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Point Mutations of the $\alpha1\beta2\gamma2$ γ-Aminobutyric Acid$_A$ Receptor Affecting Modulation of the Channel by Ligands of the Benzodiazepine Binding Site

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SUMMARY

Clinically relevant benzodiazepines allosterically stimulate neurotransmitter-evoked chloride currents at the γ-aminobutyric acid type A (GABA$_A$) receptor. Rat wild-type or mutated $\alpha1$, $\beta2$, and $\gamma2S$ subunits were coexpressed in *Xenopus* oocytes and investigated with electrophysiological techniques. Point mutations in two subunits were identified that affect the response of γ-aminobutyric acid (GABA)-induced currents by benzodiazepines. Mutation of one of three amino acid residues to alanine (αTyr^{161} and αThr^{206}) or leucine (γPhe^{77}) resulted in a ~3-fold increase in potentiation by diazepam. The response to zolpidem was increased in two mutant channels containing the mutated α subunit but was nearly absent in channels containing the mutated γ subunit. In the former cases, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) acted as a negative allosteric modulator of the channel, much stronger than in the wild-type channel, whereas there was no significant difference to the wild-type channel in the latter case. Thus, the mutant γ subunit has different functional consequences for the various types of ligand of the benzodiazepine binding site. All three amino acid residues, αTyr^{161}, αThr^{206}, and γPhe^{77}, are close or identical to homologous residues that are implicated in GABA binding. If the residues binding the channel agonist GABA are located at subunit interfaces, the residues influencing the benzodiazepine effects must also be located at subunit interfaces.

Responsiveness to benzodiazepines is a distinctive property of the GABA-gated receptor channels, which are ubiquitous and abundant in the mammalian brain. The structural determinants of GABA$_A$ receptors underlying benzodiazepine action are of significant interest due to the widespread clinical use of some benzodiazepines for their anxiolytic, sedative, muscle relaxant, and anticonvulsive properties. The GABA$_A$ receptor has been purified (1), and after the initial cloning of two subunits (2), 16 different subunits have been cloned (for reviews, see Refs. 3–5). The receptor is believed to have a pentameric structure (6) and belongs to the superfamily of ligand-gated ion channels (2). These neurotransmitter-gated ion channels are composed of transmembrane subunits that share a common organization of their hydrophilic and hydrophobic domains. In the GABA$_A$, nicotinic acetylcholine, and glycine receptors, affinity labeling and site-directed mutagenesis have revealed that the large extracellular amino-terminal segment contains the neurotransmitter binding domain. For these receptors, it has been proposed that the neurotransmitter binding sites are formed by multiple loops at the interface of subunits (7). Supporting this model, several amino acid residues have been identified in the GABA$_A$ receptor $\beta2$ subunits (Tyr^{157}, Thr^{160}, Thr^{202}, Tyr^{205}) (8) and $\alpha1$ subunit (Phe^{64}) (9, 10). A functional γ subunit is required for modulation of the channels by benzodiazepines (11, 12). The benzodiazepine pharmacology of a GABA$_A$ receptor subtype critically depends on the particular α subunit isoform that is present (13). Thus, α and γ subunits are both thought to contribute to the benzodiazepine binding site. The imidazopyridine zolpidem, used predominately for its hypnogenic and sedative properties, is unusually selective for $\alpha1$-containing GABA$_A$ receptors (13). The $\alpha2$ and $\alpha3$ subunits confer intermediate zolpidem affinity and the $\alpha5$ subunit confers very low affinity to triple-subunit combinations $\alpha x\beta2\gamma2S$ (14–16). That the γ subunit may indeed contribute to the benzodiazepine site is substantiated by the observation that zolpidem displays very low affinity to $\gamma3$ in contrast to $\gamma2$-containing receptors (17). Also, a point mutation in the $\gamma2$ subunit affects the benzodiazepine pharmacology (18).

At the level of single-channel resolution, it has been shown that diazepam results in an increase in the channel opening

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; HEPES,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

frequency without a change in the single-channel conductance (19). Diazepam itself does not open the $GABA_A$ channels but rather acts as a positive allosteric modulator. The negative allosteric modulator acting at the benzodiazepine site, DMCM, has been shown to decrease channel open frequency (19).

Recently, Galzi and Changeux (7) hypothesized that the benzodiazepine binding site might be located at the interface of two subunits and that loops homologous to the ones that form the GABA binding site are involved in the binding. We tested this hypothesis by analyzing a series of mutants in the amino-terminal region of $GABA_A$ receptor subunits. We show here for the first time extensive functional data on the consequences of point mutations in the rat α1- and γ2S subunits (named α and γ) on benzodiazepine effects, supporting the postulate that the benzodiazepine site may be located in a homologous position to the GABA binding site. Interestingly, we show a point mutation in the γ subunit that leads to a receptor displaying an increased response to diazepam, whereas the response to zolpidem is strongly decreased.

Materials and Methods

Construction of receptor subunits. The cDNAs coding for the α1, β2, and γ2 subunits of the rat $GABA_A$ receptor channel have been described previously (20–22). Oligonucleotide-mediated mutagenesis was performed with a kit supplied by Amersham (Buckinghamshire, UK). Single-stranded DNA of $GABA_A$ receptor subunits cloned in M13 mp18 was used as starting material. Mutants were subcloned in pSPT19 (Pharmacia) and sequenced completely. Template DNAs were linearized, and capped transcripts were synthesized *in vitro* with SP6 polymerase (Promega). Approximately 25 μg of capped RNA was polyadenylated by using 250 units yeast Poly(A) polymerase (Amersham) for 10 min at 30°. After phenol extraction, the cRNA combinations were mixed in equimolar amounts, coprecipitated in ethanol, and stored at −20°.

Expression and functional characterization. *Xenopus leavis* oocytes were prepared, injected, and defoliculated, and currents were recorded as described previously (12). Briefly, oocytes were injected with 50 nl of cRNA dissolved in 5 mM HEPES, pH 6.8. This solution contained the transcripts coding for each of the different subunits at a concentration of 100 nM (calculated from the UV absorption) to allow injection of stoichiometric amounts.

Electrophysiological experiments were performed according to the two-electrode voltage-clamp method at a holding potential of −80 mV with either a home-built or a modified OC-725 (Warner instrument Corp.) amplifier. Allosteric potentiation and inhibition via the benzodiazepine site were measured at a GABA concentration eliciting 5–15% of the maximal current amplitude, measured at 10 mM GABA. GABA was first perfused alone and then in combination with diazepam, zolpidem, or DMCM, respectively. The two current amplitudes were compared. GABA was applied for 20 sec, and a washout period of 4–15 min was allowed to ensure full recovery from desensitization. Unless otherwise indicated, oocytes were only exposed to a single drug in addition to GABA to avoid contamination. For the same reason, the perfusion system was cleansed with dimethylsulfoxide after every experiment with allosteric modulators acting at the benzodiazepine site.

Results

Functional expression of mutant channels. A total of 18 different point mutations have been introduced into the amino-terminal half of α, β, and γ subunits of the $GABA_A$ receptor (Fig. 1 and Table 1). Triple-subunit combinations $\alpha\beta\gamma$ were functionally expressed in *Xenopus* oocytes. All mutants analyzed expressed GABA-activated chloride currents. The current amplitude elicited by GABA was similar to that of wild-type channels for most of the mutants. The mutant α_{Y161A} resulted in the expression of currents amounting to only ~8% of the response from wild-type subunit combinations, with a slow time course of expression, suggesting an effect on the assembly of subunits or gating of the channel.

Only a small decrease in the apparent GABA affinity was

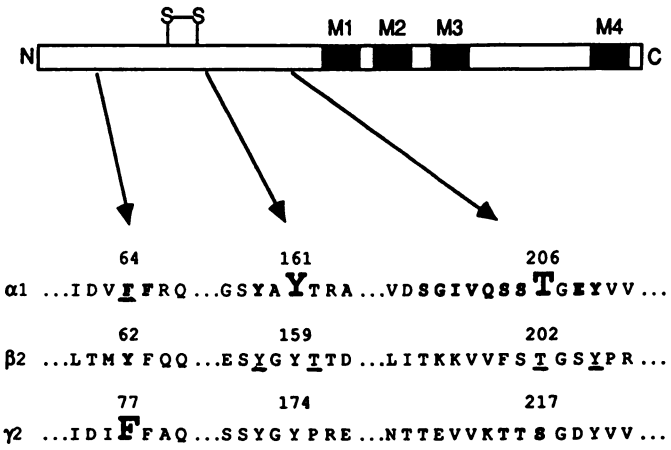

Fig. 1. Localization of amino acid residues (α_{Y161}, α_{T206}, and γ_{F77}) involved in enhanced sensitivity toward benzodiazepine ligands. Channels containing the mutant γ_{F77L} display an increased sensitivity to diazepam but a decreased sensitivity to zolpidem. These three residues are located in the amino-terminal domains between the disulfide bridge and the first membrane spanning domain (*M1*). *Bold*, amino acid residues mutated (except α_{H101}, which is not shown). *Underlined*, residues implicated in GABA binding (8–10).

TABLE 1

Modulation of the GABA current

Modulation of currents by diazepam, zolpidem, or DMCM has been measured at a GABA concentration eliciting ~10% of the maximal current amplitude. Data are given as mean ± standard deviation (number of oocytes/number of different batches used). Data have been analyzed with the *U* test of Wilcoxon, Mann and Whitney, by comparing the stimulation of the individual mutant with that of wild-type receptors in the same batch of oocytes. Mutants displaying a significantly different stimulation ($\alpha \leq 0.1$, in at least three different batches of oocytes) are indicated in bold.

Subunit combination	Relative current amplitude in the presence of		
	1 μM Diazepam	1 μM Zolpidem	0.3 μM DMCM
	%		
$\alpha\beta$	103 ± 7 (16/6)	105 ± 8 (16/6)	
$\alpha\beta\gamma$	149 ± 29 (63/17)	158 ± 46 (44/16)	86 ± 8 (8/3)
$\alpha_{F64L}\beta\gamma$[a]	118 ± 7 (6/2)	117 ± 8 (3/1)	87 ± 15 (2/2)
$\alpha_{F65L}\beta\gamma$	139 ± 33 (13/4)	129 ± 26 (10/4)	75 ± 15 (2/2)
$\alpha_{H101A}\beta\gamma$	124 ± 10 (3/1)	114 ± 11 (3/1)	
$\alpha_{Y159A}\beta\gamma$	176 ± 4 (3/1)	139 ± 21 (3/1)	
$\alpha_{Y161A}\beta\gamma$	**260 ± 36 (13/4)**	**329 ± 87 (12/4)**	**65 ± 4 (4/1)**
$\alpha_{S199A}\beta\gamma$	137 ± 9 (3/1)	161 ± 45 (4/2)	
$\alpha_{G200A}\beta\gamma$	148 ± 6 (3/1)	187 ± 65 (3/1)	
$\alpha_{I201A}\beta\gamma$	158 ± 37 (3/1)	176 ± 41 (3/1)	
$\alpha_{V202A}\beta\gamma$	152 ± 18 (3/1)	132 ± 19 (3/1)	
$\alpha_{Q203A}\beta\gamma$	127 ± 14 (11/4)	122 ± 16 (5/2)	
$\alpha_{S204A}\beta\gamma$	174 ± 34 (6/2)	151 ± 47 (6/2)	
$\alpha_{S205A}\beta\gamma$	179 ± 35 (7/2)	177 ± 64 (6/3)	
$\alpha_{T206A}\beta\gamma$	**231 ± 18 (12/3)**	**263 ± 50 (11/4)**	**59 ± 6 (4/1)**
$\alpha_{E208A}\beta\gamma$	199 ± 45 (8/2)	186 ± 37 (7/2)	
$\alpha_{Y209A}\beta\gamma$	122 ± 13 (13/4)	106 ± 12 (7/2)	
$\alpha\beta_{Y62L}\gamma$[a]	196 ± 62 (19/5)	223 ± 96 (13/5)	85 ± 7 (5/2)
$\alpha\beta\gamma_{F77L}$[a]	**216 ± 27 (29/6)**	**111 ± 5 (19/6)**	**90 ± 2 (5/2)**
$\alpha\beta\gamma_{S217A}$	188 ± 33 (6/2)	154 ± 30 (5/2)	

[a] These mutants have been described by Sigel *et al.* (9).

observed between wild-type (~5 μM) and most mutant triple combinations (less than a factor of three). Exceptions are α_{F64L} and homologous mutations in the β (Tyr^{62} to Leu^{62}) and γ (Phe^{77} to Leu^{77}) subunits, which display a 200-, 30-, and 6-fold shift toward lower affinities, respectively, and have been described previously (9). Desensitization of the GABA currents did not visibly differ between wild-type and mutant channels.

Channels with altered diazepam responses. Most of the mutants constructed (Fig. 1) behaved in a manner similar to that of the wild-type channel with respect to allosteric stimulation by diazepam and zolpidem (Table 1). The stimulation of the GABA current by diazepam is compared between the wild-type and the subunit combination containing the point mutation α_{Y161A}. Representative current traces are shown in Fig. 2. Remarkably, three mutants (α_{Y161A}, α_{T206A}, and γ_{F77L}) showed a much stronger potentiation by diazepam than by the wild-type in the same batch of oocytes ($\alpha \leq 0.05$, *U* test of Wilcoxon, Mann and Whitney; Fig. 3). Channels containing the mutant β_{Y62L} subunit behaved variably in different batches of oocytes. In two of five injections, the potentiation was significantly increased, but in three experiments, it was similar to the wild-type control. To ensure that the concentration of diazepam used in the screening (1 μM) was sufficiently high, diazepam dose-response curves were measured, and data in Fig. 4 confirm that this concentration was near-saturation for all subunit combinations tested. There was no significant effect of the mutations on the apparent affinity for diazepam. Diazepam did not affect the maximal current amplitude elicited by GABA or induce by itself any current.

At the beginning of the present work, we expected to identify mutants with a reduced benzodiazepine modulation, and indeed the point mutations α_{H101}, α_{Q203A}, and α_{Y209A} apparently result in a reduced stimulation by diazepam. However, the apparent reduction in each of these mutant channels was not in every case significant ($\alpha > 0.1$) compared with the wild-type subunit combination in the same batch of oocytes (see below). This could be due to the low number of oocytes that can be examined in a single batch. Therefore, it cannot be excluded that the benzodiazepine modulation is reduced in channels containing α_{H101A}, α_{Q203A}, or α_{Y209A}. More sensitive methods are required to prove this.

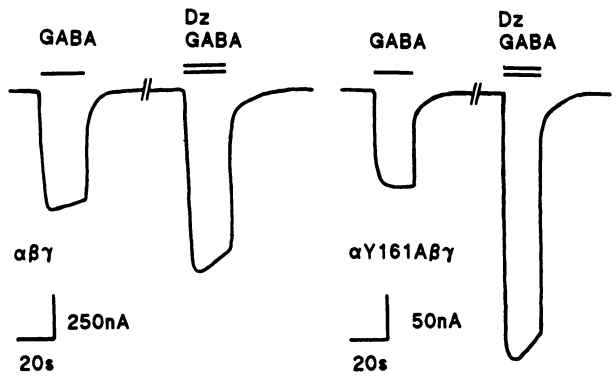

Fig. 2. Stimulation of GABA elicited currents by diazepam. Sample tracings of currents recorded under voltage-clamp conditions from oocytes injected with wild-type αβγ and the mutant $\alpha_{Y161A}\beta\gamma$ $GABA_A$ receptor. Because diazepam (*Dz*) enhancement of the currents depends on the GABA concentration used, the concentration of GABA eliciting ~10% of the maximal current amplitude was determined first. Stimulation was calculated by comparison of the current amplitudes backextrapolated to the time of half-maximal response.

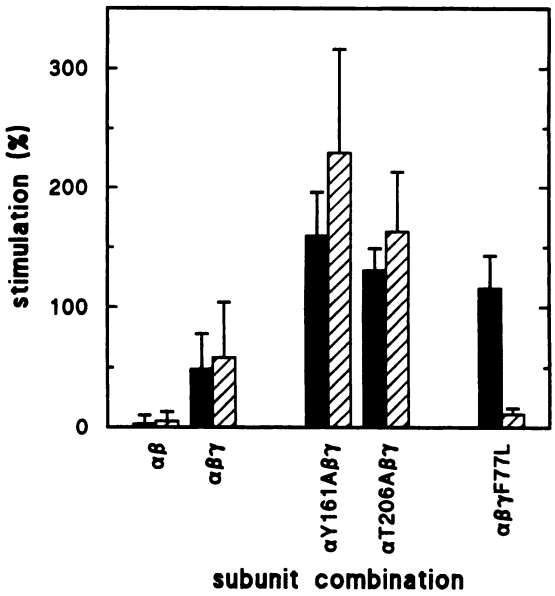

Fig. 3. Comparison of wild-type with channels carrying an interesting point mutation. Stimulation of the GABA currents by diazepam (*filled bars*) and by zolpidem (*striped bars*). *Error bars*, standard deviation for ≥11 oocytes.

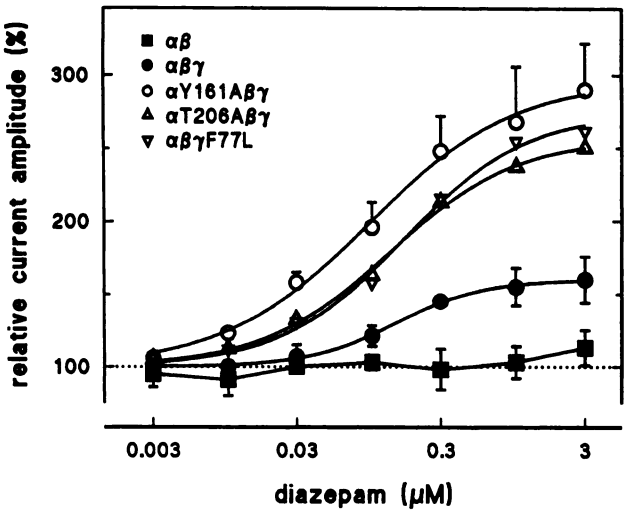

Fig. 4. Cumulative diazepam dose-response curves for mutant and wild-type channels. After establishment of the GABA concentration eliciting ~10% of the maximal current amplitude, the same concentration of GABA was coapplied with increasing amounts of diazepam. *Error bars*, standard deviation for three oocytes. In some cases, error bars fall within the symbols. A second experiment with three oocytes from a different donor animal gave similar data. After standardization and subtraction of the control current response elicited by GABA (100%), data points from the triple-subunit combinations were fitted to the equation $I(c) = I_{max}/(1 + (SC_{50}/c)^n)$, where $I(c)$ is the relative current amplitude elicited, c is the diazepam concentration, I_{max} is the maximal current in the presence of diazepam, SC_{50} is the diazepam concentration eliciting half-maximal stimulation, and n is the Hill coefficient.

Channels with altered zolpidem responses. Two of the mutants studied here, which show a ~3-fold enhancement by diazepam (α_{Y161A} and α_{T206A}), are also important for zolpidem stimulation (Table 1 and Figs. 2 and 3). Most remarkably, zolpidem potentiation of channels containing γ_{F77L} is nearly abolished, whereas diazepam potentiation is increased (Fig. 5). Dose-response curves for zolpidem with the wild-type receptor or the subunit combination containing

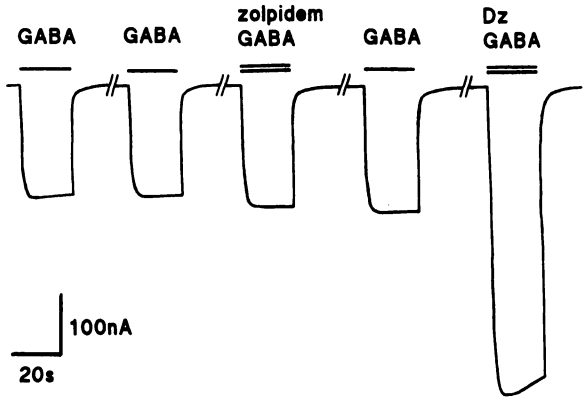

Fig. 5. An amino acid change in the γ subunit leading to supersensitivity toward diazepam (*Dz*) fails to respond to zolpidem. The channel containing the point mutation γ_{F77L} was expressed and exposed twice to an application of GABA. Subsequently, zolpidem was coapplied with GABA, washed out for 10 min. GABA was then applied alone and then in combination with diazepam. Although zolpidem had little effect on the GABA response, diazepam resulted in a large stimulation of the current amplitude.

α_{Y161A} indicated that here the concentration used in the screening (1 μM) was near-saturation (data not shown). The mutants that show a nonsignificantly reduced potentiation by diazepam (α_{H101A}, α_{Q203A}, and α_{Y209A}) show a significantly reduced potentiation by zolpidem ($\alpha < 0.05$).

Effect of other substances. The negative allosteric modulator DMCM was applied in combination with GABA to determine whether an increased sensitivity for diazepam is paralleled by an increased or a decreased sensitivity toward DMCM. Of the three point mutations resulting in channels with enhanced sensitivity toward diazepam, two resulted also in an enhanced sensitivity toward DMCM (Table 1). Interestingly, the mutation γ_{F77L}, which resulted in channels showing a decreased zolpidem enhancement, showed no altered response compared with the wild-type channel (Table 1).

With 1 μM concentration of the benzodiazepine antagonist Ro 15–1788 coapplied with GABA, no potentiation could be observed in the mutant channels containing either of these three mutants (α_{Y161A}, α_{T206A}, or γ_{F77L}) (data not shown). Thus, the amino acid residues identified here have different functional consequences than the mutant γ_{T142S} identified by Mihic *et al.* (18).

There are a number of other classes of compounds that affect GABAergic function by interacting with the $GABA_A$ receptor at sites other than the benzodiazepine binding site (for reviews, see Refs. 5 and 23). Thus, it was interesting to see whether these types of modulation were affected by the mutations affecting the benzodiazepine stimulation. The mutations α_{Y161A}, α_{T206A}, and γ_{F77L} did not affect pentobarbital (1 mM) activation or 5α-pregnan-3α-ol-20-one (1 μM) potentiation measured at a GABA concentration eliciting 10% of the maximal current amplitude (data not shown). This suggests that the stimulation seen in receptors containing these mutants is specific to benzodiazepines and does not generalize to other classes of modulators of $GABA_A$ receptor function.

Discussion

Mutations with an altered response to ligands of the benzodiazepine binding site. In this study, we used functional expression of $GABA_A$ receptor channels carrying a point mutation to find amino acids important for benzodiazepine effects. Stimulation of GABA-induced currents by benzodiazepines depends on the concentration of GABA relative to the concentration of GABA that elicits half-maximal current amplitude. Therefore, modulation was determined at a GABA concentration eliciting 5–15% of the maximal current amplitude in a given channel. Mutation of at least three amino acid residues (α161, α206, and γ77) were found to enhance stimulation by diazepam and also show different responses toward other ligands of the benzodiazepine binding site (see Results). Mutations resulting in a strong phenotype were verified in several independent experiments.

That the altered response to ligands of the benzodiazepine binding site is not simply due to a general change in channel conformation is also indicated by the fact that these mutants showed either a small or no effect on the apparent affinity for GABA for the channel gating. Also, pentobarbital activation of $GABA_A$ receptors and 5α-pregnan-3α-ol-20-one potentiation of GABA currents remained unaffected.

Two of the amino acids identified here are located in the α subunit, Tyr^{161} and Thr^{206}, and one is located in the γ subunit, Phe^{77}. The α1 subunit is the principal site of [^{3}H]flunitrazepam photoaffinity labeling (24). In agreement with this finding, *in vitro* binding studies identified several amino acid residues in α1, α3, α4, and α6 as being involved in benzodiazepine binding (25–28). All residues identified in these studies correspond to either His^{101}, Thr^{162}, Gly^{200}, or Val^{211} of the α1 sequence.

Separation of diazepam and zolpidem effects by a mutation in the γ subunit. In two cases (α_{Y161A} and α_{T206A}), an enhanced sensitivity to diazepam paralleled an enhanced sensitivity to zolpidem (Table 1). However, in the case of γ_{F77L}, diazepam effects were enhanced, whereas zolpidem effects were nearly abolished. Preliminary results from binding experiments indicate that indeed the binding affinity for zolpidem is drastically reduced.[1] This is a surprising finding, as the type of benzodiazepine pharmacology has so far been mainly ascribed to the α subunit isoform (25–28). An exception is the mutant γ_{T142S}, in which the benzodiazepine antagonist flumazenil acts as a positive allosteric modulator (18) and potentiation of the GABA response by diazepam is rather increased, whereas stimulation by zolpidem is decreased.

Supersensitivity to diazepam parallels, in most cases, supersensitivity to DMCM. In the two cases (α_{Y161A} and α_{T206A}) in which an enhanced sensitivity to diazepam paralleled an increased sensitivity to zolpidem, the mutant channels also showed a supersensitivity to DMCM (Table 1). This indicates that in each case the alanine replacement made the channels more sensitive to allosteric modulatory compounds regardless of whether it was positively or negatively acting. Again, the mutation in the γ subunit (γ_{F77L}) was an exception. The behavior in response to DMCM could not be differentiated from the behavior of the wild-type channel.

Homology of the benzodiazepine site with the GABA site. At least three amino acid residues have been identified here that markedly affect stimulation via the benzodiazepine site. They are either in direct contact with diazepam, zolpidem, and DMCM or are involved in allosteric interaction with the

[1] A. Buhr and E. Sigel, unpublished observations.

benzodiazepine site. The residue α_{T206} is located in a region in which residues have already been identified as important for the binding affinity of benzodiazepine to other α subunit isoforms (homologous to α1 Gly^{200}, Val^{211}; Refs. 25 and 28). Furthermore, it is close to Gln^{203} and Tyr^{209}, which are important for a reduction in zolpidem effects. Residue α_{T206} is in fact exactly homologous to β_{T202}, which is assumed to directly interact with the agonist GABA (8). Interestingly, an additional residue, γ_{F77}, has been identified here as important for benzodiazepine function. This residue is located in a subunit region that has never before been implicated in benzodiazepine function. Residue γ_{F77} is homologous to α_{F64} (Fig. 1), which can be photolabeled by the agonist muscimol (10) and is important for apparent GABA affinity of the channel gating (9). Similarly, the residue α_{Y161} is in close proximity to Tyr^{159} and Thr^{162}, amino acid residues homologous to residues in the β subunit that have been identified to be important for GABA affinity by Amin and Weiss (8).

Thus, the amino acid residues identified in this study as well as the ones identified in binding studies (25–28) are located in regions homologous to regions suggested to form the GABA binding site. As the latter binding site is thought to be located at subunit interfaces, the regions influencing benzodiazepine effects must also be located at subunit interfaces. The amino acid residues thought to be involved in the binding of GABA are located in homologous regions to the agonist binding site in nicotinic acetylcholine receptors and the glycine receptor (for a review, see Ref. 7). Photoaffinity labeling experiments have indicated a direct contact of these amino acid residues with agonist or antagonists of the respective channels. It is likely that at least some of the residues identified here directly interact with benzodiazepines.

Mutagenesis studies introducing new residues in the identified positions and including new positions as well as binding studies are under way in our laboratory. It will also be interesting to see which type of pharmacology each modification causes. Preliminary results with the γ mutant identified here indicate that not only type I benzodiazepines are affected by this mutation.[2] Photoaffinity labeling experiments will contribute further information. However, final verification of all structural predictions has to await crystallization and structural resolution of the $GABA_A$ receptor.

Acknowledgments

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