



Dual mode of stimulation by the β -carboline ZK 91085 of recombinant GABA_A receptor currents: molecular determinants affecting its action

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1 In electrophysiological measurements the β -carboline ethyl 6-benzyloxy- β -carboline-3-carboxylate (ZK 91085) acts as a positive allosteric modulator on rat recombinant $\alpha 1\beta 2\gamma 2$ GABA_A receptors and binds with high affinity ($IC_{50} = 1.5$ nM) to the [³H]-flunitrazepam site. Flumazenil was able to partially counteract the current modulation. These observations indicate an action of ZK 91085 at the benzodiazepine binding site.

2 At the dual subunit combination $\alpha 1\beta 2$, which lacks the γ subunit required for benzodiazepine modulation, we still observed a potentiation of GABA currents. Thus ZK 91085 acts *via* an additional site on the channel. At the subunit combination $\alpha 1\beta 1$, ZK 91085 potentiation is strongly reduced as compared to $\alpha 1\beta 2$. In binding studies, ZK 91085 was able to decrease [³⁵S]-TBPS binding in $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 2$ but not in $\alpha 1\beta 1$. This selectivity of ZK 91085 for receptors containing the $\beta 2$ isoform over those containing the $\beta 1$ isoform is reminiscent of the action of loreclezole.

3 To identify amino acid residues important for the second type of modulation, we functionally compared wild type $\alpha 1\beta 2$ and mutant receptors for stimulation by ZK 91085. The mutation $\beta 2N265S$, that abolishes loreclezole effects, also abolishes ZK 91085 stimulation. The mutation $\beta 2Y62L$ increased stimulation by ZK 91085 3–4 fold, locating an influencing entity of the second type of action of ZK 91085 at an α/β subunit interface.

4 Structural intermediates of ZK 91085 and the β -carboline abecarnil, the latter of which only slightly potentiated GABA currents in $\alpha 1\beta 2$, were analysed to determine structural requirements for modulation.

5 ZK 91085 thus allosterically stimulates the GABA_A receptor through two sites of action: the benzodiazepine site and the loreclezole site in contrast to classical β -carbolines, that confer negative allosteric modulation through the benzodiazepine site.

Keywords: GABA_A receptor; ZK 91085; β -carbolines; DMCM; loreclezole

Abbreviations: DMCM, methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate; GABA, γ -aminobutyric acid; GABA_A, γ -aminobutyric acid type A receptor; HEK, human embryonic kidney; TBPS, t-butylbicyclophosphorothionate; ZK 38622, ethyl β -carboline-3-carboxylate; ZK 91085, ethyl 6-benzyloxy- β -carboline-3-carboxylate; ZK 92610, ethyl 6-diallylamino- β -carboline-3-carboxylate; ZK 93423, ethyl 6-benzyloxy-4-methoxy-methyl- β -carboline-3-carboxylate; ZK 111403, ethyl 6-benzyloxy-4-ethyl- β -carboline-3-carboxylate; ZK 113630, isopropyl 6-benzyloxy-4-methyl- β -carboline-3-carboxylate; ZK 115304, isopropyl 6-benzyloxy- β -carboline-3-carboxylate; ZK 135988, 6-benzyloxy-4-methoxy-methyl-3-(1H-1,2,4-triazol-3-yl)- β -carboline

Introduction

The GABA_A receptor (γ -aminobutyric acid type A receptor) is one of the major inhibitory ion channels in the central nervous system. So far six isoforms of its subunits (α , β , γ , δ , ϵ , π) and 15 subtypes have been discovered (Burt & Kamatchi, 1991; Macdonald & Olsen, 1994; Dunn *et al.*, 1994; Rabow *et al.*, 1995; Davies *et al.*, 1997; Hedblom & Kirkness, 1997). The transmembrane subunits of the GABA_A receptor show homology to the subunits of the nicotinic acetylcholine receptor, glycine receptor and the serotonin type 3 receptor and belong to the same superfamily of ligand gated ion channels. It is believed that the GABA_A receptor forms a pentameric structure (Nayem *et al.*, 1994) and the major isoform consists of $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits (McKernan &

Whiting, 1996). A stoichiometry of two α , two β and one γ has been proposed (Chang *et al.*, 1996; Tretter *et al.*, 1997). Many classes of drugs interact with this channel, including benzodiazepines, barbiturates and neurosteroids (Sieghart, 1995). The GABA_A receptor has been targeted for pharmacological control of anxiety, sleep and epilepsy. Expression of different recombinant subunit combinations results in channels displaying different pharmacological and electrophysiological properties (Sigel *et al.*, 1990; Rabow *et al.*, 1995). *In situ* hybridization techniques demonstrate that different receptor subunits are expressed in distinct regions of the brain (Wisden *et al.*, 1992; Laurie *et al.*, 1992). Thus, drugs which exhibit a subunit selectivity *in vitro* may consequently act on GABA_A receptor subtypes of specific regions in the brain.

β -carbolines are known to bind with high affinity to the benzodiazepine binding site of the GABA_A receptor. One of the best characterized β -carbolines is DMCM, which acts as a negative allosteric modulator by reducing the frequency of

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channel opening (Rogers *et al.*, 1994). At low concentrations (nM range) DMCM inhibits GABA currents, whereas at high concentrations (μ M range) the dose-response curve interestingly reverses and DMCM stimulates submaximal GABA responses (Sigel & Baur, 1988; Sigel *et al.*, 1990; Im *et al.*, 1995). Recently, this potentiation by DMCM in the μ M range has been ascribed to a positive allosteric modulation at the loreclezole site (Stevenson *et al.*, 1995). In comparison to the benzodiazepine binding site, the apparent affinity of DMCM for the loreclezole site is three orders of magnitude lower. *In vivo* DMCM exerts a proconvulsant action. New anti-convulsant drugs with a β -carboline structure acting at both, the benzodiazepine and the loreclezole site, should either be antagonists or positive allosteric modulators at the benzodiazepine site to avoid such a convulsant effect.

Using functional analysis combined with binding studies, we have found that the β -carboline ZK 91085 acts as a positive allosteric modulator at the benzodiazepine site. Furthermore, we observed an additional component of potentiation by ZK 91085 that might be due to an interaction *via* the loreclezole site. We also show that two point mutations in the β subunit affect this modulation. At least one of the two identified amino acid residues is located at subunit interfaces and is homologous to residues participating in the formation of the agonist site.

Methods

Oocyte expression and electrophysiology

Xenopus laevis oocytes were removed from anaesthetized frogs and manually separated from the ovary by a platinum loop under a light microscope. Prior to defolliculation by a collagenase treatment, the oocytes were injected with 50 nl of 5 mM HEPES-KOH (pH 6.8), containing a mixture of cRNA coding for each of the different subunits. For dual subunit combinations a concentration of 100 nM was used for each transcript. For triple subunit combinations 50 nM of the cRNA coding for α 1, 50 nM for β 2 and 150 nM for γ 2 were injected. A detailed description of the isolation, culturing, injection and defolliculation of the oocytes is given elsewhere (Sigel *et al.*, 1990). The point mutants used were constructed as described previously (Buhr *et al.*, 1997). Numbers of the amino acid residues indicate the position in the corresponding mature rat subunit isoform.

Oocytes were placed on a nylon grid in a 0.4 ml bath and perfused by a gravity-driven system throughout the experiment at 6 ml min⁻¹ with a modified Barth's medium consisting of (in mM) NaCl 90, KCl 1, MgCl₂ 1, CaCl₂ 1, HEPES-NaOH 5, pH 7.4. Cells were impaled with two 0.3–1 M Ω resistance microelectrodes containing 3 M KCl and the membrane potential was clamped at –80 mV (two-electrode voltage-clamp). All experiments were performed at room temperature (22–29°C). Allosteric modulation depends on the GABA concentration that is coapplied with the modulating drug. Therefore modulation was quantified at a GABA concentration eliciting alone approximately 5% (EC₅) of the maximal current amplitude measured at 10 mM GABA. EC₅ was established by determining I_{max} first and subsequently titrating with GABA until the desired relative response was obtained. A full GABA concentration-response curve was not determined for each of the analysed subunit combinations. But our measurements of the respective EC₅ of different subunit combinations are in line with previous observations that dual subunit combinations have an about 7 fold higher

apparent affinity of GABA compared to triple combinations (Sigel *et al.*, 1990). Unless indicated otherwise, experiments were carried out using a new oocyte for every compound analysed to prevent contamination. Drugs were bath applied 30–40 s at least until the peak of the response was observed. Between two applications, oocytes were perfused with the modified Barth's medium (see above) for at least 3 min to ensure full recovery from desensitization. Concentration-response curves were fitted to the logistic equation: $I = I_{\max} / (1 + (EC_{50}/c))$, where c is the drug concentration, EC₅₀ is the concentration of drug eliciting half maximal response and I_{max} is the maximal response. All β -carbolines were a kind gift from Dr Schneider (Schering, Germany), loreclezole was obtained from Janssen and all other compounds were from Sigma or Fluka.

Binding assays

HEK-293 cells (American type of Culture Collection, Rockville, MD, U.S.A., CRL 1573) were maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, NY, U.S.A.) supplemented with 10% foetal calf serum (JHR Biosciences, Lenexa, KS, U.S.A.), 2 mM glutamine, 50 μ M β -mercaptoethanol, 100 units ml⁻¹ penicillin G and 100 μ g ml⁻¹ streptomycin in 75-cm² Petri dishes by using standard cell culture techniques.

3×10^6 HEK-293 cells were transfected with a total of 21 μ g cDNA encoding for the rat α 1-, β 2-, and γ 2-subunits (ratio 1 : 1 : 1), or with 20 μ g cDNA encoding for α 1- and β 2-, or α 1- and β 1- subunits (ratio 1:1) subcloned individually into pCDM8 expression vectors, using the calcium phosphate precipitation method (Chen & Okayama, 1988). The medium was changed 20 h after transfection and the cells were harvested 48 h after transfection by scraping into phosphate buffered saline. Cells were centrifuged at 12,000 $\times g$ for 10 min and the cell pellet was homogenized in 50 mM Tris-citrate buffer, pH 7.4 by using an Ultraturrax, followed by three centrifugation (200,000 $\times g$ for 20 min) resuspension cycles, and were then used for ligand binding studies or were stored at –20°C.

For binding studies, membranes were centrifuged and resuspended in 50 mM Tris-citrate buffer, pH 7.4, at a protein concentration of about 1 mg ml⁻¹ as measured by the BCA-protein assay kit of Pierce Chem. Co. with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 150 mM NaCl and 2 nM of [³H]-flunitrazepam in the absence or presence of 10 μ M diazepam or various concentrations of ZK 91085 for 90 min at 4°C (Zezula *et al.*, 1996). For [³⁵S]-TBPS binding studies, membranes were incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 200 mM NaBr and 2 nM [³⁵S]-TBPS in the absence or presence of 10 μ M picrotoxin or various concentrations of ZK 91085 for 3 h at room temperature (Zezula *et al.*, 1996). Membranes were then filtered through Whatman GF/B filters. For [³H]-flunitrazepam binding, the filters were rinsed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer. When [³⁵S]-TBPS binding was investigated, the filters were rinsed three times with 3.5 ml of this buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml Hydrofluor (National Diagnostics, NJ, U.S.A.) scintillation fluid. Non-specific binding determined in the presence of 10 μ M diazepam or 10 μ M picrotoxin was subtracted from total [³H]-flunitrazepam or [³⁵S]-TBPS binding, respectively, to result in specific binding.

Results

Expression of wild type and mutant GABA_A receptors in *Xenopus oocytes* and in HEK 293 cells

Wild type $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$, $\alpha 1\beta 1$ and mutant $\alpha 1\beta 2Y62L\gamma 2$, $\alpha 1Y209A\beta 2\gamma 2$, $\alpha 1\beta 2\gamma 2F77L$, $\alpha 1\beta 2Y62L$, $\alpha 1Y209A\beta 2$, $\alpha 1F64L\beta 2$, $\alpha 1T206A\beta 2$, $\alpha 1\beta 2N265S$ rat recombinant GABA_A receptors were functionally expressed in *Xenopus laevis* oocytes. All of the wild type and mutant receptors produced chloride inward currents of comparable amplitude in response to application of GABA. GABA concentrations required to elicit EC₅ indicate no significant change in the apparent GABA affinity for most of the mutated dual and triple combinations relative to wild type receptors. Exceptions were $\alpha 1F64L\beta 2$, $\alpha 1\beta 2Y62L$ and $\alpha 1T206A\beta 2$, which displayed a shift amounting to about 60-, 3- and 3 fold lower affinities, respectively. $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$ and $\alpha 1\beta 1$ rat recombinant GABA_A receptors were also expressed in HEK 293 cells for binding studies.

Modulation by ZK 91085 via the benzodiazepine site

Displacement of the ligand [³H]-flunitrazepam by ZK 91085 (Figure 1) indicated an α subunit specific affinity for the benzodiazepine site (data on file, Schering, Germany). Functional studies with ZK 91085 were undertaken to confirm this finding. They demonstrated a stimulation by various concentrations of ZK 91085 of currents elicited by GABA in oocytes expressing $\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 2\gamma 2$ and $\alpha 5\beta 2\gamma 2$ rat recombinant GABA_A receptors (representative current traces for the subunit combination $\alpha 1\beta 2\gamma 2$ are shown in Figure 2), but we failed to observe a subunit specificity (not shown). To understand this apparent contradiction, we further characterized the action of ZK 91085 on $\alpha 1\beta 2\gamma 2$ receptors. 10 μ M ZK 91085 alone induced currents amounting to less than 0.3% of the maximum current amplitude elicited by GABA, indicating

that the observed potentiation is due to allosteric modulation. Dose-response curves of ZK 91085 were bi-phasic (Figure 3) and could be best fitted with a half maximal stimulation of

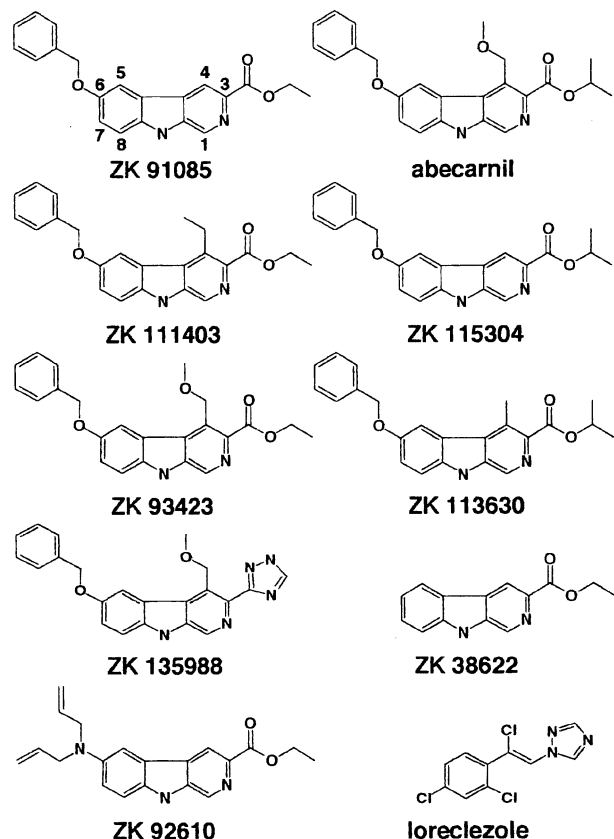


Figure 1 Chemical structure of the β -carbolines used in this study and of the anticonvulsant compound loreclezole.

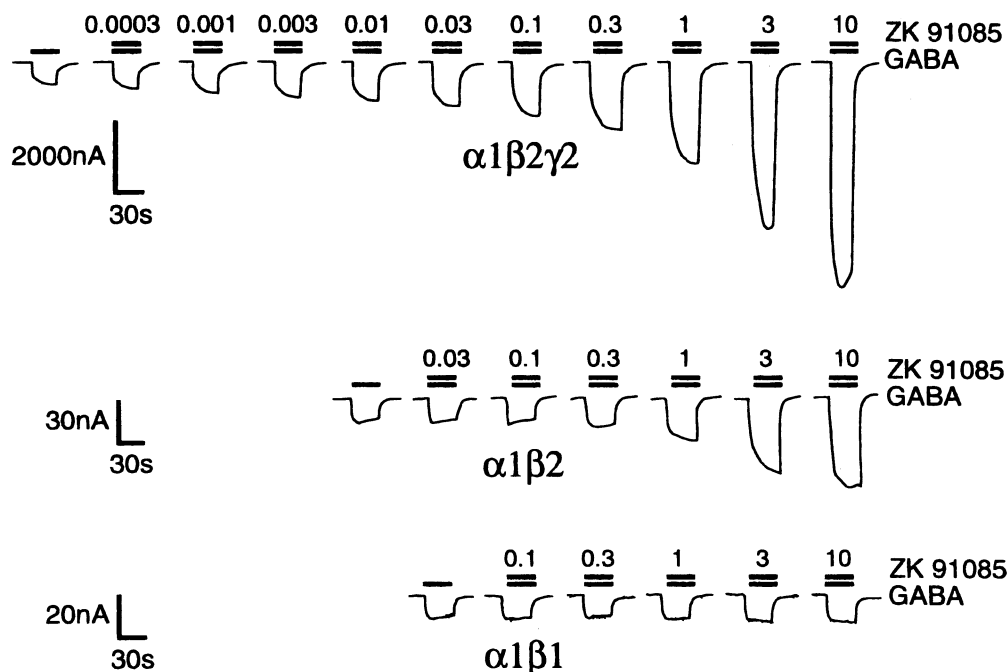


Figure 2 Potentiation of GABA responses by ZK 91085 at recombinant rat $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$ and $\alpha 1\beta 1$ GABA_A receptors expressed in *Xenopus laevis* oocytes. Application of GABA alone resulted in approximately 5% of the maximal current amplitude. Increasing concentrations of ZK 91085 were coapplied with GABA. Periods of drug application are indicated by horizontal bars above the current records. Concentrations of ZK 91085 are shown in μ M. Three additional experiments for each receptor combination carried out with oocytes from 1–2 batches gave comparable results.

10 ± 5 nM (mean \pm s.e.mean of four oocytes from one batch) and the maximal stimulation amounting to $59 \pm 3\%$ for the high affinity phase and 1.6 ± 0.7 μ M (mean \pm s.e.mean of four oocytes from one batch) and $694 \pm 180\%$ for the lower affinity phase. Solutions containing ZK 91085 at higher concentrations than 10 μ M could not be prepared due to solubility problems.

We first concentrated on the high affinity phase. Significant stimulation of currents elicited by GABA was first observed at 3 nM ZK 91085. In combination with 1 μ M Ro 15-1788 potentiation by 0.1 μ M and 0.7 μ M ZK 91085 could be reduced by about 70% (Figure 4) and 51% ($n=3$; not shown), respectively, but the stimulation was not abolished completely. Higher concentrations of ZK 91085 cannot be expected to be inhibited by 1 μ M Ro 15-1788 due to the high affinity of ZK 91085 to the benzodiazepine binding site, which is similar to the affinity of Ro 15-1788. Binding studies using [³H]-flunitrazepam as a ligand namely demonstrated that ZK 91085 displaced this ligand with an IC_{50} value of 1.5 nM (Figure 5). These experiments confirmed a high affinity interaction of ZK 91085 with the [³H]-flunitrazepam binding site.

Additional modulation by ZK 91085

The lacking α subunit specificity, the bi-phasic stimulation of $\alpha 1\beta 2\gamma 2$ receptors and their only partial blockade by Ro 15-1788 all indicated an interaction of ZK 91085 *via* an additional site. To separate the two effects, we functionally expressed receptors lacking a $\gamma 2$ subunit, which is required for benzodiazepine modulation (Pritchett *et al.*, 1989; Sigel *et al.*, 1990; Günther *et al.*, 1995). Representative current traces obtained with $\alpha 1\beta 2$ are shown in Figure 2. Concentration-response curves for ZK 91085 at receptors consisting of either $\alpha 1\beta 2\gamma 2$ or $\alpha 1\beta 2$ subunits are compared in Figure 3. A potentiation by ZK 91085 was still present at $\alpha 1\beta 2$ receptors but smaller as compared to the triple subunit combination. The EC_{50} value at $\alpha 1\beta 2$ was > 1.8 μ M. Additionally, 10 μ M ZK 91085 induced an approximately 5 fold shift to the left of the GABA dose-response curve, without affecting the maximal current amplitude (data not shown, two experiments each). As

expected, stimulation by 1 μ M ZK 91085 amounting to $136 \pm 13\%$ (mean \pm s.e.mean of 22 oocytes from nine batches) proved to be insensitive to 1 μ M of the benzodiazepine antagonist Ro 15-1788 at $\alpha 1\beta 2$ receptors ($142 \pm 11\%$, mean \pm s.e.mean of four oocytes from one batch). 10 μ M ZK 91085 alone induced currents amounting to maximally 0.1% of the maximum current amplitude elicited by GABA, demonstrating that the observed stimulation is due to a positive allosteric modulation.

At $\alpha 1\beta 1$ receptors, the current stimulation by 10 μ M ZK 91085 amounted to only 24% of that at the subunit

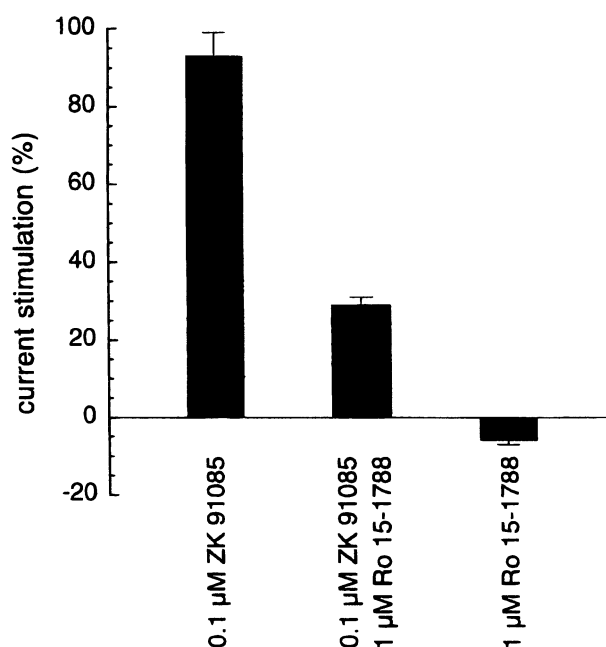


Figure 4 The benzodiazepine antagonist Ro 15-1788 (1 μ M) partially inhibits the stimulation by 0.1 μ M ZK 91085 at recombinant rat $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Results are standardized to control GABA applications that elicited approximately 5% of the maximal current amplitude. Bars show mean \pm s.e.mean of three oocytes.

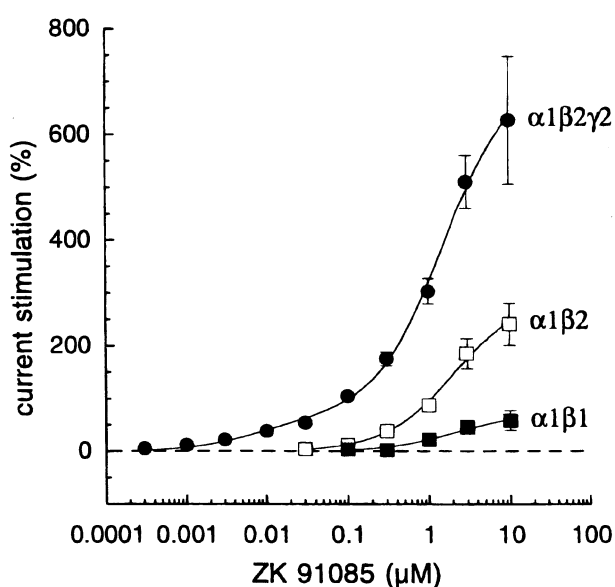


Figure 3 Concentration response curves for ZK 91085 at recombinant rat $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$ or $\alpha 1\beta 1$ GABA_A receptors. Experiments were carried out as described under Figure 2. Values are shown as mean \pm s.e.mean of four oocytes from 1–2 batches.

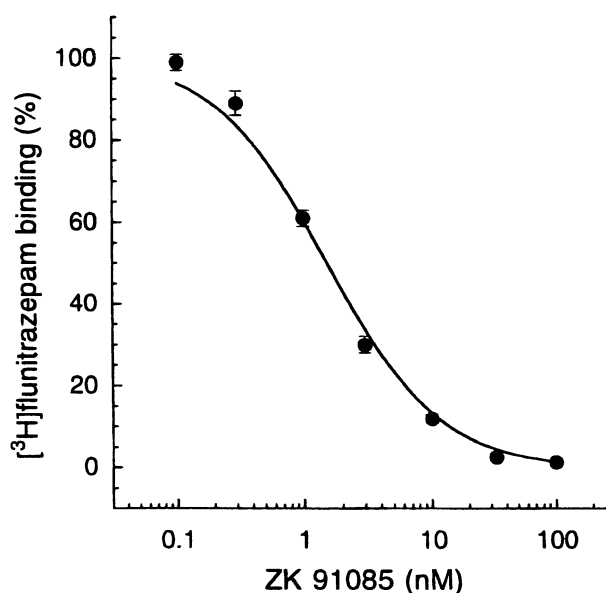


Figure 5 Displacement by ZK 91085 of binding of [³H]-flunitrazepam to membranes from HEK 293 cells transiently transfected with rat recombinant $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Bars show mean \pm s.e.mean of six determinations (two experiments, triplicates each).

combination $\alpha 1\beta 2$ (Figure 3). This selectivity of ZK 91085 for receptors containing the $\beta 2$ isoform as compared to those containing the $\beta 1$ isoform is reminiscent of the loreclezole action (Wafford *et al.*, 1994; Wingrove *et al.*, 1994). The point mutant $\beta 2N265S$, that replaces the amino acid residue found in the $\beta 2$ subunit by the corresponding residue in the $\beta 1$ subunit, and results in the removal of loreclezole potentiation also abolished stimulation by ZK 91085 (Table 1). We tested also whether the potentiation by ZK 91085 is additive with that of loreclezole. Representative current traces are shown in Figure 6. A saturating concentration of 10 μM loreclezole (Wafford *et al.*, 1994; Stevenson *et al.*, 1995; determined here) stimulated GABA currents by $786 \pm 35\%$ (mean \pm s.e.mean of four oocytes from two batches) at $\alpha 1\beta 2$ receptors. No additional potentiation was observed when loreclezole was coapplied with 10 μM ZK 91085 ($767 \pm 36\%$, mean \pm s.e.mean of four oocytes from two batches).

Table 1 Effect of point mutations on current stimulation by 1 μM ZK 91085

Subunit combination	Stimulation \pm s.e.mean (%)	n	Dunn (Two-tail)
$\alpha 1\beta 2$	136 ± 13	22	
$\alpha 1\beta 1$	2 ± 4	4	
$\alpha 1\beta 2N265S$	-2 ± 2	4	$P < 0.02$
$\alpha 1F64L\beta 2$	107 ± 22	6	n.s.
$\alpha 1\beta 2Y62L$	509 ± 77	12	$P < 0.001$
$\alpha 1T206A\beta 2$	168 ± 55	6	n.s.
$\alpha 1Y209A\beta 2$	83 ± 12	13	n.s.
$\alpha 1\beta 2\gamma 2$	526 ± 101	7	
$\alpha 1\beta 2Y62L\gamma 2$	730 ± 94	5	n.s.
$\alpha 1Y209A\beta 2\gamma 2$	424 ± 81	7	n.s.
$\alpha 1\beta 2\gamma 2F77L$	682 ± 51	3	n.s.

Current modulation by 1 μM ZK 91085 was measured at EC₅. Values are shown as mean \pm s.e.mean. Subsequent to a Kruskal-Wallis test, statistical significance was evaluated using the procedure by Dunn (n.s.: not significant at the 80% confidence level). Dual mutant subunit combinations are compared with $\alpha 1\beta 2$, triple mutant subunit combinations are compared with $\alpha 1\beta 2\gamma 2$.

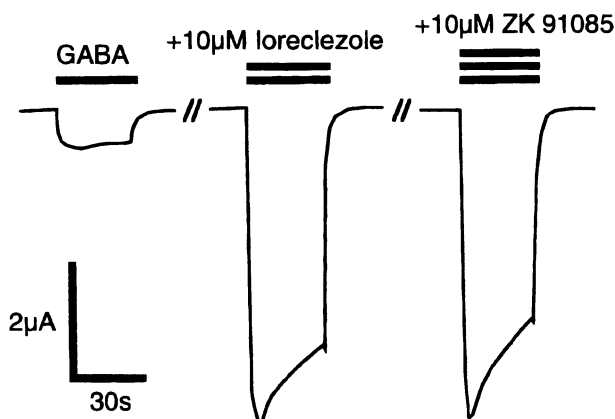


Figure 6 Potentiation of GABA currents by loreclezole and ZK 91085 is not additive at recombinant rat $\alpha 1\beta 2$ GABA_A receptors. The concentration of GABA eliciting approximately 5% of the maximal current amplitude was determined first. Ten μM loreclezole strongly stimulated these GABA currents. When 10 μM ZK 91085 was coapplied with 10 μM loreclezole, no additional stimulation was observed. Bars above the current records indicate the periods of drug application. Three additional experiments carried out with oocytes of two separate batches gave comparable results.

Recently, loreclezole was demonstrated to inhibit [³⁵S]-TBPS binding to cells expressing $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 3\gamma 2$ receptors, but not to those expressing the $\alpha 1\beta 1\gamma 2$ receptors (Wafford *et al.*, 1994). Similarly, ZK 91085 also decreased [³⁵S]-TBPS binding to membranes from HEK 293 cells transiently transfected with $\alpha 1\beta 2$ or $\alpha 1\beta 2\gamma 2$ receptors (Figure 7). The IC₅₀ value was decreased about 3 fold at $\alpha 1\beta 2\gamma 2$ receptors as compared to $\alpha 1\beta 2$ channels. At the dual subunit combination $\alpha 1\beta 1$, ZK 91085 failed to decrease [³⁵S]-TBPS binding.

Effects of the drug structure on current stimulation

Stimulation of GABA currents by 10 μM ZK 91085 amounted to $271 \pm 52\%$ (mean \pm s.e.mean of four oocytes from one batch). 10 μM of the β -carboline abecarnil was over 10 fold less potent than ZK 91085 at $\alpha 1\beta 2$ receptors (Figure 8). Both β -carbolines are structurally closely related. Thus, it was interesting to see which structural entities are required for modulation. We therefore measured concentration response curves of the structural intermediates available to us (Figures 1 and 8). Maximal stimulation could not be determined as solutions containing these drugs at higher concentrations than 10 μM could not be prepared due to solubility problems. EC₅₀ values could therefore not be determined. As an exception, ZK 135988 and ZK 38622 were soluble up to 100 μM . However, the concentration response curves of these compounds were not saturated at 100 μM either.

Specifically, the role of the substituents at position three, four and six were studied. Introduction of an ethyl group at position four of ZK 91085 provides ZK 111403 and reduced stimulation at 10 μM about 5 fold. ZK 93423 has a methoxymethyl group instead of the ethyl group at the same position. This resulted even in a larger reduction in current stimulation. Interestingly, replacement of the ester moiety in ZK 93423 by a triazole group to give ZK 135988 largely restored current stimulation. Introduction of an additional methyl group into the ester moiety at position three of ZK 91085 to yield ZK 115304 interfered less with the current stimulation, but the reduction still amounting to about 2 fold.

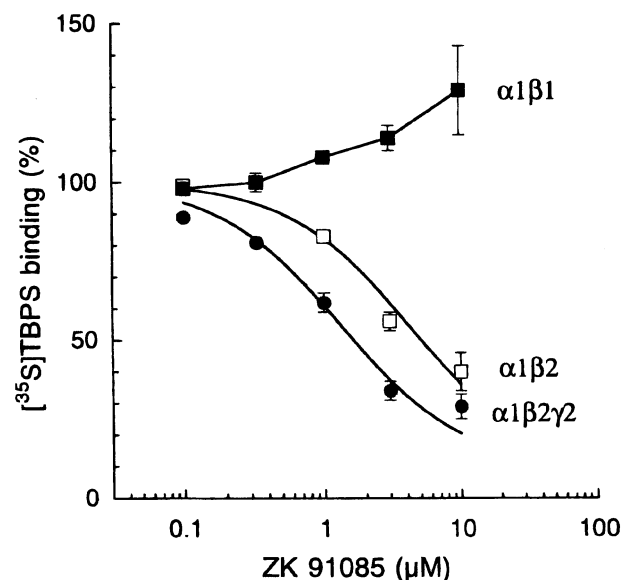


Figure 7 Effect of ZK 91085 on [³⁵S]-TBPS binding to transiently transfected HEK 293 cells expressing recombinant rat $\alpha 1\beta 2$, $\alpha 1\beta 1$ or $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Bars show mean \pm s.e.mean of six determinations (two experiments, triplicates each).

When an additional methyl group was introduced in ZK 115304 at position four (providing ZK 113630) the potentiation again was nearly abolished.

Replacement of the benzyloxy group at position six of ZK 91085 by the diallylamino group in ZK 92610 resulted only in a small reduction in current stimulation. Compound ZK 38622, lacking a substituent at this position, showed almost the same potentiation as ZK 91085. However, the last two compounds seem to stimulate currents induced by GABA only at much higher concentrations as ZK 91085.

In addition concentration response curves at the dual subunit combination containing the point mutant $\beta 2N265S$, that abolishes loreclezole effects, were carried out to determine which of the intermediates act similar to loreclezole (Figure 8). In comparison with the wild type, current stimulation of all intermediates was reduced at the mutated receptor with the exception of abecarnil, ZK 93423 and ZK 135988. These three β -carbolines showed a slightly enhanced potentiation at $\alpha 1\beta 2N265S$ receptors.

Effects of point mutations on the modulation by ZK 91085

As mentioned previously the mutation $\beta 2N265S$ abolished the current stimulation at $\alpha 1\beta 2$ by ZK 91085. The potentiation of GABA currents by $1 \mu M$ ZK 91085 was compared between wild type and further mutant receptors (Table 1). Representative current records for the mutant receptor $\alpha 1\beta 2Y62L$ as well as the wild type $\alpha 1\beta 2$ subunit combination are shown in Figure 9. At dual subunit combinations containing the mutant $\beta 2Y62L$ subunit, the potentiation by ZK 91085 was significantly increased. Mutations $\alpha 1F64L$ and $\alpha 1T206A$ did not result in a significant change of the potentiation by ZK 91085. Channels containing the mutant $\alpha 1Y209A$ subunit behaved variably in different batches of oocytes. In two of four injections, the potentiation by ZK 91085 was significantly decreased about 2 fold, but in the other two experiments, potentiation was similar to the wild type control.

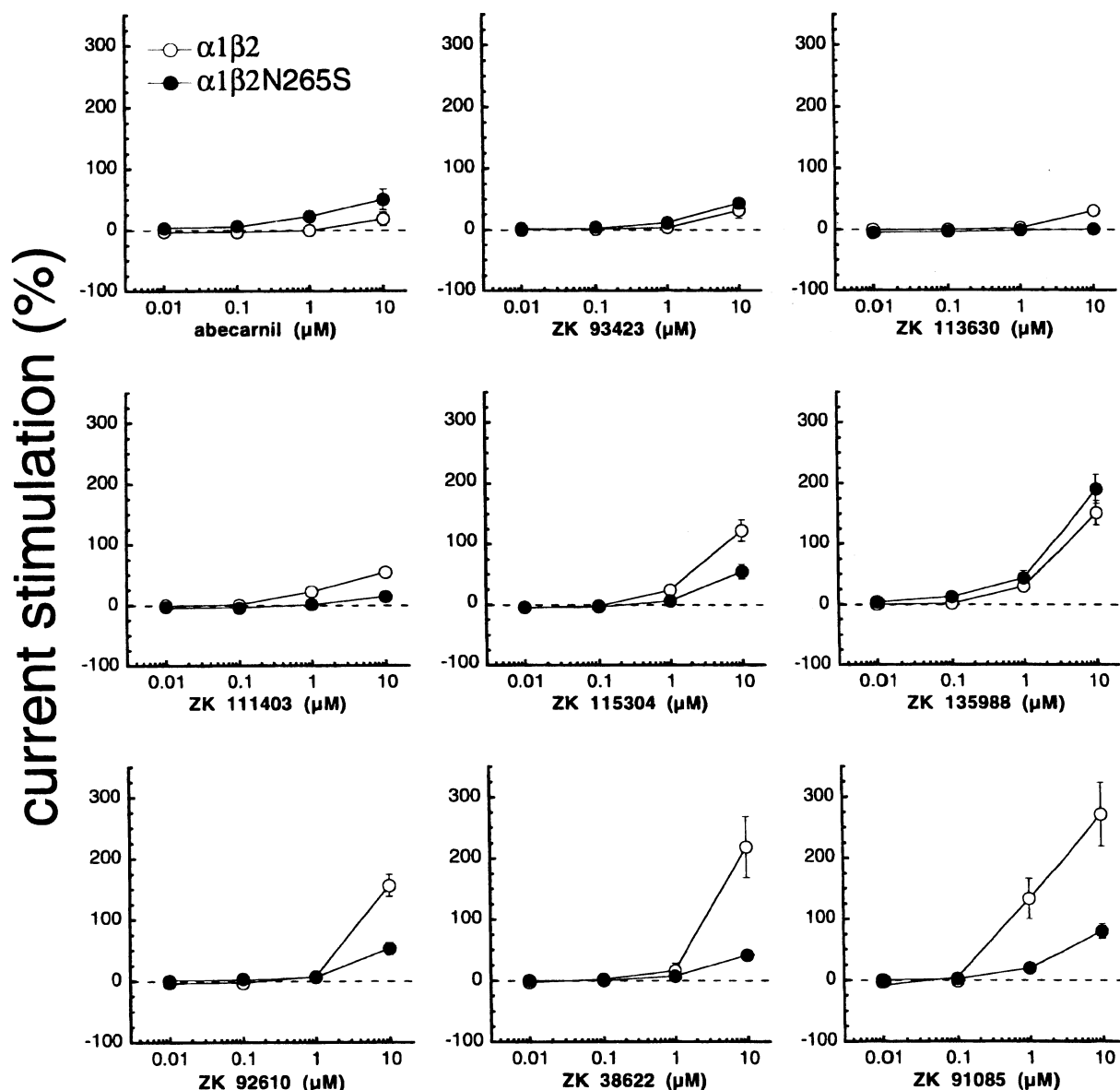


Figure 8 Comparison of the concentration response curves of ZK 91085, abecarnil and structurally related compounds at $\alpha 1\beta 2$ and $\alpha 1\beta 2N265S$ receptors. Results are standardized to control GABA applications that elicited approximately 5% of the maximal current amplitude in the same experiments. Bars show mean \pm s.e. mean of three oocytes.

In triple subunit combinations $\alpha 1\beta 2\gamma 2$ the mutated receptors $\alpha 1\beta 2\gamma 62L\gamma 2$, $\alpha 1\gamma 209A\beta 2\gamma 2$ and $\alpha 1\beta 2\gamma 2F77L$ did not result in any significant change as compared to wild type receptors. From the results obtained with the dual subunit

combinations, we expected for the mutation $\beta 2Y62L$ a current increase. Intriguingly, such an increase was not found. Possibly a current increase by the mutation is being masked by the large current stimulation by ZK 91085 seen in the triple subunit combination as compared to the dual subunit combination (Figure 3) found prior to mutation.

Figure 10 compares concentration response curves for ZK 91085 in $\alpha 1\beta 2$ and $\alpha 1\beta 2Y62L$ channels. As expected, the maximal stimulation by ZK 91085 observed in these experiments was higher for $\alpha 1\beta 2Y62L$ as compared to wild type receptors. Due to solubility problems with ZK 91085, the EC_{50} values could unfortunately not be determined. Current stimulation in $\alpha 1\beta 2Y62L$ by $10\ \mu M$ DMCM ($408 \pm 44\%$, mean \pm s.e. mean of four oocytes from two batches), which stimulates GABA currents *via* the loreclezole site (Stevenson *et al.*, 1995), was also significantly increased ($P < 0.025$, Mann-Whitney, one-tailed) as compared to the wild type receptor ($170 \pm 15\%$, mean \pm s.e. mean of four oocytes from two batches). Interestingly, the stimulation of the current elicited in $\alpha 1\beta 2$ by $1\ \mu M$ loreclezole ($95 \pm 24\%$, mean \pm s.e. mean of six oocytes from two batches) was not significantly affected by the mutation in the β subunit ($70 \pm 28\%$, mean \pm s.e. mean of four oocytes from two batches).

Discussion

ZK 91085 stimulates GABA_A receptor currents via two different sites

ZK 91085 stimulated currents elicited by GABA in rat recombinant $\alpha 1\beta 2\gamma 2$ channels in a concentration dependent manner in two phases. Action of ZK 91085 *via* the benzodiazepine site was demonstrated on a functional level by antagonism by Ro 15-1788. About 70% of the current stimulation of $0.1\ \mu M$ ZK 91085 were inhibited by $1\ \mu M$ Ro 15-1788 (Figure 4). Binding studies with ZK 91085 also revealed a high affinity for the benzodiazepine site (Figure 5), clearly demonstrating an interaction at this site. In contrast to the classical β -carbolines, which allosterically inhibit GABA_A receptor function through this site, ZK 91085 stimulates here. The two-phasic stimulation and the only partial blockade by Ro 15-1788 indicated that ZK 91085 interacts with an additional site on the receptor.

To characterize this second site of action, we analysed the dual subunit combination $\alpha 1\beta 2$. This receptor lacks the $\gamma 2$ subunit, which is required for benzodiazepine modulation (Pritchett *et al.*, 1989; Sigel *et al.*, 1990; Günter *et al.*, 1995). ZK 91085 still stimulated GABA responses at the dual subunit combination $\alpha 1\beta 2$, although at higher concentrations only (Figures 2 and 3). The β -carboline DMCM has previously been described to not only interact with the benzodiazepine site but also with the loreclezole binding site (Stevenson *et al.*, 1995). To test whether the present stimulation of $\alpha 1\beta 2$ receptors by the β -carboline ZK 91085 can also be ascribed to an interaction at the loreclezole site, we investigated the modulation at a dual subunit combination containing the $\beta 1$ isoform, which shows a strongly reduced loreclezole stimulation (Wafford *et al.*, 1994). Indeed, ZK 91085 stimulation at $\alpha 1\beta 1$ receptors was also nearly abolished (Figures 2 and 3) and this selectivity for the $\beta 2$ over the $\beta 1$ subunit could be confirmed by displacement studies of [³⁵S]-TBPS by ZK 91085 (Figure 7).

The β -carboline DMCM and the general anaesthetic etomidate display the same β subunit specificity (Stevenson

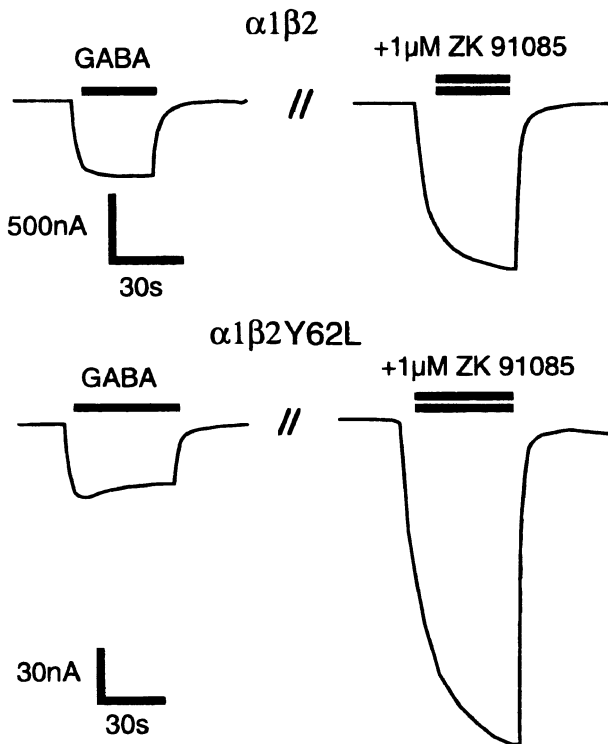


Figure 9 Effect of the $\beta 2Y62L$ point mutation on the current stimulation by $1\ \mu M$ ZK 91085. After establishment of a GABA concentration eliciting approximately 5% of the maximum current amplitude, this GABA concentration was coapplied with $1\ \mu M$ ZK 91085. Similar results were obtained in additional experiments for each subunit combination (Table 1). Bars above the current records indicate the periods of drug application.

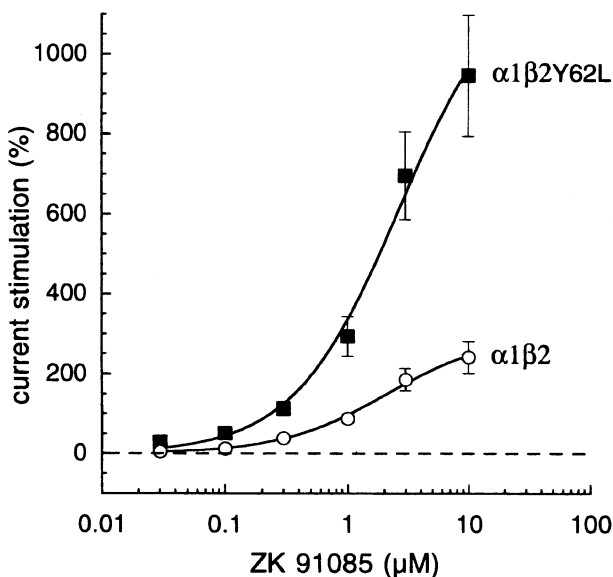


Figure 10 Effect of the mutation $\beta 2Y62L$ on the concentration response curve. GABA eliciting about 5% of the maximal current amplitude was applied either alone or in combination with increasing concentrations of ZK 91085 to wild type $\alpha 1\beta 2$ and mutated $\alpha 1\beta 2Y62L$ receptors. Values are shown as mean \pm s.e. mean of at least four oocytes. Data for the subunit combination $\alpha 1\beta 2$ were taken from Figure 3.

et al., 1995; Hill-Venning *et al.*, 1997). Site-directed mutagenesis studies previously demonstrated, that the β isoform specificity of loreclezole, DMCM and etomidate is determined by a single asparagine residue in $\beta 2$ and $\beta 3$ ($\beta 2$ Asn-265 and $\beta 3$ Asn-265), the corresponding amino acid in $\beta 1$ being a serine (Stevenson *et al.*, 1995; Belelli *et al.*, 1997; Wingrove *et al.*, 1994). Mutation $\beta 2N265S$ also determined the observed β subunit specificity for ZK 91085.

Functional competition experiments showed, that GABA currents potentiated by loreclezole are further stimulated by coapplication of either zolpidem, pentobarbitone or the steroid 5α -pregnan- 3α -ol-20-one, suggesting separate binding sites (Wafford *et al.*, 1994). In contrast, such experiments with loreclezole and ZK 91085 did not show any additional stimulation by ZK 91085, in line with an interaction of both ligands at the same binding site (Figure 6). In summary, our results are consistent with the hypothesis, that potentiation by ZK 91085 at $\alpha 1\beta 2\gamma 2$ receptors is actually an addition of the modulation at the benzodiazepine and most probably the loreclezole binding site.

Structural features of β -carbolines important for modulation

Due to solubility problems, only a limited concentration range of the β -carboline derivatives could be studied (Figure 8). It is not entirely clear from our data whether potency or efficacy is affected by the structural changes. Therefore we compare here in first instance the extent of current stimulation at $10\ \mu\text{M}$.

The structurally related abecarnil only slightly stimulated GABA currents at $\alpha 1\beta 2$ receptors as compared to ZK 91085 (Figure 8). Thus, it was interesting to determine structural entities required for this modulation. An additional methyl group in the ethyl ester moiety (ZK 115304) reduced current stimulation about 2 fold and additional substituents at position four (ZK 111403, ZK 93423 and ZK 113630) strongly interfered with current stimulation. Interestingly, replacement of the ester by a triazole group in abecarnil (ZK 135988) restored stimulation for the most part in spite of the methoxymethyl substituent present. A triazole group is also present in loreclezole. Whether or not this fact is important for the current stimulation is not known. Replacement of the ester by a triazole group in ZK 91085 may enhance its stimulatory effect.

When the benzyloxy group of ZK 91085 at position six is replaced or even omitted (ZK 92610 and ZK 38622), the extent of potentiation at $10\ \mu\text{M}$ was not much affected. However, stimulation was only evident at high concentrations of the substances, such that the benzyloxy substituent may be important for apparent affinity of a substance.

To see whether the observed potentiation displays similar properties as the current stimulation by loreclezole, we also tested all these compounds for modulation at the channel containing the point mutant $\beta 2N265S$, which strongly interferes with loreclezole potentiation. All compounds studied showed indeed a behaviour reminiscent of the action of loreclezole with the exception of abecarnil, ZK 93423 and ZK 135988. Interestingly, all three compounds carry a methoxymethyl group at position four. This substituent may abolish the sensitivity of current stimulation to the point mutation $\beta 2N265S$.

ZK 91085 was tested for an anticonvulsant action in mice (Hollinshead *et al.*, 1990). This β -carboline displayed no effect at $40\ \text{mg kg}^{-1}$. In the same study ZK 91085 revealed an IC_{50} value of $8.9\ \text{nM}$ for the benzodiazepine site, which is in

agreement with our results (Figure 5). These authors discussed an ester hydrolysis *in vivo* as responsible for the lack of activity of ZK 91085 *in vivo*.

$\beta 2Y62L$ influences ZK 91085 modulation of $\alpha 1\beta 2$

Amino acid residues located at subunit interfaces affect GABA and benzodiazepine action (Sigel & Buhr, 1997). We were interested to see whether residues homologous or identical to these residues affect the modulation of ZK 91085. The dual subunit combinations carrying the point mutation $\beta 2Y62L$ markedly increased potentiation by ZK 91085 (Table 1, Figures 9 and 10). It is not clear from our data, whether the binding site for ZK 91085 is directly affected or whether the conformational changes occurring after binding are altered by the mutation. A general change in channel conformation in the mutant receptor $\alpha 1\beta 2Y62L$ appears unlikely, as this mutant showed only a small effect on the apparent affinity of GABA (see results). DMCM stimulation also was markedly increased in $\alpha 1\beta 2Y62L$ as compared to the wild type (see results). In contrast, loreclezole action was unaffected in $\alpha 1\beta 2Y62L$ receptors (see results). This finding is surprising, as all results discussed so far for ZK 91085 clearly paralleled the behaviour of loreclezole. This apparent contradiction may be explained by partially overlapping binding sites of ZK 91085 (or DMCM) and loreclezole, where $\beta 2Y62$ is located in the binding pocket for ZK 91085 (or DMCM) but not that for loreclezole (Figure 1).

A second surprise was the absence of any functional effect by the $\beta 2Y62L$ mutation in receptors composed of triple subunit combinations. Stimulation by ZK 91085 is much smaller in dual subunit combinations than in triple subunit combinations. It is conceivable that addition of a γ subunit to the dual subunit combination removes a similar constraint as the mutation $\beta 2Y62L$ in the dual subunit combination. This removal of the constraint leads then to a larger current stimulation by ZK 91085 (Table 1).

The residue identified in this work is located at a position directly homologous to regions forming the GABA and the benzodiazepine site in $\alpha 1\beta 2\gamma 2$ receptors. The residue $\beta Y62$ is directly homologous to $\alpha F64$, which has been shown to be important for GABA competitive antagonist affinity (Sigel *et al.*, 1992) and can be photolabelled by the agonist muscimol (Smith & Olsen, 1994). It is also directly homologous to $\gamma F77$, influencing binding of ligands of the benzodiazepine site (Buhr *et al.*, 1997). In the pentamer model of the $\alpha 1\beta 2\gamma 2$ receptor, the two agonist binding sites and the benzodiazepine site are believed to be located at α/β and α/γ subunit interfaces, respectively (Galzi & Changeux, 1994; Sigel & Buhr, 1997). The tyrosine residue identified here as important for ZK 91085 modulation may be located at the remaining α/β subunit interface of the $\alpha 1\beta 2\gamma 2$ receptor, the $\beta 2$ subunit contributing residue 62. In case our mutation affects the binding site for ZK 91085, this binding site would have to be located at this remaining subunit interface. Of course we cannot exclude an allosteric effect of the mutation $\beta 2Y62L$ on the binding site.

In conclusion, we show that the β -carboline ZK 91085 acts as a positive allosteric modulator at two sites of the GABA_A receptor, namely the benzodiazepine site and most probably the loreclezole site. Two point mutations, $\beta 2N265S$ and $\beta 2Y62L$, markedly affected stimulation by ZK 91085 *via* the latter site. A structure activity study with structural intermediates of ZK 91085 and abecarnil identified at least positions three and four of β -carbolines as important for the loreclezole like modulation.

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