Detection and Binding Properties of GABA<sub>A</sub> Receptor Assembly Intermediates

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Density gradient centrifugation of native and recombinant γ-aminobutyric acid, type A (GABA<sub>A</sub>) receptors was used to detect assembly intermediates. No such intermediates could be identified in extracts from adult rat brain or from human embryonic kidney (HEK) 293 cells transfected with α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits and cultured at 37 °C. However, subunit dimers, trimers, tetramers, and pentamers were found in extracts from the brain of 8–10-day-old rats and from α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> transfected HEK cells cultured at 25 °C. In both systems, α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits could be identified in subunit dimers, indicating that different subunit dimers are formed during GABA<sub>A</sub> receptor assembly. Co-transfection of HEK cells with various combinations of full-length and C-terminally truncated α<sub>1</sub> and β<sub>3</sub> or α<sub>1</sub> and γ<sub>2</sub> subunits and co-immunoprecipitation with subunit-specific antibodies indicated that even subunits containing no transmembrane domain can assemble with each other. Whereas α<sub>1</sub>γ<sub>2</sub>, α<sub>1</sub>N<sub>γ</sub>γ<sub>2</sub>, α<sub>1</sub>γ<sub>2</sub>N, and α<sub>1</sub>Nγ<sub>2</sub>N combinations exhibited specific <sup>3</sup>H]Ro 15-1788 binding, specific <sup>3</sup>H]muscimol binding could only be found in α<sub>1</sub>β<sub>3</sub> and α<sub>1</sub>β<sub>3</sub>N, but not in α<sub>1</sub>Nβ<sub>3</sub> or α<sub>1</sub>Nβ<sub>3</sub>N combinations. This seems to indicate that a full-length α<sub>1</sub> subunit is necessary for the formation of the muscimol-binding site and for the transduction of agonist binding into channel gating.

Members of the ligand-gated ion channel family, such as the nicotinic acetylcholine receptor (nAChR),<sup>1</sup> the GABA<sub>A</sub> receptor, the glycine receptor, or the 5-hydroxytryptamine, type 3 receptor, are heteromeric proteins composed of five subunits (1). The subunits of these proteins are co-translationally inserted into the membrane, lumen, or both, of the endoplasmatic reticulum, after which the subunits fold and oligomerize (2–4). During these folding and oligomerization events, ligand-binding sites of the receptors are formed. Proteins, once properly folded and oligomerized, are transported to their proper destination, whereas misfolded or improperly oligomerized subunits are retained in the endoplasmatic reticulum and degraded (2, 3, 5). Little is known about the molecular events involved in subunit oligomerization and formation of ligand-binding sites. In the present study the first steps of these events are investigated for GABA<sub>A</sub> receptors.

GABA<sub>A</sub> receptors are chloride channels that can be opened by GABA (6) and are the site of action of various pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants. These drugs modulate GABA-induced chloride flux by interacting with separate and distinct allosteric binding sites (7). So far, at least 19 GABA<sub>A</sub> receptor subunits belonging to several subunit classes (six α, three β, three γ, one δ, one ε, one π, one θ, and three ρ) have been identified in the mammalian brain (8, 9). Expression studies indicated that β<sub>1</sub>, γ<sub>1</sub>, and γ<sub>2</sub> subunits have to combine to form GABA<sub>A</sub> receptors with a pharmacology resembling that of the majority of native receptors (7). Most reports agree that these receptors are composed of two α, two β, and one γ subunit (10–13).

Density gradient centrifugation studies indicated that recombinant GABA<sub>A</sub> receptors composed of α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> subunits almost exclusively sediment as subunit pentamers. α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> subunit combinations sediment as tetramers and pentamers, whereas combinations of α<sub>1</sub>γ<sub>2</sub> or β<sub>3</sub>γ<sub>2</sub> subunits predominantly form heterodimers (12). These results suggested a subunit arrangement in GABA<sub>A</sub> receptors in which four alternating α and β subunits are connected by a γ subunit (12).

Presently, however, nothing is known about the processes that lead from single subunits to completely assembled and pharmacologically functional receptors. Because no assembly intermediates could be identified in HEK cells transfected with α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> subunits under the conditions used and because not all of the subunit dimers that can be formed in HEK cells transfected with two different subunits might be formed when all three subunits are co-expressed, it is not clear whether αβ, αγ, or βγ subunit dimers or some or all of these dimers are the starting point for GABA<sub>A</sub> receptor synthesis.

The pentameric receptor possesses binding sites for the endogenous neurotransmitter GABA, presumably located at the interface between α<sub>1</sub> and β<sub>3</sub> subunits (14), for benzodiazepines, located between the α<sub>1</sub> and γ<sub>2</sub> subunit (15), as well as for TPBS, presumably located either within or close to the channel formed by these subunits (16–18). Presently, nothing is known about the events leading to the formation of the various binding sites on GABA<sub>A</sub> receptors.

By lowering the temperature during culture of HEK cells transfected with α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits, in the present study we were able to detect assembly intermediates of GABA<sub>A</sub> receptors using sucrose density gradient centrifugation. Results
indicated that different subunit dimers are formed during GABA<sub>A</sub> receptor assembly. Studies investigating the dimerization of complete and truncated subunits additionally suggested that the binding sites for [3H]muscimol or the benzodiazepine [3H]Ro 15-1788 can already be formed by the proper subunit dimers or trimers.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The antibodies anti-peptide <i>a</i>(1–9) (19), anti-peptide <i>b</i>(1–13) (12), anti-peptide <i>b</i>(345–408) (20), anti-peptide <i>y</i>(319–366) (12), and anti-peptide <i>y</i>(15–33) (21) were generated and affinity purified as described previously. The monoclonal antibodies b17, recognition subunits (22), were purchased from Roche Molecular Biochemicals.

**Generation of cDNA Constructs**—For the generation of recombinant receptors, <i>a</i>, <i>b</i>, and <i>y</i> subunits of GABA<sub>A</sub> receptors from rat brain were cloned and subcloned into pCDM8 expression vectors (Invitrogen, San Diego, CA) as described previously (12, 23).

Truncated subunits were constructed by polymerase chain reaction amplification using the full-length subunit as template. The polymerase chain reaction primers contained EcoRI and HindIII restriction sites, which were used to clone the fragments into pCDM8 vectors (Invitrogen). The truncated subunits were confirmed by sequencing.

**Culture and Transfection of HEK 293 Cells**—Transformed HEK 293 cells were cultivated in 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine, 50 μg/ml β-mercaptoethanol, 100 units/ml penicillin G, and 100 μg/ml streptomycin in 75-cm<sup>2</sup> culture dishes using standard cell culture techniques. HEK 293 cells (3 × 10<sup>7</sup>) were transfected with a total amount of 20 μg of subunit cDNAs via the calcium phosphate precipitation method (24).

**Density Gradient Centrifugation**—Transfected HEK cells were incubated 44 h at 37 °C or 8 h at 37 °C followed by 16 h at 25 °C. Cells from eight culture dishes were harvested and extracted in 1.6 ml of Lubrol extraction buffer (1% Lubrol PX, 0.18% phosphatidylcholine, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing 0.3 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 100 μg/ml bacitracin) for 8 h at 4 °C. This buffer was used rather than a Triton X-100 or a deoxycholate buffer. Because of its low solubilizing ability it did not dissociate assembly intermediates and, thus, allowed their identification (3). Membranes from adult rat brains (preparation described in Ref. 25) were extracted in 3.5 ml of Lubrol extraction buffer. The extract was centrifuged for 40 min at 150,000 <i>x</i> g at 4 °C, and the clear supernatant was incubated overnight at 4 °C under gentle shaking with 15 μg β(345–408) or γ(319–366) antibodies. After addition of Immunoprecipitin (preparation described in Ref. 12 and 0.5% nonfat dry milk powder and shaking for additional 3 h at 4 °C, the precipitate was washed three times with IP buffer consisting of 0.5% nonfat dry milk powder and 0.1% Tween 20 for 10 min and then incubated with 1 ml of 0.24 μg/ml CSPD or CPD-star reagent (Tropix, Bedford, MA) diluted in assay buffer. After 5 min the fluid was removed, and the membranes were washed in a foil and exposed to x-ray films (X-Omat S, Eastman Kodak Co.) for various time periods. Signals were quantified by a gel documentation system (Docu Gel 2000i; software: RFLP-Scan; MWG Biotech, Ebersberg, Germany).

**Immunoprecipitation and Co-immunoprecipitation of GABA<sub>A</sub> Receptor Subunits**—Transfected HEK cells were incubated 44 h at 37 °C. Cells from four culture dishes were extracted with 800 μl of Lubrol extraction buffer for 8 h at 4 °C. The extract was centrifuged for 40 min at 150,000 <i>x</i> g at 4 °C, and the clear supernatant was incubated overnight at 4 °C under gentle shaking with 15 μg β(345–408) or γ(319–366) antibodies. After addition of Immunoprecipitin (preparation described in Ref. 12 and 0.5% nonfat dry milk powder and shaking for additional 3 h at 4 °C, the precipitate was washed three times with IP buffer and then incubated with 1 ml of IP buffer consisting of 0.5–1 mg/ml of protein A-Sepharose (Amersham Pharmacia Biotech) and 0.2% Tween 20 for 30 min at 4 °C. The precipitated proteins were dissolved in sample buffer and subjected to SDS-PAGE and Western blot analysis.

**Radioligand Binding Studies**—For binding studies frozen membranes from untransfected or transfected HEK cells were thawed, and cells were homogenized in 50 mM Tris/citrate buffer, pH 7.4, by using an UltraTurrax, followed by three centrifugation (200,000 <i>x</i> g for 20 min at 4 °C) resuspension cycles. Cell pellets were resuspended in 50 mM Tris/citrate buffer, pH 7.4, at a protein concentration in the range of 0.5–1 mg/ml as measured with BCA protein assay kit (Pierce) with bovine serum albumin as standard. Membranes were then incubated for 90 min at 4 °C in a total of 1 ml of a solution containing 50 mM Tris/citrate buffer, pH 7.4, and various concentrations (range, 0.1–1000 nM) of [3H]Ro 15-1788 (87.0 Ci/mmol; Amersham Pharmacia Biotech) in the absence or presence of 100 μM diazepam (Hoffmann La Roche, Basle, Switzerland). For muscimol binding assays, the membranes were incubated for 60 min at 4 °C in a total of 1 ml of a solution containing 50 mM Tris/citrate buffer, pH 7.4, at a protein concentration in the range of 0.5–1 mg/ml as measured with BCA protein assay kit (Pierce) with bovine serum albumin as standard. Membranes were then filtered through Whatman GF/B filters, and the lipid extraction of filtration papers was performed by 3.5 ml of ice-cold 50 mM Tris/citrate buffer and were then subjected to scintillation counting. Unspecific binding in the presence of 100 μM diazepam, 10 μM GABA, or 40 μM ITPBO was subtracted from total [3H]Ro 15-1788, [3H]muscimol, or [3H]ITPBO binding, respectively, to result in specific binding (23).

**Immunofluorescence—HEK cells were fixed with 2% paraformaldehyde in PBS 30–35 h after transfection, followed by a 10-min wash in 50 mM NH<sub>4</sub>Cl in PBS. Washes between incubation steps were performed in PBS containing 5 mM Na<sub>2</sub>HPO<sub>4</sub>. Immunofluorescence was performed with 0.1% Triton X-100 for 5 min. Blocking was performed in 5% bovine serum albumin in PBS for 10 min, followed by an incubation with primary antibody in 1% bovine serum albumin in PBS. Primary antibodies were detected with goat anti-rabbit IgG<sub>1</sub>, Bodipy FL (Molecular Probes, Eugene, OR) or donkey anti-mouse IgG<sub>1</sub>, Cy3 (Amersham Pharmacia Biotech) in 1% bovine serum albumin in PBS. Immunofluorescence was visualized using a Zeiss Axiosvert 135 M microscope attached to a confocal laser system (Carl Zeiss LSM 5, 80×) equipped with a argon laser and a helium-neon laser and suitable filter sets. To verify that labeling of cells without permeabilization was restricted to the cell surface, parallel samples were stained with antibodies directed against the intracellular loop of GABA<sub>A</sub> receptor subunits (experiments not shown). These antibodies detected GABA<sub>A</sub> receptor subunits only after permeabilization of transfected cells. Results obtained from double labeling experiments were compared with single labeling experiments to demonstrate that the labeling pattern in double labeling experiments was not caused by cross-bleeding artifacts (experiments not shown).
RESULTS

Detection of Assembly Intermediates—In an attempt to identify GABA<sub>A</sub> receptor assembly intermediates, extracts from adult rat brain or from HEK 293 cells transfected with α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits were subjected to sucrose density gradient centrifugation. Under these conditions, depending on their molecular mass, monomeric and multimeric proteins migrate into the gradient with different sedimentation coefficients. Gradients were fractionated, and the proteins in individual fractions were precipitated and subjected to SDS-PAGE and Western blot analysis with subunit specific antibodies. s<sub>o</sub> values of receptors and receptor intermediates were determined by analyzing the sedimentation of standard proteins with known s<sub>o</sub> values added to each gradient. OD, optical density (arbitrary units); s<sub>o</sub>, sedimentation value. The experiments were performed four times with comparable results.

In other experiments, the culture temperature was first kept at 37 °C for the first 8 h after transfection of HEK cells with α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits to normally initiate transcription and translation and was then reduced to 25 °C for the following 16 h to slow down the assembly of recombinant GABA<sub>A</sub> receptors. Receptors formed were extracted from the cells and were subjected to density gradient centrifugation as described in the legend to Fig. 1. Individual fractions of the gradients were analyzed in Western blots using α<sub>1</sub>(1–9) (A), β<sub>3</sub>(345–408) (B), and γ<sub>2</sub>(319–366) (C) antibodies. OD, optical density (arbitrary units); s<sub>o</sub>, sedimentation value. The experiments were performed eight times with comparable results.
of the antibodies, because none of the antibodies used for these experiments exhibited any cross-reactivity with other subunits as demonstrated by Western blot analysis of various recombinant receptors (27, 28).

In other experiments, the sedimentation properties of GABA<sub>A</sub> receptors extracted from the brain of 8–10-day-old rats were investigated. In this developing tissue, GABA<sub>A</sub> receptors are continuously synthesized in different neurons, and it was hoped that amounts of assembly intermediates sufficient to be detected would be present. As shown in Fig. 3, this actually was the case: α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2</sub> subunits extracted from 8–10-day-old rats, in contrast to those from adult rat brain, sedimented in multiple, overlapping peaks. Whereas the sedimentation pattern of α<sub>1</sub> and γ<sub>2</sub> subunits was again similar, showing overlapping peaks and shoulders at 5.5, 6.8, and 8.7 s, the sedimentation pattern of β<sub>3</sub> subunits was slightly different, showing prominent peaks at 3.3 and 5.5 s and overlapping peaks and shoulders between 6.8 and 8.7 s (Fig. 3).

**Formation of the [<sup>3</sup>H]Muscimol-binding Site**—Because the [<sup>3</sup>H]muscimol-binding site on GABA<sub>A</sub> receptors is located at the interface of α and β subunits (14), it was interesting to investigate whether this binding site could already be formed by GABA<sub>A</sub> receptor assembly intermediates containing α<sub>1</sub> and β<sub>3</sub> subunits. Because co-transfection of HEK cells with α<sub>1</sub> and β<sub>3</sub> subunits leads to the formation of α<sub>1</sub>β<sub>3</sub> tetramers and pentamers (12), truncated α<sub>1</sub>(α<sub>1</sub>N) and β<sub>3</sub>(β<sub>3</sub>N) subunits containing the complete extracellular N-terminal domain but no transmembrane domains were cloned to investigate whether they can assemble with full-length β<sub>3</sub> and α<sub>1</sub> subunits, respectively, forming smaller assembly intermediates. α<sub>1</sub> and β<sub>3</sub> subunits and α<sub>1</sub>N and β<sub>3</sub>N fragments were then co-transfected into HEK cells in various combinations, and expressed subunits were extracted from these cells and were immunoprecipitated with β<sub>3</sub>(1–13) antibodies. The precipitate was subjected to SDS-PAGE and Western blot analysis using digoxigenized α<sub>1</sub>(1–9) antibodies. The experiment was performed three times with comparable results.

When extracts from HEK cells co-transfected with α<sub>1</sub>N and full-length β<sub>3</sub> subunits or α<sub>1</sub>N and β<sub>3</sub>N constructs were precipitated with β<sub>3</sub>(1–13) antibodies, three protein bands with apparent molecular masses 30, 33, and 36 kDa could be detected in Western blots (Fig. 4A). The protein band with identical molecular mass could be precipitated by α<sub>1</sub>(1–9) antibodies from these cells as well as from untransfected HEK cells (experiments not shown), indicating that this protein band represents the α<sub>1</sub> subunit of GABA<sub>A</sub> receptors. The weakly labeled lower molecular weight bands varied in labeling intensity in different experiments and could not be detected in untransfected HEK cells. They thus seemed to represent degradation products of the α<sub>1</sub> subunit. The precipitation of α<sub>1</sub> subunits by β<sub>3</sub>(1–13) antibodies was not due to a cross-reactivity of these antibodies because it could not be observed in HEK cells transfected with α<sub>1</sub> subunits only (experiment not shown).

![Figure 3. Sucrose density gradient centrifugation of GABA<sub>A</sub> receptors from the brain of young rats. Extracts from the brain of 8–10-day-old rats were analyzed as described in Fig. 1. OD, optical density (arbitrary units); s, sedimentation value. The experiments were performed five times with comparable results.](image1)

![Figure 4. Co-immunoprecipitation of full-length and truncated α<sub>1</sub> and β<sub>3</sub> or α<sub>1</sub>N and γ<sub>2</sub>N subunits. HEK cells were co-transfected with α<sub>1</sub>N and β<sub>3</sub>N, α<sub>1</sub>N and γ<sub>2</sub>N, α<sub>1</sub>N and β<sub>3</sub>N, or γ<sub>2</sub>N (A) or with α<sub>1</sub>N and γ<sub>2</sub>N (B). Cell extracts were immunoprecipitated with β<sub>3</sub>(1–13) (A) or γ<sub>2</sub>(1–33) (B) antibodies. The precipitate was subjected to SDS-PAGE and Western blot analysis using digoxigenized α<sub>1</sub>(1–9) antibodies. The experiment was performed three times with comparable results.](image2)
demonstrated that these subunits formed receptors expressed on the cell surface. Permeabilization of the cells indicated the additional presence of a large number of intracellular subunits with an identical subcellular distribution (Fig. 5, C and D). In HEK cells transfected with α1N constructs and β3 subunits, only β3 subunits could be detected on the cell surface (Fig. 5, E and F). These results are in agreement with previous reports demonstrating that β3 subunits are able to form homo-oligomeric receptors that are expressed on the cell surface (21, 30). The observation that the truncated and the full-length subunit could be detected in the permeabilized cells in the same subcellular compartments (Fig. 5, G and H) indicates that β3 subunits that assembled with truncated α1 subunits were retained within the cell. No cell surface labeling was observed when HEK cells were co-transfected with α1 and β3N or α1N and β3N constructs (Fig. 5, I, J, M, and N). However, α1 and β3N constructs (Fig. 5, K and L) or α1N and β3N subunits could be localized in the same subcellular compartments (Fig. 5, O and P).

To investigate whether assembly products from full-length and truncated subunits are able to form specific [3H]muscimol-binding sites, membranes from nontransfected HEK cells or from cells transfected with α1 and β3, α1N and β3, α1 and β3N, or α1N and β3N were incubated with 5 nM of [3H]muscimol in the absence or presence of 10 μM GABA. For HEK cells transfected with α1 and β3 subunits, a specific [3H]muscimol binding of 328 ± 53 fmol/mg protein was found (Table I), whereas in cells co-transfected with α1 and β3N constructs, a specific [3H]muscimol binding of 21 ± 4 fmol/mg protein was detected (Table I). In nontransfected HEK cells (not shown), however, and in cells co-transfected with α1N and β3 or with α1N and β3N, no specific [3H]muscimol binding could be identified. Scatchard analysis of equilibrium binding data indicated a high affinity [3H]muscimol binding to HEK cells co-transfected with α1 and β3N constructs (KD of 12.1 ± 4.1 nM, Bmax of 78 ± 29 fmol/mg protein, mean ± S.E., n = 4), and to cells transfected with α1 and β3 subunits (KD of 7.9 ± 3.2 nM, Bmax of 805 ± 53 fmol/mg protein, mean ± S.E., n = 4). Whereas the affinity for [3H]muscimol of cells transfected with α1 and β3N constructs or with α1 and β3 subunits was comparable (p = 0.45, unpaired Student’s t test), the Bmax values were significantly different (p < 0.0001, unpaired Student’s t test). These results indicate that even intracellular and incomplete assembly intermediates can form specific high affinity [3H]muscimol-binding sites. For the formation of this binding site, however, a full-length α1 subunit is necessary.

Density gradient centrifugation of constructs formed after transfection of HEK cells with α1 and β3N combinations indicated broad peaks at 5.0 and 6.1 s. Because dimers composed of full-length subunits sediment at 5.5 s and trimers at 6.7 s, these data are compatible with the formation α1β3N dimers and trimers (Fig. 6). The lower sedimentation coefficients might have been due to the lower molecular mass of the truncated β3N construct. The broad peak at 6.1 s might have been due to the formation of a mixture of intermediates composed of (α1)2β3N and α1β3Nβ3N subunits.

**Formation of the Benzodiazepine-binding Site**—Because the benzodiazepine-binding site on GABA<sub>α</sub> receptors is located at the interface of α1 and γ2 subunits (15), it was interesting to investigate whether this site could already be formed by α1γ2 dimers. Previous studies have indicated that HEK cells transfected with α1 and γ2 subunits form high affinity [3H]flunitrazepam-binding sites (23, 31) although predominantly forming subunit dimers (12). But the formation of minor amounts of higher oligomers and even completely assembled subunit pentamers could not be excluded by these studies.

To eliminate the possibility of formation of completely assembled subunit pentamers, in addition to the truncated α1N construct a truncated γ2 subunit (γ2N), was cloned that again contained the complete extracellular N-terminal domain but no transmembrane domains. HEK cells were then co-transfected either with α1 and γ2N subunits, α1 and γ2, α1N and γ2, or α1N and γ2N subunits. Expressed subunits were extracted from these cells and were immunoprecipitated with γ2 (1–33) antibodies. As shown in Fig. 4B, the full-length α1 or the truncated α1N construct could be co-precipitated by γ2 (1–33) antibodies from extracts of the appropriately co-transfected HEK cells. This was not due to a cross-reactivity of the γ2 (1–33) antibody because this antibody (in contrast to α1 (1–9) antibodies) could not precipitate α1 subunits from HEK cells transfected with α1 subunits only (experiments not shown). These results therefore indicate that not only full-length α1 and γ2 subunits but also α1N and γ2N, α1 and γ2N, and even α1N and γ2N are able to form hetero-oligomers.
FIG. 6. Sucrose density gradient centrifugation of extracts from HEK cells co-transfected with α1 and β2N subunits. OD, optical density (arbitrary units); s, sedimentation value. The experiments were performed two times with comparable results.

To investigate whether the structures formed from α1 and γ2, α1N and γ2, α1 and γ2N, or α1N and γ2N subunits were transported to the cell surface, appropriately transfected HEK cells were again investigated by immunofluorescence and confocal laser microscopy. As shown in Fig. 7 (A and B) for intact cells and in agreement with previous reports (4, 21) no GABA<sub>A</sub> receptor subunits could be detected on the cell surface. In permeabilized cells, however, a similar subcellular distribution of subunits was observed as in cells transfected with full-length α1 and γ2 subunits (experiments not shown).

To investigate whether assembly products composed of full-length and truncated or of two truncated subunits are able to form benzodiazepine-binding sites, membranes from nontransfected HEK cells or from cells co-transfected with α1 and γ2, or α1N and γ2N, or α1N and γ2N, or α1N and γ2N, or α1N and γ2N, or α1N and γ2N, or α1N and γ2N, and the number of [35S]TPBS binding (23) was determined as described under “Experimental Procedures.” Values are given as the means ± S.E. from three separate experiments performed in triplicate.

FIG. 7. Immunofluorescence of HEK cells co-transfected with full-length and truncated α1 and γ2 subunits. HEK cells were transfected with α1 and γ2 subunits. α1 subunits were labeled on the cell surface (A) or in permeabilized cells (C) using α1(1–9) antibodies. γ2 subunits were labeled on the cell surface (B) or in permeabilized cells (D) using γ2(1–33) antibodies. Rabbit antibodies were detected using anti-rabbit IgG Bodipy FL antibodies. Immunofluorescence was investigated by confocal laser microscopy (single sections). The experiment was performed five times with similar results.

| Transfected subunits | Co-precipitation | Cell surface expression | [3H]Ro 15-1788 binding |
|----------------------|------------------|-------------------------|------------------------|
| α1 + γ2             | +                | –                       | 17.6 ± 1.2             |
| α1N + γ2            | +                | –                       | 16.3 ± 2.3             |
| α1 + γ2N            | +                | –                       | 18.1 ± 1.4             |
| α1N + γ2N           | +                | –                       | 16.2 ± 2.1             |

Formation of the TPBS-binding Site—The TPBS-binding site of GABA<sub>A</sub> receptors can be identified in receptors composed of homo-oligomeric β3 subunits, α1β3 and α1β3γ2 subunits (23) and for the formation of this site the presence of the second transmembrane domain of the β3 subunit in a receptor is essential (18). It therefore was no surprise that only HEK cells transfected with α1 and β3 subunits but not those transfected with α1 and β3N, or α1N and β3 subunits exhibited a specific [35S]TPBS binding (experiments not shown). HEK cells transfected with α1N and β3 subunits were not investigated for [35S]TPBS binding because in these cells homo-oligomeric β3 receptors are formed (see above) that in any case exhibit high affinity [35S]TPBS binding (23).

To investigate whether the TPBS-binding site can be formed by assembly intermediates containing α1 and β3 subunits, a β3 fragment was cloned (β3(TM3)) that not only contained the extracellular N-terminal domain but also the first three transmembrane domains of the β3 subunit. The β3(TM3) fragment could be co-precipitated with α1 subunits from HEK cells co-transfected with α1 and β3(TM3) (experiments not shown). Double staining of intact HEK cells transfected with full-length α1 and β3(TM3) constructs (Fig. 8, A and B) demonstrated that none of these subunits were expressed on the cell surface, but both subunits could be detected in the permeabilized cells in the same subcellular compartments (Fig. 8, C and D). However, no specific [35S]TPBS binding could be identified in these cells.
This failure to detect specific \(^{35}\text{S}\)TBPS binding was not due to an improper folding of the \(\beta_3\)TM3 fragment, because in the same cells a specific \(^{3}\text{H}\)muscimol binding of 50 ± 4 fmol/mg protein could be observed. The quantitative difference between \(^{3}\text{H}\)muscimol binding in HEK cells transfected with \(\alpha_1\beta_2\), \(\alpha_1\beta_3\)TM3 was significant (\(p = 0.007\), unpaired Student’s \(t\) test) and reproducible. Because the \(K_D\) for \(^{3}\text{H}\)muscimol binding to cells transfected with \(\alpha_1\) and \(\beta_2\) subunits (7.9 ± 3.2 nM) was not significantly different from that of cells transfected with \(\alpha_1\) and \(\beta_3\) subunits (12.1 ± 4.1 nM), the increase in \(^{3}\text{H}\)muscimol binding, thus, presumably was due to an increase in the number of binding sites. This could have been caused by an increased stabilization of the \(^{3}\text{H}\)muscimol-binding site because of the presence of the three \(\beta_3\) transmembrane domains in the assembly product of \(\alpha_1\) and \(\beta_3\)TM3 or by the formation of a second muscimol-binding site in a possible assembly product composed of two \(\alpha_1\) and two \(\beta_3\)TM3 subunits.

**DISCUSSION**

**Different Subunit Dimers Are Formed during \(\text{GABA}_A\) Receptor Assembly**—The present study aimed to detect subunits or subunit combinations that could form the starting point of the \(\text{GABA}_A\) receptor assembly process. However, neither in the adult rat brain nor in HEK cells transfected with \(\alpha_1\beta_2\gamma_2\) subunits and kept under standard tissue culture conditions could assembly intermediates be identified by density gradient centrifugation. This indicated that receptor synthesis in these tissues is either low and/or assembly of receptors is too fast to allow intermediates to be identified. When protein folding and subunit oligomerization of recombinant \(\text{GABA}_A\) receptors was slowed down by reducing the culture temperature to 25 °C, however, subunit monomers, dimers, trimers, tetramers, and pentamers could be detected by sucrose density gradient centrifugation. Interestingly, \(\alpha_1\), \(\beta_2\), as well as \(\gamma_2\) subunits could be identified in subunit dimers and all other oligomers. A similar result was obtained from brains of young rats, where a high expression of \(\text{GABA}_A\) receptor subunits caused by ongoing development of the tissue leads to a constant high concentration of assembly intermediates. An identification of the subunit composition of the dimers was not possible, because the peaks for dimers and trimers were overlapping and could not be completely separated by density gradient centrifugation. A possible co-immunoprecipitation of two subunits in the dimer peak thus could have been caused by the respective subunit dimer or by a contamination with subunit trimers. In addition, the similarity of the apparent molecular masses of the \(\alpha_1\), \(\beta_2\), and \(\gamma_2\) subunits and the microheterogeneity of the labeled protein bands (12) prevented an identification of the exact dimers formed after radiolabeling of subunits by culturing with \(^{35}\text{S}\)methionin.

Although the formation of \(\alpha_1\beta_2\), \(\alpha_1\gamma_2\), and \(\beta_2\gamma_2\) heterodimers has been demonstrated previously in cells co-transfected with these subunit combinations (12), the presence of all three subunits in the dimer peak of brains from young rats or of cells transfected with \(\alpha_1\), \(\beta_2\), and \(\gamma_2\) subunits does not necessarily mean that all possible heterodimers are formed in these tissues. The data could also be explained by the formation of two different heterodimers or by the formation of heterodimers and/or homodimers. In addition, some of the dimers could be dead end products for the assembly of \(\text{GABA}_A\) receptors and be subsequently degraded (5). The present data therefore cannot clarify the question of whether assembly of \(\text{GABA}_A\) receptors can start from more than one possible dimer.

This question so far has also not been unequivocally answered for the \(\text{nAChR}\). Thus, it has been reported that \(\alpha\) subunits of the \(\text{nAChR}\) first form heterodimers with \(\gamma\) and \(\delta\), but not with \(\beta\) subunits. The \(\alpha\gamma\) and \(\alpha\delta\) heterodimers then were proposed to assemble with the \(\beta\) subunit and with each other to form the complete \(\alpha\beta\gamma\delta\) receptor (32). In another study, \(\alpha\beta\gamma\) trimers were the first stable assembly intermediates identified (3), and it was proposed that the complete receptor is then formed by a stepwise addition of the \(\delta\) and the second \(\alpha\) subunit. In any case, the complete assembly of the \(\text{nAChR}\) is a complex, slow, and inefficient process (33), and its mechanism is still not entirely clarified.

\(^{3}\text{H}\)Muscimol but No \(^{35}\text{S}\)TBPS-binding Sites Are Formed by \(\alpha_1\beta_3\) Dimers and/or Trimers—Although during the early steps of assembly of the \(\text{nAChR}\), subunit oligomerization and folding events lead to the formation of ligand-binding sites. Thus, a monomeric but properly folded \(\alpha\) subunit is sufficient for binding of the competitive antagonist \(\alpha\)-bungarotoxin, whereas the formation of binding sites for agonists and low molecular weight antagonists occurs in \(\alpha\gamma\) and \(\alpha\delta\) dimers (34). In the present study we therefore investigated the formation of ligand-binding sites on \(\text{GABA}_A\) receptor intermediates.

In agreement with previous studies it was demonstrated that full-length \(\alpha_1\) and \(\beta_3\) subunits on co-transfection into HEK cells form \(^{3}\text{H}\)muscimol as well as \(^{35}\text{S}\)TBPS-binding sites (23). These subunits are able to form pentameric receptors (12) and are expressed on the cell surface (4, 21, 30). C-terminally truncated \(\alpha_1\) or \(\beta_3\) subunits, containing only the extracellular N-terminal domain also could assemble with each other or with full-length \(\beta_3\) or \(\alpha_1\) subunits, respectively, but the assembly products remained in intracellular compartments and could not be detected on the cell surface. Specific \(^{3}\text{H}\)muscimol but no \(^{35}\text{S}\)TBPS-binding sites could be observed in HEK cells co-transfected with full-length \(\alpha_1\) subunits and \(\beta_3\)N constructs. The absence of \(^{35}\text{S}\)TBPS-binding sites in these cells as well as in cells co-transfected with \(\alpha_1\)N and \(\beta_3\)N subunits is not surprising, because recently it has been demonstrated that the presence of a TM2 region of the \(\beta_3\) subunit is essential for the formation of these sites (18).

Although similar amounts of subunits were expressed in \(\alpha_1\beta_3\)N- or \(\alpha_1\beta_3\)-transfected cells and although the affinity of \(^{3}\text{H}\)muscimol for the sites formed was comparable, the number of \(^{3}\text{H}\)muscimol-binding sites in \(\alpha_1\beta_3\)-transfected cells was small compared with that in \(\alpha_1\beta_3\)-transfected cells. Immunoprecipitation studies and density gradient centrifugation indicated that most of the \(\alpha_1\) subunits and \(\beta_3\)N fragments formed in the cells were assembled into heterodimers and heterotrimers.
and only small amounts of these subunits remained unassembled. The comparatively small number of high affinity [3H]muscimol-binding sites, thus, indicates that only a small part of the α₁β₂N heterodimers or heterotrimers contained these sites. This could have been due to a partially incorrect assembly of subunits or a low probability of formation of high affinity [3H]muscimol-binding sites caused by the lacking transmembrane regions of the β₂N construct or the incomplete assembly of the receptor. The latter suggestion is supported by the finding that only a small number of unassembled nAChR α subunits exhibited α-bungarotoxin binding but that the number of binding sites increased with additional assembly steps (3).

Because β₂ subunits alone can form subunit pentamers exhibiting high affinity [35S]TBPS-binding sites, a co-transfection with α₁N and β₂ subunits could not be used to investigate whether [35S]TBPS binding can be formed by assembly intermediates. Therefore, a β₂TM3 construct containing the N-terminal domain and three of the four transmembrane domains of the β₂ subunit was transfected into HEK cells together with full-length α₁ subunits. The observed absence of α₁ subunits and β₂TM3 fragments on the cell surface suggests that neither α₁β₂TM3 hetero-nor β₂TM3 homo-pentamers were formed in these cells or that pentamers formed were retained within the cells. The finding that no [35S]TBPS sites could be detected in α₁β₂TM3-transfected cells then either indicates that this site cannot be formed by an incompletely assembled receptor or that a complete β₂ subunit in any case is essential for the correct formation of the [35S]TBPS site.

[3H]Ro 15-1788-binding Sites Are Formed on α₁γ₂ Dimers—In the present work we demonstrated that α₁ and γ₂, α₁N and γ₂N, as well as α₁N and γ₂N subunits are able to form hetero-oligomers that are not expressed on the cell surface but form specific [3H]Ro 15-1788-binding sites. It has been reported previously that assembly of GABA_A receptor α₁ and γ₂ subunits on co-transfection into HEK cells predominantly stops at the stage of dimers (12). Because it is unpredictable that higher oligomers are formed in the presence of truncated subunits, these results indicate that the formation of the benzodiazepine-binding site already occurs at the stage of heterodimers and that even intracellular and truncated α₁ and γ₂ subunits lacking transmembrane domains are capable of binding benzodiazepines. The comparatively low affinity and number of the binding sites formed, however, indicates that [3H]Ro 15-1788-binding sites formed by heterodimers do not significantly contribute to the total number of these binding sites formed in the brain.

Implications for the Function of GABA_A Receptors—Although the [3H]muscimol-binding site is formed by the N-terminal domain of the GABA_A receptor α and β subunits (14), transmembrane domains seem also to support its formation. This is indicated by the observation that in HEK cells co-transfected with α₁N and β₂N constructs, in contrast to those transfected with α₁ and β₂N constructs, no [3H]muscimol-binding sites could be identified. Because binding of GABA to the [3H]muscimol-binding site in intact GABA_A receptors causes a conformational change in the transmembrane domains leading to the opening of the chloride ion channel (7), a close conformational interaction of the two subunit domains is to be expected. In addition, studies have indicated that point mutations within the second transmembrane domain (35) or the first extracellular loop between TM2 and TM3 (36) of subunits strongly influence gating of the channel.

Interestingly, however, α₁ transmembrane domains seem to be more important than the corresponding β₂ domains for the formation of a [3H]muscimol-binding site. This is indicated by the observation that HEK cells transfected with full-length α₁ subunits and β₂N constructs but not those transfected with full-length β₂ subunits and α₁N constructs exhibit specific [3H]muscimol-binding sites. Because α subunits not only contribute to the formation of the [3H]muscimol site (14) but also to the formation of the benzodiazepine-binding site (15), it is tempting to speculate that conformational changes in the chloride channel induced by GABA as well as the modulation of the GABA-induced current by benzodiazepines might predominantly be mediated by the α subunit.

In contrast to the [3H]muscimol-binding site that is expressed in cells transfected with α₁β₂ or α₁β₂N combinations only, comparable amounts of [3H]Ro 15-1788-binding sites are expressed in α₁γ₂, α₁Nγ₂, α₁γ₂N, or α₁Nγ₂N transfected cells. Binding affinity was similar for all these combinations but was more than 100-fold lower than that of α₁β₂γ₂ receptors, suggesting that the affinity of the [3H]Ro 15-1788-binding site is influenced by the presence of additional subunits in a completely assembled receptor. Interestingly, however, the affinity of the benzodiazepine-binding site formed on subunit dimers is not influenced by the absence of α₁ and γ₂ transmembrane domains, possibly reflecting an absence of a direct interaction between the benzodiazepine-binding site and the channel forming transmembrane domains. This conclusion is supported by the observation that binding of benzodiazepines does not cause direct opening of the chloride channel in the absence of GABA but enhances the frequency of GABA-induced channel opening (6). It thus can be speculated that binding of benzodiazepines to its site at the αγ interface strongly influences the conformation of the GABA-binding site located at the other side (αβ interface) of the same α subunit. This could either enhance the affinity of GABA for its binding site (6, 7) or produce a conformational change similar to that produced by binding of GABA and thus reduce the number of GABA molecules necessary for opening the channel, as indicated by a previous study (37). Each of these mechanisms would enhance the frequency of channel opening by GABA. Further studies will have to decide between these possibilities.

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Detection and Binding Properties of GABA<sub>A</sub> Receptor Assembly Intermediates

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