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Alterations of the benzodiazepine site of rat $\alpha6\beta2\gamma2$-$GABA_A$ receptor by replacement of several divergent amino-terminal regions with the $\alpha1$ counterparts

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1 The benzodiazepine site of the $\alpha6\beta2\gamma2$ subtype of γ-aminobutyric $acid_A$ ($GABA_A$) receptors is distinguishable from that of the $\alpha1\beta2\gamma2$ subtype by its inability to interact with classical benzodiazepines (i.e., diazepam) and its agonistic response to Ro 15-1788, which behaves as an antagonist in the $\alpha1\beta2\gamma2$ subtype.

2 The point mutation of Arg 100 of the $\alpha6$ subunit to histidine (the corresponding residue in $\alpha1$) has been shown to enable the $\alpha6\beta2\gamma2$ subtype to interact with diazepam but failed in this study to abolish the ability of Ro 15-1788 to enhance GABA-induced Cl^- currents.

3 Here we identified the segment of P161 to L187 of $\alpha6$ to contain the functional region responsible for the agonistic action of Ro 15-1788. Its replacement with the corresponding $\alpha1$ sequence abolished the ability of Ro 15-1788 to enhance GABA currents without appreciable effects on its binding affinity to the benzodiazepine site or on the functionality of the other benzodiazepine site ligands such as diazepam, U-92330 and 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate. These data support the evidence that the functionality of a given ligand could arise from a single region of the benzodiazepine site, not shared by others.

4 In addition we have learned that several residues in the N-terminal region of $\alpha6$, such as R100, V142 and N143, have the ability to influence GABA-dependent Cl^- current induction probably by allosterically modulating low affinity GABA sites.

Keywords: Benzodiazepine site; GABA sensitivity; cloned $GABA_A$ receptors; $GABA_A$ receptor mutants; Ro 15-1788

Introduction

γ-Aminobutyric $acid_A$ ($GABA_A$) receptors are supramolecular receptor-Cl^- channel complexes and exist in combination of various subunits (α, β, γ and δ), each consisting of several isoforms in mammalian brains (Bateson *et al*., 1991; DelOrey & Olsen, 1992; Wisden & Seeburg, 1992; Barnard *et al*., 1993; Harvey *et al*., 1993). Among numerous possible $GABA_A$ receptor subtypes, the $\alpha x\beta2\gamma2$ subtypes, when expressed in human embryonic kidney cells, display many functional properties similar to those of native neuronal receptors and express the benzodiazepine site which is an allosteric modulator site of therapeutic importance (Pritchett *et al*., 1989; Draguhn *et al*., 1990; Verdoorn *et al*., 1990; Puia *et al*., 1991; Im *et al*., 1993). Different α isoforms alter the properties of the benzodiazepine site of the $GABA_A$ receptor subtypes. The point of interest to us is that the benzodiazepine site of the $\alpha6\beta2\gamma2$ subtype of $GABA_A$ receptors is distinguishable from that of the $\alpha1\beta2\gamma2$ subtype by its inability to interact with classical benzodiazepines (e.g., diazepam) (Lüddens *et al*., 1990) and its agonistic response to Ro 15-1788 (Im *et al*., 1993), which behaves as an antagonist in the $\alpha1\beta2\gamma2$ subtype. Recently, mutation of arginine 100 (Arg 100) in the amino (N) terminal region (extracellular) of $\alpha6$ to histidine (the corresponding residue in $\alpha1$) has been shown to enable the $\alpha6\beta2\gamma2$ subtype to interact with a classical benzodiazepine, diazepam (Wieland *et al*., 1992). However, our preliminary study with the mutant showed that Ro 15-1788 still enhanced GABA-currents. This raises the possibility that a N-terminal region(s) of the $\alpha6$ other than Arg 100 is responsible for the functionality of Ro 15-1788. Upon alignment of the N-terminal residues near Arg 100 of α isoforms, three regions have attracted our attention (Figure 1): two of these represent electrostatic charge differences (HN121 for $\alpha6$ vs ED for $\alpha1$ and VN143 for $\alpha6$ vs ED for $\alpha1$) and the third one a hypervariable region from P161 to L187, where 17 out of the 27 residues are divergent. As an initial attempt to map the putative functional region for Ro 15-1788, Arg 100 and the three N-terminal regions were successively mutated to the corresponding residues in the $\alpha1$ subunit; Mutant 1 (R100H), Mutant 2 (R100H, HN121ED), Mutant 3 (R100H, HN121ED and VN142ED), and Mutant 4 (including the segment from P161 to L187 replaced with the corresponding $\alpha1$ sequence in addition to those mutations in Mutant 3). Each mutant was expressed in combination with $\beta2$ and $\gamma2$ subunits in human embryonic kidney cells (HEK 293 cells). From this study we found that the segment from P161 and L187 of $\alpha6$ contains the functional region responsible for abolishing the ability of Ro 15-1788 to enhance GABA-currents without appreciable effect on its binding affinity.

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Methods

Preparation of mutants

Single point mutations were produced by site-directed mutagenesis by use of a Promega kit. Briefly, a fragment (bp 1–1390) of the $\alpha6$ subunit cDNA of $GABA_A$ receptors was isolated with BamHI and PstI restriction enzymes and was cloned into pAlter-1 (a mutagenesis vector from Promega). Mutants were generated by use of appropriate mutagenic oligonucleotides (21 MERs prepared by Genosys), in combination with an oligonucleotide restoring ampicillin resistance in pAlter-1, following the procedures described in the kit. Three mutants were obtained from successive point mutations of R100H, HN121ED and VN142ED. The mutated insert was subcloned back to the original eukaryotic expression vector (Wieland *et al*., 1992). In the fourth mutant, the region from P161 to L187 of $\alpha6$ was replaced with the corresponding $\alpha1$ segment, via

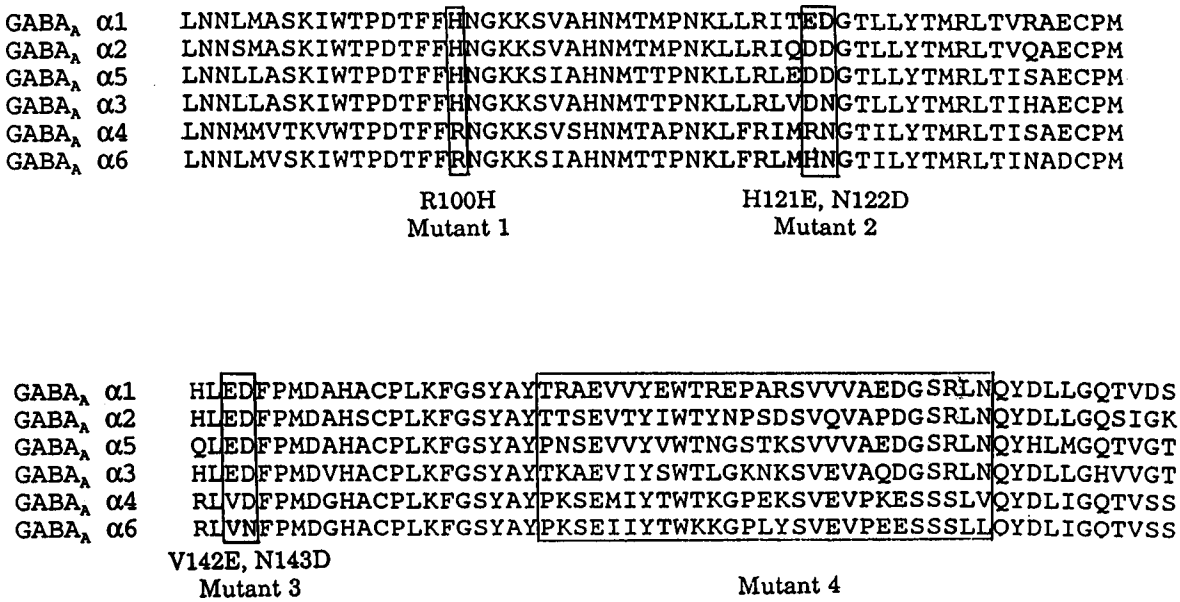

Figure 1 Alignment of the amino acid sequences of the α isoforms of GABA$_A$ receptors near arginine 100. Each mutant was produced from the preceding mutant; Mutant 1 (R100H), Mutant 2 (R100H, HN121ED), Mutant 3 (R100H, HN121ED and VN142ED) and Mutant 4 (including the segment from P161 to L187 replaced with the corresponding α1 sequence in addition to those mutations in Mutant 3).

polymerase chain reaction (PCR). Briefly, a sense oligonucleotide, 5′ TGGATGGACACGCATGTCCCACTAAATTTGGGAGCTAT 3′, contains the α6 sequence from the base 860 to 880, and the α1 sequence from the base 601 to 615. The antisense oligonucleotide, 5′ GTCTCAATAGAAACTGTTTGCCCAATAAGGTCATACTGGTT 3′, contains the α6 sequence from the base 1022 to 997 and the α1 sequence from the base 712 to 699. PCR with the two primers and α1 as the template yielded a product of expected size. The product was digested with DrdI and BfaI, and ligated with the plasmid containing Mutant 3 with matching cohesive ends. All mutations were confirmed by cDNA sequencing and all other procedures for DNA analysis, construction and purification were as described previously (Sambrook *et al*., 1989).

Human embryonic kidney cells (HEK 293 cells, ATCC CRL 1573) were transfected with the vector carrying mutant cDNAs or the wild type α6 in combination with the vectors carrying β2 and γ2 cDNAs, in an equal ratio, in the presence of a transfection-reagent, DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammoniumsulphate, Boehringer Mannheim Gmbh). Preparation of baculovirus constructs (AcNPV) carrying rat cDNAs for GABA$_A$ receptor subunits and the growth of Sf-9 cells in the presence of the recombinant baculovirus were carried out as described previously (Carter *et al*., 1992).

Electrophysiology

The whole cell patch clamp technique (Hamill *et al*., 1981) was used to record the GABA-mediated Cl^- currents in human embryonic kidney cells (HEK293) expressing various combinations of GABA$_A$ receptor subunits. Briefly, the pipette solution contained (in mM): CsCl 140, EGTA 11, $MgCl_2$ 4, ATP 2 and HEPES 10; pH 7.3. Cells were bathed in an external solution containing (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8 and HEPES 5; pH 7.2. GABA and drugs were dissolved in the external solution and were applied through a U-tube placed within 100 μm of the target cell. The recording chamber was superfused with the extracellular solution at a rate of 3 ml min^{-1}. Also the gravitational flow through the U-tube drew the solution from the chamber at a rate of 1.5 ml min^{-1} except for the period of drug application. The current was recorded at room temperature (21–24°C) with an Axopatch 1D amplifier, a CV-4 headstage (Axon Instrument Co.) and a Gould Recorder 220.

Binding studies

Binding of radioactive ligands was measured in membranes obtained from Sf-9 cells expressing recombinant receptors, by use of filtration techniques as described previously (Pregenzer *et al*., 1993). Briefly, binding of [^{3}H]-muscimol or [^{3}H]-Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) was measured in a medium containing (in mM): NaCl 118, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES/Tris 20 (pH 7.3), the radioactive ligand and 30 μg membrane proteins in a total volume of 500 μl. The mixtures were incubated at 4°C for 60 min and filtered over a Whatman GF/B filter under vacuum. The filter was washed three times with 4 ml of the above solution and counted for the radioactivity. Non-specific binding was measured in the presence of excess amounts of cold ligands and was used to compute specific binding. In some experiments, [^{3}H]-Ro 15-4513 binding was measured in the presence of test compounds at various concentrations. The IC_{50} value for a test compound was obtained from dose-response profiles consisting of at least 6 different points and converted to the K_i value with the equation of $K_i = IC_{50}/(1 + [\text{Test ligand}]/K_d$ for Ro 15-4513).

Results

Before the functional characterization of benzodiazepine site ligands, we examined GABA dose-response profiles for Cl^- current induction in the α6β2γ2 subtype and those containing individual mutant α6 subunits. The currents were measured by use of the whole cell patch-clamp technique at a holding potential of −30 mV under a symmetrical Cl^- gradient. The amplitude of Cl^- currents increased as a function of GABA concentrations (Figure 2) and was analysed with a logistic equation, $E/E_{max} = [GABA]^{n_H}/(K_{0.5} + [GABA]^{n_H})$ where $K_{0.5}$ is the half maximal GABA concentration, n_H is the slope factor and E_{max} is the maximal GABA response. Figure 2 shows the data fit after normalization of the current amplitude to the E_{max}. The $K_{0.5}$ for GABA decreased from 2.1±0.3 μM in the wild type to 0.33±0.03 and 0.11±0.01 μM in Mutant 1 (R100H) and 2 (R100H, H121E and N122D), respectively, but approached the wild type value in Mutant 3 (R100H, H121E, N122D, V142E and N143D) and Mutant 4 with the $K_{0.5}$ of 1.2±0.2 and 1.7±0.3 μM, respectively. The slope factor was

not appreciably affected in any of the mutants, ranging from 1.2 to 1.4.

The changes in the half maximal GABA concentration for Cl^- current induction led us to examine whether the mutations alter the high affinity GABA site, which was measured with [^{3}H]-muscimol binding in the membranes of Sf-9 cells infected with the appropriate recombinant baculoviruses. The K_d for muscimol was not appreciably affected by the mutations, being 4.8 ± 0.3, 7.0, 4.2 ± 0.8, 3.0 and 4.4 ± 2 nM for the wild type, Mutant 1, 2, 3 and 4, respectively. These results underscore the view that the high affinity GABA site is not directly related to the half maximal GABA concentration for Cl^- current induction. Probably, low affinity GABA sites may be directly responsible for channel openings.

To characterize the benzodiazepine site of the mutants, we examined the effects of several key benzodiazepine site ligands on GABA-induced Cl^- currents (Figure 3). Diazepam, U-92330 (5-acetyl-3-(5′-cyclopropyl-1′,2′,4′-oxadiazole-3′-yl)-7-chloro-4,5-dihydro [1,5-a] quinoxaline), Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxylate) and 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) have already been established as a classical benzodiazepine agonist, a non-benzodiazepine agonist, an antagonist and an inverse agonist, respectively, for the benzodiazepine site of the $\alpha1\beta2\gamma2$ subtype (Pritchett *et al*., 1989; Puia *et al*., 1992; Petke *et al*., 1992). Thus, no additional data on their action in the $\alpha1\beta2\gamma2$ subtype is included here. In the $\alpha6\beta2\gamma2$ subtype (wild type) (Figure 3), diazepam produced no effect, Ro 15-1788 and U-92330 markedly enhanced the currents as described earlier (Im *et al*., 1993) and DMCM partially increased GABA currents. With the R100H mutation (Mutant 1), diazepam enhanced GABA currents and DMCM inhibited the currents. These properties of Mutant 1 are analogous to those of the $\alpha1\beta2\gamma2$ subtype, as shown in an earlier study (Wieland *et al*., 1992). However, interestingly, Ro 15-1788 still enhanced GABA currents in Mutant 1, suggesting that a region(s) of α6 other than Arg 100 is responsible for the functionality of Ro 15-1788. U-92330 also enhanced the currents in Mutant 1, but this dihydroimidazoquinoxaline analogue has been established as a benzodiazepine-site agonist in both the $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ subtypes (Im *et al*., 1993), indicating its functional pharmacophore(s) insensitive to the α isoforms.

For quantitative comparison of the Ro 15-1788 action in different batches of cloned receptors, we used the ratio of the net, maximal GABA-current increase by Ro 15-1788 to that by diazepam in the same patch. The ratio should be independent of batch to batch variations, because they share the common binding site (see below). The currents were measured in the presence of the drugs at a saturating con-

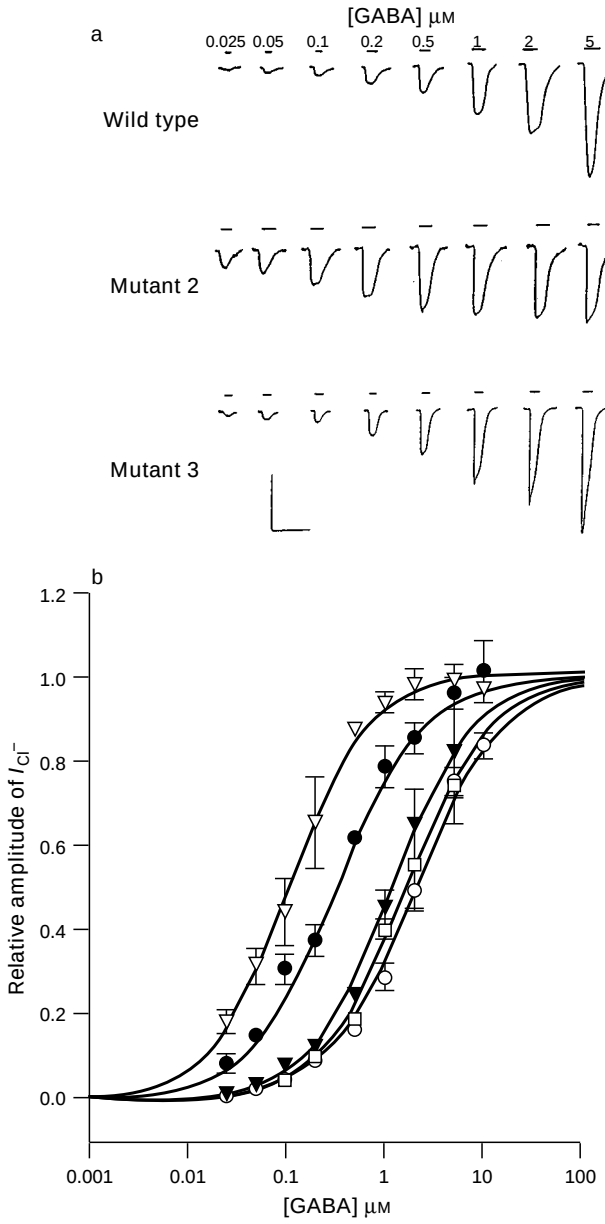

Figure 2 Current traces and plots showing dose-response profiles for GABA-induced Cl^- currents in the $\alpha6\beta2\gamma2$ subtype of $GABA_A$ receptors and those containing α6 mutants. (a) Representative traces for GABA-induced Cl^- currents measured by use of the whole cell patch clamp technique at a holding potential of −30 mV under a symmetrical Cl^- gradient. The concentration of GABA was varied from 0.025 to 5 μM. The vertical calibration bar represents 250 pA and the horizontal bar 30 s. The relative magnitude of the current at the peak was plotted as a function of GABA concentration (b); the $\alpha6\beta2\gamma2$ (wild type) (○), Mutant 1 (●), Mutant 2 (▽), Mutant 3 (▼) and Mutant 4 (□). The data represent the mean and vertical lines s.e. from three representative dose-response profiles (three cells) from two or more transfections and were fitted (solid lines) to a logistic equation,

$$E = E_{max} * [GABA]^{n_H} / (K_{0.5} + [GABA]^{n_H})$$

where $K_{0.5}$ is a half-maximal GABA concentration, ^{nH} is a slope factor and E_{max} is a maximal GABA response.

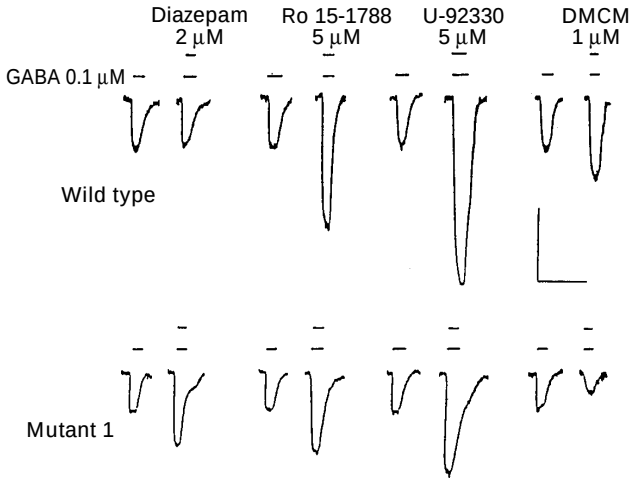

Figure 3 Effects of several representative benzodiazepine ligands on GABA-induced Cl^- currents in the $\alpha6\beta2\gamma2$ subtype of $GABA_A$ receptors and Mutant 1. Cl^- currents were induced with GABA 0.1 μM in the absence or presence of diazepam 2 μM, Ro 15-1788 5 μM, U-92330 5 μM or DMCM 1 μM. The traces were obtained from typical cells, and the drugs were tested one after another after the patch had been washed until the GABA response was restored to the original level. The vertical calibration bar represents 100 pA for wild type and 250 pA for Mutant 1. The horizontal bar represents 30 s.

centration (diazepam at 2 μM and Ro 15-1788 at 5 μM) to obtain their relative maximal efficacy, and in the presence of GABA at subsaturating concentrations, at which allosteric ligands are highly effective (0.1 to 0.5 μM, see below). The efficacy ratio of Ro 15-1788 to diazepam was 1.1±0.1, 1.0±0.2 and 0.95±0.2 in mutant 1, 2 and 3, respectively, and as expected, was fairly constant between different batches for a given receptor type. Only in Mutant 4, the ratio dropped to 0.1±0.2, indicating a loss of the ability of Ro 15-1788 to enhance GABA currents. This functional change appears to be limited to Ro 15-1788 because the efficacy ratio of U-92330 to diazepam remained unchanged in all the mutants including Mutant 4. The ratio was 1.6±0.4, 1.3±0.2, 1.0±0.3 and 1.2±0.2 in Mutant 1, 2, 3 and 4, respectively. Also DMCM reduced GABA currents by 55 to 60% in all the mutants including Mutant 4.

In order to confirm the alteration of the Ro 15-1788 action in Mutant 4, we examined its effect on Cl^- currents at various GABA concentrations and compared the data with those in the wild type (Figure 4). In Mutant 4, Ro 15-1788 (5 μM, a saturating concentration, see below) largely failed to enhance the currents induced with GABA at various concentrations.

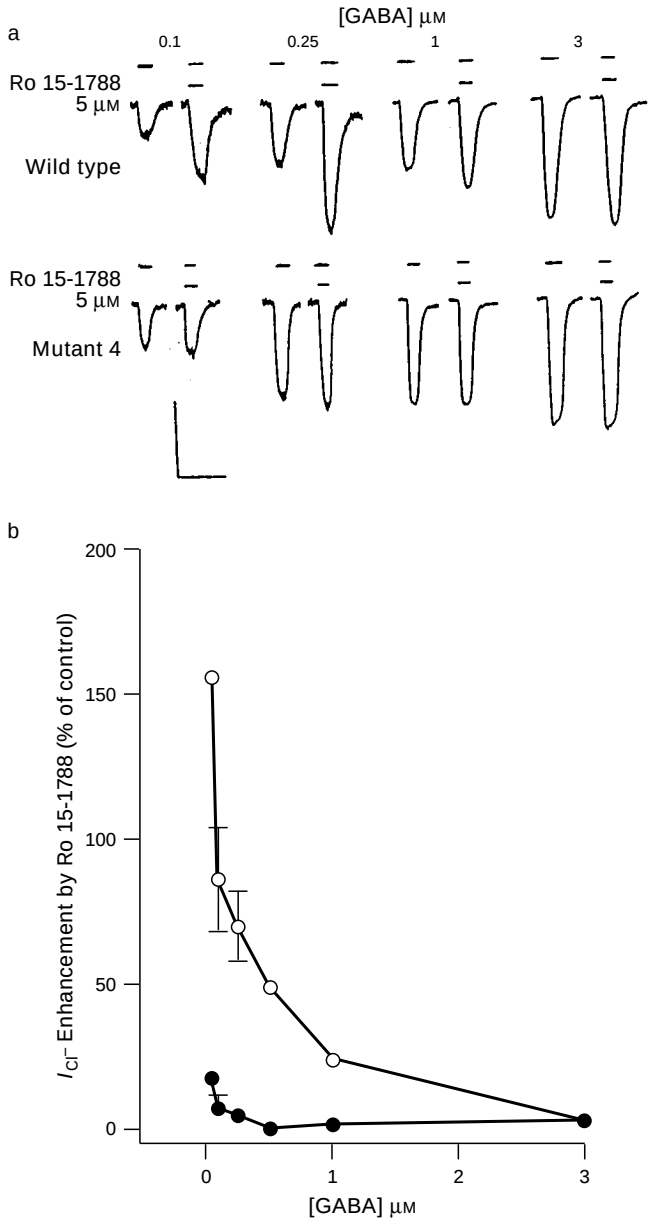

Figure 4 Comparison of the ability of Ro 15-1788 to enhance Cl^- currents induced with GABA at various concentrations in the $\alpha6\beta2\gamma2$ subtype and Mutant 4. Cl^- currents were induced with GABA 0.05, 0.1, 0.25, 0.5, 1 and 3 μM in the presence of Ro 15-1788 5 μM. (a) Representative current traces. (b) The net current increase by Ro 15-1788 was normalized to the current level observed without Ro 15-1788 at a given GABA concentration and the data were plotted as a function of GABA concentration; $\alpha6\beta2\gamma2$ (○) and Mutant 4 (●). The data represent the mean and vertical lines s.e. from three representative dose-response profiles (three cells). In the representative traces, the vertical bar represents 200 pA for the traces obtained with GABA 0.1 and 0.25 μM and 400 pA for those obtained with GABA 1 and 3 μM. The horizontal bar represents 30 s.

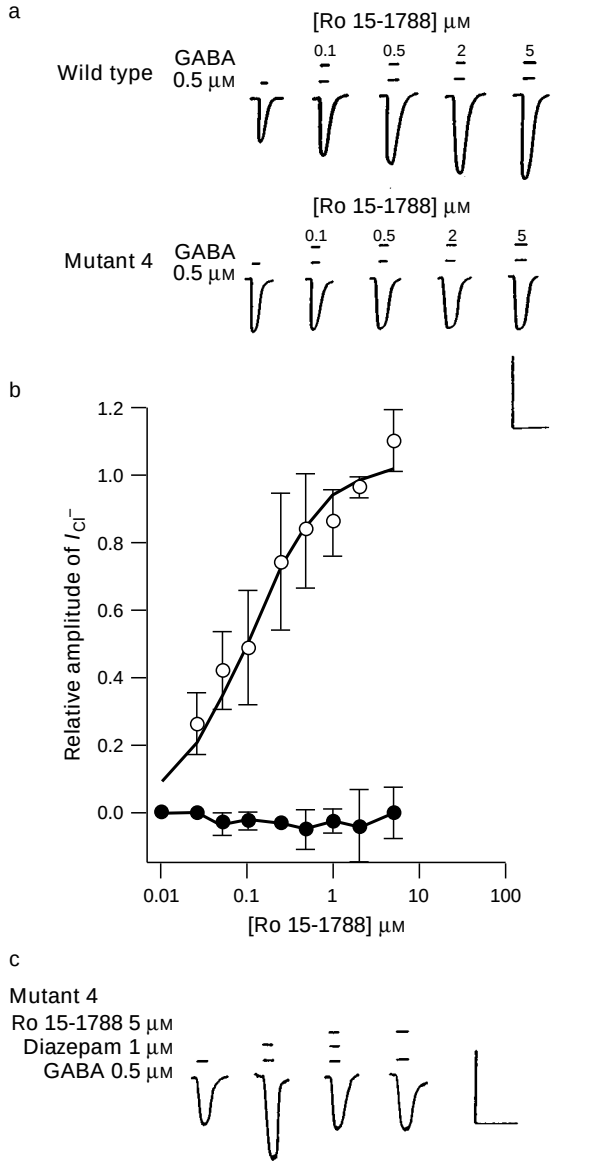

Figure 5 Comparison of dose-response profiles for the ability of Ro 15-1788 to enhance Cl^- currents induced by GABA at a fixed concentration (0.5 μM) in the $\alpha6\beta2\gamma2$ and Mutant 4. Cl^- currents were induced by GABA (0.5 μM) in the presence of Ro 15-1788 at 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2 and 5 μM in the $\alpha1\beta2\gamma2$ subtype and Mutant 4. (a) Representative current traces. (b) The net increase of the currents by Ro 15-1788 at a given concentration was normalized to the maximal increase and plotted as a function of Ro 15-1788 concentration; $\alpha6\beta2\gamma2$ (○) and Mutant 4 (●). The solid line represents the data fitted to the logistic equation (see text). The half-maximal concentration for Ro 15-1788 was 0.11 μM with the slope factor of 1. The data represent the mean and vertical lines s.e. from at least three dose-response profiles (three cells). (c) In Mutant 4, diazepam (1 μM) enhanced GABA (0.5 μM)-induced Cl^- currents and its enhancement was abolished by Ro 15-1788 5 μM. In the traces, the vertical bar represents 400 pA and the horizontal bar represents 30 s.

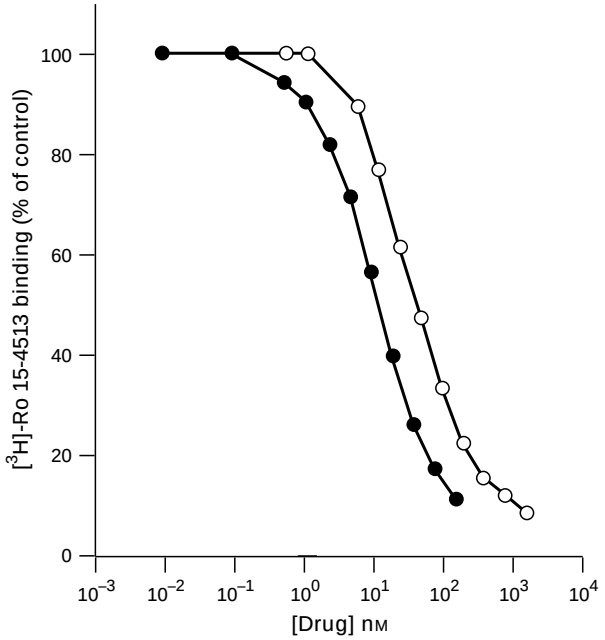

Figure 6 Comparison of the ability of Ro 15-1788 and diazepam to displace [^{3}H]-Ro 15-4513 in Mutant 4. Binding of [^{3}H]-Ro 15-4513 (10 nM) to the benzodiazepine site in Mutant 4 was measured in the presence of Ro 15-1788 (●) and diazepam (○) in the concentration range 0.01 to 200 nM. We obtained K_i values of 6.3 ± 0.6 and 22 ± 3 nM for Ro 15-1788 and diazepam, respectively, by use of the equation,

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where [L] is the ligand concentration (10 nM) and K_d is the dissociation constant for [^{3}H]-Ro 15-4513, 7.7 nM.

For example, the drug marginally enhanced the currents induced by GABA 0.05 μM (by only 18 ± 9%) and produced no appreciable effects on the currents induced by GABA at higher concentrations (0.1 to 3 μM). In the wild type, Ro 15-1788 enhanced the currents in a GABA-concentration-dependent manner; it enhanced the currents by 155, 95, 75, 60 and 0% with GABA at 0.05, 0.1, 0.25, 0.5 and 3 μM, respectively.

Dose-response profiles for Ro 15-1788 action on GABA(0.5 μM)-induced Cl^- currents were also compared in Mutant 4 and the wild type (Figure 5). In Mutant 4, Ro 15-1788 at the concentration from 0.01 to 5 μM failed to enhance Cl^- currents whereas in the wild type the drug dose-dependently enhanced the currents with a half-maximal concentration of 0.11 ± 0.02 μM. This value is close to its K_i of 0.096 ± 0.019 μM in the $\alpha 6\beta 2\gamma 2$ subtype obtained from competition binding studies with [^{3}H]-Ro 15-4513. Diazepam, on the other hand, maintained its ability to enhance GABA currents in Mutant 4, and its enhancement of the currents was abolished by Ro 15-1788 5 μM (Figure 5c). This is consistent with their competition for a common binding site. Finally, competition binding experiments with [^{3}H]-Ro 15-4513, which is a well established ligand for the benzodiazepine site of the $\alpha 6\beta 2\gamma 2$, showed that Ro 15-1788 and diazepam displaced the radioactive ligand with a K_i of 6.3 ± 0.6 and 22 ± 3 nM, respectively, in Mutant 4 (Figure 6). It should be noted that the dissociation constant for [^{3}H]-Ro 15-4513 was 8.8 ± 0.5 and 7.7 ± 0.3 nM in the $\alpha 6\beta 2\gamma 2$ and Mutant 4, respectively.

Discussion

In this study we attempted to investigate the benzodiazepine site, particularly the regions contributed by the α subunit, by taking advantage of the marked phenotypic differences between the benzodiazepine site of the $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes of $GABA_A$ receptors; namely, the inability of the benzodiazepine site of the $\alpha 6\beta 2\gamma 2$ subtype to interact with classical benzodiazepines and its agonistic response to Ro 15-1788, which behaves as an antagonist in the $\alpha 1\beta 2\gamma 2$ subtype (Lüddens *et al*., 1990; Im *et al*., 1993). We observed that, as shown in an earlier study, the mutation of R100 to histidine (Mutant 1) near the N-terminus of α6 enables the receptor to interact with a classical benzodiazepine, diazepam (Wieland *et al*., 1992). Furthermore, the mutation conferred on diazepam the ability to enhance GABA currents as a high affinity agonist to the benzodiazepine site and DMCM the ability to inhibit the currents, as observed in the $\alpha 1\beta 2\gamma 2$ subtype. However, the mutation did not abolish the ability of Ro 15-1788 to enhance GABA currents. Our current mutagenic study on the N-terminal residues near R100 of α6 revealed that the segment from P161 to L187 (Mutant 4) contains the functional region responsible for the agonistic response of Ro 15-1788. Replacement of the segment with the corresponding α1 sequence abolished its ability to enhance GABA currents, with little effect on its binding affinity. This mutational effect is highly selective, because it produced no appreciable effect on the functionality of the other agonists (diazepam and U-92330) or inverse agonist (DMCM). This segment of α6 represents a hypervariable region where 17 out of the 27 residues are divergent from α1. Further studies are needed to pinpoint the residue(s) in the region responsible for the functionality of Ro 15-1788.

These data provide evidence that the functionality of a given ligand could arise from its interaction with a single region, not shared with other ligands. It is reasonable to propose, therefore, that ligands which are selective for a particular α isoform can be discovered more readily on the basis of their functional characteristics, rather than on the basis of their binding affinity, because multiple regions seem to be involved in binding while a single region appears to be responsible for functionality. For instance, Ro 15-1788 appears to be the α6-specific agonist, because the drug behaves as an antagonist in the $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ subtypes. Interestingly, mutation on the two other α6-unique regions, HN121ED and VN142ED, had no noticeable effect on the functionality of the ligands we used in this study. If a variety of ligands were to be examined, we might find ligands which are selectively interacting with the mutated regions.

Our current mutational study also revealed that R100H mutation altered GABA efficacy; lowering the $K_{0.5}$ for GABA-induced Cl^- currents which probably represents low affinity GABA sites (see the Results section). This effect further intensified in Mutant 2 (R100H and HN121ED), but largely disappeared in Mutant 3 (R100H, HN121ED and VN142ED) and Mutant 4. This suggests that the two mutated residues of Mutant 3, E142 and D143, interact with H100 either directly within the binding site or indirectly through low affinity GABA sites. It is reasonable to propose that certain key residues of the benzodiazepine site (e.g. R100, V142 and N143) upon contact with ligands are able to influence allosterically GABA efficacy (via low affinity GABA sites), although ligand-induced changes might not be necessarily the same as observed with the mutations. It should also be pointed out that the mutations we examined here did not alter the high affinity GABA site as measured with [^{3}H]-muscimol. Thus, the high affinity GABA site seems to be not directly involved in channel openings, but its occupancy may induce conformational changes which are prerequisite for channel openings upon binding of GABA to low affinity sites. The same role has been proposed for the high affinity site for acetylcholine receptors (Jackson, 1989). Furthermore, the insensitivity of the high affinity GABA site to the α isoform mutations strengthens the earlier proposal that the high affinity GABA site is on the β subunit (DelOrey & Olsen, 1992).

We would like to note an earlier study (Kleingoor *et al*., 1993) in which no appreciable difference was found in the $K_{0.5}$ for GABA to induce Cl^- currents in the $\alpha 6\beta 2\gamma 2$ subtype and in the R100H mutant (Mutant 1), 0.34 and 0.3 μM, respectively.

The discrepancy arises from the $K_{0.5}$ value for the wild type which is only one sixth of our value (2.1 ± 0.3 μM). As seen with our current data (Figure 2), GABA 0.3 μM induced Cl^- currents to much less than 10% of the maximal level in the wild type and accordingly its $K_{0.5}$ value should be much greater than the value obtained (0.34 μM). At present we do not know how to explain the difference.

In summary, the segment of P161 to L187 of α6 has been identified to contain the functional region responsible for the agonistic action of Ro 15-1788 in the α6β2γ2 subtype, since its replacement with the corresponding α1 sequence selectively abolished the ability of the drug to enhance GABA currents with little effect on its binding affinity or the functionality of the other ligands such as diazepam, U-92330 and DMCM. In addition we found that several key residues in the N-terminal region of α6, such as R100, V142 and N143, have the ability to influence GABA-dependent Cl^- current induction probably by altering GABA affinity to its low affinity sites.

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