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Pharmacology of recombinant γ-aminobutyric acid$_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits

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Abstract Amino acids in the α- and γ-subunits contribute to the benzodiazepine binding site of GABA$_A$-receptors. We show that the mutation of a conserved histidine residue in the N-terminal extracellular segment (α1^{H101R}, α2^{H101R}, α3^{H126R}, and α5^{H105R}) results not only in diazepam-insensitivity of the respective αxβ2,3γ2-receptors but also in an increased potentiation of the GABA-induced currents by the partial agonist bretazenil. Furthermore, Ro 15-4513, an inverse agonist at wild-type receptors, acts as an agonist at all mutant receptors. This conserved molecular switch can be exploited to identify the pharmacological significance of specific GABA$_A$-receptor subtypes in vivo.

Key words: Neurotransmitter; Receptor; γ-Aminobutyric acid; Benzodiazepine; Recombinant; Central nervous system

1. Introduction

Classical benzodiazepines (BZ) such as diazepam are in wide clinical use as anxiolytics, hypnotics, myorelaxants and anticonvulsants. These activities are based on the enhancement of GABAergic transmission at BZ-sensitive GABA$_A$-receptors [1], which are composed of an α-subunit variant (α1, α2, α3, or α5) in combination with a β-subunit (β1–3) and a γ-subunit (γ1–3) [2–4]. The receptor subtypes α1β2,3γ2, α2β2,3γ2, α3β2,3γ2 and α5β2,3γ2 are considered to be the major mediators of BZ actions in the brain [5]. To identify the pharmacological significance of individual receptor subtypes, point mutations were sought by which individual receptor subtypes might be rendered diazepam-insensitive in vivo.

In previous mutational analyses, a histidine to arginine substitution in position 101 of the α1-subunit (α1^{H101R}) strongly reduced the diazepam response of the recombinant α1β2γ2-receptor expressed in human embryonic kidney (HEK) 293 cells in vitro [6,7]. Since a homologous histidine residue also occurs in the α2-, α3- and α5-subunits (Fig. 1), we investigated whether the histidine to arginine mutation would represent a common molecular switch to render the respective receptor subtypes diazepam-insensitive. A common molecular switch could be exploited for the analysis of the contribution of individual receptor subtypes to the pharmacological spectrum of diazepam by generating knock-in mice carrying this point mutation. In the present study, the BZ-responsiveness of point-mutated receptors was tested electrophysiologically following the co-expression of the mutated α1-, α2-, α3- and α5-subunits with the wild-type β2- (or β3-) and γ2-subunits in HEK 293 cells.

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Abbreviations: GABA$_A$, γ-aminobutyric acid type A; BZ, benzodiazepine; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate

2. Materials and methods

2.1. Construction of GABA$_A$ receptor subunit expression vectors

The rat α1-subunit cDNA [6] (1.6-kb *Xho*I fragment in *Xho*I site of pSK-α1) was subcloned as a *Hin*dIII/*Kpn*I fragment into M13mp19 and point-mutated using the Amersham Sculptor kit and oligonucleotide UR39: 5′-GAC TTT TTT CCA TTC CGG AAA AAT GTA TCT-3′. The mutated cDNA was resequenced, subcloned first into pKS (*Hin*dIII/*Kpn*I), then with a complete *Bgl*II and partial *Bam*HI digest into the *Bam*HI site of the expression vector pBC12/CMV [8]. The α2-cDNA (1.4-kb fragment in M13PICH-rα2) underwent oligonucleotide-directed mutagenesis with KL4: 5′-TGA CTT TTT CCC GTT CCG GAA GAA GGT GTC AGG AGT-3′, and was resequenced and subcloned as a *Bam*HI/*Bgl*II fragment into the *Bam*HI site of pBC12/CMV. The α3-cDNA [9] (3.4-kb *Eco*RI fragment in M13mp18) was mutated using oligonucleotide UR40: 5′-AGA TAC CTT CTT CCG GAA CGG TAA AAA ATC-3′. The mutated cDNA was sequenced and subcloned into pKS with *Sac*I (now as a 1.7-kb fragment). From there it was partially digested with *Sst*I and completely with *Bam*HI and subcloned into pSP72 (Promega), from where it was recovered as a *Bam*HI/*Bgl*II fragment and subcloned into the *Bam*HI site of pBC12/CMV. The α5-cDNA [9] (*Eco*RI partial fragment in M13PIC-19H) was mutated using oligonucleotide UR41: 5′-GAC TTC TTC CCA TTC CGG AAG AAT GTG TCT-3′, sequenced and subcloned into pKS as a 2.1-kb *Hin*dIII/*Sst*I fragment. It was then subcloned into pSP72 with *Sst*I/Asp^{718}, from where it was recovered as a *Bam*HI/*Bgl*II fragment and subcloned into the *Bam*HI site of pBC12/CMV. Corresponding expression vectors containing the wild-type α1-, α2-, α3- and α5-subunit cDNAs were prepared in parallel. The β2-, β3- and γ2-subunits were also used in pBC12/CMV.

2.2. Cell culture and transfection

Human embryonic kidney cells 293 were grown in MEM medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin and 20 mM L-glutamine (all from Life Technologies, Basel, Switzerland) and transformed transiently with rat cDNA αβγ combinations (ratio 1:1:1) by calcium phosphate precipitation [10].

2.3. Electrophysiology and data analysis

The whole-cell configuration of the patch-clamp technique was used to record GABA-induced Cl^- currents. The GABA dose-response curves were obtained by applying 2-s pulses of GABA every 2 min to the patch-clamped HEK-293 cells, using a multibarrelled microapplicator pipette, as previously described [11]. The maximum current amplitudes from individual cells were first fitted separately using the equation $I/I_{max} = 1/(1+(EC_{50}/[GABA])^{Hill})$, where I = GABA-evoked current, I_{max} = the maximum of the fit, EC_{50} = the GABA concentration evoking the half maximal response, and Hill = the Hill coefficient. The individual dose-response curves were then normalized to I_{max}, and the data replotted using the mean values for each concentration. For each experiment, at least three GABA control responses were evoked and only cells showing stable GABA responses were selected for the drug testing. Prior to microapplication of a GABA-drug mixture, the same concentration of the drug alone was applied by bath

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perfusion for at least 2 min. Stock solutions of the test compounds were prepared in 100% DMSO and diluted 1000-fold before use. During the experiment, all bath solutions contained 0.1% DMSO, which by itself was without detectable effect on the GABA responses. The benzodiazepine ligands bretazenil, Ro 15-4513, and diazepam were kindly provided by F. Hoffmann-La Roche Ltd. (Basel).

3. Results

3.1. GABA-sensitivity of the point-mutated recombinant $GABA_A$-receptors

A histidine to arginine codon substitution was introduced into homologous positions of rat α1-, α2-, α3- and α5-subunit cDNAs (positions 101, 101, 126 and 105, respectively) (Fig. 1) by site-directed mutagenesis. The wild-type and point-mutated α-subunits were co-expressed in HEK 293 cells with the γ2-subunit and either the β2- or the β3-subunit, depending on the associations most commonly detected in rat brain [12–14], in order to generate the following subunit combinations: $\alpha1\beta2\gamma2$, $\alpha1^{H101R}\beta2\gamma2$, $\alpha2\beta3\gamma2$, $\alpha2^{H101R}\beta3\gamma2$, $\alpha3\beta3\gamma2$, $\alpha3^{H126R}\beta3\gamma2$, $\alpha5\beta2\gamma2$ and $\alpha5^{H105R}\beta2\gamma2$.

The EC_{50} values for GABA for the receptors incorporating wild-type α-subunits were 23 ± 2 μM for $\alpha1\beta2\gamma2$, 74 ± 12 μM for $\alpha2\beta3\gamma2$, 165 ± 68 μM for $\alpha3\beta3\gamma2$ and 14 ± 1 μM for $\alpha5\beta2\gamma2$ (Fig. 2). These values largely correspond to the potencies of GABA reported previously: the published EC_{50} values are 4.5–20 μM for $\alpha1\beta2\gamma2$ [15–17], 25 μM for $\alpha2\beta3\gamma2$ [18], and 4.2–16 μM for $\alpha5\beta2\gamma2$ [9,11,15,17]. Only the EC_{50} value determined for the $\alpha3\beta3\gamma2$ (165 ± 68 μM) differed appreciably from the published values, 28 μM and 33 μM [17,18]. The GABA EC_{50} values for receptors incorporating the point-mutated α-subunits were all modestly higher than the EC_{50} values for the wild-type receptors: $\alpha1^{H101R}\beta2\gamma2$ yielding an EC_{50} value of 31 ± 1 μM (compared to 23 ± 2 μM for $\alpha1\beta2\gamma2$); $\alpha2^{H101R}\beta3\gamma2$ yielding 154 ± 4 μM (compared to 74 ± 12 μM for $\alpha2\beta3\gamma2$); $\alpha3^{H126R}\beta3\gamma2$ yielding 253 ± 51 μM (compared to 165 ± 68 μM for $\alpha3\beta3\gamma2$) and $\alpha5^{H105R}\beta2\gamma2$ yielding 27 ± 1 μM (compared to 14 ± 1 μM for $\alpha5\beta2\gamma2$) (Fig. 2).

3.2. Modulation of point-mutated $GABA_A$-receptors by ligands of the benzodiazepine site

To assess the modulation of the GABA-evoked currents by benzodiazepine site ligands, GABA concentrations of 3 μM (α1-, α3- and α5-receptors) and 30 μM (α2-receptor) were chosen, corresponding to maximally EC_{30} values. The modulation of the GABA response by the full agonist diazepam is summarized in Fig. 3; representative individual traces for the $\alpha2\beta3\gamma3$ subtype are demonstrated in Fig. 4. The benzodiazepine full agonist diazepam (1 μM) potentiated GABA-induced

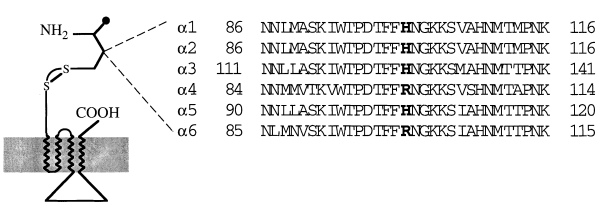

Fig. 1. Alignement of partial N-terminal extracellular sequences of the rat $GABA_A$-receptor α-subunits (α1–α6) (modified from [6]). The conserved histidine residue in the α1-, α2-, α3- and α5-subunits (positions 101, 101, 126, and 105, respectively) are highlighted. The α4- and α6-subunits contain an arginine residue at the corresponding positions (99 and 100, respectively).

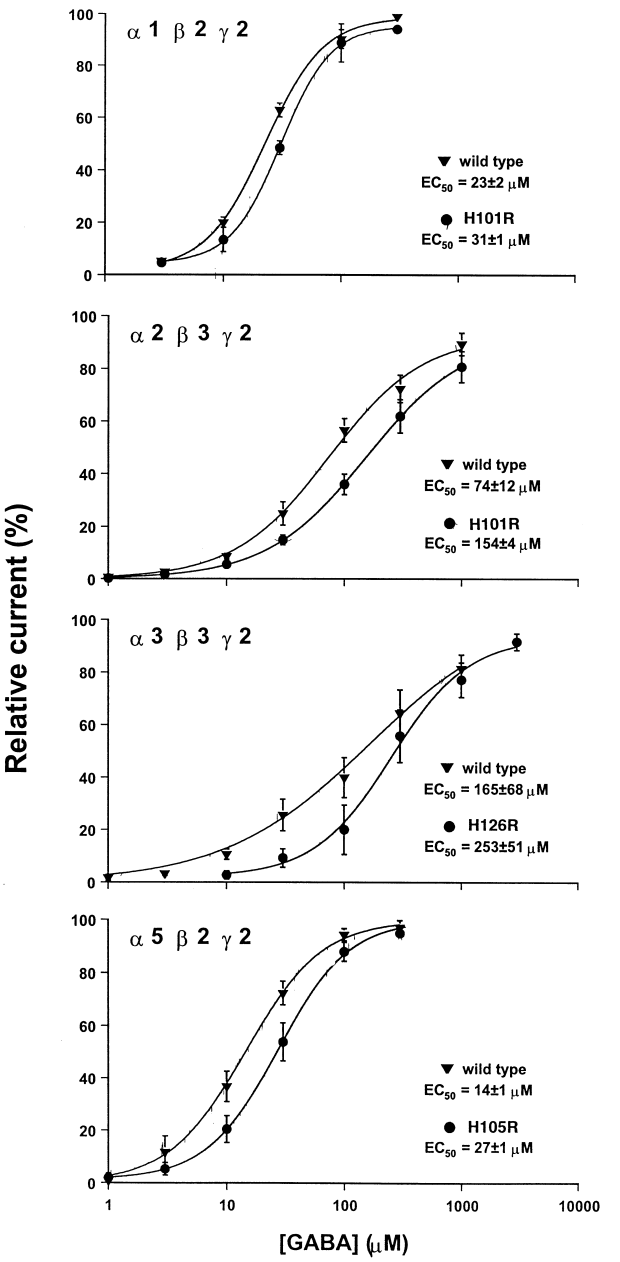

Fig. 2. GABA dose-response curves for wild-type and point-mutated $GABA_A$-receptor subtypes expressed in HEK 293 cells transiently transfected with the cDNAs coding for the wild-type (wt) or mutated α-subunits in combination with the β2- or β3- and γ2-subunits ($\alpha1\beta2\gamma2$, $\alpha1^{H101R}\beta2\gamma2$; $\alpha2\beta3\gamma2$, $\alpha2^{H101R}\beta3\gamma2$; $\alpha3\beta3\gamma2$, $\alpha3^{H126R}\beta3\gamma2$; $\alpha5\beta3\gamma2$, and $\alpha5^{H105R}\beta3\gamma2$). The data points and error bars represent the means and standard errors for the different GABA concentrations ($n = 3$–5).

currents of receptors incorporating wild-type α1-, α2-, α3- and α5-subunits by 29 ± 11%, 72 ± 5%, 108 ± 29% and 118 ± 19%, respectively (Figs. 3 and 4). In contrast, the GABA-evoked responses mediated by receptors containing the point-mutated α1-, α2-, α3- and α5-subunits were diazepam-insensitive (Figs. 3 and 4) at doses that produced maximum enhancement at wild-type receptors. These results demonstrate that α2-, α3- and α5-$GABA_A$-receptors can be rendered diazepam-insensitive by a histidine to arginine point mutation. Thus, the structure-activity relationship for the in-

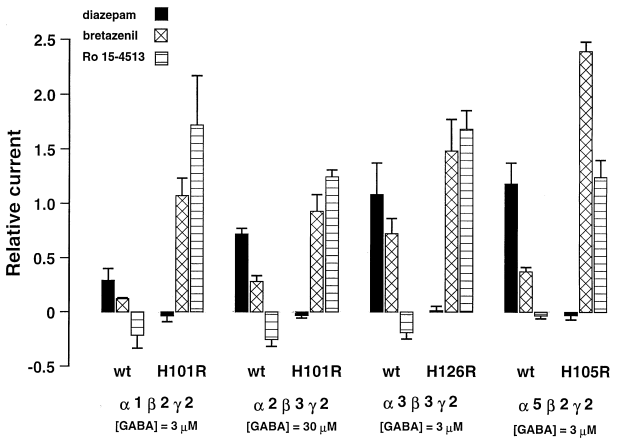

Fig. 3. Potentiation of the GABA-evoked currents by BZ site ligands for wild-type and point-mutated $GABA_A$-receptor subtypes expressed in HEK 293 cells transiently transfected with the cDNAs coding for the wild-type (wt) or mutated α-subunits in combination with the β2- or β3- and γ2-subunits (for details see legend to Fig. 2). The modulation of GABA-evoked currents by the BZ site ligands diazepam (1 μm), bretazenil (1 μm) and Ro 15-4513 (1 μm) is expressed relative to the control currents at the given GABA concentration (mean ± standard error, $n = 3–7$).

teraction of diazepam with the BZ site appears to be conserved among the receptor subtypes tested.

To assess the influence of the histidine to arginine point mutation on the responsiveness to other benzodiazepine site ligands, the effect of the partial agonist bretazenil was analyzed. Application of bretazenil (1 μM) to the receptors containing the wild-type α1-, α2-, α3- and α5-subunits characteristically potentiated the GABA-evoked response, but to a lesser degree than the full agonist diazepam applied at the same concentration (α1β2γ2, potentiation: 12 ± 1%; α2β3γ2, potentiation: 28 ± 6%; α3β3γ2, potentiation: 72 ± 14%; and α5β2γ2, potentiation: 37 ± 4%) (Figs. 3 and 4). However, application of bretazenil (1 μM) to the receptors containing the point-mutated α-subunits resulted in a potentiation of the GABA-evoked response that was 2- to 9-fold higher than that observed for receptors incorporating wild-type α-subunits ($\alpha1^{H101R}\beta2\gamma2$, potentiation: 107 ± 16%; $\alpha2^{H101R}\beta3\gamma2$, potentiation: 93 ± 15%; $\alpha3^{H126R}\beta3\gamma2$, potentiation: 148 ± 29%, and $\alpha5^{H105R}$, potentiation: 239 ± 8%) (Figs. 3 and 4). Bretazenil thus showed an increased potentiating effect when tested on the diazepam-insensitive receptors compared to wild-type receptors. Its potency was about equal to ($\alpha2^{H101R}\beta3\gamma2$, $\alpha3^{H126R}\beta3\gamma2$) or significantly higher ($\alpha1^{H101R}\beta2\gamma2$, $\alpha5^{H105R}\beta2\gamma2$) than that of diazepam (1 μM) at the corresponding wild-type receptors. Thus, the point mutation which renders the α1-, α2-, α3- and α5-receptors diazepam-insensitive does not abolish the effectiveness of bretazenil at any of the receptors.

The partial inverse agonist Ro 15-4513 is known to reduce the amplitude of the GABA-evoked responses at α1β2γ2, α1β1γ2, α2β1γ2, α5β3γ2 and α5β3γ3 receptors [19–22]. Here, we show that the inverse agonism of Ro 15-4513 extends to the α2β3γ2-, α3β3γ2-, and α5β2γ2-receptors (Figs. 3 and 4). However, on receptors containing the point-mutated α1-, α2-, α3- and α5-subunits, Ro 15-4513 (1 μM) was found to strongly potentiate the GABA-evoked currents. The percent enhancement of the GABA-evoked currents by Ro 15-4513 applied to the point-mutated receptors (α1β2γ2, potentiation: 171 ± 45%; α2β3γ2, potentiation: 124 ± 6%; α3β3γ2, potentiation: 168 ± 17%; α5β2γ2, potentiation: 124 ± 16%) was equal to (α5) or greater than (α1, α2, α3) the potentiation induced by the same dose of diazepam applied to the wild-type receptors (Figs. 3 and 4). The point mutation thus changed the mode of interaction of Ro 15-4513 from inverse agonism to agonism. These results suggest that the mode of interaction of Ro 15-4513 with the benzodiazepine binding site differs from that of either diazepam and bretazenil.

A switch in the apparent efficacy of a benzodiazepine site ligand has previously been observed for methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), based, however, on an unusual dose-response behavior on various wild-type receptors (α1β1γ2, α1β2γ2, and α5β2γ2); DMCM acted as an inverse agonist at low doses and as an agonist at high doses [9]. To exclude the possibility that the agonism observed for Ro 15-4513 on the point-mutated receptors is due to a dose-response phenomenon, an entire dose-response curve was recorded for the effect of Ro 15-4513 on one of the mutant receptors, $\alpha2^{H101R}\beta3\gamma2$ (Fig. 5). For each dose tested over the range of 1 nM to 1 μM, Ro 15-4513 displayed an agonistic effect on the mutated receptors. Thus, the agonistic activity of Ro 15-4513 on receptors containing the mutated α-subunits cannot be attributed to a dose-response phenomenon. The histidine to arginine mutation in the α1- α2-, α3- and α5-subunits rather switches the efficacy of Ro 15-4513 from inverse agonism to agonism.

4. Discussion

The BZ binding site is considered to be located at the interface of the α- and the γ2-subunit of the $GABA_A$ receptor (reviewed in [23]). Here we demonstrate that the histidine residue in the BZ site, originally identified in the α1-subunit as a photoaffinity target [24,25], is a functional characteristic common to all major BZ-sensitive $GABA_A$-receptors. Mutation of this residue to arginine in either the α1-, α2-, α3- or the α5-subunit leads to a complete loss of potentiation of GABA-induced currents by diazepam when co-expressed with β2- or β3- and γ2-subunits, in accordance with the diazepam-insensitivity of the α4β2γ2 and α6β2γ2 wild-type receptors which contain an arginine residue in the corresponding position [19,26–28]. Since diazepam does not bind to either the α4- and α6-receptors [26,27,29] or the $\alpha1^{H101R}\beta2\gamma2$-receptors [6], the lack of diazepam-responsiveness of the point-mutated α2-, α3- and α5-receptors can most likely be attributed to a lack of affinity for diazepam.

In contrast to diazepam, the potentiation of GABA-evoked responses of the point-mutated receptors by bretazenil is uniformly increased compared to the respective wild-type receptors. This is unlikely to be due to an increase in the affinity of bretazenil, since bretazenil displays a nanomolar affinity to the diazepam-insensitive α6β3γ2-receptor ($K_i = 12.7$ nM) which is lower than its affinity to the diazepam-sensitive α1β3γ2-receptor ($K_i = 0.35$ nM) [27]. Thus, the histidine to arginine point mutation does not appear to increase the affinity but rather to affect the signal transduction process in the interaction between bretazenil and the mutant receptors. These differences in the interaction of diazepam and bretazenil appear to be common to all receptor subtypes tested and point to distinct

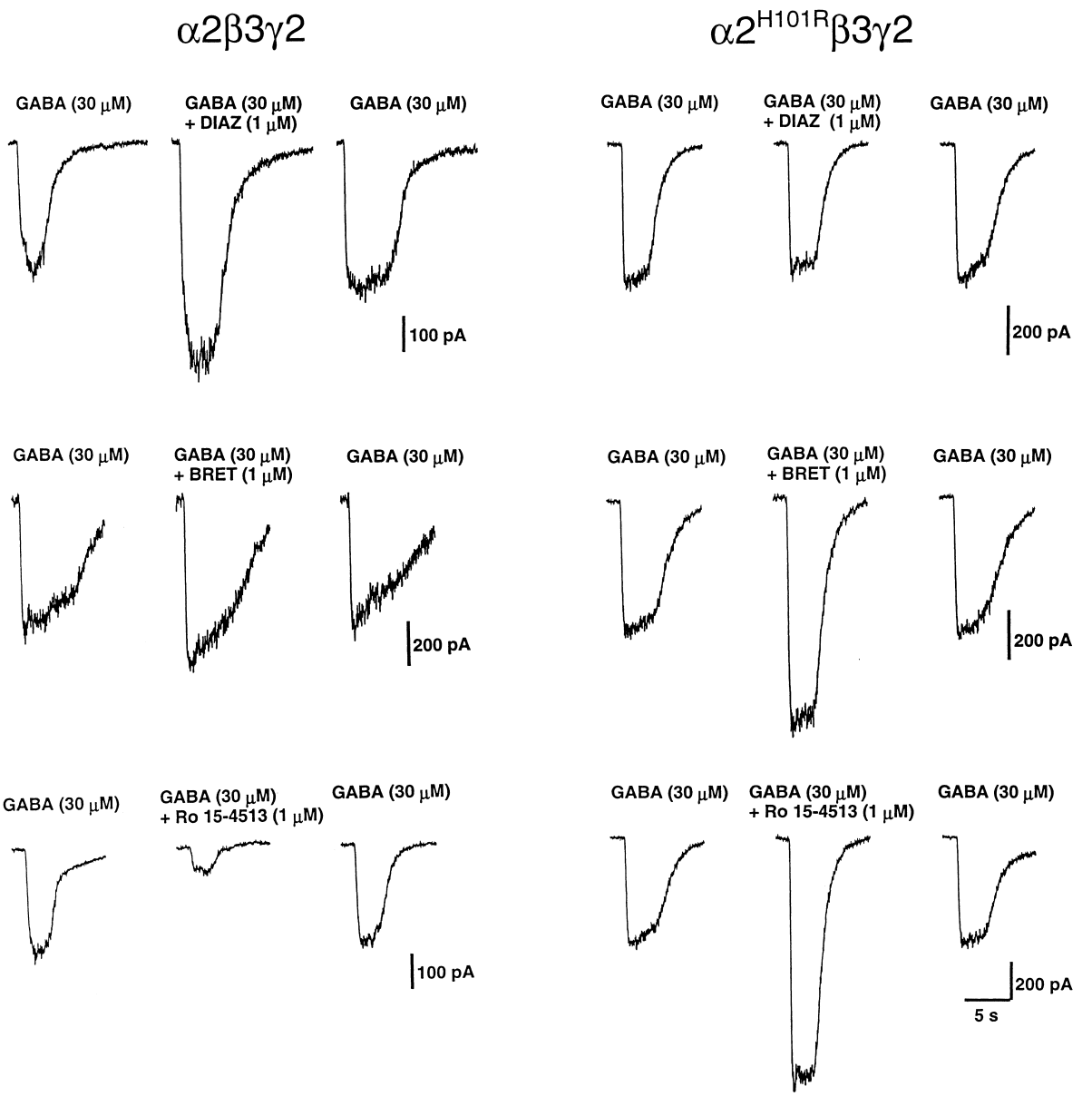

Fig. 4. Single traces of the effects of diazepam (DIAZ), bretazenil (BRET) and Ro 15-4513 on the GABA-evoked currents are exemplified for wild-type α2β3γ2 and point-mutated $\alpha2^{H101R}\beta3\gamma2$ $GABA_A$-receptors. In α2β3γ2-receptors the agonist diazepam (1 μm) and to a lesser degree the partial agonist bretazenil (1 μm) potentiated the current, while the inverse agonist Ro 15-4513 (1 μm) reduced the current. In $\alpha2^{H101R}\beta3\gamma2$ receptors, diazepam was without effect and the current was strongly potentiated by bretazenil and Ro 15-4513.

domains for interaction of diazepam and bretazenil at the BZ binding site.

The inverse agonist Ro 15-4513 interacts in yet another manner with the benzodiazepine binding site of $GABA_A$-receptors. In the point-mutated receptors, its efficacy is switched from inverse agonism to agonism. This is in line with the agonistic action of Ro 15-4513 on the diazepam-insensitive α4- or α6-subunit-containing receptors α4β1γ2, α4β2γ2, α6β1γ2 and α6β2γ2 [19,26–28] and the retention of its high affinity to the diazepam-insensitive α6-receptors (K_D=10 nM) [6]. Ro 15-4513 has previously been shown to act as a partial agonist at α1β1γ2-receptors containing a threonine to serine substitution in position 142 of the γ2-subunit [21]. Therefore, critical residues in both the α-subunits and the γ2-subunit determine the efficacy of Ro 15-4513.

In summary, the mutation of a single histidine to arginine in the α1- α2-, α3- and α5-subunits induces a conformational change with distinct pharmacological consequences common to all $GABA_A$-receptors tested. The results underline the notion that the $GABA_A$-receptor subtypes contain common and highly conserved structural determinants influencing the affinity and efficacy of BZ site ligands.

Our findings open the possibility to define the pharmacological significance of $GABA_A$-receptor subtypes in vivo. By individually mutating the conserved histidine residue in the α1-, α2-, α3- and α5-subunits to arginine by gene targeting, individual $GABA_A$-receptor subtypes will be rendered insensitive to diazepam. The resulting mouse lines are expected to display characteristic deficits in the pharmacological spectrum of diazepam. The present results support the validity of this in

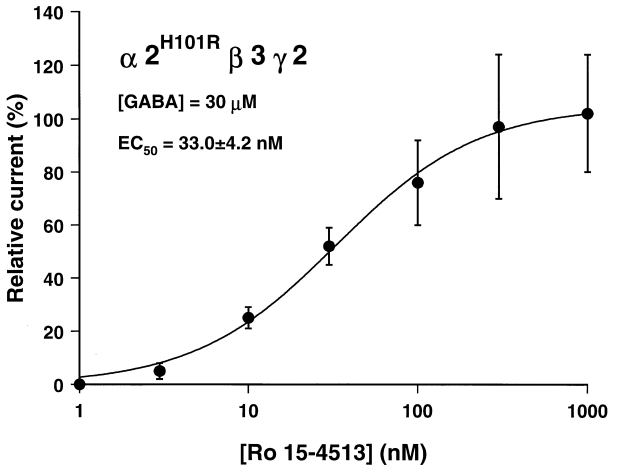

Fig. 5. Dose-response curve for the effect of Ro 15-4513 on the recombinant $\alpha 2^{H101R}\beta 3\gamma 2$ receptor-mediated current evoked by 30 μM GABA. The mean amplitudes of the potentiated currents relative to the control currents were normalized to a control value set at 1.0, and plotted as a function of the drug concentration. The data points and error bars represent the means and standard errors for different drug concentrations ($n = 3$–4).

vivo approach by verifying three major preconditions. (i) The point mutation affects the diazepam response at the corresponding four $GABA_A$-receptor subtypes in the same manner. (ii) The mutation does not appreciably affect the potency of GABA in activating the four receptor subtypes, suggesting that the physiological responsiveness of the receptors to GABA is not altered. (iii) The mutation does not interfere with subunit synthesis and assembly, which is in keeping with the expression of diazepam-insensitive α4- and α6-receptors in the brain. The point-mutated mouse lines will provide important insights into the neuronal circuits mediating selective actions of diazepam-induced behavior.

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