Subunit Arrangement of γ-Aminobutyric Acid Type A Receptors*

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The GABA_2 receptors are ligand-gated chloride channels. The subunit stoichiometry of the receptors is controversial; four, five, or six subunits per receptor molecule have been proposed for αβ receptors, whereas αβγ receptors are assumed to be pentamers. In this study, α-β and β-α tandem cDNAs from the α1 and β2 subunits of the GABA_A receptor were constructed. We determined the minimal length of the linker that is required between the two subunits for functional channel expression for each of the tandem constructs. 10- and 23-amino acid residues are required for α-β and β-α, respectively. The tandem constructs either alone or in combination with each other failed to express functional channels in Xenopus oocytes. We can exclude tetrameric or hexameric αβ GABA_A receptors. We can also exclude proteolysis of the tandem constructs. In addition, the tandem constructs were combined with single α, β, or γ subunits to allow formation of pentameric arrangements. In contrast to the combination with α subunits, the combination with either β or γ subunits led to expression of functional channels. Therefore, a pentameric arrangement containing two α1 and three β2 subunits is proposed for the receptor composed of α and β subunits. Our findings also favor an arrangement αβγα for the receptor composed of α, β, and γ subunits.

GABA_A receptors mediate fast synaptic inhibition in the mammalian brain. They are believed to form heterooligomers composed of subunits from six classes with several isoforms (α1–6, β1–3, γ1–3, δ, ε, δ, π) (1–5). These subunits belong to the gene superfamily of ligand-gated ion channels, which includes nicotinic acetylcholine receptors, GABA_A receptors, glycine receptors, and the serotonin type 3 (5HT_3) receptor. The major GABA_A receptor isoform is likely to be composed of α1, β2, and γ2 subunits (1, 2, 6, 7). Heterologous expression demonstrated that the combination of α and β subunits produces GABA-gated currents, but coexpression of a γ subunit is required for benzodiazepine sensitivity of the expressed receptors (8).

GABA_A receptors composed of α and β subunits differ from receptors that additionally contain the γ subunit in regard to Zn^{2+} and benzodiazepine sensitivity and to single channel conductance (9–13). Some populations of neuronal GABA_A receptors show high Zn^{2+} sensitivity coupled with low single-channel conductance as described for αβ receptors (14, 15). Although receptors made from α, β, and γ subunits are thought to be pentameric (16–18), the subunit stoichiometry of receptors composed of α and β is still controversial. Recombinantly expressed receptors have been reported as possibly tetrameric (19, 20) as well as pentameric (18, 21). Unitary dose-response curves for αβ receptors, single IC_{50} values for Zn^{2+} inhibition, and unitary single channel properties (1) provide evidence against the formation of two populations of receptors, e.g. 2αβ and 3αβ. A tetrameric rather than a pentameric structure has been proposed as one of several explanations for the lower average single channel conductance for the αβ receptor as compared with the αβγ receptor (22, 23).

A powerful way to gain insight into the arrangement of subunits in a multimeric channel is to predetermine the alignment of subunits by gene fusion and to analyze whether the linked subunits are able to form functional channels. This approach was first successfully applied to potassium channels (24–26). Later it was also used to study subunit stoichiometry of other ion channels, e.g. a cyclic nucleotide-gated channel (27), the mechanosensitive channel MscL of Escherichia coli (28), and the cystic fibrosis conductance regulator channel (29). All these channels have their N and C termini on the cytoplasmic side so that the linkage occurs intracellularly. Up to now it has only been used once with limited success in the field of ligand-gated ion channels, which have both C and N termini on the extracellular side. Applying it to a GABA_A receptor, Im et al. (30) prepared a tandem construct where the α6 subunit is linked to the β2 subunit via 10 glutamine residues and studied functional expression in HEK293 cells. The connection between the two subunits included the signal sequence of the β2 subunit of 24-amino acid residues in length. The consequences of such a signal sequence in the middle of a protein are difficult to predict.

We constructed here tandem constructs of α1 and β2 subunits for the first time in both arrangements α1-β2 and β2-α1. We determined the minimal length of the linkers necessary for the formation of functional channels. The constructs were expressed in Xenopus laevis oocytes either alone or in combination with single subunits to establish subunit stoichiometry and arrangement of GABA_A receptors. We provide novel information on the architecture of GABA_A receptors.

EXPERIMENTAL PROCEDURES

Construction of the Tandem cDNAs—Several α-β tandem cDNAs encoding a single polypeptide αβ with linkers of differing length were established in the pCMV vector. The tandem constructs consisted of the modified rat α1 subunit (31) including its signal sequence at the N terminus and the mature rat β2 subunit at the C terminus. The modified α1 subunit differs from the original rat subunit by insertion of one amino acid residue. Insertion of this residue confers reactivity to the monoclonal antibody bd24 (32, 31), which was essential for Western blot analysis (shown in the “Results” section). The α1 subunit was amplified by polymerase chain reaction using the pCHAI vector as template and the primers CATAGAAGACACCGGGACGA as a vector-specific primer and XTTGATGGGTGGTGGGGGCTTTT as a gene-specific primer. The latter was complementary to the last codons before the stop codon and had the first part of the sequence coding for the respective linker attached (X). The β2 subunit was amplified using pCB2 as template and

* This study was supported by Swiss National Science Foundation Grant 3100-053599.98/1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GABA, γ-aminobutyric acid; GABA_A, GABA type A; HEK, human embryonic kidney; K_{app}, apparent affinity.
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The primers ACTGACACACATCTCAGCT as vector-specific primer and YCAGAGTCAATCGCCCTTGT as a gene-specific primer. The latter was complementary to the first codons of the sequence of the mature protein and had the second part of the sequence coding for the respective linker attached (Y). The obtained fragments contained the open reading frame of the gene and some additional vector-derived sequence preceding or succeeding. The fragments were cut in the vector-derived sequence by EcoRI or XbaI, respectively, ligated to be ligated in a three-frAGMENT-ligation into the pCMV vector cut with EcoRI and XbaI. The sequence of the resulting plasmids was verified. In the α-0-β tandem, the last amino acid residue of the α1 subunit is directly attached to the first amino acid residue of the mature β2 subunit. In the other tandems the following amino acid sequences are present between the N-terminal α1 subunit and the C-terminal β2 subunit: α-7-β; α-10-β, Q<sub>16</sub>. The β-α tandem cDNAs were prepared similarly. The β subunit was amplified using CATAGAAAGACCCCGAAGCA as vector-specific and XGTTCACATGAAACGCAATAGAC as gene-specific primer. The mature α subunit was amplified using ACTGACACATCTCAGCT as vector-specific and YCAGAGTCAATCGCCCTTGT as gene-specific primer. The linkers introduced into the different β-α tandems are the following: β10α, Q<sub>16</sub>; β15α, Q<sub>20</sub>AQP; β20α, Q<sub>24</sub>AQP; β32α, Q<sub>20</sub>(Q<sub>2</sub>A,P)<sub>4</sub>α.Q<sub>16</sub>. A long sequence of consecutive glutamine residues might exhaust the respective tRNA pool during protein synthesis and therefore lead to an early termination of the synthesized protein. Therefore, other amino acid residues were introduced. Alanine and proline residues were chosen for their properties to form no distinct secondary structure elements.

Expression of Tandem Constructs and Wild Type Subunits in Xenopus Oocytes—Capped cRNAs were synthesized (Ambion, Austin, Texas) from the linearized pcMV vectors containing the different tandem constructs, the single α1, β2, and γ2 subunits, and from the vector pVA2580 (33) encoding a neuronal voltage-gated sodium channel (Na<sup>+</sup>). A poly(A) tail of about 400 residues was added to each transcript using yeast pol(A) polymerase (U. S. Biochemical Corp.). The concentration of the cRNA was quantified on a formaldehyde gel pool during protein synthesis and therefore lead to an early termination of the synthesized protein. Therefore, other amino acid residues were introduced. Alanine and proline residues were chosen for their properties to form no distinct secondary structure elements.

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The Tandem Constructs Are Not Proteolyzed in the Linker Sequence—To evaluate whether the expressed tandem constructs were intact or subjected to proteolysis we analyzed the newly formed GABA<sub>A</sub> receptors by Western blotting. The monoclonal antibody bd24 against the N-terminal of the α1 subunit (31, 32) was used. Fig. 3 shows that single α1 subunits of wild type receptors migrate at 50 kDa (lane 1). This specific band is missing in the α-10-β/β combination (lane 2), thus indicating the absence of monomeric α1 subunit and, therefore, of significant proteolysis of the linker. A very faint unspecific signal at the 50-kDa position is also seen for non-injected oocytes. With a linker of 7 residues in length (α-7-β), we found standardized maximal current amplitudes that remained below those expressed from wild type receptors, whereas the tandem construct with a linker of 10 residues (α-10-β) resulted in similar standardized maximal current amplitudes. The dose-response curves of the α-7-β and the α-10-β tandem constructs were close to that of the wild type receptors (Fig. 5A). The two constructs resulted in channels with similar K<sub>i</sub> values of 9 ± 3 and 11 ± 2 μM, respectively, comparable with the combination of single α and β subunits with a K<sub>i</sub> of 9 ± 2 μM, pointing to an unchanged apparent affinity for GABA despite the covalent linkage.

On the right panel of Fig. 4 the results of the analogue examination for the β-α construct are shown. There was almost no detectable current when we combined the constructs with linkers of 10- and 15-amino acid residues with single β2
subunits. A tandem construct with a linker of 20 residues produced receptors with maximized apparent current amplitudes similar to those of wild type receptors. However, the dose-response curve (Fig. 5B) was shifted to the right, i.e. the apparent affinity for GABA was reduced. With $64 \pm 3 \mu M$, the $K_a$ was about 7-fold higher than that of the wild type receptors. A tandem construct containing a linker of 23 residues also reached maximized current amplitudes similar to wild type receptors. The GABA dose-response curve for these channels (Fig. 5B) is characterized by a $K_a$ of $20 \pm 2 \mu M$, which is close to the wild type receptor, with a $K_a$ of $9 \pm 2 \mu M$.

**GABA$_A$ Receptors Made from a1 and b2 Subunits Are Pentamers Containing 2 a and 3 b Subunits**—The two functional tandem constructs $\alpha$-10-$\beta$ and $\beta$-23-$\alpha$ were analyzed further. When either the $\alpha$-10-$\beta$ or the $\beta$-23-$\alpha$ constructs were expressed alone, we hardly detected GABA-evoked currents (Fig. 6A). The co-expressed voltage-gated sodium channel showed the same expression levels in oocytes expressing tandem constructs or wild type receptors. Thus, the absence of RNAase activity and the capability of protein expression in the individual oocyte was confirmed. Moreover we exclude proteolysis for either construct because proteolysis of the linker would in each case liberate $a1$ and $b2$ subunits, which in turn should result in functional channels. When $\alpha$-10-$\beta$ and $\beta$-23-$\alpha$ constructs were expressed in the same oocyte, the standardized maximal current amplitudes remained below 10% of the wild type current (Fig. 6C).

These results led to the conclusion that tetrameric receptors of the arrangement $a\beta\alpha\beta$, which is equal to the arrangement $\beta\alpha\beta\alpha$ (see Fig. 2B, I and II) or of the arrangement $a\beta\alpha\beta$ (see Fig. 2B, III) do not correspond to a functional receptor made from single $\alpha$ and $\beta$ subunits.

The tandem constructs $\alpha$-10-$\beta$ and $\beta$-23-$\alpha$ were also coex-
Fig. 6D shows that both the α-10-β and the β-23-α tandem constructs could be complemented with single β2 subunits to form functional channels. This result matches the theoretical consideration that both tandem constructs yield the same arrangement when complemented with a single β2 subunit (compare Fig. 2B, VI and VII).

Coexpression of the Tandem Constructs with a Single γ2 Subunit—When the α-10-β tandem construct is complemented with a single γ2 subunit, the standardized maximal current amplitude amounts to about 26% compared with the wild type receptor (Fig. 6E). Submaximal current amplitudes can be stimulated by diazepam by 134 ± 8% (mean ± S.D., n = 3) (not shown). The β-23-α tandem construct complemented with a single γ subunit results in functional channels with standardized maximal current amplitudes similar to wild type receptors (Fig. 6E). Submaximal current amplitudes of these receptors are also stimulated by diazepam by 360 ± 10% (mean ± S.D., n = 3) (not shown).

DISCUSSION

In this study we have demonstrated the feasibility of covalent subunit linkage α1-β2 and β2-α1 for the GABA<sub>A</sub> receptor channel. We have also established the minimal linker lengths required for functional expression. Our results strongly suggest a pentameric structure of the GABA<sub>A</sub> receptor composed of α1 and β2 subunits and exclude a tetramer. The technique described here may also be applied to the study of other ligand-gated ion channels.

Tandem linkage of subunits is a powerful strategy to extract information about stoichiometry and arrangement of multimeric proteins. This approach has first been applied to the study of potassium channels (24). Later, Im et al. (30) made a tandem construct consisting of the GABA<sub>A</sub> receptor subunit precursors α6 and β2. They found that their α6-β2 tandem construct alone failed to produce functional GABA channels, but combination with either single α6 or γ2 subunits, but not β2 subunits, restored receptor function after expression in HEK293 cells. Functional expression was, however, very low in all these cases and did not exceed 0.2 nA even for the wild type subunit combination α6 and β2 (30).

In the present tandem constructs we omitted the signal sequence stretch of the second subunit, which might have unimportant conformational effects on e.g. protein folding, insertion of the protein into the membrane, subunit assembly, or proteolysis of the connection between the subunits. We linked the α1 and the β2 subunits of the GABA<sub>A</sub> receptor in both arrangements and expressed the resulting tandem constructs α-β and β-α in Xenopus oocytes. They were both shown to result in functional channels when complemented with β2 subunits. When the tandem constructs were expressed either alone or in combination with each other, no functional receptors were formed. Therefore, our most important conclusion here is that the GABA<sub>A</sub> receptor made from α1 and β2 subunits is not composed of an even number of subunits. We can exclude tetrameric receptors of the subunit arrangements alone and the arrangement αββα from their co-expression. Only arrangements of a 1:1 stoichiometry of α and β subunits have been tested here because stoichiometries for αβ receptors of 3:1 or 1:3 have been shown to be unlikely (19, 20). These findings confirm the conclusion drawn from Western blot analysis that proteolytic cleavage in the sequence of the linker (Fig. 7A) does not occur to a significant extent. The participation of only one subunit of the tandem construct in the functional receptor (Fig. 7B) can also be excluded. If either one or both of these events had occurred, the formation of functional pentameric receptors from the tandem constructs alone would have been observed.

The observation that both tandem constructs form functional channels in combination with single β2 subunits but fail to do so in combination with single α1 subunits supports the view that a receptor made from α and β subunits is a pentamer composed of two α and three β subunits. This has also been proposed based on immunoprecipitation experiments in HEK293 cells expressing αβ3 receptors (18). A receptor stoichiometry of three α6 and two β2 subunits has also been suggested (30). This might indicate that the subunit stoichiometry of an αβ receptor depends on the specific subunit isoforms expressed together and/or on differences in the expression systems used.

A further aim of this study was the design of optimal linkers between the subunits. The linkage of two subunits should position both next to each other in the receptor. When no functional channels can be detected, the forced neighborhood of the two subunits either prohibits proper channel formation, or the linker is too short. When, in contrast, functional channels can be expressed from linked subunits, their neighborhood may be assumed unless the linker is very long. In this case the two linked subunits do not necessarily locate next to each other in the receptor multimer. It is then possible for another subunit to position itself between the two linked subunits. We therefore determined the minimal linker length for both, the α-β and the β-α tandem constructs, necessary for the formation of functional channels. We found this length to be 10 and 23 amino acid residues, respectively. Shorter linkers altered the apparent affinity for GABA or the maximal current amplitude of the channel, probably by distorting the conformation of the resulting receptor. It should be noted that the α-7-β and β-20-α tandem constructs, which have linkers that are 3 amino acid residues or about 11 Å shorter, performed nearly as well as wild type receptors. Therefore, the optimal linker length may be somewhat shorter than 10 or 23 amino acid residues, respectively. In our calculation of the actual linker length we included the synthetic linker as well as the C- and N-terminal elongations of the respective subunits (Table I). We assumed an extended conformation of both with 3.6-Å per amino acid residue. In this case the total length of the subunit connection may be estimated to be maximally 83 and 97 Å in the α-β and the β-α tandem construct, respectively, which might be diminished by the existence of secondary structure elements. For the reasons mentioned above, we assume that the actual linker length is substantially shorter. It is of interest to estimate whether these respective linker lengths allow interspersing of an additional subunit. We can consider the nicotinic acetylcholine receptor an appropriate model for the structure of the GABA<sub>A</sub> receptor.
receptor, as they both belong to the same superfamily of ligand-gated ion channels. The three-dimensional structure of the nicotinic acetylcholine receptor has been resolved to 4.6 Å (37). All the members of the superfamily share a high sequence homology and the same topology, and it is assumed that they also have a very similar overall shape. From the dimensions of the receptor we can estimate the minimal length of a peptide passing along the perimeter of one subunit to be about at least 54 Å if the N terminus is located at the membrane surface. This minimal length of 54 Å is unrealistic for the following reasons. First, the receptor surface is certainly not smooth, but irregular. Second, the N terminus of the second subunit of the tandem construct is not necessarily located at the membrane surface as the beginning of the connection is predicted to be. Most importantly, location of either the N terminus or the C terminus away from the opposed edges of the linked subunits would both result in a corresponding increase of the required minimal length. Comparing the maximal length of the subunit connections and the minimal length such a connection must have to surround an additional subunit and the restrictions made to these values, we consider it unlikely that another subunit is interspersing, but we cannot entirely exclude this possibility.

In initial experiments we combined the two tandem constructs α-10-β and β-23-α each with single γ subunits. In the case of the β-23-α tandem construct, the resulting channel exhibited the same maximal current amplitude as wild type receptors, whereas in the case of the α-10-β tandem construct, maximal current amplitudes remained below that of wild type receptors. The fact that both tandem constructs exhibited the same maximal current amplitude as wild type receptors, whereas in the case of the tandem construct, the γ subunits alone are no more formed (23), but the γ subunit would not result in additional subunit linkage for a ligand-gated ion channel. For the first time we have established the minimal linker lengths required for functional expression. Our results strongly suggest a pentameric structure of the α1β2 GABA<sub>A</sub> receptor and exclude a tetramer. This work provides a new perspective for the study of subunit arrangement also of other ligand-gated ion channels.

**Acknowledgment**—We thank Dr. V. Niggl for carefully reading the manuscript.

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J. Biol. Chem. 2001, 276:36275-36280.
doi: 10.1074/jbc.M105240200 originally published online July 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105240200

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