_3 subunits. Membranes from HEK transfected with β3 subunits of $GABA_A$ receptors were incubated with increasing concentrations of [^{35}S]TBPS in the absence or presence of 10 μM IPTBO. Radioactivity bound to membranes was determined after rapid filtration on Whatman GF/B filters. Binding in the presence of IPTBO was subtracted from total [^{35}S]TBPS binding to result in specific [^{35}S]TBPS binding. Data were then subjected to Scatchard analysis (*inset*). Data shown represent a single experiment performed in duplicate. The experiment was performed three times with comparable results.

results indicating that the assembly of homo-oligomeric β3 receptors was less efficient than that of α1β3γ2 receptors (7).

Previous investigations have indicated that GABA and a variety of allosteric modulators of $GABA_A$ receptors are able to inhibit [^{35}S]TBPS binding to $GABA_A$ receptors in brain membranes (18–22). Therefore, in the present study, the actions were investigated of some of these modulators on [^{35}S]TBPS binding to membranes from HEK transfected with β3 subunits. As shown in Fig. 2A, in contrast to GABA, which was not able to inhibit the binding of [^{35}S]TBPS to these membranes, (+)-etomidate, alphaxalone, and pentobarbital dose-dependently inhibited specific [^{35}S]TBPS binding. Similar inhibition curves were obtained for Ro 5–4864, etazolate, propofol, and chlormethiazole (Fig. 2B).

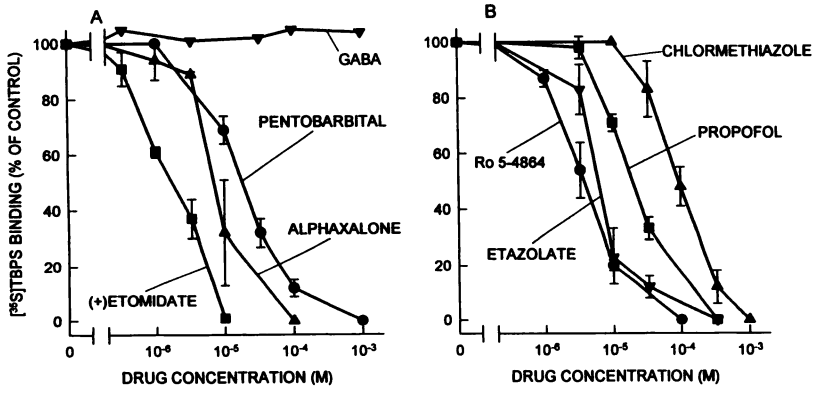

Fig. 2. Inhibition of specific [^{35}S]TBPS binding to membranes from HEK transfected with β3 subunits by various compounds. Membranes from HEK transfected with β3 subunits were incubated with 2 nM [^{35}S]TBPS in the absence or presence of 10 μM IPTBO or various concentrations of compounds as indicated. Membranes were then filtered through Whatman GF/B filters and radioactivity bound to membranes was determined as described in Experimental Procedures. Data shown represent mean ± SD values from three or four separate experiments performed in duplicate.

In other experiments, the potencies of these compounds in inhibiting [^{35}S]TBPS binding were compared in membranes from HEK transfected with β3 subunits (shown in Fig. 2) and in cerebellar membranes. As shown in Table 2, the potency of pentobarbital, (+)-etomidate, or Ro 5–4864 in inhibiting [^{35}S]TBPS binding was higher and that of alphaxalone and etazolate was lower in membranes from HEK transfected with β3 subunits than in cerebellar membranes. The potencies of propofol and chlormethiazole, however, were similar in both membranes (Table 2).

Previous studies have indicated that some of the allosteric modulators of $GABA_A$ receptors were able to accelerate the dissociation of [^{35}S]TBPS from its binding sites on brain membranes (23, 24). To further investigate the similarity between [^{35}S]TBPS binding sites in brain membranes and in membranes from HEK transfected with β3 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 (23, 24). As shown in Fig. 3, dissociation initiated by a completely displacing concentration of TBPS or picrotoxin was slow and monophasic. This was in contrast to the rapid biphasic dissociation observed when completely displacing concentrations of etazolate, propofol, and pentobarbital were added. Although alphaxalone, chlormethiazole, and Ro 5–4864 also were able to significantly accelerate the dissociation of [^{35}S]TBPS from its binding site, the change in dissociation velocity induced by these compounds was less than that caused by etazolate, propofol, or pentobarbital (Fig. 3).

Recently, small amounts of mRNA for α1, β3, and γ2 subunits of $GABA_A$ receptors have been identified in nontransfected HEK (25, 26). It was therefore of interest to investigate whether these endogenous subunits could be translated into proteins and could coassemble with exogenous β3 subunits transfected into HEK. For this, membranes from transfected cells were extracted with desoxycholate, and $GABA_A$ receptor subunits were isolated by chromatography on immunoaffinity columns containing antibodies directed against the transfected subunit.

In control experiments, it was demonstrated that in eluates from the anti-peptide γ2(316–352) immunoaffinity column used to purify $GABA_A$ receptors from HEK cotransfected with α1, β3, and γ2 subunits, the α1 subunit could be identified by the staining of a protein with an apparent molecular mass of 51 kDa (13) with the anti-peptide α1(1–9) antibody (Fig. 4, *lane 10*). The second protein band, with an apparent molecular mass of 47 kDa, stained by this antibody could also be detected in extracts from HEK transfected with α1 subunits only (Fig. 4, *lane 1*). This protein band thus might represent either a nonglycosylated isoform or a degradation product of the α1 subunit. The conclusion that both the 51-kDa and the 47-kDa protein represent α1 subunits expressed in these cells after transfection with the respective cDNA is supported by the finding that these proteins could not be isolated by the anti-peptide γ2(316–352) antibody from HEK transfected with γ2 subunits only (Fig. 4, *lane 7*).

In the same eluates, β3 subunits, previously demonstrated to exhibit an apparent molecular mass of 53–55 kDa (27), could be identified by staining with the anti-peptide β3(345–408) antibodies (Fig. 4, *lane 11*). Again, the protein with a molecular mass of less than 50 kDa that also was stained by these antibodies might represent a deglycosylated isoform or a degradation product of this subunit. The specificity of the antibody reaction is demonstrated by the fact that these proteins could not be detected in eluates from anti-peptide γ2(316–352) immunoaffinity columns used to isolate $GABA_A$ receptors from HEK transfected with γ2 subunits only (Fig. 4, *lane 8*).

Last, γ2 subunits, previously shown to exhibit an apparent molecular mass of 45–49 kDa (13), were identified in these eluates by the anti-peptide γ2(316–352) antibodies (Fig. 4, *lane 12*). The protein smear with an apparent molecular mass of slightly less than 80 kDa identified by all three antibodies might represent aggregation products of these subunits de-

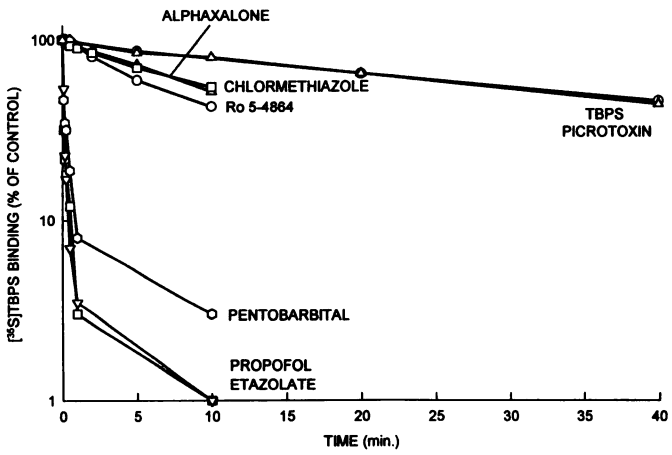

Fig. 3. Displacement of [^{35}S]TBPS from its binding sites on membranes from HEK transfected with β3 subunits by various $GABA_A$ receptor ligands. Membranes from HEK transfected with cDNA encoding for the β3 subunit were incubated with 2 nM [^{35}S]TBPS until equilibrium. Then 2 μM TBPS, 100 μM picrotoxin, 1 mM chlormethiazole, 100 μM Ro 5–4864, 100 μM alphaxalone, 1 mM pentobarbital, 333 μM propofol or 100 μM etazolate 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 three times with similar results.

TABLE 2

Potency of various allosteric ligands of $GABA_A$ receptors for inhibition of [^{35}S]TBPS binding to membranes from HEK transfected with β3 subunits and to membranes from cerebellum

Membranes from HEK transfected with β3 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. Membranes were then filtered through Whatman GF/B filters, and radioactivity specifically bound to membranes was determined as described in Experimental procedures. Values are given as mean ± SD with the number of experiments performed in duplicate in brackets.

	IC_{50}	
	β3	Cerebellum
	μM	
GABA		0.24 ± 0.08 (4)
Pentobarbital	19.5 ± 4.0 (3)	100 ± 22 (4)
(+)-Etomidate	1.6 ± 0.5 (3)	4.6 ± 0.4 (4)
Etazolate	3.8 ± 0.3 (3)	1.6 ± 0.5 (3)
Alphaxalone	11.7 ± 9.0 (3)	1.8 ± 0.4 (4)
Propofol	20.2 ± 0.9 (3)	16 ± 7 (3)
Chlormethiazole	84 ± 20 (4)	53 ± 7 (3)
Ro 5–4864	8.8 ± 3.8 (4)	19 ± 5 (3)

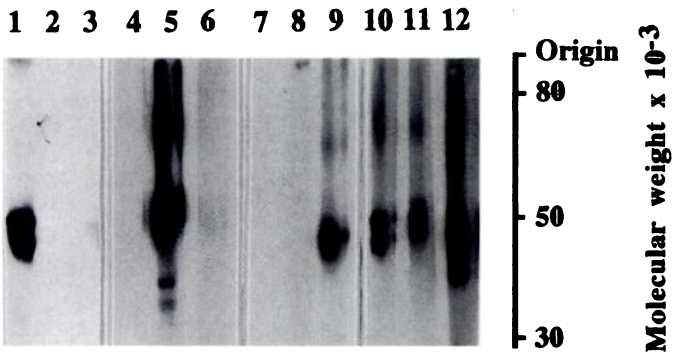

Fig. 4. Identification of GABA$_A$ receptor subunits in membranes from HEK transfected with cDNAs for α1, β3, or γ2 subunits or with a mixture of these cDNAs. *Lanes 1–3*, Immunoaffinity chromatography on anti-peptide α1(1–9) antibodies was used to isolate GABA$_A$ receptors from cells transfected with α1 subunits; *lanes 4–6*, anti-peptide β3(345–408) antibodies were used to isolate receptors from cells transfected with β3 subunits; *lanes 7–9*, anti-peptide γ2(316–352) antibodies were used to isolate GABA$_A$ receptors from cells transfected with γ2 subunits; *lanes 10–12*, anti-peptide γ2(316–352) antibodies were used to isolate GABA$_A$ receptors from cells transfected with with α1β3γ2 subunits. Eluates obtained from the chromatography of the respective cell membrane extracts from 4 or 0.6 culture plates were applied to each of the *lanes 1–9* or *lanes 10–12*, respectively, and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each lane was then subjected to Western blot analysis, using the anti-peptide α1-antibodies (*lanes 1, 4, 7,* and *10*), anti-peptide β3-antibodies (*lanes 2, 5, 8,* and *11*), or anti-peptide γ2-antibodies (*lanes 3, 6, 9,* and *12*) for the identification of the respective GABA$_A$ receptor subunits. Antibodies bound to proteins were detected using anti-digoxigenin-alkaline phosphatase Fab fragments and a sensitive chemiluminescence detection system, as described in Experimental procedures. Anti-peptide β3(345–408) and anti-peptide γ2(316–352) antibodies are able to recognize the corresponding rat and human subunits. Anti-peptide α1(1–9) antibodies predominantly recognize rat α1 subunits. Therefore, in control experiments, blots parallel to those shown in *lanes 1, 4,* and *7* were incubated with the monoclonal antibody bd 24, which selectively recognizes the human α1 subunit of GABA$_A$ receptors (14). This antibody slightly cross-reacted with the large amounts of rat α1 subunit present in the blot performed in parallel to *lane 1* but did not react with the blots produced in parallel to *lanes 4* and *7* (experiments not shown). This indicates that human α1 subunits did not significantly coassemble with the rat β3 or γ2 subunits exogenously transfected into HEK. The gel was calibrated with proteins of known molecular mass. The experiment was performed twice with similar results.

tected by the very sensitive chemiluminescence detection system that was used. These results thus demonstrate that in HEK transfected with α1, β3, and γ2 subunits, all three subunits were expressed and could be coisolated by an antibody directed against one of these subunits, indicating a coassembly of these subunits into heteromeric GABA$_A$ receptors.

As shown in Fig. 4 (*lanes 1–3*), large amounts of α1 subunits but no significant amounts of β3 or γ2 subunits could be identified in eluates from the anti-peptide α1(1–9) immunoaffinity column used to purify GABA$_A$ receptors from HEK transfected with α1 subunits. Similarly, only the β3 subunits (Fig. 4, *lane 5*) or the γ2 subunits (Fig. 4, *lane 9*) could be isolated by anti-peptide β3(345–408) or anti-peptide γ2(316–352) antibodies from cells transfected with β3 or γ2 subunits, respectively, although the gel was heavily overloaded to detect even small amounts of endogenous subunits. These results appear to indicate that HEK transfected with a single type of subunit actually do express this subunit and that endogenous subunits present in these cells either are not significantly expressed or do not coassemble with exogenous subunits under these conditions.

Discussion

In the present study, we demonstrated that membranes isolated from HEK transfected with β3 subunits of GABA$_A$ receptors exhibited significant and specific high affinity [^{35}S]TBPS binding. Because the presence of mRNAs for the α1, β3, and γ2 subunit of GABA$_A$ receptors has recently been demonstrated in nontransfected HEK (25, 26), a possible coexpression of these endogenous subunits with exogenously transfected β3 subunits was investigated. By using subunit-specific antibodies, the presence of β3 subunits but not of α1 or γ2 subunits could be demonstrated in β3 subunit-transfected HEK. This appears to indicate that endogenous α1 or γ2 subunits were not expressed or were only weakly expressed in these cells under the conditions used. This conclusion is consistent with previous results indicating that the absolute amount of the endogenous mRNAs for these subunits was less than 2% of that produced in these cells after transfection with plasmids encoding for the respective exogenous subunits (26). A possible coassembly of unidentified endogenous subunits with β3 subunits appears to be excluded by the observation that [^{3}H]muscimol or [^{3}H]flunitrazepam was not able to specifically bind to membranes from β3 subunit-transfected HEK, although these compounds are able to bind to recombinant receptors consisting of αβ or $\alpha\beta\gamma_2$ subunits, respectively (28).[2] All of these results indicate that endogenous subunit proteins, if they are expressed at all in HEK, do not combine to a significant extent with exogenously transfected subunits to form heterologous GABA$_A$ receptors (26).

It is thus reasonable to conclude that the high affinity [^{35}S]TBPS binding sites observed in the present study were formed by homo-oligomeric β3-channels assembled in these cells. This conclusion is supported by immunocytochemical studies that demonstrated that β3 subunit proteins were present in the outer membrane of HEK transfected with these subunits (29), presumably reflecting an incorporation of homo-oligomeric β3 channels into this membrane (30). Electrophysiological studies indicated that HEK but also *Xenopus* oocytes or insect cell lines transfected with β subunits of GABA$_A$ receptors were able to form GABA-gated chloride ion channels that could be modulated by pentobarbital and picrotoxin (4–8). Also, the present biochemical study indicates that binding of [^{35}S]TBPS, which is assumed to occur to the picrotoxin binding site located either within or close to the chloride ion channel of the GABA$_A$ receptor (18, 23, 24, 31, 32), could be modulated by pentobarbital and picrotoxin. In addition, [^{35}S]TBPS binding to membranes from β3 subunit-transfected HEK, similar to that observed in cerebellar membranes (17, 18), was absolutely dependent on the presence of chloride or bromide ions in the incubation solution. The halide ion dependence of [^{35}S]TBPS binding previously has been attributed to a close association of this binding site with the GABA$_A$ receptor-associated chloride ion channel (18).

In contrast to membranes from HEK transfected with β3 subunits, no specific high affinity [^{35}S]TBPS binding could be

[2] J. Zezula, A. Slany, and W. Sieghart. Identification of allosleric binding sites on recombinant γ-aminobutyric acid$_A$ receptors containing one, two or three different subunits. Submitted for publication.

observed in membranes from cells transfected with $\alpha 1$ or $\gamma 2$ subunits, although the extent of expression of these subunits was similar to that of $\beta 3$ subunits (Fig. 4). Because all homo-oligomeric $GABA_A$ receptors that have been investigated could be blocked by picrotoxin (1, 4–6, 8), these results might indicate that picrotoxin binding sites have structural requirements that are slightly different from those of TBPS binding sites. Because picrotoxin in electrophysiological experiments exhibits a much faster (in milliseconds) onset of activity than TBPS (30 min to peak effect; see Ref. 31), it is tempting to speculate that picrotoxin might block GABA-activated chloride ion flux by binding close to the mouth of the chloride ion channel, whereas TBPS might bind to a slightly overlapping site within the channel.

The absence of [^{3}H]flunitrazepam binding sites in membranes from HEK transfected with $\alpha 1$, $\beta 3$, or $\gamma 2$ subunits of $GABA_A$ receptors is in agreement with previous reports indicating that only receptors consisting of $\alpha\gamma$ (33, 34), $\beta\gamma$ (34), or $\alpha\beta\gamma$ (3, 28, 34) subunits can be modulated by benzodiazepines. Interestingly, however, no significant induction of high affinity binding sites for the GABA agonist [^{3}H]muscimol could be observed in membranes from HEK transfected with $\alpha 1$, $\beta 3$, or $\gamma 2$ subunits. Because at high concentrations GABA was able to activate chloride ion flux in homo-oligomeric receptors consisting of one of these subunits (4, 6, 8), high affinity [^{3}H]muscimol binding sites appear to have structural requirements different from those of low affinity GABA binding sites involved in opening of $GABA_A$ receptor-associated chloride ion channels. Nevertheless, the observation that β subunits can be photolabeled by [^{3}H]muscimol in native $GABA_A$ receptors (35) and the presence of [^{3}H]muscimol binding sites on $GABA_A$ receptors consisting of $\alpha 1$ and $\beta 2$ subunits but not on those consisting of $\alpha 1$ and $\gamma 2$ subunits (28)[2] suggest a close association of [^{3}H]muscimol binding sites with β subunits of $GABA_A$ receptors. These binding sites therefore might be localized between the α and β subunits of native $GABA_A$ receptors.

In the present study, however, even high concentrations of GABA were unable to modulate [^{35}S]TBPS binding to membranes from HEK transfected with $\beta 3$ subunits. This appears to indicate either that homo-oligomeric rat $\beta 3$ receptors do not bind GABA or that the binding of GABA to these receptors, in contrast with native $GABA_A$ receptors (19) or with recombinant receptors containing $\alpha 1\beta 3\gamma 2$ subunits,[2] does not allosterically inhibit [^{35}S]TBPS binding. These results are consistent with a study indicating that mRNA encoding for rat $\beta 1$ subunits injected into *Xenopus* oocytes gave rise to chloride ion channels that were open in the absence of GABA but could be inhibited by picrotoxin (5). Because $\beta 1$ subunits from bovine brain appear to be able to form GABA-activated chloride ion channels (4, 6, 8), the formation of channels that cannot be modulated by GABA might be due to specific structural features present in rat but not in bovine β subunits.

In contrast to GABA, a variety of compounds that have been demonstrated previously to allosterically modulate $GABA_A$ receptors (19–24) were able to inhibit [^{35}S]TBPS binding to membranes from HEK transfected with $\beta 3$ subunits, with a potency similar to that observed in cerebellar membranes. This observation is in agreement with results from electrophysiological studies that indicated the presence of picrotoxinin, pentobarbital, steroid, and propofol binding sites on homo-oligomeric $\beta 1$ receptors (4, 8, 36, 37). So far, no systematic electrophysiological study investigating the presence of allosteric binding sites for etazolate, (+)-etomidate, Ro 5–4864, and chlormethiazole on these receptors has been published.

In agreement with previous investigations on brain membranes (23, 24), maximally inhibiting concentrations of pentobarbital, etazolate, propofol, chlormethiazole, Ro 5–4864, and alphaxalone, but not those of picrotoxin or unlabeled TBPS, accelerated the dissociation of [^{35}S]TBPS from HEK membranes. This accelerated dissociation of [^{35}S]TBPS has been interpreted as an allosteric interaction of these compounds with the TBPS binding site of $GABA_A$ receptors (23, 24). The difference in the extent of acceleration of [^{35}S]TBPS dissociation induced by these compounds then might have been due to their differential ability to induce an appropriate conformational change in the homo-oligomeric $\beta 3$ receptors.

An allosteric interaction with the TBPS binding site of at least some of the ligands investigated is supported by indirect evidence. Thus, as mentioned, the extremely slow association of TBPS with its binding site on brain membranes, which could be enhanced under conditions that at least partially opened the chloride ion channels associated with $GABA_A$ receptors (18, 23, 24), indicated that TBPS might block these receptors by binding within the channel. Most of the compounds that inhibited [^{35}S]TBPS binding in the present study, however, are able to enhance GABA-induced chloride ion flux. Because binding of these compounds within the channel would inhibit rather than enhance chloride ion flux, their binding sites probably are located on the outside of the channel.

In addition, the ability of barbiturates to potentiate the GABA-induced chloride ion flux in homo-oligomeric $GABA_A$ receptors consisting of $\alpha 1$ or $\gamma 2$ subunits (4, 11), which did not exhibit high affinity [^{35}S]TBPS binding sites as demonstrated in the present study, indicates that at least the barbiturate binding sites differ from [^{35}S]TBPS binding sites. Similarly, the stimulation of the GABA-induced chloride ion flux by barbiturates and inhalation anesthetics and its inhibition by Ro 5–4864 in $GABA_A$ receptors consisting of α and γ subunits (38, 39) appears to indicate that binding sites for barbiturates, anesthetics, and Ro 5–4864 are also present on $GABA_A$ receptors in the absence of β subunits. Therefore, these sites might be formed by amino acid sequences highly conserved in different $GABA_A$ receptor subunits.

In summary, the present study indicates that most of the allosteric binding sites present on $GABA_A$ receptors can be formed by the β subunit of these receptors. Homo-oligomeric $\beta 3$ subunit-containing receptors are therefore an excellent model system for $GABA_A$ receptors in the brain and can be used for the investigation of the molecular structure and localization of these allosteric binding sites.

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Send reprint requests to: Dr. Werner Sieghart, Department of Biochemical Psychiatry, University Clinic for Psychiatry, Währinger Gürtel 18–20, 1090 Vienna, Austria.