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Gaboxadol: in vitro interaction studies with benzodiazepines and ethanol suggest functional selectivity

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Abstract

The interaction of gaboxadol (THIP; 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol), ethanol and a series of benzodiazepine site agonists including diazepam, flunitrazepam, lorazepam, indiplon, zaleplon and zolpidem has been characterized at human $\alpha_1\beta_3\gamma_{2S}$ γ-aminobutyric acid type A ($GABA_A$) receptors expressed in *Xenopus* oocytes and in the rat cortical wedge preparation. At the expressed receptors, gaboxadol and the benzodiazepine site agonists interacted synergistically and ethanol did not further enhance this potentiation. In contrast, in the rat cortical wedge preparation, where the inhibition of spontaneous activity was assessed, much weaker effects of the benzodiazepine site agonists were seen. Furthermore, ethanol did not further potentiate the effects of gaboxadol. These findings suggest that gaboxadol in functionally intact tissue may interact with a receptor population, which is insensitive to the modulation by benzodiazepines.

Keywords: Gaboxadol; THIP; Benzodiazepine; Ethanol

1. Introduction

γ-Aminobutyric acid type A ($GABA_A$) receptors are the molecular substrates for the clinically widely used and abused benzodiazepines, benzodiazepine site agonists, barbiturates, neurosteroids and anaesthetics like propofol. In addition to these pharmacologically defined classes of compounds, $GABA_A$ receptors are selectively activated by directly acting $GABA_A$ receptor agonists such as muscimol, isoguvacine and 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol (THIP, gaboxadol).

Synergism between benzodiazepine site agonists and ethanol or, in general, between central nervous system (CNS) depressants and ethanol is thought to be one of the hallmarks for a high abuse potential for novel compounds. Thus, in order to obtain a CNS depressant with a relatively low abuse potential, the synergistic interaction between the compound and, e.g., ethanol or benzodiazepines should, in principle, be minimized.

Gaboxadol is currently in development as a novel hypnotic. Clinical studies have shown that gaboxadol primarily affects the quality of sleep with increased non-rapid eye movement (NREM) sleep and a fewer number of awakenings. However, in contrast to the benzodiazepines, the latency to persistent sleep is only slightly reduced by gaboxadol (Faulhaber et al., 1997; Lancel et al., 2001; Mathias et al., 2001).

A possibility for concomitant use of benzodiazepines and gaboxadol could therefore arise if an insomniac patient suddenly decided to seek instant sedation with one of the standard hypnotics or, alternatively, with ethanol. In order to address the potential for synergistic interaction of gaboxadol and benzodiazepines or ethanol, we have in *Xenopus* oocytes expressing $\alpha_1\beta_3\gamma_{2S}$ human $GABA_A$ receptors and in slices from rat neocortex characterized the consequences of co-treatment with gaboxadol, ethanol and a series of benzodiazepine site agonists.

Previous studies with gaboxadol have shown that gaboxadol (THIP) binds to all $GABA_A$ receptors with a relatively low selectivity (Ebert et al., 1997). However, the functional consequences of this binding are highly dependent on subunit composition of the individual $GABA_A$ receptor. Thus, at α_1 or $\alpha_3\beta_{2/3}\gamma_2$ receptors gaboxadol is a partial agonist, at $\alpha_5\beta_{2/3}\gamma_{2S}$ a full agonist (Ebert et al., 1994) and at $\alpha_4\beta_3\delta$-containing receptors a superagonist with high potency and a maximal response 60% larger than that of GABA (Adkins et al., 2001; Brown et al., 2002).

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Pharmacological characterization of gaboxadol and a series of agonists, partial agonists and a competitive antagonist in the rat cortical wedge preparation suggested that these compounds primarily activated extrasynaptic receptors, although no final conclusion could be drawn (Ebert et al., 2002).

If, as suggested, a functionally relevant proportion of the extrasynaptic $GABA_A$ receptors in the neocortex contain $\alpha_4\beta_3\delta$ subunits and the majority of synaptic $GABA_A$ receptors are composed of $\alpha_1\beta_{2/3}\gamma_2$ subunits, the pharmacological consequences would be significant. Since $\alpha_4\beta_3\delta$-containing $GABA_A$ receptors are insensitive to modulation by any benzodiazepine site agonist, whereas the converse is the case for $\alpha_1\beta_{2/3}\gamma_2$-containing receptors, the interaction between gaboxadol and benzodiazepines could be additive.

2. Materials and methods

2.1. cDNAs

Cloning and sequencing of cDNAs encoding human α_1, β_3 and γ_{2S} $GABA_A$ receptor subunit proteins have been described elsewhere (Hadingham et al., 1993a,b). α_1 encoding cDNA was engineered into a pCDM8 vector and β_3 and γ_{2S} into a pcDNAI/Amp vector. DNA was a gift from Dr. Paul Whiting, Merck Sharp and Dohme, Terlings Park, Harlow, UK.

2.2. Xenopus oocyte expression

An adult female *Xenopus laevis* was anaesthetized by immersion in a 0.4% (w/v) 3-aminobenzoic acid ethyl ester solution (Sigma Diagnostics, St. Louis, MO, USA) for 15–20 min. Through an incision in the abdominal wall, two to three ovarian lobes were removed and stages V and VI oocytes manually defolliculated with watchmaker's fine forceps. After mild collagenase treatment (type IA (Sigma), 0.5 mg/ml, for 6 min) to remove follicle cells, the oocyte nuclei were then directly injected with 13.8 nl of injection buffer [88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0, filtered through nitrocellulose] containing cDNAs encoding human $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptor subunits (1.67 ng/μl/subunit). Oocytes were incubated for 24 h in Modified Barth's Saline [88 mM NaCl, 1 mM KCl, 15 mM HEPES, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$] supplemented with 2 mM sodium pyruvate, 0.1 U/l penicillin and 0.1 μg/l streptomycin and filtered through nitrocellulose.

2.3. Two-electrode voltage clamping

Oocytes were placed in a 60-μl bath and perfused with Ringer [115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.5]. Cells were impaled with agar plugged 0.5–1 MΩ electrodes containing 3 M KCl and voltage clamped at −40 to −70 mV by a GeneClamp 500B amplifier (Axon Instruments). The cells were continuously perfused with Ringer buffer at 4–6 ml/min, and the drugs were applied in the perfusate. Gaboxadol-containing solutions were applied until the peak of the response was observed, usually after 30 s or less. The effects of $GABA_A$ receptor modulators were examined on control gaboxadol responses using a concentration that elicited 20% of the maximal response (EC_{20}) determined for each individual oocyte. To ensure complete binding, modulators were preapplied 60 s before co-application of gaboxadol EC_{20} and the modulators. Six minutes of wash time between gaboxadol EC_{20} (co)-applications were allowed in order to prevent receptor desensitisation. Data were normalized with respect to the gaboxadol EC_{20} control response of each oocyte (defining the EC_{20} response as 100%) and presented as arithmetic mean ± S.E.M.

2.4. Cortical wedge preparation

The rat cortical wedge preparation was carried out according to previously published methods (Ebert et al., 2002; Harrison and Simmonds, 1985). In short: an adult male Sprague–Dawley rat (150–175 g; M&B, Ry, Denmark) was decapitated and the brain rapidly removed and placed in ice-cold O_2/CO_2 (95%/5%) saturated Ca^{2+}–Krebs medium (118 mM NaCl; 2.1 mM KCl; 1.2 mM KH_2PO_4; 11 mM D-glucose; 25 mM $NaHCO_3$; 2.5 mM $CaCl_2$). With a coronal section, the cerebellum was freed from the brain, using a handheld razor blade. The cut surface of the remaining block of the brain was fixed to a Teflon pad with a cyanoacrylate glue (Loctite 401, Loctite, Dublin, Ireland) (the anterior part of the brain pointing upwards). The pad was mounted in a vibratome chamber that was immediately filled with ice-cold Ca^{2+}–Krebs medium. Using a vibratome (Vibroslice model HA752, Campden Instrument, Leicester, UK), successive coronal sections were made and discarded until the corpus callosum appeared as an unbroken line connecting the two hemispheres of the brain. Subsequently, three to four slices (500 μm thick) were cut and carefully transferred to a Petri dish containing ice-cold Ca^{2+}–Krebs medium. With two handheld razor blades, the slices were divided at the midline, thereby separating the hemispheres. Parallel with the midline, a rod ("wedge") of brain tissue, approximately 1.5 mm thick, was cut from each hemisphere and the striatal tissue was trimmed off.

The recording arrangement consisted of a stand holding a slide (angled 45°) containing four two-compartment baths. The two compartments of each bath were separated by a wall supplied with a slot for placement of the wedge. Along the wall in each compartment, a strip of double-layered, nonwowen dishcloth tissue was placed which when wetted provided contact to the Ag/AgCl electrodes (Dri-Ref™, World Precision Instruments, Sarasota, FL, USA) situated in the top of the slide. The electrical potential between the two compartments was measured by the electrodes and

displayed on a Yokogawa LR 4220E chart recorder (Yokogawa Electric, Tokyo, Japan).

With a pair of forceps, the wedge was mounted across the slot, with the cortex part of the wedge situated in the left compartment and the corpus callosum part in the right compartment. The gap between the two compartments was insulated with grease (a mixture of high vacuum silicone grease, Wacker-Chemie, Munich, Germany, and heavy white mineral oil, Sigma) beneath and above the wedge traversing the slot. In order to prevent the wedge from drying out, another wetted strip of dishcloth tissue was placed on top of the wedge. The two compartments were independently and continuously superfused with Krebs medium at 1 ml/min/compartment (provided by a peristaltic pump model 110, Ole Dich Instrumentmakers, Hvidovre, Denmark). In order to ensure that the impulses would propagate only from the left to the right, Ca^{2+}–Krebs was supplied to the left compartment and Krebs medium devoid of Ca^{2+} to the right compartment.

The wedges were left for development of spontaneous activity for 2–3 h.

2.5. Cortical wedge experimental procedure

Characterization of effects on the spontaneous activity was initiated when the spontaneous activity was more frequent than 30 spikes per 12 min and stable over a 30-min period. The number of population responses (spikes) per 12 min was counted. Compounds were applied in superfusion buffer and the wedges were superfused for 20 min. Solutions of GABA or gaboxadol EC_{20}, GABA or gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist and GABA or gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist + 1 ‰ (v/v) ethanol were applied cumulatively. Thereafter, the wedges were washed with superfusion buffer until the control frequency of spontaneous activity was regained, and finally, GABA or gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist + 1000 nM of the competitive benzodiazepine site antagonist, Ro 15-1788 (flumazenil), was applied. The number of spikes during the last 12 min of each drug application were counted and the frequency (spikes per minute) was calculated. The relative frequency, calculated as the ratio between frequencies in the presence and absence, respectively, of compound was calculated (the outcome measure being % spontaneous activity). Obtained data were presented as arithmetic means ± S.E.M.

In control experiments investigating synergism, the data were presented as % inhibition of spontaneous activity (= 100% − % spontaneous activity).

2.6. Drug solutions

The chemical structures of GABA, the partial $GABA_A$ receptor agonist, gaboxadol, the three classical benzodiazepines, diazepam, flunitrazepam and lorazepam, the three novel benzodiazepine site agonists with non-benzodiazepine structure, indiplon (NBI 34060), zaleplon (pyrazolopyrimidines) and zolpidem (an imidazopyridine), and the benzodiazepine site antagonist Ro 15-1788 (flumazenil) are depicted in Fig. 1.

All drugs were purchased from ordinary commercial sources except from gaboxadol, indiplon and zaleplon which were obtained from Department of Medicinal Chemistry, H. Lundbeck, Valby, Denmark.

Stock solutions (10 mM) of the benzodiazepine site ligands were made in dimethyl sulfoxide (DMSO). The concentration of DMSO in the final solutions never exceeded 0.1% of which no effect was seen, neither when applied alone nor when co-applied with the GABA/gaboxadol EC_{20} solution. GABA was dissolved in water and stored in aliquots at −20 °C. Solutions of gaboxadol (in Ringer or Ca^{2+}–Krebs) were made fresh on a daily basis.

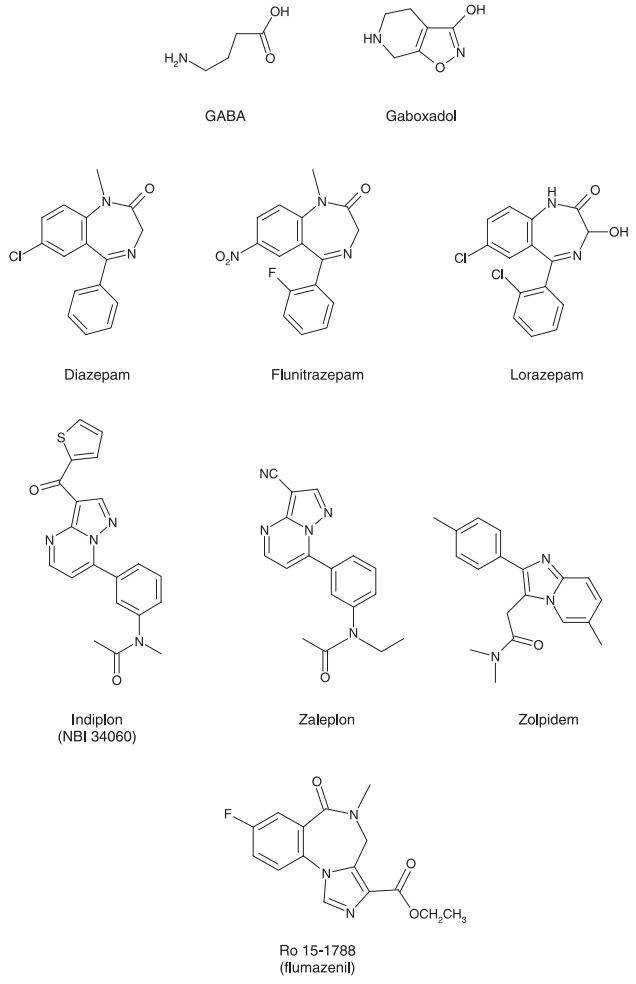

Fig. 1. Structures of GABA, the partial $GABA_A$ receptor agonist, 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol (gaboxadol) and seven benzodiazepine site ligands; the three classical 1,4-benzodiazepines, diazepam, flunitrazepam and lorazepam, the three novel benzodiazepine site agonists, indiplon (NBI 34060), zaleplon (pyrazolopyrimidines) and zolpidem (an imidazopyridine), and the benzodiazepine site antagonist, Ro 15-1788 (flumazenil).

2.7. Statistical data analysis

Using SigmaStat 2.03 (SPSS, Chicago, IL, USA), the results obtained from *Xenopus* oocytes and the rat cortical wedge preparation were compared statistically using a one-way analysis of variances (ANOVA) combined with a pairwise multiple comparison procedure (Student–Newman–Keuls Method) and were considered significant if $P < 0.05$. If normality or equal variance tests failed, a Kruskal–Wallis one-way analysis of variance on Ranks was performed instead.

For analysis of results obtained from control experiments investigating synergism between GABA/gaboxadol and 1000 nM benzodiazepine site agonist or 1 ‰ (v/v) ethanol in the rat cortical wedge model, the responses to GABA/gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist/1 ‰ ethanol were compared to the sum of the responses to the agonist and to the modulator when applied alone, using a paired *t*-test.

3. Results

3.1. Investigation of positive modulators in Xenopus oocytes

Xenopus oocytes intranuclearly injected with cDNAs encoding $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptor subunit proteins were tested using Two-Electrode Voltage Clamp. The potentiation of gaboxadol EC_{20} responses by positive modulators of the $GABA_A$ receptor complex, including six benzodiazepine site agonists and ethanol, was investigated. The three classical 1,4-benzodiazepines, diazepam, flunitrazepam and lorazepam, and three novel benzodiazepine site agonists with non-benzodiazepine structure, indiplon (NBI 34060), zaleplon and zolpidem, were tested in concentrations of 10, 100 and 1000 nM. It was examined whether 1 ‰ (v/v) ethanol could contribute to further potentiation of the gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist response. Furthermore, to verify that the potentiation induced by 1000 nM benzodiazepine site agonist was mediated via the benzodiazepine site, it was investigated whether the benzodiazepine site antagonist, Ro 15-1788 (flumazenil), could counteract the benzodiazepine site agonist action. A representative example of a trace recorded from one of these experiments is seen in Fig. 2 and the summarized results from these experiments are depicted in Fig. 3.

From Fig. 3, it appears that all benzodiazepine site agonists produced an increase of the gaboxadol EC_{20} response. The largest potentiation was brought about by zolpidem, for which the response to gaboxadol EC_{20} + 1000 nM zolpidem was 308%, corresponding to 208% potentiation (% potentiation=% response – 100%). Flunitrazepam and lorazepam potentiated gaboxadol EC_{20} responses less than zolpidem (159% and 124%, respectively), whereas diazepam and zaleplon only exhibited ~ 100% potentiation. However, the weakest degree of potentiation was observed with indiplon, which potentiated gaboxadol EC_{20} responses by only 47%.

The results reveal no obvious difference in the degree of potentiation in the concentration of 1000 nM between the classical 1,4-benzodiazepines and novel agonists with non-benzodiazepine structure. In addition, the mode of action of all the benzodiazepine site agonists tested, regardless of chemical structure, was purely modulatory, since no effect of the benzodiazepine site agonists was observed during the pretreatment period.

Indiplon turned out to be the weakest modulator, yielding only a statistically significant potentiation of the gaboxadol EC_{20} response in concentrations of 100 and 1000 nM.

For the remaining benzodiazepine site agonists, 10, 100 and 1000 nM of the compounds all potentiated the gaboxadol EC_{20} response significantly ($P < 0.005$). However, 1 ‰ (v/v) ethanol was not able to bring about a further potentiation for any of the benzodiazepine site agonists. For lorazepam, indiplon, zaleplon and zolpidem, no significant difference was found between gaboxadol EC_{20} and gaboxadol/benzodiazepine site agonist/Ro 15-1788 responses, indicating that the benzodiazepine site antagonist, Ro 15-1788, abolished the benzodiazepine site agonist potentiation

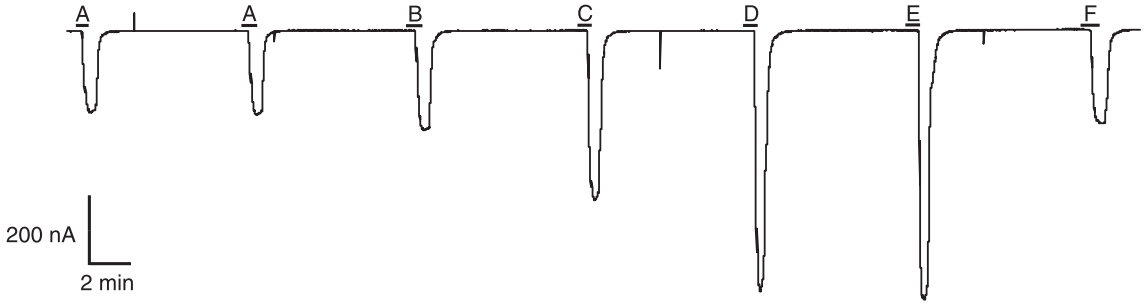

Fig. 2. Example of a typical trace recorded from a *Xenopus* oocyte expressing $GABA_A$ receptors composed of $\alpha_1\beta_3\gamma_{2S}$ subunits. The modulatory effects of flunitrazepam (FLU) and ethanol were tested by application of: A: Gaboxadol EC_{20}. B: Gaboxadol EC_{20} + 10 nM FLU. C: Gaboxadol EC_{20} + 100 nM FLU. D: Gaboxadol EC_{20} + 1000 nM FLU. E: Gaboxadol EC_{20} + 1000 nM FLU + 1 ‰ (v/v) ethanol. F: Gaboxadol EC_{20} + 1000 nM FLU + 10 μM Ro 15-1788. Gaboxadol EC_{20} was applied until a stable response was obtained (the last two applications are shown in the figure). The oocyte was superfused with a solution containing the modulator(s) 60 s prior to co-application of gaboxadol EC_{20} + modulator(s).

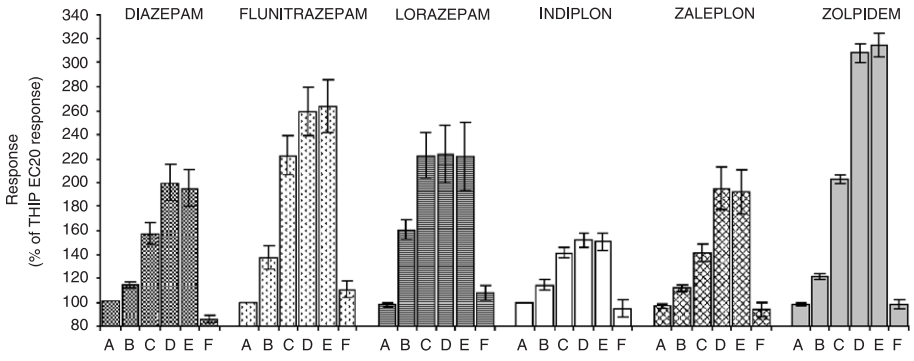

Fig. 3. Potentiation of gaboxadol EC_{20} responses by benzodiazepine site ligands and ethanol in *Xenopus* oocytes expressing $GABA_A$ receptors composed of $\alpha_1\beta_3\gamma_{2S}$ subunits. The bars represent responses to: A: Gaboxadol EC_{20}. B: Gaboxadol EC_{20} + 10 nM benzodiazepine site agonist. C: Gaboxadol EC_{20} + 100 nM benzodiazepine site agonist. D: Gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist. E: Gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist + 1 ‰ (v/v) ethanol. F: Gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist + 10 μM Ro 15-1788. Data were normalized with respect to the gaboxadol EC_{20} control response (defined as 100%) and presented as arithmetic means ± S.E.M. Data were obtained from five to eight individual oocytes.

completely. For diazepam and flunitrazepam a small, however significant, difference was found, gaboxadol/diazepam/Ro 15-1788 being 15% smaller than gaboxadol EC_{20} response and gaboxadol/flunitrazepam/Ro 15-1788 being 10% larger than gaboxadol EC_{20} response.

The statistically significant potentiation of gaboxadol EC_{20} responses combined with the fact that benzodiazepine site agonists are incapable of gating the receptor complex when applied alone (during the pretreatment period) points to a significant synergistic interaction between the $GABA_A$ receptor agonist (gaboxadol) and the benzodiazepine site agonists at the molecular level in *Xenopus* oocytes.

3.2. Investigation of positive modulators in the rat cortical wedge model

Due to the inhibitory nature of the GABAergic system in the CNS, $GABA_A$ receptor agonists reduce the frequency of NMDA receptor mediated spontaneous activity when applied to the rat cortical wedge preparation. Thus, in the rat cortical wedge model, GABA and gaboxadol EC_{20} values are defined as the concentrations needed to reduce the frequency of spontaneous activity to 80% of the control frequency. Since modulators augment the responses to these agonists, a further decrease in the frequency can be obtained with these compounds when co-applied with GABA or gaboxadol Fig. 4.

Preliminary experiments revealed that only modest and statistically insignificant degrees of potentiation of GABA and gaboxadol EC_{20} responses were brought about by 10 and 100 nM of the benzodiazepine site agonists (0–40%) (data not shown). In order to ensure ligand saturation of all benzodiazepine binding sites and thus elicitation of a detectable potentiation, a benzodiazepine site agonist concentration of 1000 nM (although not physiologically relevant) was chosen for all subsequent experiments. Again, it was investigated whether 1 ‰ (v/v) ethanol could induce a further potentiation of the GABA/gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist response. After a washout period, the benzodiazepine antagonist, Ro 15-1788, was used to confirm that the potentiating effect of the benzodiazepine site agonists was mediated via the benzodiazepine site. The results are seen in Fig. 5. To obtain comparability of these inhibitory responses with the results from *Xenopus* oocytes, the degrees of potentiation of GABA and gaboxadol EC_{20} responses induced by 1000 nM benzodiazepine site agonist have been calculated in Table 1.

As anticipated from the preliminary studies, lower degrees of potentiation (0–120%) than in the oocytes were obtained. The data were tested statistically, using a one-way ANOVA.

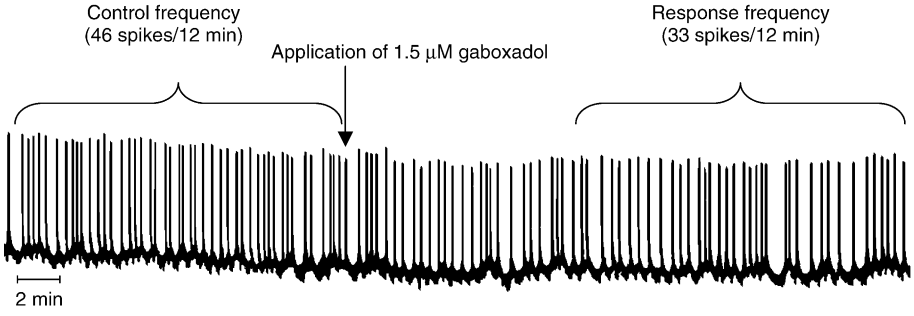

Fig. 4. Excerpt from a trace recorded from the rat cortical wedge model showing the control and GABA EC_{20} response frequencies. The number of spikes during the last 12 min of each drug application was counted and the frequency (spikes per minute) was calculated. The relative frequency, calculated as the ratio between frequencies in the presence and absence, respectively, of compound was calculated. In the presented example, the relative frequency is 72%.

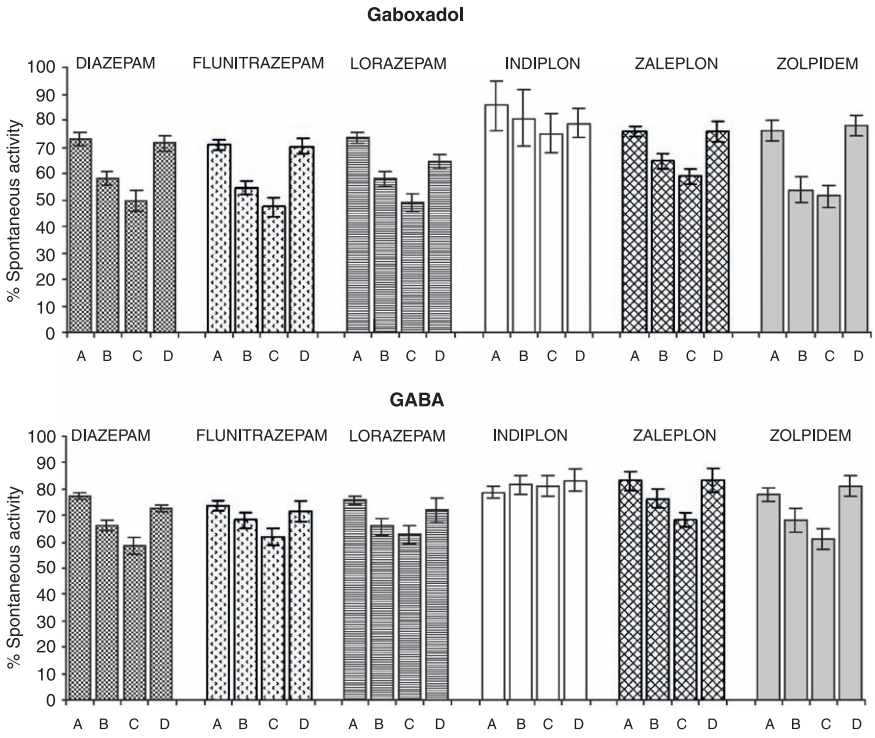

Fig. 5. Potentiation of gaboxadol (top panel) and GABA (bottom panel) EC_{20} responses (inhibition of spontaneous activity) by benzodiazepine site ligands and ethanol in the rat cortical wedge model. The bars represent responses to: A: Gaboxadol or GABA EC_{20}. B: Gaboxadol or GABA EC_{20} + 1000 nM benzodiazepine site agonist. C: Gaboxadol or GABA EC_{20} + 1000 nM benzodiazepine site agonist + 1 ‰ (v/v) ethanol. D: Gaboxadol or GABA EC_{20} + 1000 nM benzodiazepine site agonist + 1000 nM Ro 15-1788. Data were normalized with respect to the control frequency of each wedge and presented as arithmetic means ± S.E.M ($n = 6–14$).

For indiplon, no difference between responses to the four different applications (A–D in Fig. 5) was found, neither with GABA nor gaboxadol, though a slight potentiation of the gaboxadol EC_{20} responses was observed. For the remaining benzodiazepine site agonists, the groups proved to be different and a post hoc multiple comparison procedure (Student–Newman–Keuls method) was carried out. Except from diazepam, none of the benzodiazepine site agonists in a concentration of 1000 nM potentiated the GABA EC_{20} response significantly. However, co-application of GABA EC_{20}, 1000 nM benzodiazepine site agonist and 1 ‰ (v/v) ethanol yielded a response that was significantly different from that elicited by GABA alone. For diazepam, this potentiation was very clear ($P < 0.001$), and in addition, different from that obtained by GABA + 1000 nM diazepam. For the remaining benzodiazepine site agonists, the GABA/benzodiazepine site agonist response was statistically indistinguishable from that of GABA/benzodiazepine site agonist/ethanol, indicating that ethanol did not potentiate the response further. In the diazepam experiments, the benzodiazepine site antagonist, Ro 15-1788, was able to abolish the effect of diazepam completely, since the GABA/diazepam/Ro 15-1788 response was not significantly different from the GABA response but significantly different from the GABA/diazepam response ($P < 0.05$). For flunitrazepam, lorazepam, zaleplon and zolpidem, GABA/benzodiazepine site agonist/Ro 15-1788 responses were not significantly different from neither GABA nor GABA/benzodiazepine site agonist

Table 1
% Potentiation of GABA/gaboxadol EC_{20} responses by co-application of 1000 nM benzodiazepine site agonist using the rat cortical wedge preparation

	% Potentiation of GABA/gaboxadol EC_{20} response (mean ± S.E.M.)					
	Diazepam	Flunitrazepam	Lorazepam	Indiplon	Zaleplon	Zolpidem
GABA	51 ± 8.9%	18.6 ± 5.7%	40 ± 7.3%	~ 0%	21 ± 8.6%	49 ± 18.4%
Gaboxadol	62 ± 7.3%	60 ± 10%	55 ± 9.9%	26 ± 24%	53 ± 15%	120 ± 25%

For each wedge, the potentiation was calculated as follows:

$$\%\ \text{potentiation} = \frac{\text{GABA/gaboxadol response} - \text{GABA/gaboxadol} + \text{BZD response}}{100 - \text{GABA/gaboxadol response}} \times 100\%,$$

where the GABA/gaboxadol ± benzodiazepine site agonist responses are the relative frequencies of spontaneous activity in the presence of GABA/gaboxadol ± benzodiazepine site agonist.

responses, suggesting that benzodiazepine site agonist potentiation was only partially antagonized or that the effect is mediated via a nonspecific mechanism.

Statistical data analyses of experiments using gaboxadol as agonist revealed a clear potentiation of gaboxadol EC_{20} responses with both benzodiazepine site agonist and benzodiazepine site agonist + ethanol. With exception from lorazepam, ethanol, however, was not able to increase the gaboxadol/benzodiazepine site agonist response further. For diazepam, flunitrazepam, zaleplon and zolpidem, Ro 15-1788 completely antagonized the benzodiazepine site agonist potentiation while no significant difference between the responses of gaboxadol/lorazepam and gaboxadol/lorazepam/Ro 15-1788 was observed, indicating lack of complete antagonization.

Since the benzodiazepine site agonists were applied to functionally intact brain tissue with an already on-going GABA system, it is possible that the effect of the benzodiazepine site agonists to some extent is due to modulation of endogenous GABAergic activity. Thus, in an attempt to investigate whether GABA/gaboxadol and the benzodiazepine site agonists interacted synergistically or the effects were simply additive, a series of control experiments for three selected benzodiazepine site agonists (diazepam, flunitrazepam and zolpidem) was undertaken.

These experiments, in which GABA or gaboxadol EC_{20} and 1000 nM benzodiazepine site agonist were applied alone and together, revealed that the response to GABA or gaboxadol EC_{20} + 1000 nM benzodiazepine site agonist (column C in Fig. 6) was not greater than the sum of the responses to GABA or gaboxadol EC_{20} and 1000 nM benzodiazepine site agonist (column A + B). Furthermore, no difference in the activity of GABA and gaboxadol in the presence of any the benzodiazepines was seen.

By comparison of column A + D and E in Fig. 6, it is seen that the same is the case for 1‰ (v/v) ethanol (P>0.05).

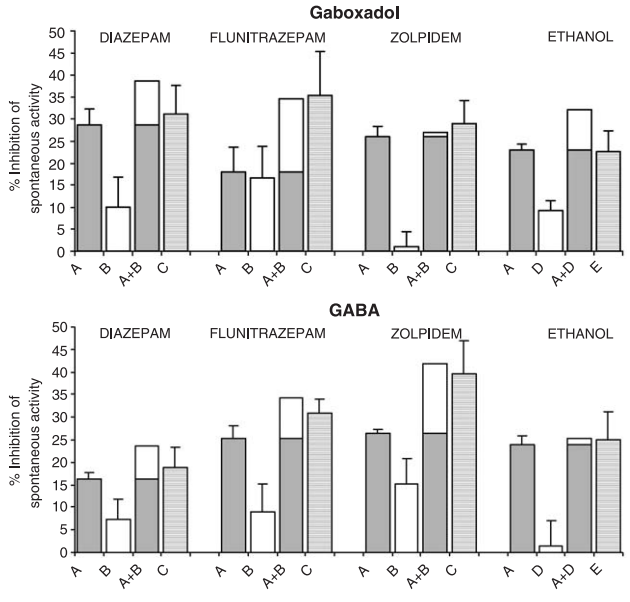

Fig. 6. Investigation of synergistic interaction between GABA/gaboxadol and 1000 nM benzodiazepine site agonist or 1‰ (v/v) ethanol in the rat cortical wedge model. Note the data are quoted as % inhibition of spontaneous activity (= 100% − % spontaneous activity). The bars represent responses to: A: Gaboxadol or GABA EC_{20} (grey bars). B: 1000 nM benzodiazepine site agonist (white bars). C: Gaboxadol or GABA EC_{20} + 1000 nM benzodiazepine site agonist (hatched bars). D: 1‰ (v/v) ethanol (white bars). E: Gaboxadol or GABA EC_{20} + 1‰ (v/v) ethanol (hatched bars). Data presented as arithmetic means ± S.E.M. (n = 4–6).

4. Discussion

In the present study, large differences between gaboxadol and benzodiazepine site agonist interaction in *Xenopus* oocytes and the rat cortical wedge model were seen. The relative benzodiazepine site agonist potentiation of agonist EC_{20} responses was three- to fourfold lower in the rat cortical wedge preparation than in *Xenopus* oocytes. Furthermore, control experiments in the rat cortical wedge model revealed that both GABA/gaboxadol EC_{20} and 1000 nM benzodiazepine site agonists, when each of them was applied alone, reduced the frequency of the spontaneous activity. The effect elicited by the benzodiazepine site agonists when applied alone to the cortical wedge preparation stems from potentiation of endogenous activity at $GABA_A$ receptors. Since the most abundant $GABA_A$ receptor subtype within the CNS is composed of $\alpha_1\beta_{2/3}\gamma_2$ subunits (Fritschy and Möhler, 1995; Sperk et al., 1997; Wisden et al., 1992), this benzodiazepine site agonist-sensitive receptor subtype (mainly located in the synaptic junction) presumably is the major contributor to the endogenous GABA activity. In contrast to the findings in *Xenopus* oocytes, no statistical evidence of a significant synergistic effect was found when gaboxadol and the six benzodiazepine site agonists were co-applied in the cortical wedge preparation. As has been suggested in a previous study using the rat cortical wedge model (Ebert et al., 2002), gaboxadol might primarily interact with extrasynaptic $\alpha_4\beta_x\delta$-containing receptors, which are known to be insensitive to benzodiazepine site agonists such as flunitrazepam and zolpidem (Adkins et al., 2001; Brown et al., 2002). In contrast, the classical 1,4-benzodiazepines interact potently and efficaciously with α_1-, α_2-, α_3- and α_5-containing receptors (Smith et al., 2001), whereas indiplon, zaleplon and zolpidem preferentially interact with α_1-containing receptors (Dämgen and Lüddens, 1999; Smith et al., 2001). Thus, the findings from the rat cortical wedge preparation may be explained by the idea that gaboxadol and the benzodiazepine site agonists could interact with two distinct receptor populations and the effects exerted at these separate populations would sum up in an additive rather than supra-additive manner.

α_1 is the most abundant isomer from the α class of $GABA_A$ receptor subunits. It has been estimated that 70–90% of all $GABA_A$ receptors within the brain contain one or

more α_1 subunit(s) (Sieghart and Sperk, 2002). It can and has been questioned which receptor population should be targeted in order to obtain a drug devoid of side effects. On one hand, the most abundant receptor populations represent the quantitatively most important and as such are more likely to induce functional effects upon targeting. On the other hand, the less abundant receptor populations are more specifically located and as such more likely to produce selective effects. $\alpha_4\beta_x\delta$-containing receptors are enriched in thalamus, which is known to play a key role in sleep. The presumed α_4 selectivity of gaboxadol might be part of the explanation why gaboxadol exhibits a novel soporific profile compared to benzodiazepine site agonists. Results from preclinical as well as clinical studies in healthy young volunteers and geriatric patients have revealed that gaboxadol displays many characteristics of an "ideal" hypnotic. For instance, gaboxadol has been shown to promote NREM sleep (leaving the time spent in rapid eye movement (REM) sleep unaffected), enhance delta activity in the electroencephalography (EEG), decrease the number of awakenings and increase the subjective sleep quality. Furthermore, no hangover effect is observed on the following morning (Faulhaber et al., 1997; Lancel, 1997; Lancel and Faulhaber, 1996; Lancel et al., 2001; Mathias et al., 2001). In contrast, in a variety of mammalian species, classical 1,4-benzodiazepines and novel benzodiazepine site agonists such as zolpidem and zopiclone have been found to reduce sleep latency, increase the amount of NREM sleep (during which the delta activity in the EEG is attenuated and spindling is augmented) and inhibit REM sleep (Lancel, 1999). Many of these effects are generally considered undesirable.

The present study therefore is yet another suggestion of an extrasynaptic α_4 selective activity of gaboxadol in intact systems. However, it still remains to be established if the main site of action for gaboxadol indeed is extrasynaptic receptors and if this is the explanation for the clinical findings.

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