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The γ3-subunit of the $GABA_A$-receptor confers sensitivity to benzodiazepine receptor ligands

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The γ3-subunit of the $GABA_A$-receptor in rat brain has been identified by molecular cloning. When co-expressed with the α5- and β2-subunits in transfected cells a high potency for GABA (K_a = 4.9 ± 1.2 μM) and a strong cooperativity in gating the channel (H = 1.9 ± 0.2) was observed. The GABA response was potentiated in the presence of flunitrazepam and reduced by βCCM. An analogous bi-directional modulation of the GABA response was observed with diazepam and DMCM as tested with the subunit combinations $\alpha1\beta2\gamma3$ and $\alpha3\beta2\gamma3$ expressed in *Xenopus* oocytes. Since the benzodiazepine receptor ligands were virtually inactive in the absence of the γ3-subunit, as tested with the $\alpha3\beta2$- and $\alpha5\beta2$-subunit combinations, the γ3-subunit is a prerequisite for the benzodiazepine receptor sensitivity of the expressed $GABA_A$-receptors. The γ3-subunit could functionally replace the γ2-subunit with regard to the bi-directional allosteric drug modulation.

$GABA_A$-receptor heterogeneity; Recombinant $GABA_A$-receptor; γ3-Subunit expression

1. INTRODUCTION

The $GABA_A$-receptor, which mediates the main inhibitory synaptic event in the CNS, is a gated chloride channel whose function is modulated by various clinically important drugs, in particular by barbiturates and ligands of the benzodiazepine receptor. Structurally, the $GABA_A$-receptor is a hetero-oligomeric protein which is made up, in unknown stoichiometry, of the members of at least 5 different classes of subunits [1–4]. The γ2-subunit is of special relevance for the pharmacology of the $GABA_A$-receptor since this subunit, in combination with α- and β-subunits, is necessary to convey benzodiazepine receptor sensitivity to recombinant $GABA_A$-receptors [5]. The pattern of expression of the γ2-subunit, mapped in the brain by in situ hybridization histochemistry [5–7] and by immunocytochemistry [8], revealed a marked regional variation suggesting that subunits other than the γ2-subunit may exist which confer the benzodiazepine receptor sensitivity to $GABA_A$-receptors. We now show that the recently identified γ3-subunit [9] is able to convey benzodiazepine receptor sensitivity to $GABA_A$-receptors when co-expressed with α- and β-subunits.

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2. MATERIALS AND METHODS

2.1. *Molecular cloning*

A cDNA-library in LZap II (Stratagene) from mRNA of newborn rat brain was screened [10] with 2 oligonucleotides derived from the mouse γ3-subunit sequence, bp 61–26 and bp 1190–1131 [9]. One clone which hybridized to both probes was isolated and the cDNA-insert subcloned into the *Bam*HI site of a pBC expression vector [11]. The γ3-subunit cDNA was sequenced by the dideoxynucleotide chain-termination method [10].

2.2. *Electrophysiological analysis*

Recordings were performed 2–4 days after transfection of human embryonic kidney cells (ATCC CRL 1573, 293) with rat subunit cDNAs using the whole-cell configuration of the patch-clamp technique [12] at −30 mV as described previously [11]. Expression of cRNAs and electrophysiological analysis of drug effects on GABA currents expressed in *Xenopus* oocytes were performed as described previously [13].

3. RESULTS

3.1. *Cloning of the rat γ3-subunit cDNA*

Since the α- and β-subunit cDNAs used in the present study were of rat origin [13,14], the γ3-subunit cDNA was cloned from the same species by hybridization screening using 2 oligonucleotide probes from the mouse γ3-subunit cDNA sequence [9]. A 1.9 kb rat cDNA clone, which hybridized with both probes, contained the entire open reading frame displaying a nucleotide and protein sequence identity of 96 and 99%, respectively, to the mouse cDNA clone (Fig. 1).

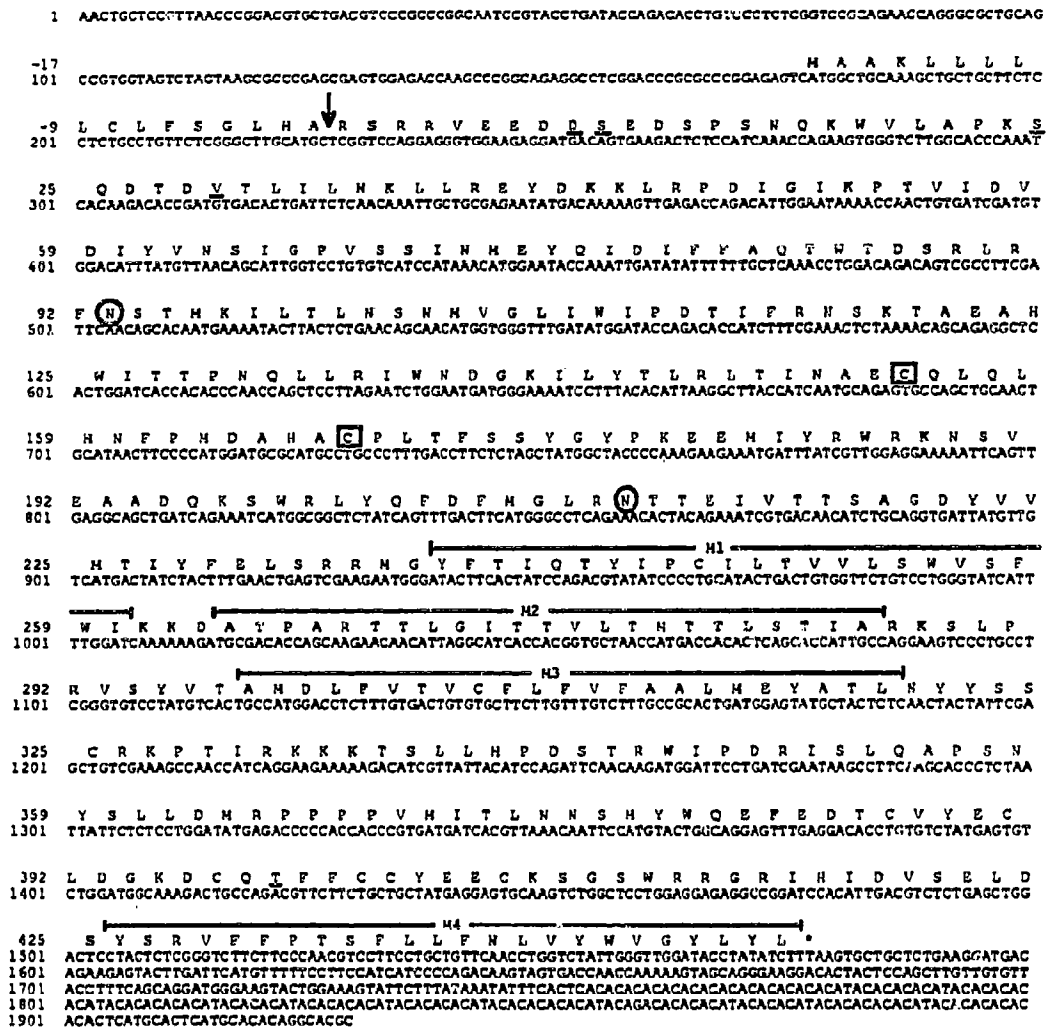

Fig. 1. cDNA sequence and deduced amino acid sequence of the γ3-subunit of the $GABA_A$-receptor from rat brain. The putative signal sequence cleavage site is indicated by an arrow; sites for *N*-linked glycosylation are circled; the 2 cysteines thought to form a disulfide bridge are boxed in; the proposed membrane spanning domaines (M1–M4) are indicated by solid lines; the amino acids which differ from those of the mouse γ3-subunit sequence [9] are underlined.

3.2. *GABA dose–response curve for the $\alpha5\beta2\gamma3$ subunit combination*

The dose–response curve for GABA was generated by applying increasing concentrations of GABA (1, 3, 10, 30, 100 μM) for 2 s followed by a resting interval of 1 min to mammalian cells transfected with the $\alpha5\beta2\gamma3$-subunit cDNAs (Fig. 2). The peak amplitudes of the GABA-induced currents were measured and fitted separately for each cell with the logistic equation

$$I/I_{max} = 1/\{1 + (EC_{50}/\{GABA\})^{n}\} \quad (1)$$

where EC_{50} is the GABA concentration eliciting the half maximal current (1/2 I_{max}) and H the Hill coefficient. The results from 4 experiments yielded values of EC_{50} = 4.9 ± 1.2 μM and H = 1.9 ± 0.2.

3.3. *Modulation of the GABA-response by drugs*

In the presence of pentobarbital (50 μM) the GABA-induced current (3 μM GABA) was increased by 47 ± 13% in cells transfected with the $\alpha5\beta2\gamma3$-subunit cDNAs (n = 3). This potentiation was not dependent on the presence of the γ3-subunit, since the GABA-response was likewise potentiated (by 106%, n = 2) in cells co-transfected only with the $\alpha5$- and the $\beta2$-subunit cDNAs. To determine whether the recombinant receptors expressed from the $\alpha5\beta2\gamma3$ cDNAs displayed sensitivity to benzodiazepine receptor ligands, the influence of the agonist flunitrazepam and the inverse agonist βCCM (methyl-4-ethyl-β-carboline-3-carboxylate) on the GABA-response was tested. Drug concentrations were chosen which were previously shown to induce a maximum response in recombinant receptors [11,15]. In the presence of flunitrazepam (1 μM) the current induced by GABA (3 μM) was potentiated by 86 ± 12% (n

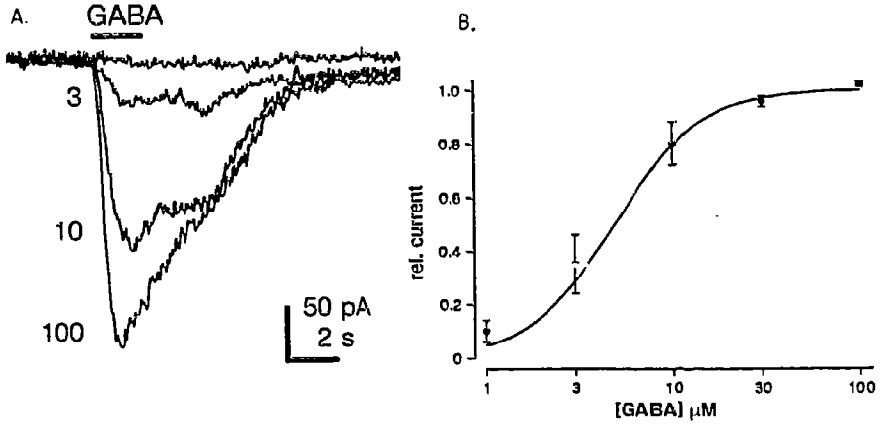

Fig. 2. GABA dose-response curve of cells co-transfected with the $\alpha5\beta2\gamma3$-subunit cDNAs. (A) Superimposed whole cell currents evoked by different GABA concentrations indicated by the numbers (μM) at each trace. (B) Plot of the relative current amplitude as a function of increasing GABA concentrations. The points represent mean values with standard deviations from 4 cells.

= 3) at the $\alpha5\beta2\gamma3$-receptors. The inverse agonist βCCM (1 μM) decreased the current amplitude elicited by GABA (10 μM) by 29 ± 5% (n = 5) in the $\alpha5\beta2\gamma3$-combination (Fig. 3). Only negligible drug modulation was observed in the absence of the $\gamma3$-subunit. When the $\alpha5\beta2$-subunit cDNAs were expressed only a slight reduction of the GABA response was recorded in the presence of flunitrazepam (1 μM) or βCCM (1 μM), by 8 ± 5% (n = 3) and 3 ± 4% (n = 4), respectively.

The effects of agonists and inverse agonists were further tested on various other subunit combinations containing the $\gamma3$-subunit in the *Xenopus* oocyte expression system and compared to subunit combinations containing the $\gamma2$ instead of the $\gamma3$-subunit (Fig. 4). In all combinations tested the $\gamma3$-subunit could functionally replace the $\gamma2$-subunit with regard to the positive allosteric modulation of the GABA response by the benzodiazepine agonist diazepam and the negative allosteric modulation by the inverse agonist DMCM (methyl-4-ethyl-6, 7-dimethoxy-β-carboline-3-carboxylate) (Fig. 4). The depression of the GABA-gated current by DMCM was even more pronounced in the subunit combinations containing $\gamma3$ rather than $\gamma2$. When $\alpha3\beta2$ was expressed in the absence of a γ-subunit the stimulation by diazepam was reduced to 7 ± 1% (n = 5) as compared to >110% in the presence of either $\gamma2$ or $\gamma3$, while the inhibition by DMCM was lost completely (Fig. 4).In the combinations containing the $\gamma2$-subunit ($\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$) the effect of diazepam was somewhat smaller than found previously [13].

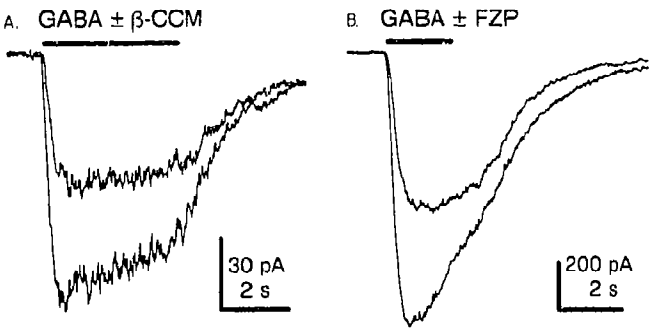

Fig. 3. Effects of flunitrazepam and βCCM on GABA-induced currents in cells co-transfected with the $\alpha5\beta2\gamma3$-subunit cDNAs. (A) The GABA-induced current (10 μM, bottom trace) was reduced in the presence of βCCM (1 μM, top trace). (B) Flunitrazepam (FZP, 1 μM) potentiated (bottom trace) the current elicited by GABA (3 μM) alone (top trace).

4. DISCUSSION

Three types of γ-subunits of the $GABA_A$-receptor have so far been cloned [5,9,16]. The $\gamma1$-subunit has been reported to give rise to an untypical pharmacology in that the recombinant receptors displayed agonistic actions for βCCM when co-expressed with some α- and

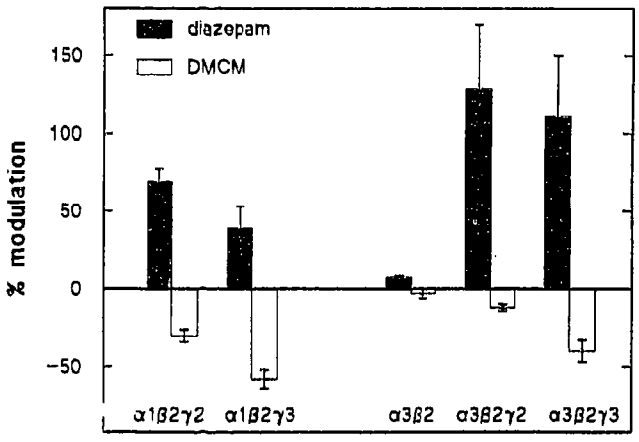

Fig. 4. Diazepam and DMCM modulation of different subunit combinations expressed in *Xenopus* oocytes. Drug effects were tested at a GABA concentration eliciting about 10% of the maximal current amplitude in the respective subunit combinations. Each value is an average and SD of at least 7 cells originating from 2 different donor animals, except for the $\alpha3\beta2$ combination, for which 5 cells from a single donor were analysed. The diazepam concentration was 1 μM; the DMCM concentration was 0.1 μM and 0.3 μM for the subunit combinations containing $\alpha3$ and $\alpha1$, respectively.

β-subunit combinations [17,18]. In contrast, the γ2-subunit conveyed a bi-directional modulation of the GABA response, being enhanced by an agonist and reduced by an inverse agonist of the benzodiazepine receptor [5]. This ability of the γ2-subunit is shared by the γ3-subunit [9] as now shown by an analysis of the $\alpha5\beta2\gamma3$-subunit combination expressed in mammalian cells from rat subunit cDNAs. These findings are substantiated by an investigation of additional subunit combinations ($\alpha1\beta2\gamma3$, $\alpha3\beta2\gamma3$) expressed in *Xenopus* oocytes, using a different benzodiazepine agonist and inverse agonist. As shown in transfected cells and in the oocyte expression system, neither benzodiazepine agonists nor inverse agonists appreciably modulate the GABA response when only the $\alpha5\beta2$ or the $\alpha3\beta3$ subunits, respectively, are co-expressed. This suggests that the γ2- or γ3-subunits are essential for a functional benzodiazepine receptor site in the triple subunit combinations.

Both the γ2- and γ3-subunits support a strong cooperativity of GABA in gating the channel. This is shown by the Hill coefficients of $H = 1.9 \pm 0.2$ recorded for the $\alpha5\beta2\gamma3$-subunit combination and $H = 1.7 \pm 0.1$ reported earlier for the $\alpha5\beta2\gamma2$-combination [13]. However, the γ2- and the γ3-subunits may confer a different GABA-sensitivity to recombinant receptors. While a half-maximal response to GABA was obtained at a K_a-value of 4.9 ± 1.2 μM for the $\alpha5\beta2\gamma3$-subunit combination, a K_a value of 14 ± 3 μM was reported for the $\alpha5\beta2\gamma2$-subunit combination expressed in *Xenopus* oocytes [13]. Preliminary analysis of GABA dose–response curves of the subunit combinations $\alpha1\beta2\gamma2$, $\alpha1\beta2\gamma3$, $\alpha3\beta2\gamma2$ and $\alpha3\beta2\gamma3$ expressed in *Xenopus* oocytes indicates that γ3 confers a 3–4-fold higher GABA-sensitivity to the recombinant $GABA_A$-receptors than the γ2-subunit (S.K., unpublished observations). Thus, it is conceivable that the γ3-subunit gives rise to receptors in situ with a higher sensitivity to GABA than those containing the γ2-subunit.

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