Subtle Changes in Residue 77 of the γ Subunit of α1β2γ2
GABA<sub>A</sub> Receptors Drastically Alter the Affinity for Ligands
of the Benzodiazepine Binding Site

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Recombinant α1β2γ2 γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors were functionally expressed in Xenopus oocytes. Upon the mutation F77L, diazepam and Ro 15-1788 retained the ability to interact with the benzodiazepine binding site, but zolpidem lost this ability. To quantify these data, radioligand binding experiments were performed using membrane preparations of transiently transfected human embryonic kidney 293 cells. The amino acid γ77, phenylalanine, was also mutated to tyrosine, tryptophan, and isoleucine. Although there was little effect on Ro 15-1788 binding upon mutation to tyrosine, the loss in affinity for diazepam was from 12 to 2,720 nM. The change to leucine, in contrast, resulted in little change in the diazepam affinity, whereas there was a strongly reduced affinity for zolpidem from 17 to 4,870 nM and for methyl 6,7-dimethoxy-4-ethyl-carboline-3-carboxylate (DMCM) from 1.9 to 1,780 nM, respectively. The change to tryptophan resulted in two-phasic displacement curves, and only about 50% of the [3H]flunitrazepam binding could be displaced by zolpidem, DMCM, and Ro 15-1788, respectively, whereas midazolam and diazepam still resulted in 100% displacement, indicating the presence of two sites upon expression of this mutant receptor. Functional expression in Xenopus oocytes showed that all mutant channels displayed a comparatively small change (<4.3-fold) in their apparent agonist affinity and that these channels could still be functionally modulated by ligands of the benzodiazepine binding site. We conclude that subtle changes in γ77 drastically affect benzodiazepine pharmacology and that this residue probably interacts directly with most ligands of the benzodiazepine binding site and therefore defines part of the benzodiazepine binding pocket.

GABA<sub>A</sub> receptors are the major ion channels in mammalian brain conferring neuronal inhibition. Two subunits have initially been purified (1), and their coding DNA has been cloned (2). Later, a total of 15 mammalian subunits have been cloned (for reviews, see Refs. 3–7). They are homologous to subunits of the nicotinic acetylcholine receptor, of the glycine receptor, and the serotonin type 3 receptor, and it is assumed that the natural receptor is a pentameric protein (8).

The GABA<sub>A</sub> receptor is the site of action of benzodiazepines and related compounds (Fig. 1; for review, see Ref. 6). There is a widespread use of some benzodiazepines for their anxiolytic, sedative, muscle relaxant, and anticonvulsive properties, and the structural determinants underlying benzodiazepine action are of interest. Clinically used, sedative benzodiazepines act as positive allosteric modulators of the receptor. The α1 subunit has been described as the major subunit that is photoaffinity labeled by [3H]flunitrazepam (9), and one major labeled amino acid has been identified (10). In agreement with these observations, in vitro binding studies identified several amino acid residues in α1, α3, α4, and α6 to be involved in benzodiazepine binding (11–14). In addition, a γ subunit is required for functional modulation of the channels by benzodiazepines (15, 16). Point mutations in the γ2 subunit also affect the benzodiazepine pharmacology (17, 18) in these functional experiments. The view that γ subunits are essential for the formation of the benzodiazepine binding site was recently confirmed with γ2-less mice (19). Thus, α and γ subunits are both thought to contribute to the benzodiazepine binding site, but its structural localization remains to be described.

The imidazopyridine zolpidem selectively binds to α1-containing GABA<sub>A</sub> receptors and is able to displace diazepam there (20). The α2 and α3 subunits confer intermediate zolpidem affinity and the α6 subunit very low affinity to triple subunit combinations α3β2γ2 (21–23). That the γ subunit may indeed contribute to the zolpidem site is substantiated by the observation that zolpidem displays very low affinity to γ3, in contrast to γ2-containing receptors (24, 25).

Recently, we have identified the amino acid side chains α1 161 and α1 206, and γ2 77 as important for increased stimulatory effects of diazepam in α1β2γ2 receptor channels expressed in Xenopus oocytes at the functional level (18). Interestingly, γF77L resulted in an almost complete loss of zolpidem effects. We attempted to understand this phenomenon better. We report drastic consequences of four different point mutations of the wild type phenylalanine in the γ2 subunit for ligand specificity of the benzodiazepine binding site. Some binding data indicate the presence of two benzodiazepine binding sites. A probable way of interaction of the wild type channel with different ligands is discussed. Our work represents an initial step leading to a rational design based on the identification of structural determinants on the receptor protein.

MATERIALS AND METHODS

Construction of Receptor Subunits—The cDNAs coding for the α1, β2, and γ2 subunits of the rat GABA<sub>A</sub> receptor channel have been described elsewhere (26–28). The mutant γF77L has a phenylalanine to leucine substitution at position 77 of the mature peptide and has been described before (18, 29). The mutant subunits γF77V, γF77I, and γF77W.
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**FIG. 1. Chemical structure of benzodiazepine binding site ligands.** Diazepam, flunitrazepam, midazolam, and Ro 15-1788 have a benzodiazepine structure. Midazolam and Ro 15-1788 have an imidazole ring substituent, and Ro 15-1788 lacks the phenyl substituent. Zolpidem, CI 218872, and DMCN have a non-benzodiazepine structure.

were prepared using the QuikChange™ mutagenesis kit (Stratagene). For cell transfection, the cDNAs were subcloned into the polylinker of pBICMV (30). This expression vector allows for high level expression of a foreign gene under control of the cytomegalovirus promoter. The α subunit was cloned into the EcoRI, and the β and γ subunits were subcloned into the SalI site of the polylinker by standard techniques. Electrophysiological experiments were performed by the two-electrode voltage-clamp method at a holding potential of −80 mV. To quantify GABA sensitivity, agonist concentrations between 0.03 and 10,000 M were applied for 20 s, and a washout period of 4–15 min was allowed to ensure full recovery from desensitization. Current responses were fitted to the Hill equation: \( I = I_{\text{max}} \left(1 + \frac{[L]}{EC_{50}}\right)^n \), where \( I \) is the current amplitude at a given concentration of GABA (A), \( I_{\text{max}} \) is the maximum current amplitude, \( EC_{50} \) is the concentration of agonist yielding half-maximal current amplitudes, and \( n \) is the Hill coefficient. Allosteric potentiation via the benzodiazepine site was measured at a GABA concentration eliciting 5–15% of the maximal GABA current amplitude by coapplication of GABA and the drugs acting at the benzodiazepine binding site. Ro 15-1788, in contrast to the other ligands of the benzodiazepine binding site used here, developed its effects not instantaneously. Therefore, it was preapplied for 30 s to ensure a rapid onset of action upon perfusion with GABA. Unless mentioned otherwise, oocytes were only exposed to a single drug in addition to GABA, to avoid contamination, and the perfusion system was cleaned by washing with dimethyl sulfide for the same reason.

**Transfection of Recombinant GABA\(_{\gamma}\) Receptor in Cultured Cells**—The cells were maintained in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin by standard cell culture techniques. Equal amounts (total of 20 µg of DNA/90-mm dish) of plasmids coding for GABA\(_{\gamma}\) receptor subunits were transfected into human embryonic kidney 293 cells (ATCC CRL 1573) by the calcium phosphate precipitation method (32). After overnight incubation, the cells were washed twice with serum-free medium and refed with medium. Wild type receptor and triple subunit combinations expressed well as indicated by the respective \( B_{\text{max}} \) of 1.1–6.0 pmol binding sites/mg of protein, with an average of 2.9 ± 1.4 pmol, as determined by radioligand binding assays. An exception was the tryptophan mutant, which resulted in the expression of 0.59–0.64 pmol binding sites/mg of protein.

**Membrane Preparation**—Approximately 60 h after transfection the cells were harvested by washing with ice-cold phosphate-buffered saline (pH 7.0) and centrifuged at 150 × g. Cells were washed and stored with buffer containing 10 mM potassium phosphate, 0.1% BSA, 0.1 mM EDTA (pH 7.4). Cells were homogenized by sonication in the presence of 10 µM phenylmethylsulfonyl fluoride and 1 mM EDTA. Membranes were collected by three centrifugation-resuspension cycles (100,000 × g for 20 min) and then used for GABA ligand binding stored at −20 °C.

**Binding Assays**—Resuspended cell membranes (0.2–0.5 ml) were incubated for 90 min on ice in the presence of \([\text{3H}]\text{Ro 15-1788}\) (87 Ci/mmol, DuPont NEN) or \([\text{3H}]\text{flunitrazepam}\) (86 Ci/mmol, DuPont NEN) and various concentrations of competing ligands. Membranes (20–80 µg of protein/filter) were collected by rapid filtration on GF/C filters presoaked in 0.3% polyethyleneimine. After three washing steps with 4 ml of buffer, the filter-retained radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 100 µM Ro 15-1788 or 10 µM flunitrazepam, respectively. Data were fitted by using a nonlinear least squares method to the equation \( B(c) = B_{\text{max}} \cdot c(1 + [K_d] + c) \) for displacement curves with a single component, where \( c \) is the concentration of ligand; \( B \), binding; \( B_{\text{max}} \), maximal binding; \( K_d \), dissociation constant; and \( n \), Hill coefficient. Displacement curves \( IC_{50} \) values were converted to \( K_i \) values according to the Cheng-Prusoff equation (33). In the case of the tryptophan mutant, for some ligands assuming two components yielded the better fit. Protein concentration was determined with the Bio-Rad protein assay kit with bovine serum albumin as standard.

**RESULTS**

**Zolpidem Cannot Compete with Diazepam in the γF77 Mutant**—The dual subunit combination α1β2 or triple subunit combinations α1β2γ2 were functionally expressed in *Xenopus* oocytes and characterized using electrophysiological techniques. Diazepam displays approximately a 3-fold enhancement of the stimulation of GABA currents after the point mutation F77L in the γ subunit, whereas zolpidem alone almost lost the ability to affect GABA currents (18; Table I). The effects of all compounds tested were independent of the size of the current amplitude expressed from a given subunit combination or the time point after injection of the oocytes with the corresponding cRNAs.

It was interesting to know whether zolpidem fails to bind mutated channels or whether the zolpidem still binds and only fails to stimulate GABA currents in these channels. To answer this question, we performed electrophymiological experiments and tried to counteract the stimulatory effects of 1 µM diazepam with increasing amounts of zolpidem or of the antagonist Ro 15-1788 in cumulative dose-response curves, respectively (data not shown). 1 µM Ro 15-1788 did not alter GABA responses in mutant and wild type channels. In Fig. 2A it is shown that 1 µM Ro 15-1788 can completely reverse the stimulatory effect of

**TABLE I**

| Substance(s)          | α1 | α1γ | α1γF77L |
|-----------------------|----|-----|--------|
| 1 µM diazepam         | 103 ± 7 | 149 ± 29 | 216 ± 27 |
| 1 µM zolpidem         | 105 ± 8 | 158 ± 46 | 111 ± 5  |
| 1 µM diazepam + 1 µM Ro 15-1788 | ∼200 ± 8 | 100 ± 8  |
| 0.3 µM diazepam + 3 µM zolpidem | ∼200 ± 8 | 100 ± 8  |

* a, not determined.

The effect of benzodiazepine binding site ligands on GABA currents was measured as indicated under “Materials and Methods.” Data are given as mean ± S.D. of at least five determinations performed in at least two batches of oocytes.
diazepam. In contrast to this observation, the effect of 0.3 μM diazepam cannot be reduced by 3 μM zolpidem (Fig. 2B and Table I). This lower concentration of diazepam was chosen from a dose-response curve for the stimulation of GABA currents by diazepam and results in about 70% of the maximal stimulation in mutant channels (18). Based on our experiments, we conclude that Ro 15-1788 can but zolpidem cannot compete with diazepam in mutant αβγF77L channels.

Point Mutation of γF77L Drastically and Differentially Affects the Binding Affinity of Benzodiazepine Site Ligands—These electrophysiological experiments resulted in a good description of functional effects, but only in a qualitative assessment of the interaction with ligands of the benzodiazepine binding site with the GABA<sub>α</sub> receptor. A quantitative description of the binding was obtained using equilibrium binding of [3H]Ro 15-1788 to membrane preparations of human embryonic kidney cells transiently transfected with αβ2γ2 GABA<sub>α</sub> receptor cDNAs or transfected with the triple combination containing the mutant γF77L subunit. Binding occurred in each case to a single class of binding sites (Fig. 3A). The affinity for Ro 15-1788 was reduced about 28-fold in the mutant channels compared with the wild type combination (Table II). However, it should be noted that the affinity for this ligand was still relatively high (17 nM). The binding specificities of wild type and mutant receptors were analyzed for a number of other modulatory drugs acting at the benzodiazepine binding site. Data are summarized in Table II. The affinity for the non-benzodiazepine ligands tested was most affected, displaying Ki values above 1 μM. In contrast, diazepam retained medium affinity (about an 8-fold decrease). Most remarkably, the affinity of the β-carboline DMCM was decreased 940-fold for the mutant receptor (Fig. 3B and Table II). The affinities for zolpidem and Cl<sub>218872</sub> were reduced 325- and 130-fold, respectively. Best fit was obtained assuming a Hill coefficient of 1.0 in all cases. Thus, all drugs tested displaced [3H]Ro 15-1788 apparently by binding to a single, noncooperative site and displaced the radioactive ligand to the same extent as high concentrations of nonradioactive Ro 15-1788.

Point Mutations γF77I, γF77Y, and γF77W—Further point mutants were then prepared and studied in the same way. Fig. 4 shows a similar experiment comparing the binding of [3H]Ro 15-1788 to wild type receptors and receptors containing the mutated γF77L subunit. Although there was very little effect on Ro 15-1788 affinity (Fig. 4A), the displacement of [3H]Ro 15-1788 by diazepam (Fig. 4B) was drastically affected, showing a 230-fold decrease in diazepam affinity (Fig. 4B and Table II). Interestingly, the same mutation resulted in an about a 7- and
TABLE II

Binding affinities of different benzodiazepine binding site ligands

| Subunit Combination | Kd or Ki (relative affinity) |
|---------------------|-----------------------------|
| Ro 15–1788          | Dazepam | Flunitrazepam | Midazolam | Zolpidem | Cl 218872 | DMCM |
| $\alpha$F77Y        | 0.61 ± 0.24 | 12 ± 8 | 3.0 ± 0.8 | 1.8 ± 0.04 | 15 ± 3 | 46 ± 1.3 | 1.9 ± 0.03 |
| $\alpha$F77Y$\gamma$Y$\gamma$TY | 0.97 ± 0.03 | 2,716 ± 0 | 502 ± 2 | 251 ± 3 | 5.4 ± 0.1 | 6.6 ± 0.7 | 92 ± 14 |
| $\alpha$F77Y$\gamma$TY$\gamma$TL | 17 ± 2 | 92 ± 21 | 7.2 ± 3.2 | ND$^a$ | 4,870 ± 50 | 6,100 ± 1,300 | 1,780 ± 760 |
| $\alpha$F77Y$\gamma$TY$\gamma$TL Site 1 | 1,233 ± 52 | 55 ± 4.3 | 4.5 ± 0.7 | 15 ± 1.2 | >10,000 | >10,000 | >10,000 |
| Site 2 | >10,000 | >10,000 | >10,000 | >10,000 | >10,000 |

$^a$ ND, not determined.
$^b$ Data were fitted with a Hill coefficient of 0.7, maybe indicating the presence of two binding sites with different affinity.
$^c$ Saturation binding data could be fitted satisfactorily with a Hill coefficient of 1.0.

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Functional Properties of Mutant Channels—Triple subunit

way, and the results are summarized in Table II. All curves were fitted satisfactorily assuming a Hill coefficient of 1.0.

$[\text{3H}]$Ro 15–1788 displayed no specific binding after expression of the isoleucine mutant-containing triple subunit combination. Using $[\text{3H}]$flunitrazepam, we obtained evidence for the presence of a single type of binding site with almost the same affinity as wild type receptors (Table II). Comparison of receptors containing isoleucine and leucine instead of the wild type phenylalanine surprisingly shows little difference in apparent diazepam affinity, whereas there is 74-fold difference to wild type receptors (Table II). Other non-benzodiazepines displayed no measurable affinity to the isoleucine-containing mutant. The loss in DMCM affinity of >5,000-fold for the change from wild type to the isoleucine mutant is the largest effect observed here (Table II). Again, all curves were fitted satisfactorily assuming a Hill coefficient of 1.0.

Point Mutation γF77 Leads to Heterogeneity of the Binding Sites—$[\text{3H}]$Ro 15–1788 displayed again no specific binding after expression of this triple subunit combination. Saturation binding curves using $[\text{3H}]$flunitrazepam revealed at least a 6-fold reduced affinity compared with wild type receptors, with a $K_d$ of 18 nM. Displacement curves were in this case measured at 6 nM. As indicated under “Materials and Methods,” this mutant resulted in about 20% of the normally observed expression. A tryptophan residue in this position may interfere somewhat with the biosynthesis or assembly in this expression system.

Whereas midazolam and diazepam displaced radioactive flunitrazepam to the same extent as did nonradioactive flunitrazepam, zolpidem, Ro 15–1788, and DMCM displaced only 40–60%. The affinity of zolpidem for this partial displacement was about the same as in the wild type, and the affinities for DMCM and Ro 15–1788 were strongly decreased (Table II). The displacement curve for diazepam was fitted with a Hill coefficient of 0.64, which may indicate the presence of more than one site having a slightly different affinity for diazepam. Cl 218872 almost lost the ability to bind to the mutated receptor (Table II). The displacement curves for diazepam, zolpidem, and Cl 218872 are illustrated in Fig. 5. Obviously, there are two binding sites with different properties present. The implications of this heterogeneous behavior is discussed further below.
membrane preparations of transiently transfected cells. Although the affinity for zolpidem is reduced about 325-fold in the above mutant, the affinity for the antagonist Ro 15-1788 is reduced only 28-fold. From these data, it becomes clear that the concentrations of the ligands used in functional competition experiments were too low to expect displacement of diazepam in the case of zolpidem. Interestingly, the affinity for the β-carboline DMCM was almost 3 orders of magnitude lower than in wild type channels, therefore displaying the largest effect after mutation of γF77 to leucine. Of all the ligands tested, the affinity to diazepam was the least affected. Its binding affinity was reduced only about 8-fold, and it retained a medium affinity. Preliminary functional data show that 1 μM flunitrazepam similar to diazepam increases potentiation of GABA-induced currents and therefore indicate that the mutant channel still has a considerable affinity for this benzodiazepine. This suggestion was also confirmed with binding studies. Interestingly, it appears that the affinities of all benzodiazepines tested are still well below 100 nM after mutation to leucine, whereas the affinities of non-benzodiazepines are much lower (μM range).

**Effects of the γF77 Mutation on Binding Properties—**At the level of binding experiments the leucine mutant was compared with the isoleucine mutant. Although both mutant receptors displayed an affinity toward flunitrazepam similar to that of the wild type, a small decrease in diazepam affinity was observed. However, the affinity for Ro 15-1788 was affected much more strongly in the isoleucine (2,020-fold) than in the leucine (28-fold) mutant. Thus, a simple shift of a methyl group by one carbon atom leads to a 74-fold change. It would be interesting to study the alanine mutant to see whether this is the result of steric hindrance. The γ1 subunit has an isoleucine residue in the position homologous to 77 in the γ2 subunit and also has no measurable affinity for Ro 15-1788 and DMCM; but it still displays relatively high affinity for flunitrazepam. This behavior is reminiscent of our isoleucine mutation. It would be interesting to change the isoleucine in γ1 into a phenylalanine and study the consequences for the Ro 15-1788 affinity.

**Effects of the γF77 Mutation on Binding Properties—**The phenylalanine in position 77 of the γ subunit was also replaced by a more bulky amino acid. The change to tyrosine, i.e. the introduction of an additional hydroxyl group, left the affinity for Ro 15-1788 almost unaffected and even increased the affinities for zolpidem and CI 218872, whereas the affinities for diazepam, flunitrazepam, midazolam, and DMCM were drastically decreased. This indicates that an entity in the latter, but not in the former compounds is not tolerated by the substituted aromatic ring. Furthermore, the interaction of γ77 with classic benzodiazepines and the antagonist Ro 15-1788 is different.

**Effects of the γF77 Mutation on Binding Properties—**Replacement of phenylalanine by tryptophan yielded binding data that could be interpreted as the presence of two binding sites for ligands of the benzodiazepine site on a receptor pen-

### Table III

| Subunit combination | EC50 (μM) | Relative currents in the presence of 0.3 μM diazepam | 1 μM zolpidem |
|---------------------|----------|------------------------------------------------------|---------------|
| α2γ                | 7.6 ± 4.3 (3) | 156 ± 56 (5) | —a |
| α2γF77Y             | 12.4 ± 6.7 (3) | 118 ± 4 (3) | 530 ± 69 (3) |
| α2γF77L             | 3.3 ± 11.4 (3) | 192 ± 19 (3) | — |
| α2γF77T             | 17.3 ± 7.8 (3) | 180 ± 11 (3) | — |
| α2γF77W             | 18.3 ± 11.2 (3) | 196 ± 40 (7) | — |

**Fig. 5.** Evidence for the presence of two sites after the mutation γF77Y. Displacement of binding of [3H]flunitrazepam (6 nM) to membranes prepared from 293 cells transiently transfected with α2β2γ2. Displacement was achieved by including different concentrations of zolpidem (○), diazepam (●), or CI 218872 (▲). Data for zolpidem and CI 218872 were fitted assuming an Hill coefficient of 1 and 50% displacement for zolpidem. Data for diazepam were not satisfactorily fitted assuming a Hill of 1, therefore this coefficient was also fitted and was found to be 0.64, indicating the presence of more than one site. Similar to zolpidem, only about 50% displacement could be observed using Ro 15-1788 and DMCM, up to a concentration of 30,000 nM.

**DISCUSSION**

This study demonstrates that a single amino acid in position 77 of the mature γ2 subunit of α1β2γ2 GABA<sub>A</sub> receptors drastically affects the pharmacology of benzodiazepine site ligands. Interestingly, this residue is directly homologous to α1F64, which has been implied in agonist binding (29, 34). In the following we discuss results of a functional study, of binding experiments, and of displacement of binding by several compounds representative for the various classes of benzodiazepine site ligands, for four different mutations in this position.

**Competition of Ligands of the Benzodiazepine Binding Site on the Functional Level—**Expression in Xenopus oocytes of the γF77L mutant together with α1 and β2 subunits results in ion channels displaying a 3-fold increased stimulatory effect of diazepam compared with wild type channels, whereas the effect of zolpidem nearly disappears. Functional competition experiments indicate that upon mutation the antagonist Ro 15-1788 competes with diazepam in the mutated channel, but zolpidem has lost this ability. Thus, zolpidem apparently fails to bind to mutated channels, whereas Ro 15-1788 still seems to bind to these channels.

**Effects of the γF77L Mutation on Binding Properties—**This interpretation is directly supported by binding experiments to combinations α2γ were expressed functionally in Xenopus oocytes. All mutants analyzed expressed GABA-activated chloride currents of maximal current amplitude comparable to those of wild type channels. Only a small decrease (factor of 1.6–4.3) in the apparent GABA affinity (EC50) was observed between wild type and mutant channels (Table III). For the leucine mutant about a 5-fold reduced agonist affinity has been described before (29). GABA currents by wild type and mutant channels were stimulated by coapplication of 0.3 μM diazepam together with 1–3 μM GABA, indicating that all mutants studied here retain allosteric coupling to the agonist site. As expected for this concentration of diazepam, receptors containing the tyrosine substitution displayed a reduced response to diazepam but retained strong stimulation by 1 μM zolpidem (Table III).
tamer (see “Results”). This finding was surprising, as there was no indication from our binding data on other mutants for a positive or negative cooperativity between two sites. The properties of the two binding sites are very different from each other. If a γ2 subunit always takes part in the formation of a benzodiazepine binding site and there is only one drug binding site/γ subunit interface, this would indicate the presence of two γ2 subunits in a receptor pentamer. Alternatively, the alteration at the γ2 subunit containing site would have to be communicated by allosteric mechanisms to the γ2 subunit-independent site.

An alternative explanation for our data would be the existence of a second pool of receptors with different binding properties. It is however intriguing that the relative abundance of the two populations was about 1:1 in all experiments. On a functional level, after expression of the mutant channels in Xenopus oocytes, no evidence was obtained pointing to a heterogeneity of this site. Namely, cumulative dose-response curves of the stimulation of GABA currents by diazepam did not point to the presence of multiple sites (data not shown).

The presence of more than one binding site on a receptor has also been postulated on the basis of an altered rate of dissociation by the same or other ligands in cerebellum (35). However, as shown by Prinz and Striessnig (36), this cannot be taken as conclusive evidence for the presence of multiple sites. The best indication for the presence of multiple sites has been provided by photoaffinity labeling with [3H]flunitrazepam of brain extracts, where it has been shown that covalent binding of one molecule destroys about four sites for reversible binding (37, 38). The presence of more than one binding site on a receptor for negative allosteric modulators is also suggested from the two-phasic response to these agents in functional measurements of receptors expressed in Xenopus oocytes (39).

Functional Properties of the Mutant Channels—After functional expression in Xenopus oocytes all mutant receptors formed channels with a slightly (1.6–4.3-fold) reduced apparent affinity for the agonist GABA. The agonist binding site is allosterically coupled to the benzodiazepine binding site. In view of the relatively small changes in the apparent binding affinities for the agonist and the huge changes in the observed binding affinities for ligands of the benzodiazepine binding site, we think it more likely that the latter site is affected directly.

Interaction of Ligands of the Benzodiazepine Binding Sites with the Receptor.—It is clear that there are large effects of the substituent present in position 77 of the γ2 subunit on the specificity of the benzodiazepine binding site. Obviously, the non-benzodiazepines prefer an aromatic substituent in position 77, and addition to the phenyl ring of a hydroxyl group even increases the affinity for zolpidem and Cl 18872. Exchange of the aromatic ring by smaller side chains results in much larger effects for non-benzodiazepines (130–5,000) than for diazepam, flunitrazepam, and midazolam (1.5–8.3). Remarkably, the loss in affinity of mutant receptors is always different between this group of three benzodiazepines and Ro 15-1788.

We suggest that γ77 interacts directly with benzodiazepine binding site ligands. Obviously, different ligands interact in a different fashion with the wild type phenyl residue, but it is of course unlikely that chemically distinct ligands have identical interactions with the receptor site. As both types of ligand compete for binding, their respective binding sites have to overlap.

Conclusion—Small chemical changes in position 77 of γ2 subunit have huge effects on the benzodiazepine pharmacology. The analysis of the molecular definition of the benzodiazepine binding sites should help in the understanding of how these ligands bind to the GABA_A receptor. Such an analysis may provide a rational basis for drug design. The phenylalanine identified here appears to be a key determinant for the activity of zolpidem, DMCM, Cl 218872, and the binding of the antagonist Ro 15-1788 and must be very close to or part of the diazepam binding site of GABAA receptors.

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