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Amino acid residue 200 on the α_1 subunit of $GABA_A$ receptors affects the interaction with selected benzodiazepine binding site ligands

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Abstract

Mutant α_1 subunits of the $GABA_A$ receptor were coexpressed in combination with the wild-type β_2 and γ_2 subunits in human embryonic kidney (HEK) 293 cells. The binding properties of various benzodiazepine site ligands were determined by displacement of ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5*a*]-[1,4]benzodiazepine-3-carboxylate ([^{3}H]Ro 15-1788). The mutation G200E led to a decrease in zolpidem and 3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine (CL 218872) affinity amounting to 16- and 8-fold. Receptors containing a conservative T206V substitution showed a 41- and 38-fold increase in methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and CL 218872 affinity combined with a decrease in diazepam and zolpidem affinity, amounting to 7- and 10-fold. Two mutations, Q203A and Q203S showed almost no effects on the binding of benzodiazepine site ligands, indicating that this residue is not involved in the binding of benzodiazepines and related compounds.

Keywords: $GABA_A$ receptor; α_1-subunit; Radioligand binding; Mutation; Benzodiazepine

1. Introduction

Benzodiazepines are widely prescribed drugs, mainly used in the treatment of insomnia and anxiety. They act via allosteric modulatory sites on $GABA_A$ receptors enhancing the γ-aminobutyric acid (GABA)-induced chloride ion flux. The $GABA_A$ receptor is a hetero-oligomeric channel protein of assumed pentameric structure (Nayeem et al., 1994). So far, a total of 15 mammalian subunits of the $GABA_A$ receptor have been cloned (Rabow et al., 1995; Sieghart, 1995). Coexpression of recombinant subunits has produced receptor channels with differing pharmacological and electrophysiological properties. For the binding of the agonist GABA α and β subunits are needed (Sigel et al., 1992; Amin and Weiss, 1993; Smith and Olsen, 1994). Combining α, β and γ subunits leads to receptors which are sensitive to the agonist and to modulatory substances acting on the benzodiazepine binding site. In vitro binding studies have identified several amino acid residues on α_1, α_3, α_4 and α_6 to be involved in benzodiazepine binding (Pritchett and Seeburg, 1991; Korpi et al., 1993; Wieland and Lüddens, 1994; Wieland et al., 1992; Amin et al., 1997; Buhr et al., 1997b). Also, two different amino acid positions on γ subunits have been identified that influence benzodiazepine affinity (Buhr et al., 1997a; Buhr and Sigel, 1997; Wingrove et al., 1997).

Interestingly, some of the amino acids residues important for benzodiazepine binding are homologous to amino acid residues on other subunits implicated in the binding of the receptor agonist (for reviews see: Galzi and Changeux, 1994; Sigel and Buhr, 1997). For example, αT206 and αY209 which are assumed to be part of the benzodiazepine binding pocket are directly homologous to βT202 and βY205 both shown to be important for binding of GABA (Amin and Weiss, 1993). Close to these two important residues on the α_1 subunit, an additional amino acid residue E225 on the α_3 subunit has been implicated in 3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine (Cl 218872) and zolpidem binding (Pritchett and Seeburg, 1991). α_3E225 is homologous to α_1G200. α_1 containing receptors are used in most studies on $GABA_A$ receptor function and results from experiments using other α subunit isoforms are often extrapolated to α_1 containing receptors. We were interested in proving the importance of α_1G200 in the interaction with benzodi-

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azepine binding site ligands. Further point mutations in the near proximity of α_1G200 were also characterized.

2. Materials and methods

2.1. Transfection of recombinant $GABA_A$ receptors in HEK 293 cells

cDNAs coding for the α_1, β_2 and γ_2 subunits of the rat $GABA_A$ receptor have been described elsewhere (Lolait et al., 1989; Malherbe et al., 1990a,b; Buhr et al., 1997a). Point mutations were constructed using the QuikChange mutagenesis kit (Stratagene). Human embryonic kidney (HEK) 293 cells (American Type of Culture Collection, MD, USA, CRL 1573) were cultured in minimum essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin and 50 μl/ml streptomycin. The cells were plated on 90-mm dishes and transfected with 20 μg of DNA per dish by the calcium phosphate precipitation method (Chen and Okayama, 1987).

2.2. 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 $150 \times g$. Cells were washed with buffer containing 10 mM potassium phosphate, 100 mM KCl, 0.1 mM K-EDTA, pH 7.4. Cells were homogenized by sonication in the presence of 10 μM phenylmethylsulfonyl fluoride and 1 mM EDTA. Membranes were collected in three centrifugation–resuspension cycles ($100\,000 \times g$ for 20 min) and then used for ligand binding or stored at −20°C.

2.3. Binding assays

Resuspended cell membranes (0.5–1 ml) were incubated for 90 min on ice in the presence of ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5*a*]-[1,4]benzodiazepine-3-carboxylate ([^{3}H]Ro 15-1788) (87 Ci/mmol, DuPont NEN, Boston, MA) and various concentrations of competing ligands. Membranes (10–25 μg of protein/filter) were collected through rapid filtration on GF/C filters presoaked in 0.3% polyethyleneimine. After three washing steps with 4 ml of buffer each, the filter-retained radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. On the basis of IC_{50} determinations, the K_i values were calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). As binding affinities of receptors containing the α_1Q203A mutation were very similar to wild type, displacement from receptors containing a serine substitution at the same position, was performed with only one concentration of competing ligand, displacing about 50% of the radioligand. For $\alpha_1\beta_2\gamma_2$ receptors 0.6–2 nM [^{3}H]Ro 15-1788 was used, for α_1G200E$\beta_2\gamma_2$ receptors the concentration of [^{3}H]Ro 15-1788 was 0.6 nM.

3. Results

3.1. Binding properties of α_1G200E$\beta_2\gamma_2$ receptors

α_1 carrying a glycine to glutamate substitution in position 200 (hereafter referred to as α_1G200E) was coexpressed in combination with the wild-type β_2 and γ_2 subunits in HEK 293 cells. Mutant receptors were compared with wild type receptors, which display a high affinity to the benzodiazepine site antagonist [^{3}H]Ro 15-1788 with a K_d smaller than 1 nM (Table 1, Fig. 1). Interestingly, binding affinities of zolpidem and Cl 218872 were strongly reduced amounting to 16- and 8-fold (Fig. 2). Diazepam and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) affinities remained almost unaffected (Fig. 3).

3.2. Binding properties of α_1T206V$\beta_2\gamma_2$ receptors

A second mutant, α_1T206V, was constructed because this mutation is more conservative than α_1T206A that has previously been described (Buhr et al., 1997b). Mutant

Table 1
Comparison of the benzodiazepine pharmacology of wild-type and mutant $GABA_A$ receptors

Subunit combination	K_d or K_i (nM)				
	[^{3}H]Ro 15-1788	Diazepam	Zolpidem	Cl 218872	DMCM
$\alpha_1\beta_2\gamma_2$	0.61 ± 0.24[a]	12 ± 8[a]	15 ± 3[a]	46 ± 1[a]	6.9 ± 1.8
α_1G200Eβ_2	0.37 ± 0.04	25 ± 1	233 ± 25	389 ± 19	4.6 ± 0.1
α_1Q203Aβ_2	0.72 ± 0.16	9.1 ± 0.2	24 ± 2	58 ± 6	2.2 ± 0.6
α_1T206Vβ_2	0.14 ± 0.01	89 ± 2	151 ± 2	1.2 ± 0.0	0.17 ± 0.02

[a] Data from Buhr et al. (1997b).
K_d values were determined by binding of [^{3}H]Ro 15-1788 to washed membranes of transiently transfected HEK 293 cells. K_i values were determined by displacement of [^{3}H]Ro 15-1788 binding and were calculated according to the Cheng–Prusoff equation. Data shown are the mean ± S.D. of two experiments each performed in duplicate.

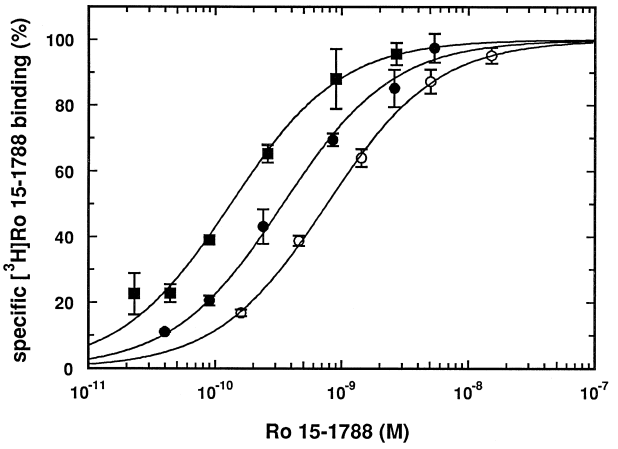

Fig. 1. Concentration dependence of specific [^{3}H]Ro 15-1788 binding to wild type and mutant receptors. HEK 293 cells were transiently transfected with $\alpha_1\beta_2\gamma_2$ (○), α_1G200E$\beta_2\gamma_2$ (●) and α_1T206V$\beta_2\gamma_2$ (■). Membranes were prepared and incubated with various concentrations of [^{3}H]Ro 15-1788. Membranes were then filtered through Whatman GF/C filters and radioactivity specifically bound to membranes was determined as described in Section 2. Values are mean ± S.D. of a measurement performed in duplicate. A second independent experiment showed similar results.

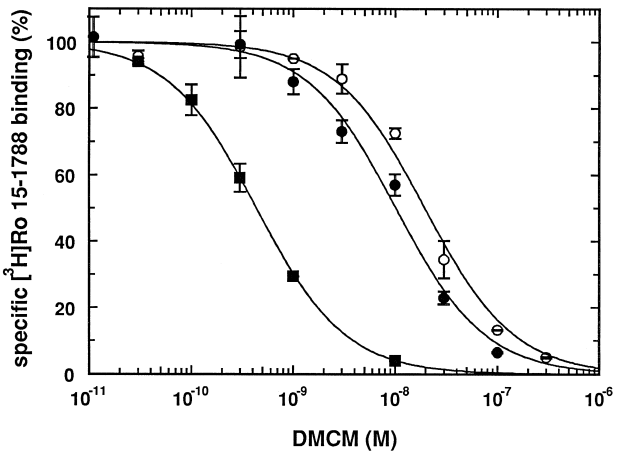

Fig. 3. Displacement of [^{3}H]Ro 15-1788 from membranes prepared from HEK 293 cells transfected with $\alpha_1\beta_2\gamma_2$ (○), α_1G200E$\beta_2\gamma_2$ (●) and α_1T206V$\beta_2\gamma_2$ (■) by DMCM. The concentrations of [^{3}H]Ro 15-1788 in this experiment were near the K_d values for the respective receptor type such that differences in IC_{50} values are comparable to differences in K_i values. Each point represents the mean ± S.D. of one determination performed in duplicate. A second independent experiment showed similar results.

α_1T206V$\beta_2\gamma_2$ receptors showed a significant increase in affinity for [^{3}H]Ro 15-1788, Cl 218872 and DMCM amounting to 4-, 38- and 41-fold compared to the wild type. In contrast diazepam and zolpidem showed a 7- and 10-fold decrease in binding affinity (Table 1). With the exception of Ro 15-1788 the changes in affinities for the above mentioned substances were qualitatively similar for α_1T206A$\beta_2\gamma_2$ receptors. It is however interesting to notice that changes in affinities for all substances, including Ro 15-1788, were quantitatively larger for the valine than for the alanine mutant.

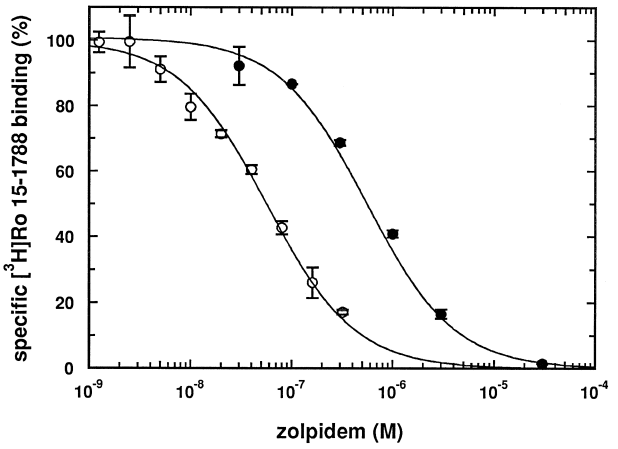

Fig. 2. Displacement of [^{3}H]Ro 15-1788 from membranes prepared from HEK 293 cells transfected with $\alpha_1\beta_2\gamma_2$ (○) and α_1G200E$\beta_2\gamma_2$ (●) by zolpidem. The concentration of [^{3}H]Ro 15-1788 was 3.4- and 1.6-fold above the K_d values of the respective receptor type. If the relative concentrations were the same, the curve for the mutant receptor would be shifted by 0.23 log units to the right, relatively to the curve obtained for the wild type receptor. Each point represents the mean ± S.D. of one determination performed in duplicate. A second independent experiment showed similar results.

3.3. Binding properties of α_1Q203A$\beta_2\gamma_2$ receptors

In a previous functional study using receptors expressed in *Xenopus laevis* oocytes and electrophysiological techniques (Buhr et al., 1996) the mutation α_1Q203A showed a nonsignificantly reduced potentiation by diazepam but a significantly reduced potentiation by zolpidem. Since mutations in the positions 200, 206 and 209 had already shown effects on benzodiazepine binding, it was interesting to see if point mutations in position 203 also lead to altered binding of benzodiazepine site ligands. Surprisingly for the Q203A mutant, binding affinities of all the ligands tested were almost the same as for wild type receptors (Table 1). Similarly, a second mutation, Q203S, also led to no change in the binding affinities of the ligands tested (data not shown).

4. Discussion

The aim of this study was to further characterize the structural properties of the binding site for benzodiazepine site ligands. From previous work it was known that α_3E225G influences the binding affinities of benzodiazepine site ligands (Pritchett and Seeburg, 1991). α_3E225 is homologous to α_1G200, thus it could be interfered that position 200 on the α_1 subunit may also be important for benzodiazepine binding. Because α_1 is the most abundant α subunit in the adult brain (Benke et al., 1991), we

wanted to test whether the findings from α_3 may be extrapolated to α_1.

α_1G200E$\beta_2\gamma_2$ receptors show strong effects on the binding of zolpidem and Cl 218872 (16- and 8-fold decrease in affinity) and minor effects on [^{3}H]Ro 15-1788, diazepam and DMCM (max 2-fold increase/decrease). The results are consistent with previous findings with the reverse analog mutation α_3E225G, which led to a 17- and a 7-fold increase in affinity for zolpidem and Cl 218872 (Pritchett and Seeburg, 1991). A glutamate instead of a glycine at position 200 (or 225 on the α_3 subunit) thus seems unfavorable for the interaction between these two ligands and the binding pocket. It might be speculated that the bulkier glutamate side-chain sterically interferes with structural elements only present on zolpidem and Cl 218872. Unless a plausible model of a 3-dimensional superposition of these two substances as well as with the classical benzodiazepines and the antagonist Ro 15-1788 becomes available, any attempt to propose a hypothetical interaction between ligands and this side-chain remains speculative.

α_1T206V$\beta_2\gamma_2$ receptors showed altered binding properties for all the ligands tested. The affinities for Cl 218872 and DMCM were strongly increased whereas the affinities for diazepam and zolpidem were reduced (Table 1). The substitution of threonine by a valine is rather conservative (the side-chains are nearly the same in size) but nonetheless shows strong effects. A possible explanation for the rise or fall in affinity for the substances discussed above could be an either favorable interaction of diazepam and zolpidem or an unfavorable interaction of Cl 218872 and DMCM with the hydroxyl group of threonine and the ligands via hydrogen-bonds. Upon replacement of this hydroxyl group by a methyl group such hypothetical interactions might get lost. Further support for this proposition comes from α_1T206A$\beta_2\gamma_2$ receptors which had qualitatively similar yet less pronounced effects (Buhr et al., 1997b) and also lack this hydroxyl group.

Apart from these results, other observations also point to the importance of this particular threonine residue: throughout the α subunits the threonine homologous to α_1T206 is strictly conserved (Fig. 4). Furthermore, the β subunits also carry a threonine residue at the homologous position. Amin and Weiss (1993) described that this particular threonine residue at position 202 on the β_2 subunit is implicated in the interaction with the channel agonist GABA. Similarly, α_1Y209, which also affects benzodiazepine binding, has its counterpart on the β_2 subunit (β_2Y205) that is involved in agonist binding (Amin and Weiss, 1993; Amin et al., 1997; Buhr et al., 1997b). This high degree in homology between the agonist- and the modulatory site indicates that these two pharmacologically distinct sites have evolved from a common ancestral binding site (Sigel and Buhr, 1997).

In these experiments, α_1G200E and α_1T206V led to altered binding affinities for benzodiazepine site ligands. Previously also α_1Y209 was implicated in benzodiazepine binding (Amin et al., 1997; Buhr et al., 1997b). G200, T206 and Y209 are spaced by three residues or multiples of it, suggestive of an α-helix. In this hypothetical α-helix, the side-chain of Q203 would point approximately in the same direction as the side-chains of either E200 or T206. If E200 or T206 are in direct contact with the ligand, we expected this also to be the case for Q203. We therefore constructed two mutants α_1Q203A and α_1Q203S to test this hypothesis. α_1Q203A$\beta_2\gamma_2$ and α_1Q203S$\beta_2\gamma_2$ receptors show no effects on the binding affinities of all ligands tested. This finding is reminiscent to the work of Pritchett and Seeburg (1991) who found no change in Cl 218872 affinity upon mutation of the homologous residue in α_3 (R228Q). Amino acid side-chains in this position, thus, do not seem to be in close contact with ligands of the benzodiazepine site, arguing against an α-helix. The possible implication of residue 203 in functional effects of zolpidem (Buhr et al., 1996) could be explained by an involvement in the allosteric coupling of channel gating rather than in binding of ligands. It is interesting to point out that although there is no obvious conservation of the amino acid residues at this position on the α subunit, all residues show a hydrophillic character (Fig. 4).

197	V	D	S	**G**	I	V	**Q**	S	S	**T**	G	E	**Y**	V	V	M	T	T	α_1
197	I	G	K	E	T	I	K	S	S	T	G	E	Y	T	V	M	T	A	α_2
222	V	G	T	**E**	I	I	R	S	S	T	G	E	Y	V	V	M	T	T	α_3
195	V	S	S	E	T	I	K	S	I	T	G	E	Y	I	V	M	T	V	α_4
201	V	G	T	E	N	I	S	T	S	T	G	E	Y	T	I	M	T	A	α_5
196	V	S	S	E	T	I	K	S	N	T	G	E	Y	V	I	M	T	V	α_6

Fig. 4. Alignment of the wild type α_1–α_6 subunits of the GABA$_A$ receptor. Amino acids corresponding to the residues 197–214 on the α_1 subunit are shown. Underlined residues indicate amino acids assumed to be involved in benzodiazepine binding. Residues printed in bold letters denominate amino acids that were mutated and characterized in this study.

In conclusion, we show here that mutations of both the amino acid residues α_1G200 and α_1T206 lead to altered specificity of the $GABA_A$ receptor for benzodiazepine site ligands. This suggests that these two amino acids form part of the benzodiazepine binding site. Surprisingly, mutations of α_1Q203, the amino acid residue located at equal distance to α_1G200 and α_1T206, do not affect ligand binding, indicating that this amino acid is not in direct contact with benzodiazepines and related compounds.

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