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Mechanism of α-subunit selectivity of benzodiazepine pharmacology at γ-aminobutyric acid type A receptors

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Abstract

Benzodiazepine pharmacology at the $GABA_A$ receptor is dependent on the α and γ subunit isoforms present. Ligands with higher affinity for certain isoforms—selective compounds—have been classified into benzodiazepine type I and II and into diazepam-sensitive and diazepam-insensitive receptors. A single amino acid position (α1G201/α3E225) has been identified which discriminates BZI and BZII receptors. The role of this residue has been explored by mutagenesis of α1 position 201 and the pharmacology of recombinant receptors examined using BZI receptor agonists. Ligand affinity is reduced by increasing side chain volume at α1G201 suggesting that steric inhibition underlies α-subunit selectivity. A second amino acid (α1H102/α6R100) determines diazepam sensitivity. The nature of the amino acid at this position was also examined by mutagenesis. Flumazenil and Ro15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate) binding affinity correlated weakly with the amino acid hydrophobicity suggesting a weak hydrophobic interaction between the ligand and α1H102. © 2002 Published by Elsevier Science B.V.

Keywords: GABA receptor; Benzodiazepine; Mutagenesis

1. Introduction

Benzodiazepines are a clinically important class of compounds used in the treatment of anxiety and sleep disorders. They exert their action by modulation of the $GABA_A$ receptor acting at a site distinct from that of the natural agonist, γ-aminobutyric acid (GABA). The $GABA_A$ receptor is a pentameric complex made up from a subset of six known subunit classes (α1–α6, β1–β3, γ1–γ3, δ, ε and θ; Barnard et al., 1998; Bonnert et al., 1999). The properties of receptors, including the benzodiazepine pharmacology, are dependent on the subunit composition with the most common receptor isoforms consisting of α, β and γ subunits (Whiting et al., 1995).

Benzodiazepine receptors have been classified pharmacologically into those which recognize the classical, 5-phenyl-1,4-benzodiazepine agonists (for example diazepam and flunitrazepam) referred to as 'diazepam-sensitive' and those which do not recognize these ligands referred to as 'diazepam-insensitive' (Malminiemi and Korpi, 1989). The cloning of cDNAs encoding subunit isoforms has allowed the expression of recombinant receptors of known composition and it is now understood that the diazepam-sensitive/diazepam-insensitive terms relate to the α subunit isoform contained within a typical αβγ2 receptor as differentiated by the displacement of [^{3}H]Ro15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate) with diazepam. Hence diazepam-sensitive receptors contain α1, α2, α3 or α5 whereas diazepam-insensitive receptors contain either α4 or α6 (Lüddens et al., 1990). The former group can be further subdivided by sensitivity to CL218,872 (3-methyl-6-(3-[trifluoromethyl]-phenyl)-1,2,4-triazolo[4,3-b]pyridazine) with the higher affinity α1-containing receptors referred to as BZI and the lower affinity BZII receptors containing α2, α3 or α5. Two amino acid positions have been identified which determine each of these α-subunit selective profiles.

The α subunits are predominantly labelled in photoaffinity labelling studies (for example, Stephenson et al., 1990). Peptide mapping of labelled subunits shows that

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[^{3}H]flunitrazepam and [^{3}H]Ro15-4513 become covalently attached to different domains of diazepam-sensitive receptors (Davies et al., 1996; Davies and Dunn, 1998; Duncalfe et al., 1996; Duncalfe and Dunn, 1996). The site of photoincorporation of [^{3}H]flunitrazepam was identified by micropeptide sequencing as histidine 102 (bovine α1 subunit numbering; Duncalfe et al., 1996). The presence of a histidine at position 101 of rat α1 (and equivalent position in other α subunits) had already been shown to determine the high affinity of the benzodiazepine agonist diazepam at α1-containing receptors over those containing α4 or α6 which have arginine at the homologous position 100 (Wieland et al., 1992).

The amino acid determining the α1 subunit selectivity of CL218,872 and of zolpidem has also been identified as α1G201 (Pritchett and Seeburg, 1991). At both positions 102 and 201, it is noticeable that the subunits conferring the higher affinity have the smaller amino acid suggesting a steric role for the α subunit in benzodiazepine selectivity. It may be speculated that a similar mechanism also underlies the greater than 1000-fold decrease in affinity for diazepam, flunitrazepam, zolpidem and CL218,872 at α6-containing receptors compared to those having α1 (Hadingham et al., 1996; Lüddens et al., 1990).

To further test the hypothesis that the α subunit negatively affects affinity of benzodiazepine binding and to investigate the role of residues at positions 102 and 201, a number of amino acid substitutions of the α1 subunit were made by site-directed mutagenesis. The mutant α1 subunits were coexpressed transiently with β and γ2 subunit cDNAs in human embryonic kidney (HEK) 293 cells and the recombinant receptors characterized by radioligand binding assays to determine the affinities for selective and non-selective benzodiazepine site ligands. A functional analysis was also made for recombinant receptors expressed in *Xenopus* oocytes. The results suggest that steric and charge effects underlie the role of the α subunit in determining the selectivity and affinity of benzodiazepine site agonists. Results similar to parts of this study have been reported by Davies et al. (1998) and Dunn et al. (1999) as discussed below. These studies of the effect of mutations at rat α1H101 also conclude that the nature of the amino acid at this position influences ligand recognition and efficacy without finding any correlation with amino acid properties.

2. Materials and methods

2.1. Site-directed mutagenesis

Cloning of human cDNAs encoding the α1, α3, α6, β1, β3 and γ2S subunits has been described previously (Hadingham et al., 1993a,b, 1996). Oligonucleotide-directed mutagenesis was performed as described previously incorporating a diagnostic restriction site (Wingrove et al., 1994). Mutants were identified by the presence of the diagnostic site and confirmed by DNA sequencing. The numbering of the residues discussed uses that appropriate for the species used in the corresponding communications. The rat α1 peptide sequence has a single amino acid deletion relative to the human and bovine sequences and hence the position of equivalent residues differs; thus rat α1H101/G200 are equivalent to human and bovine α1H102/G201.

2.2. Tissue culture and transfection

HEK293 cells were plated out 24 h prior to transfection. Calcium phosphate-mediated transfection was as Chen and Okayama (1988) using 2 μg each of α1, β1 and γ2 cDNAs with 6 μg pAdVAntage (Promega) per 10-cm dish. Cells were incubated at 37 °C in an atmosphere containing 3% CO_2 prior to harvesting. After 2–3 days the cells were harvested by scraping into phosphate-buffered saline (PBS) and pelletted by centrifugation. The cell pellet was washed twice by resuspension in 10 mM KH_2PO_4, pH7.4 before being resuspended in assay buffer (10 mM KH_2PO_4, pH7.4, 100 mM KCl) and homogenized by passing through a 27-gauge needle.

2.3. Radioligand binding

Saturation binding curves were obtained by incubating membrane homogenates with [^{3}H]flumazenil (Ro15-1788, α1G201 mutants; NEN; 87.0 Ci/mmol) or [^{3}H]Ro15-4513 (α1H102 mutants; NEN; 21.7 Ci/mmol) at eight concentrations ranging from 0.1 to 32 nM in 0.5 ml volume. Non-specific binding was determined in the presence of 10 μM flunitrazepam or 10 μM Ro15-4513, respectively. After incubation at 4 °C for 90 min, the assay was terminated by filtration onto GF/B filters using a Tomtech cell harvester (Receptor Technologies, Oxon, UK). Filters were washed three times with assay buffer and dried before detection of filter-retained radioactivity by liquid scintillation counting. Dissociation constants (K_d) were calculated by Scatchard plot. Inhibition constants (IC_{50}) were obtained by competition with a sub-K_d concentration of [^{3}H]benzodiazepine by test compound at eight concentrations using an otherwise identical assay paradigm. Test compounds were CL218,872 (Lederle, Belgium), flumazenil, flunitrazepam (Sigma) and zolpidem (Synthelabo, France). Experimental data points were fitted to a single-site dose–response curve and K_i values calculated from the Cheng–Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1+[\text{Radioligand}]/K_d)$. Statistical analyses were performed using GraphPad Prism version 2.01 (GraphPad Software, CA, USA). Values for amino acid properties were obtained from Creighton (1983) and Kyte and Doolittle (1982).

2.4. Electrophysiology

Ovary tissue was removed from an anaesthestized toad (*Xenopus laevis*), stage V and VI oocytes manually dis-

sected and subsequently defolliculated using a mild collagenase treatment (type IA, 0.5 mg/ml for 6 min). The oocyte nuclei were directly injected using the 'blind' method with 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH7.0) containing α and β subunits and γ2S (concentration of each individual subunit was 6.66μg/ml injection buffer). The injected oocytes were maintained at 20 °C and used 24–96 h later.

For electrophysiological recording, oocytes were placed in a 50-μl bath and perfused at 4–6 ml/min with modified Barth's medium (88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH7.5). Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage-clamped at −70 mV. The effects of $GABA_A$ receptor modulators were examined on control GABA responses using a concentration of agonist that elicited 20% of the maximum GABA response (EC_{20}) determined for each individual oocyte. In order to ensure complete binding, the modulators to be tested were applied for 30 s before co-application of the modulator and the GABA EC_{20} concentration. Concentration–response curves were fitted by use of GraphPad Prism using a non-linear square fitting programme to the equation $y = \min + ((\max - \min)/(1 + 10^{(\log EC50 - \log X)nH}))$ where Y is the response (as a percentage change of the control GABA EC_{20} response); min and max are the minimum and maximum responses; EC_{50} is the concentration of X eliciting a half maximal response; X is the concentration of drug, nH is the Hill coefficient.

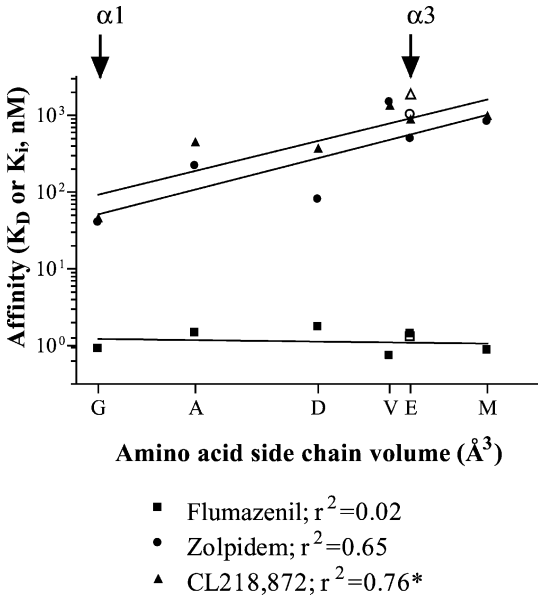

Fig. 1. Correlation between amino acid volume at α1 position 201 and ligand affinity. Receptors containing α1 subunits mutated at G201 were coexpressed with β1γ2 in HEK293 cells and affinities determined for [^{3}H]flumazenil, zolpidem and CL218,872. These affinities are shown plotted against amino acid side chain volumes (Creighton, 1983) as indicated by the ticks on the x-axis in single letter code. Values for the wild-type α3-containing receptor are shown in open symbols (these data omitted from the regression analysis) and both wild-type receptors are indicated by arrows. Correlations are given (r^2) with asterisks indicating slopes which are significantly different from zero.

3. Results

3.1. Radioligand binding data for receptors containing α1G201 mutant subunits

The α1 subunit was mutated to change α1G201 to alanine, aspartate, glutamate, methionine or valine and expressed in combination with cDNAs encoding β1 and γ2S. Saturation analysis was performed using the non-BZI/BZII receptor selective radioligand [^{3}H]flumazenil (Table 1). Affinities (K_i) were determined by displacement of this binding for CL218,872 and zolpidem (Table 1). These compounds were chosen since their affinity for α3-containing receptors is known to be affected by mutation of α3E225G (Pritchett and Seeburg, 1991). Amino acid side chain volume was plotted against ligand affinity in order to assess their relationship (Fig. 1). None of the α1G201 mutations have any affect on [^{3}H]flumazenil affinity consistent with its non-selective pharmacological profile. For both the triazolopyridazine, CL218,872, and the imidazopyridine, zolpidem, there is a trend to lower affinity with increasing amino acid side chain volume at position 201 of

Table 1
Affinity of selected benzodiazepine site ligands for receptors containing subunits mutated at α1G201

α Subunit	Amino acid volume/Å^3	[^{3}H]Flumazenil	Zolpidem	CL218,872
α1G201	G-48	0.91 ± 0.22	40.2 ± 12.6	46.8 ± 11.3
α1G201A	A-67	1.46 ± 0.67	220.0 ± 16.5	454.0 ± 53.0
α1G201D	D-91	1.75 ± 0.33	80.4 ± 11.1	376.0 ± 70.1
α1G201V	V-105	0.74 ± 0.16	1490.0 ± 180.0	1370.0 ± 234.0
α1G201E	E-109	1.42 ± 0.35	496.0 ± 14.7	898.0 ± 148.0
α3E225	E-109	1.29 ± 0.24	1020.0 ± 130	1910.0 ± 144.0
α1G201M	M-124	0.87 ± 0.30	828.0 ± 83.0	1000.0 ± 121.0

The α subunits were coexpressed with β1 and γ2 cDNAs in HEK293 cells. Affinities are K_i values in nM (except for [^{3}H]flumazenil which is K_d) using [^{3}H]flumazenil as displaced radioligand. Values are mean ± S.E.M. of at least three independent determinations.

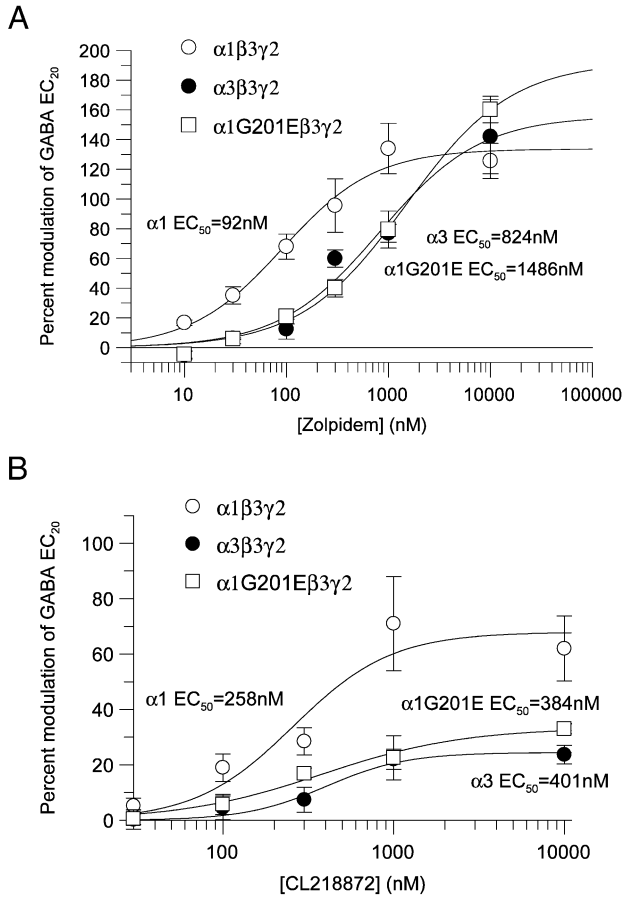

Fig. 2. Modulation of GABA-induced responses in α1G201Eβ3γ2S receptors by (A) zolpidem and (B) CL218,872. Receptors containing α1, α1G201E or α3 were coexpressed with β3γ2 in *Xenopus* oocytes. Concentration–response curves were generated for the modulation of a GABA EC_{20} response by (A) zolpidem and (B) CL218,872. Each data point represents a mean ± S.E.M. from three or more determinations. Maximum efficacy of CL218,872 was significantly reduced for α3β3γ2 and α1G201Eβ3γ2 relative to α1β3γ2 ($P<0.05$, one-way ANOVA followed by Newman–Kuels post hoc test).

α1 although the correlation is weak ($r^2=0.76$ and 0.65, respectively). Linear regression indicates a parallel increase in affinities for both of these non-benzodiazepines although the slope is only significantly different from zero for CL218,872.

3.2. Electrophysiological characterization of α1G201Eβ3γ2S

The mutant α1G201E was expressed with β3γ2S in *Xenopus* oocytes and concentration–effect curves constructed for the modulation of GABA EC_{20} currents by CL218,872 and zolpidem (Fig. 2). Previous studies have demonstrated that the β-subunit does not affect the affinity or extent of benzodiazepine modulation (Hadingham et al., 1993b). The EC_{50} for both compounds was shifted towards their value at α3-containing receptors. In the case of CL218,872 this two-fold shift was accompanied by a reduction in maximum response from 68% to 33%, similar to that for α3-containing receptors (24%) suggesting this residue may also affect benzodiazepine efficacy. While the two-fold change in EC_{50} did not reach statistical significance, the decrease in maximum efficacy was significant ($P<0.05$) for the α3β3γ2 and α1G201Eβ3γ2 relative to α1β3γ2 receptors. A larger increase was observed with the EC_{50} for zolpidem.

3.3. Radioligand binding data for receptors containing α1H102 mutant subunits

The α1 subunit was mutated at histidine position 102 to alanine, arginine, glutamate, glutamine, lysine, methionine, tyrosine or valine and coexpressed with cDNAs encoding β1 and γ2S. The radioligand [^{3}H]Ro15-4513 was chosen for this study because of its relative lack of selectivity for receptors containing α1 or α6 subunits. The affinities (K_d) of [^{3}H]Ro15-4513 for receptors containing mutant subunits were either the same as wild-type α1 or up to 10-fold higher (Table 2); the hydrophobic amino acids (namely Y, V, M and A) conferring the higher affinity. Displacement of [^{3}H]Ro15-4513 by flumazenil (Ro15-1788) or flunitrazepam allowed calculation of their K_i values (Table 2). Plotting affinity against amino acid size for substitutions at position 102 of α1 gave no clear correlation for either flumazenil or flunitrazepam (Fig. 3).

Table 2
Affinity of selected benzodiazepine ligands for receptors containing subunits mutated at α1H102

α Subunit	Amino acid volume/Å^3	[^{3}H]Ro15-4513	Flumazenil	Flunitrazepam
α1H102A	A-67	0.50 ± 0.04[a]	6.48 ± 0.22	629.0 ± 103.3
α1H102V	V-105	0.74 ± 0.10[a]	7.85 ± 1.99	587.0 ± 83.0
α1H102E	E-109	7.80 ± 2.31[a]	125.0 ± 61.0	> 5000
α1H102Q	Q-114	1.10 ± 0.30[a]	44.7 ± 19.7	>5000
α1H102	H-118	6.94 ± 0.73[a]	0.91 ± 0.22[a]	3.90 ± 0.80[a]
α1H102M	M-124	0.56 ± 0.08[a]	1.74 ± 0.38	1660.0 ± 770.0
α1H102K	K-135	4.07 ± 0.29[a]	14.0 ± 4.8	4390.0 ± 850.0
α1H102Y	Y-141	0.71 ± 0.04[a]	4.32 ± 1.10	1380.0 ± 300.0
α1H102R	R-148	26.3 ± 6.6[a]	115.0 ± 49.6	>5000
α6R100	R-148	15.0 ± 3.1[a]	124.5 ± 41.4	>5000

The α subunits were coexpressed with β1 and γ2 cDNAs in HEK293 cells. Affinities are K_i values in nM (or K_d where indicated by [a]) by displacement of [^{3}H]Ro15-4513. Values are mean ± S.E.M. of at least three independent determinations.

Any substitution at this position is detrimental to flunitrazepam binding—shifting the K_i by at least 100-fold in the direction of α6. Flumazenil binding is less affected by these substitutions. Plotting affinity against amino acid hydrophobicity gives a weak correlation for [^{3}H]Ro15-4513 ($r^2 = 0.63$) and flumazenil ($r^2 = 0.52$). The affinity for [^{3}H]Ro15-4513 also correlated with two other measures of hydrophobicity—hydrophilicity and hydropathy index (Fig. 3). Flunitrazepam affinity did not correlate with any of these four properties. Despite differences in the correlation coefficients, it is interesting that for each measure the slopes are not significantly different from one another (Fig. 3).

3.4. Electrophysiological characterization of receptors containing α1H102 subunit mutants

The α1H102 subunit mutants were expressed in combination with β1γ2S in *Xenopus* oocytes. The functional con-

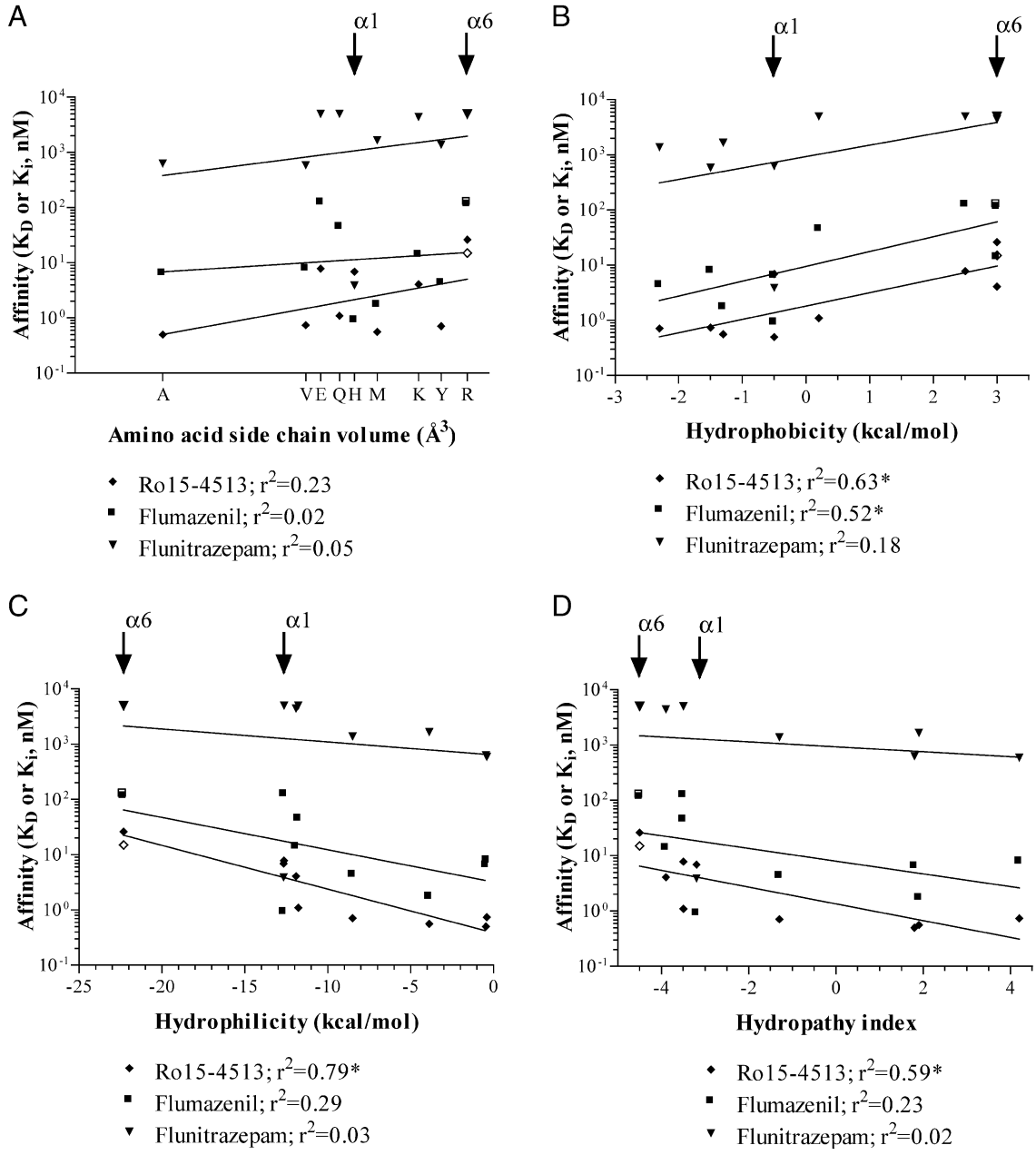

Fig. 3. Correlations between amino acid properties at α1 position 102 and ligand affinity. Receptors containing α1 subunits mutated at H102 were coexpressed with β1γ2 in HEK293 cells and affinities determined for [^{3}H]Ro15-4513, flumazenil and flunitrazepam. These affinities are shown plotted against amino acid side chain (A) volumes as indicated by the ticks on the *x*-axis in single letter code; (B) hydrophobicity; (C) hydrophilicity and (D) hydropathy index (Creighton, 1983; Kyte and Doolittle, 1982). Values for the wild-type α6-containing receptor are shown in open symbols (these data omitted from the regression analysis) and both wild-type receptors are indicated by arrows. Correlations are given (r^2) with asterisks indicating slopes which are significantly different from zero.

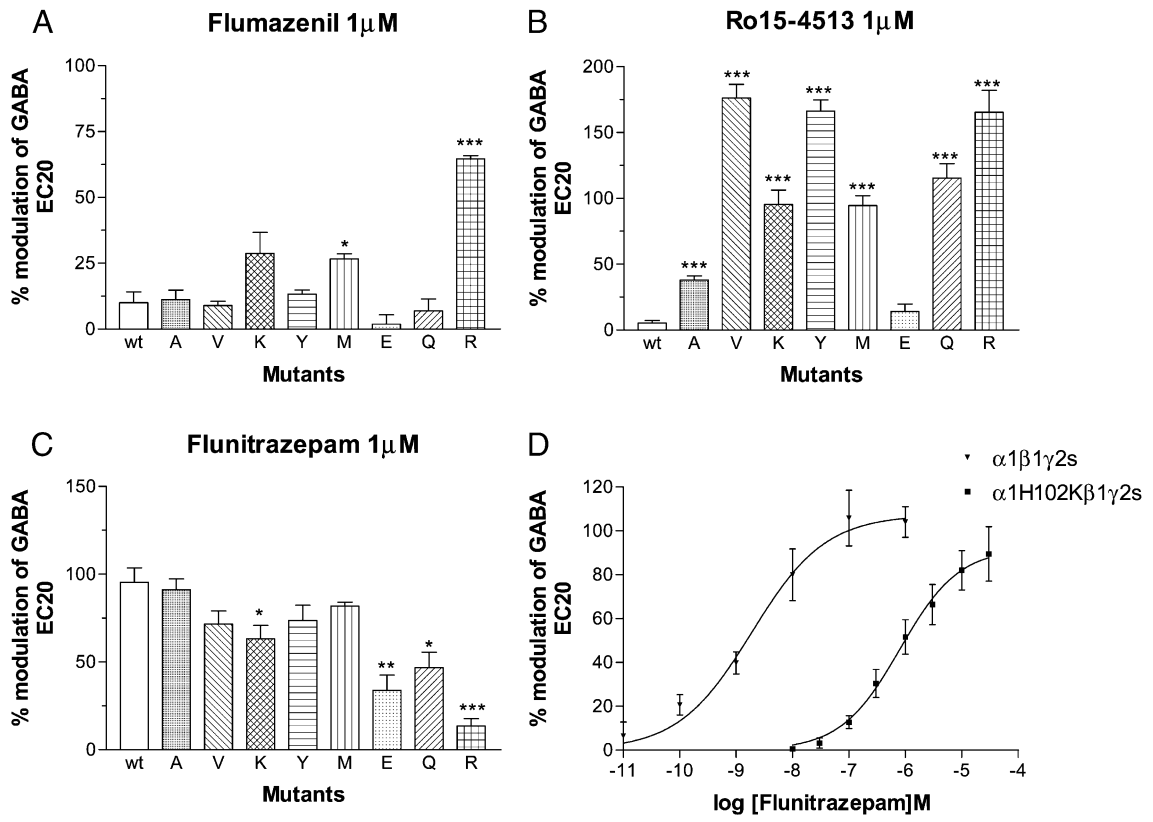

Fig. 4. Modulation of GABA currents in receptors containing α1H102 subunit mutants by (A) 1 μM flumazenil; (B) 1 μM Ro15-4513 and (C) 1 μM flunitrazepam. Receptors containing α1 subunits mutated at H102 were coexpressed with β1γ2 in *Xenopus* oocytes. Modulation of a GABA EC_{20} response was determined for each ligand at 1 μM concentration. Bars represent the modulation as a percentage of the EC_{20} response with the amino acid substituted on the *x*-axis in single letter code. Statistical differences from wild type α1β1γ2 were analysed by Student's *t*-test and levels of significance are indicated above each bar. (D) Concentration response curves for flunitrazepam on α1β1γ2 and α1H102Kβ1γ2S receptors expressed in *Xenopus* oocytes. Data represents the percent potentiation of an EC_{20} response to GABA determined for each oocyte; each point represents the mean ± S.E.M. of at least three determinations.

sequences of these changes on modulation of a GABA EC_{20} response was analysed by application of 1 μM Ro15-4513, flumazenil (Ro15-1788) or flunitrazepam (Fig. 4). With the exception of lysine, methionine and arginine, most of the mutations produced receptors at which flumazenil remained as an antagonist (Fig. 4A), Ro15-4513 on the other hand became an agonist with high efficacy at all the mutants apart from alanine and glutamate (Fig. 4B). No direct correlation between percentage modulation and amino acid side chain properties could be found. Flunitrazepam at 1μM still elicited potentiation at the majority of mutations, the lysine and methionine mutations in particular maintained a high degree of potentiation despite conferring very low affinity for flunitrazepam in the radioligand binding assay. To resolve this apparent anomaly, a concentration–response curve was generated for flunitrazepam at α1H102Kβ1γ2S receptors (Fig. 4D). The maximum potentiation was 91 ± 6.7% with an EC_{50} of 813 nM ($pEC_{50} = -6.09 \pm 0.06$) and a Hill coefficient of 0.90 ± 0.08 ($n = 5$). When compared to the wild type receptor (maximum potentiation 104 ± 7.0; EC_{50} of 1.9 nM), it became apparent that the lysine mutation resulted in a 400-fold reduction in EC_{50} but no change in maximum efficacy.

4. Discussion

4.1. Determination of BZI/BZII receptor pharmacology

The amino acid determining the α1 subunit selectivity of CL218,872 and of zolpidem has previously been identified (Pritchett and Seeburg, 1991). Here α1/α3 chimeric subunits and subsequently mutations of the α3 subunit were made and displacement of [^{3}H]flumazenil by CL218,872 used to identify α1G201 as the determinant of high affinity binding. Substitution of the corresponding position (E225) of α3 with glycine resulted in increased affinity for CL218,872 and zolpidem. Hence, this position is responsible for the phenomenon of BZI/BZII receptor selectivity. From studies of the contribution of the γ subunit to ligand binding at the benzodiazepine site (Buhr and Sigel, 1997; Wingrove et al., 1997), we hypothesized that this subunit and not the α subunit makes the major contribution to the binding energy of benzodiazepine site ligands. It has previously been suggested that the γ subunit contributes substantially to the binding energy of benzodiazepine site ligands by the identification of two amino acids in the binding site. High affinity zolpidem binding in particular

requires the simultaneous presence of γ2F77 and γ2M130 and probably a third unidentified residue (Buhr and Sigel, 1997; Sigel and Buhr, 1997; Wingrove et al., 1997). However, zolpidem is thought of as 'α-subunit selective.' It is difficult to understand how the side chain of glycine (a hydrogen atom) could contribute significantly to zolpidem binding. On the other hand, the carboxylic acid side chain of the glutamate residue present in α2/3 is both larger than glycine and also carries a charge suggesting that the lower affinity of zolpidem for α3-containing receptors may be due to inhibition of binding by either steric hindrance or charge repulsion. Hence, it was hypothesized that the α subunit could have a negative impact on ligand binding resulting in the observed α-subunit selectivity. This hypothesis was tested by the substitution of a variety of amino acids (A, D, E, M and V) at α1 position 201 and determination of ligand affinities. The non-selective benzodiazepine flumazenil is unaffected by substitution at this position. The affinity of the non-benzodiazepine agonists CL218,872 (a triazolopyridazine) and zolpidem (an imidazopyridine) are however adversely affected by increased amino acid side chain volume, the correlation for both compounds giving a similar slope. This suggests that BZI receptor selective compounds impinge on this part of the binding site resulting in a reduction in affinity by steric inhibition at BZII-type receptors. Conversely, non-selective compounds have no part of their structure in the immediate vicinity of α1G201 and hence are unaffected by any amino acid substitution at this position.

Functional responses of zolpidem and CL218,872 on receptors containing α1G201E were similar to those containing α3. The apparent change in both EC_{50} and maximum efficacy are in agreement with a possible steric effect of this mutation on benzodiazepine function, possibly indicating a role for α1G201 in the transduction of benzodiazepine binding.

4.2. Determination of diazepam-sensitive/diazepam-insensitive pharmacology

Receptors containing α6 (or α4) have an atypical benzodiazepine pharmacology (Hadingham et al., 1996; Lüddens et al., 1990; Wafford et al., 1996; Wisden et al., 1991; Yang et al., 1995). In particular, these receptors have reduced affinity for certain benzodiazepine agonists and hence have been referred to as diazepam-insensitive compared to diazepam-sensitive receptors which contain α1, α2, α3 or α5. Wieland et al. (1992) used chimeric α1/α6 subunits and site-directed mutagenesis to demonstrate the importance of α1H102 and the homologous position α6R100 in the determination of benzodiazepine agonist affinity. It has recently been demonstrated that mutation of the homologous position of α's 2, 3 or 5 to arginine also renders these isoforms diazepam-insensitive (Benson et al., 1998). As with α1G201/α3E225, the subunit conferring the higher affinity has a smaller amino acid side chain. These authors suggested that a steric mechanism may operate since the affinity for diazepam is unaffected by the pH of the assay buffer. This would be consistent with the 'diazepam-insensitive' pharmacophore being smaller than that of the 'diazepam-sensitive' site (Zhang et al., 1995). Hence, our investigation considered the possibility that a steric mechanism underlies the pharmacological specificity by substitution of α1 position 102 with A, R, E, Q, K, M, Y and V. None of the compounds tested gave a clear correlation of binding affinity with amino acid size. Substitution with the hydrophobic residues Y, V, M and A actually increased the affinity for [^{3}H]Ro15-4513—a benzodiazepine inverse agonist with no selectivity for α1/α6 subunits. The pharmacology of receptors containing either α6 or α1H102R was similar for all three compounds. There is a weak correlation between affinity and hydrophobicity for Ro15-4513 and flumazenil. Affinity for the only agonist tested, flunitrazepam, is drastically reduced by all substitutions made in this study suggesting a direct interaction with α1H102. This residue is postulated to form a link to the pendant phenyl moiety of flunitrazepam (McKernan et al., 1998).

Interestingly, the majority of mutants maintained a degree of potentiation by 1μM flunitrazepam, despite dramatic reductions in K_i for the receptor as measured by the displacement of [^{3}H]Ro15-4513 binding. However, concentration response curves comparing α1β1γ2 wild type, and the α1H102Kβ1γ2 mutant revealed a 400-fold reduction in EC_{50} to 813 nM, similar to that observed in the binding assay. As this mutation conferred the largest shift in binding K_i it was apparent that comparing the degree of potentiation at 1 μM did not reveal large differences in the EC_{50}, and in fact the maximum efficacy on α1H102Kβ1γ2 was similar to wild type suggesting that unlike Ro15-4513, the maximum level of efficacy for flunitrazepam may be similar on mutants at this position. Mutation to arginine by contrast produces a receptor which no longer responded to flunitrazepam. This is consistent with its low affinity and also with the lack of effect of flunitrazepam at receptors containing α6 which also have arginine at the homologous position. The response to Ro15-4513 and flumazenil is also altered in this mutant to resemble that observed with α6-containing receptors (Hadingham et al., 1996). The extent of potentiation did not correlate with measures of amino acid side chain volume or hydrophobicity.

The consequences of a series of mutations of rat α1H101 (including some made in this study) have recently been reported (Davies et al., 1998). When coexpressed with β2γ2, the mutations resulted in up to a 14-fold increase in affinity for [^{3}H]Ro15-4513. The affinity for flumazenil on the other hand was reduced by all mutations with a quantitatively larger decrease for flunitrazepam. These data are generally consistent with that presented here (Table 2) with the exception of the tyrosine mutant for which the affinity differed consistently by 10-fold from our values. The authors found no correlation between affinity and either amino acid size or hydrophobicity. The functional coupling

of the GABA and benzodiazepine sites was retained on mutation of α1H101 to either glutamate, phenylalanine or tyrosine (Davies et al., 1998). The rank order of potency for the mutations made was qualitatively similar when comparing the agonists flunitrazepam and ZK93423, although quantitatively smaller for the latter, and also on comparison of the antagonists flumazenil and ZK93426 (Davies et al., 1998). Since the ZK compounds are β-carbolines, this was suggested to indicate that different chemical structures with similar efficacies may interact with the receptor in the same way and that α1H101 may be involved in the determination of efficacy of these compounds. In a follow up study with these mutants, the nature of the amino acid at position 101 was shown to influence the efficacy of benzodiazepines (Dunn et al., 1999). Consistent with this proposal, mutation of the H101 equivalent in α1, α2, α3 and α5 to arginine not only creates a diazepam-insensitive receptor but also converts Ro15-4513 into a potent agonist, as at receptors containing α4/6 (Benson et al., 1998). Intriguingly however, photoaffinity labelling of brain membranes with flunitrazepam compromises the benzodiazepine binding site but not coupling with the GABA site (Brown and Martin, 1984; Gibbs et al., 1985). Recombinant α1β3γ2 receptors photoaffinity labelled with flunitrazepam also retain functional coupling (McKernan et al., 1998). In this study, it was deduced that essentially the whole flunitrazepam molecule becomes irreversibly bound to α1H102 (human sequence numbering) and then twists out of the binding site. In this way, its presence had no effect on the affinity of benzodiazepine site ligands with the exception of those having a pendant phenyl substituent. It was suggested that the phenyl group interacts with H102 by hydrogen-bonding or π−π interactions (McKernan et al., 1998). The drastic reduction in affinity caused by any mutation of α1H102 found in our study is consistent with a direct interaction between this amino acid and the phenyl ring of flunitrazepam. This need not exclude the interaction of the phenyl ring with residues of the γ subunit already proposed. Photoaffinity labelling with [^{3}H]Ro15-4513 causes an almost complete loss in the binding of other benzodiazepine site ligands consistent with a different mode of binding of this compound compared to flunitrazepam (McKernan et al., 1998).

Benzodiazepine affinity is further affected by additional, cumulative mutations of α6 (R100H/P161T/E199G/I211V) and again at this fourth position the latter residue is smaller (Wieland and Lüddens, 1994). This fourth position is one of two residues which influences the affinity of the α5 subunit selective compound L-655,708 (ethyl (*S*)-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate; Casula et al., 2001). In this case, the higher affinity α5-containing receptors have the larger amino acid (α5I215). Modulation of $GABA_A$ receptors by benzodiazepines is also affected by mutation of rat α1Y159 and α1Y209; positions homologous to two tyrosines on the β2 subunit proposed to form part of the GABA binding site (Amin et al., 1997). Mutation of either of these positions to serine impairs potentiation by diazepam and the binding of [^{3}H]flumazenil whereas the more conservative substitution by phenylalanine has a less pronounced effect. The mutations have no effect on receptor activation by GABA and homologous mutations of γ2 do not affect benzodiazepine pharmacology (Amin et al., 1997). Mutation of α1G200 to glutamate was also reported to result in a reduction in affinity for the BZI receptor selective compounds CL218,872 and zolpidem with no change for diazepam, DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) or flumazenil (Schaerer et al., 1998). The three amino acid periodicity of identified residues within this domain (α1G200, T206 and Y209; rat sequence numbering) suggested that they may lie on an α-helix. Mutations of the intervening position α1Q203 however had no effect on ligand affinity (Schaerer et al., 1998) consistent with proposals that the homologous domain of the glycine receptor forms a β-sheet (Vandenberg et al., 1992).

In summary, the impact of substitutions at two amino acid positions known to be determinants of α-subunit selective pharmacology has been assessed by site-directed mutagenesis of the α1 subunit followed by ligand binding and functional studies. The affinity of benzodiazepine site ligands is reduced by increasing amino acid volume at position 201 of the α1 subunit suggesting that ligands whose pharmacophore is in this region may lose affinity by steric inhibition. On the other hand, benzodiazepine affinity is weakly correlated with amino acid hydrophobicity at α1 position 102. These data also strongly suggest the direct interaction of flunitrazepam with α1H102, in agreement with data from other studies.

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