Chloride Channel Expression with the Tandem Construct of α6β2 GABA<sub>A</sub> Receptor Subunit Requires a Monomeric Subunit of α6 or γ2

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Despite the presence of the multiple subunits (α, β, γ, and δ) and their isoforms for γ-aminobutyric acid, type A (GABA<sub>A</sub>) receptors in mammalian brains, the αγβ2γ2 subtypes appear to be the prototype GABA<sub>A</sub> receptors sharing many properties with native neuronal receptors. In order to gain insight into their subunit stoichiometry and orientation, we prepared a tandem construct of the α6 and β2 subunit cDNAs where the carboxyl-terminal of α6 is linked to the amino-terminal of β2 via a linker encoding 10 glutamine residues. Transfection of human embryonic kidney 293 cells with the tandem construct alone failed to induce GABA-dependent Cl<sup>-</sup> currents, but its cotransfection with the cDNA for α6 or γ2, but not β2, led to the appearance of GABA currents which were picROTOX--sensitive and, in the case of γ2 containing receptors, responded to a benzodiazepine agonist, U-92930. The high affinity GABA site, however, was detected with [3H]muscimol binding in all combinations of the receptor subunits, including the tandem construct alone or with the β2. No appreciable differences were found in their Kd (2.5 nm) and B<sub>max</sub> values (1.4 pmol/mg of protein). These data are consistent with the view that the polypeptides arising from the tandem construct were expressed with the high affinity GABA site, but unable to form GABA channels. The requirement of a specific monomeric subunit (α6 or γ2) for the tandem construct to express Cl<sup>-</sup> currents supports a pentameric structure of GABA<sub>A</sub> receptors consisting of two α6, two β2, and one γ2 for the α6β2γ2 and three α6 and two β2 for the α6β2γ2 subtype.

GABA<sub>A</sub> receptors, responsible for inhibitory neurotransmission in mammalian brains, are ligand-gated Cl<sup>-</sup> channels made of various subunits (α, β, γ, and δ) (1-3). Each subunit consists of several isoforms and contains four transmembrane spanning segments (M1 to M4) (1-5). Despite the existence of the multiple subunits and their isoforms, combinations of α<sub>x</sub>, β2, and γ2 subunits produced Cl<sup>-</sup> channels sharing many functional characteristics with native neuronal receptors and displaying the ability to respond to all the GABA<sub>A</sub> receptor ligands known up-to-date (1, 3, 6, 7). Such cloned GABA<sub>A</sub> receptors have been proposed to be of pentameric structure with M2 lining the pore in analogy with another member of the four transmembrane ligand-gated channel family, acetylcholine receptors (1-3). Recent studies, including immunoprecipitation with subunit specific antibodies, have shown the presence of two α subunits per GABA<sub>A</sub> receptor (8-11). Further experimental evidence is needed, however, about the stoichiometry of the recombinant GABA<sub>A</sub> receptors of αγβ2γ2 and their modes of association. One way to gain insight into this structural issue is to predetermine the alignment of subunits via gene fusion and to study such fused gene products. Similar approaches have been successful with potassium channels made of their subunits in concatameric or tandem linkages (12, 13). In this study we prepared a tandem construct of α6 and β2 subunit cDNAs of the GABA<sub>A</sub> receptor where the carboxyl-terminal of the α6 cDNA is linked to the amino-terminal of the β2 cDNA via a synthetic oligonucleotide encoding 10 glutamine residues. In order to study their properties, the tandem construct alone or in combination of the monomeric α6, β2, and γ2 subunit cDNA was expressed in human embryonic kidney cells (HEK293 cells). Also, recombinant baculovirus carrying the tandem construct or monomeric subunits was prepared for high level of expression in SF-9 cells.

MATERIALS AND METHODS

Construction of the α6-β2 Tandem Subunit—The coding regions of the α6 and β2 subunits were isolated upon digestion of the mammalian expression vectors containing the specific GABA<sub>A</sub> receptor subunit cDNA (14). For α6, CIC cut the vector at the molecular cloning site near the 5' end of the insert, and Scal cut the vector at a site near the 3' end of the insert, which is 9 bases upstream from the stop codon. This restriction digestion yielded a fragment of expected size, 1377 bp. For β2, KpnI cut the vector at a site near the 5' end, which is 11 bases downstream from the start codon, and EcoRI cut the vector at the 3' ends of the insert, including the stop codon. A double-stranded oligonucleotide (61-mer) was purchased (Genosys) with a sequence of 5'-ACTGTGACGAGACGACACGAAACGACACACGACACGACACGATGTG-GGAGATCGCCGAGAAAAAGG3'. In this linker, the first 9 bp represent the α6 sequence before the stop codon beginning with the distal half of the Scal recognition site, the next 30 bp represent 10 glutamine residues, and the last 22 bp represent the β2 sequence, including the start codon (ATG) and the recognition site for KpnI with an overhang at the 3' end. The fragment representing the rest of the expression vector was isolated by digestion with CIC and EcoRI. Ligation products were formed from all the four fragments with proper sticky ends and were used to transform the competent E. coli SC1 strain (Stratagene). The correct plasmid was selected with polymerase chain reaction (PCR) using a primer pair, one of which anneals to the glutamine linker, and was verified with sequencing. Human embryonic kidney cells (HEK293 cells, ATCC CRL 1573) were transfected with the vector carrying the tandem cDNA alone or with α6, β2, or γ2 cDNA in a 2:1 ratio (4 to 2 μg36-mm culture dish), in the presence of a transfection reagent, DOTAP (Boehringer Mannheim GmbH). For infection of insect SF-9 cells, recombinant baculoviruses were prepared using a vector, PVL 1393 (PharMingen), carrying the tandem α6-β2 construct or the cDNA for α6, β2, or γ2. All other procedures for DNA analysis, construction, and purification were described elsewhere (15).

Northern Blotting and 3'-RACE Assays—mRNAs were prepared in the presence of guanidium thiocyanate from HEK293 cells transfected with the carries of indicated GABA<sub>A</sub> receptor subunit cDNAs. Northern blotting was carried out with nylon transfer membranes, following the procedures provided by Schleicher and Schuell. The 32P Probes for...
from HEK293 cells transfected with the tandem construct of α6 and β2 GABA<sub>A</sub> receptor subunit cDNAs. The cells were grown to about 70% confluence in a 75-cm<sup>2</sup> culture flask and incubated in the presence of the tandem construct of α6-β2 (8 μg) or with the cDNA for γ2 (4 μg) and DOTAP (60 μl) for 24 h. The cells were harvested 24 h later, and mRNAs were prepared in the presence of guanidinium thiocyanate following the standard procedures. A, Northern blotting was carried out with nylon membranes containing 10 μg of extracted mRNAs after resolution on a 1% agarose gel, following the vendor-provided procedures. The 32P-labeled probe for the γ6 was prepared using PCR in the presence of [α-32P]dCTP. The α6, β2, and γ2 mRNAs were prepared in vitro using a Promega transcription kit. B, 3' RACE assays were carried out using the HEK from Life Technologies, Inc., the universal primer and the γ6 specific primer (a) or the β2-specific primer (b). The detailed information for the primers and PCR reaction were described under “Materials and Methods.”

α6 and β2 were prepared from PCR which were carried out in a 50-μl volume using 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer) in the vender-supplied buffer, 20 μM dNTP ([α-32P]dCTP), the respective cDNA as a template, and selective primer pairs annealing to the 5′ and 3′ end of the coding region of the template cDNA. The cycle parameters were set at 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1–3 min, depending on the expected product size, with a final extension at 72 °C for 10 min after 30 cycles. The radioactive probes were purified using a QiaGen column and used within a few days. Fig. 1 shows Northern analysis of the mRNA preparations from HEK293 cells which were transfected with the tandem construct of γ6 and β2 subunits (α6-β2) alone or in combination with γ2. For the sake of comparison, authentic mRNAs for individual subunits were prepared using a transcription in vitro system with SP6 RNA polymerase (Promega) and linearized vectors at the 3′ end of the coding region. In the analysis, stringent washing conditions were employed under which the 32P-labeled probe for the α6 subunit interacted only with α6 mRNA, but not with β2 or γ2 mRNA (Fig. 1A). In the mRNA preparations from HEK293 cells transfected with α6-β2 alone or in combination with γ2, the 32P-labeled α6 probe interacted with a single band of an estimated molecular mass of about 3.5 kDa (judging from RNA molecular mass standards and as compared with the α6 mRNA of 1.7 kDa) (Fig. 1A). The 32P-labeled β2 probe detected the same band as expected from the tandem construct (data not shown). The mRNA species for the tandem construct was further confirmed by the 3′ RACE following the procedures provided by Life Technologies, Inc. (Fig. 1B). Briefly, following reverse transcription of the mRNA preparations using 3′ RACE adaptor primer (dT<sub>12</sub>CATGATR-CAGCTGCGACCCCGG), PCR was carried out with a primer specific for the α6 or β2 and the universal amplification primer (the vender-provided). The primer for α6 was a 23-mer beginning at the base 400 of α6 (5′-CGGACACATTTTTCCACAATGGG) and that for β2 was a 21-mer beginning at the base 1051 of β2 (5′-GGTCAACTACATCTTTGG). In HEK293 cells transfected with α6, β2, and γ2 subunits, PCR with the α6 primer and the universal primer produced one product with an expected size of 1491 bp and with the β2 primer also produced a single product with an expected size of 1743 bp. In the cells transfected with the tandem construct, PCR with the α6 primer and the universal primer produced one product matching with the expected size of 3663 bp for the α6-β2, and similar PCR with the β2-specific primer and the universal primer produced the product of the same size (1743 bp) as the one found in the cells transfected with β2 monomers. These data confirmed the proper construction of α6 and β2 subunits in tandem as described above and have established its in vivo transcription.

Electrophysiology—The whole cell patch clamp technique (16) was used to record the GABA-mediated Cl<sup>−</sup> currents in HEK293 cells (48 h after transfection), with the pipette solution containing (m M) 140 CsCl, 11 EGTA, 4 MgCl<sub>2</sub>, 2 ATP, and 10 Hepes, pH 7.3, and with the external solution containing (m M) 135 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 5 Hepes, pH 7.2 (17). GABA and drugs were dissolved in the external solution and were applied through a U-tube placed within 100 μM of the target cell.

Binding Studies—Sf-9 cells were infected with the baculovirus carrying the recombinant cDNAs as described previously (18). Binding of radioactive ligands was measured in the Sf-9 cell membranes, using filtration techniques as described elsewhere (19, 31). Briefly, [3H]muscimol or [3H]Ro15–4513 binding was measured in the medium containing 118 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 20 mM Hepes/Tris, pH 7.3, the radioactive ligand at various concentrations (1–40 nM), 30 μg of membrane proteins in a total volume of 500 μl at 4 °C for 60 min. Nonspecific binding was estimated in the presence of excess unlabeled ligands and was subtracted to compute specific binding as described earlier (19, 31).

RESULTS

We examined whether GABA induces Cl<sup>−</sup> currents in HEK293 cells transfected with the GABA<sub>A</sub> receptor cDNAs, using the whole cell patch clamp technique. In the cells transfected with the tandem construct alone, no currents were detected upon application of GABA at 1–20 μM (with more than 30 patches). If GABA<sub>A</sub> receptors are pentameric as proposed, functional expression of the tandem construct of α6-β2 would require the presence of monomeric subunits. Therefore, HEK293 cells were transfected with the α6-β2 and one of the monomeric subunits. Addition of α6 or γ2, but not β2, led to the appearance of GABA-induced whole cell currents, which were sensitive to picrotoxin, a specific inhibitor of GABA<sub>A</sub> receptor Cl<sup>−</sup> currents (Fig. 2). Moreover, a benzodiazepine agonist, U-92330 (19, 31), enhanced GABA currents by 136 ± 9% in the receptor made of α6-β2 plus γ2, but had no effect on that made of α6-β2 plus α6 (0 ± 5%) (Fig. 2). The picrotoxin sensitivity and the γ2-dependent interaction with the benzodiazepine site ligand have already been known in the α6β2 and α6β2γ2 subtypes of GABA<sub>A</sub> receptors (16, 19, 31). It should be also noted that no GABA-induced Cl<sup>−</sup> currents were detected in the whole cell patch with HEK293 cells (or Sf-9 cells) transfected (or infected) with the subunit of α6, β2, or γ2 alone (19, 31). GABA dose-dependently increased Cl<sup>−</sup> currents in the cells transfected with α6-β2 plus α6 or plus γ2 (Fig. 3). Analysis of the data with a logistic equation of E<sub>max</sub> = [GABA]<sup>n</sup>/K<sub>d</sub> + [GABA]<sup>n</sup> yielded a half-maximal GABA concentration (K<sub>d</sub>) of
was observed only in the membranes from the cells infected alone or in combination with that for binding experiments (18, 20). Thus, Sf-9 cells were infected with Scatchard analysis (Table I, Fig. 4).[^3H]Muscimol binding was measured in the SF-9 cells expressing the indicated subunit cDNAs. The parameters were obtained from the Scatchard analysis of binding data and are presented with the mean ± S.E. from three experiments. ND denotes not detectable.

TABLE I

| Kd (nM) | Bmax (pmol/mg protein) | Kd (nM) | Bmax (pmol/mg protein) |
|---------|------------------------|---------|------------------------|
| α6-β2   | 2.5 ± 0.2              | 1.6 ± 0.1| ND                     |
| α6-β2, α6| 2.6 ± 0.3              | 1.2 ± 0.2| ND                     |
| α6-β2, β2| 2.7 ± 0.2              | 1.2 ± 0.2| ND                     |
| α6-β2, γ2| 2.1 ± 0.2              | 1.6 ± 0.1| 9.5 ± 0.7              |

DISCUSSION

In this study we have shown that expression of the tandem construct of α6-β2 subunit cDNA alone failed to produce Cl⁻ currents in response to GABA application, but in the presence of the monomeric subunit of α6 or γ2 subunit, its expression led to the appearance of GABA- and picrotoxin-sensitive Cl⁻ currents. This could be interpreted to mean that the tandem construct alone forms receptors with only even-numbered subunits, which are not functional (no chloride channels), but in the presence of the monomeric subunit of α6 or γ2, forms a functional pentameric receptor with Cl⁻ channels. This interpretation led us to propose that the functional receptor (Cl⁻ channel) may consist of two α6-β2 and one α6 or γ2. Since these receptors displayed similar functional and pharmacological properties with the monomeric α6β2γ2 or α6β2 subtypes, we propose that the α6β2γ2 subtype consists of two α6, two β2, and one γ2 subunits and that the α6β2 subtype consists of three α6 and two β2 subunits. This proposal is in agreement with earlier immunoprecipitation studies which indicated the presence of two α subunits in the αβγ types of cloned GABA<sub>A</sub> receptors (8–11). An alternative interpretation of our results is that the functional receptors of α6β2γ2 would be made of one α6-β2 and three monomeric γ2, leading to one α6 subunit per receptor. This is incompatible with the presence of two α subunits per receptor already shown by the immunoprecipitation studies(8–11).

We also propose the orientation of the subunits in the α6β2γ2 subtype to be α6-β2-α6-β2-γ2. Such an arrangement could minimize interactions between homologous subunits and
domains and accommodate two α6-β2 tandem subunits per receptor. This arrangement is also compatible with the assignment of the benzodiazepine site at the interface between α6 and γ2 subunits (22–24), with the N-terminal of α6 involved in the formation of the benzodiazepine site (14, 25). Future study with the tandem α6-β2, β2-α6, and β2-γ2, where the hyphen represents a C- to N-terminal linkage, will be useful to test this proposal.

Of considerable interest is the appearance of the high affinity GABA site without Cl\textsuperscript{−} channels in the cells expressing the α6-β2 construct alone. Two types of GABA sites are known to be on GABA\textsubscript{A} receptors, the high affinity site with nanomolar dissociation constants and low affinity sites with micromolar dissociation constants (1, 21). The high affinity GABA site has been reported on the β subunit (1, 26), and low affinity GABA sites were not localized yet, but their affinity was markedly affected by mutations on the β subunit (27) and different α isoforms (7, 28, 29). This indicates that low affinity GABA sites could be influenced by secondary, tertiary, and quaternary interactions among the subunits. Furthermore, its occupancy could be affected by mutations on the subunit (22–24), with the N-terminal of α6 involved in the formation of the benzodiazepine site at the interface between α6 and β2 construct alone. Two types of GABA sites are known to be on GABA\textsubscript{A} receptors, the high affinity site with nanomolar dissociation constants and low affinity sites with micromolar dissociation constants (1, 21). The high affinity GABA site has been reported on the β subunit (1, 26), and low affinity GABA sites were not localized yet, but their affinity was markedly affected by mutations on the β subunit (27) and different α isoforms (7, 28, 29). This indicates that low affinity GABA sites could be influenced by secondary, tertiary, and quaternary interactions among the subunits. Furthermore, its occupancy could be affected by mutations on the subunit (22–24), with the N-terminal of α6 involved in the formation of the benzodiazepine site at the interface between α6 and β2 construct alone.

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