0026-895X/00/040756-07$3.00/0
MOLECULAR PHARMACOLOGY
Vol. 58, No. 4
141/854114
Mol Pharmacol 58:756–762, 2000
Printed in U.S.A.

Characterization of the Interaction of Zopiclone with γ-Aminobutyric Acid Type A Receptors

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Received March 10, 2000; accepted June 27, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Zopiclone is a cyclopyrrolone that is used clinically as a hypnotic. Although this drug is known to interact with neuronal γ-aminobutyric acid type A receptors, its binding site(s) within the receptor oligomer has been reported to be distinct from that of the classical benzodiazepines. After photoaffinity labeling with flunitrazepam, receptors in rat cerebellar membranes showed differentially reduced affinity for flunitrazepam and zopiclone by 50- and 3-fold, respectively. Because histidine 101 of the α-subunit is a major site of photolabeling, we have made specific substitutions of this residue and studied the consequences on the binding properties of zopiclone and diazepam using recombinant α1β2γ2-receptors transiently expressed in tsA201 cells. Both compounds showed similar binding profiles with receptors containing mutated α-subunits, suggesting a similar interaction with the residue at position 101. At α1β2γ3-receptors, flunitrazepam affinity was dramatically decreased by approximately 36-fold, whereas the affinity for zopiclone was decreased only 3-fold, suggesting a differential contribution of the γ-subunit to the binding pocket. Additionally, we used electrophysiological techniques to examine the contribution of the γ-subunit isoform in the receptor oligomer to ligand recognition using recombinant receptors expressed in *Xenopus* oocytes. Both compounds are agonists at α1β2γ2- and α1β2γ3-receptors, with flunitrazepam being more potent but less efficacious. In summary, these data suggest that histidine 101 of the α1-subunit plays a similar role in ligand recognition for zopiclone, diazepam, and flunitrazepam.

γ-Aminobutyric acid type A ($GABA_A$) receptors are heteromeric neurotransmitter receptors that belong to a ligand-gated ion channel superfamily that also includes the nicotinic acetylcholine, glycine, and serotonin type 3 receptors. Many different subunits for this receptor have been cloned, including α1–6, β1–3, γ1–3, ρ1–3, δ, π, ϵ, and θ (for reviews, see Barnard et al., 1998; Whiting et al., 1999), and the pharmacological properties of any one receptor may be attributed to its particular combination of subunit isoforms. $GABA_A$ receptors are the site of action of many clinically important compounds, including the benzodiazepines, which interact with a specific binding site likely residing at the interface between the α- and γ-subunits (Davies et al., 1996; Sigel and Buhr, 1997). Occupation of this site can produce a full spectrum of allosteric effects ranging from positive to negative modulation of GABA-gated ion flux (for reviews, see Sieghart, 1995; Barnard et al., 1998).

When used clinically, benzodiazepines display anticonvulsant, sedative/hypnotic, myorelaxant, and anxiolytic properties that can be exploited to ameliorate a variety of medical conditions. However, although highly efficacious and relatively safe, benzodiazepines are not suitable for chronic use because of the potential for development of tolerance. Other compounds that do not have a benzodiazepine structure but also bind to this site display, in some cases, fewer of the side effects associated with classical benzodiazepines (Sanger et al., 1994; Wagner et al., 1998). Nonbenzodiazepines such as the imidazopyridine zolpidem and the cyclopyrrolone zopiclone appear to be as efficacious as traditional benzodiazepines and are in clinical use (Goa and Heel, 1986; Wagner et al., 1998). Zolpidem exhibits a lower potential for tolerance and abuse than the classical benzodiazepines, and this has been attributed to its specific recognition of benzodiazepine type 1 receptors (Benavides et al., 1988; Sanger et al., 1994; Kunovac and Stahl, 1995). Zopiclone also appears to produce fewer unwanted side effects than the benzodiazepines (Julou et al., 1985), but it is unknown whether this is due to its preferential recognition of a particular receptor subtype.

There is good evidence that the effects of zopiclone are mediated through $GABA_A$ receptors, but the mechanism by which this occurs remains unclear (for review, see Doble et

This work was supported by the Medical Research Council of Canada. J.G.N. was supported by The Neuroscience Canada Foundation, the Alberta Heritage Foundation for Medical Research (AHFMR), and the Natural Sciences and Engineering Research Council; J.M.C.D. was supported by the AHFMR.

ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid type A.

al., 1995), although there is evidence to suggest that zopiclone allosterically modulates $GABA_A$ receptors in a unique fashion. The allosteric effects of zopiclone and flunitrazepam on [^{35}S]*t*-butylbicyclophosphorothionate appear to differ, in that the flunitrazepam- but not zopiclone-induced potentiation of [^{35}S]*t*-butylbicyclophosphorothionate binding is antagonized by Ro15-1788 (Lloyd et al., 1990). In vivo binding studies have shown that the intraperitoneal injection of zolpidem can decrease [^{3}H]Ro15-1788 binding in a dose-dependent manner in various mouse brain regions, whereas the injection of zopiclone leads to increased binding of [^{3}H]Ro15-1788 (Byrnes et al., 1992), but this may be a function of differences in pharmacokinetics. Furthermore, studies of rat brain membranes showed that, unlike [^{3}H]flunitrazepam binding, [^{3}H]zopiclone binding is not enhanced by secobarbital, pentobarbital, or GABA (Blanchard et al., 1983). In addition, there is no clear consensus concerning the efficacy of zopiclone at $GABA_A$ receptors. Functional studies have shown either no effect on (Concas et al., 1994), a weak potentiation of (Skerritt and MacDonald, 1984), or a robust potentiation of (Reynolds and Maitra, 1996) GABA-gated chloride conductance.

Some groups have suggested that zopiclone produces fewer clinical side effects than the classical benzodiazepines because it recognizes a unique binding site on the $GABA_A$ receptor. The evidence supporting the existence of this site is somewhat controversial. Data from kinetic studies suggest that the two sites are allosterically linked, because zopiclone was shown to accelerate the dissociation of [^{3}H]Ro15-1788 (Trifiletti and Snyder, 1984), although later studies were unable to repeat this finding (Concas et al., 1994). In addition, equilibrium binding studies have suggested that the interaction of zopiclone and Ro15-1788 is noncompetitive (Trifiletti and Snyder, 1984), whereas others have shown it to be competitive (Concas et al., 1994). These discrepancies may be due to receptor heterogeneity in the variety of tissues and cell types used in these studies.

In photoaffinity labeling experiments, it has been shown that histidine 101 of the α1-subunit is a major site of photoincorporation of [^{3}H]flunitrazepam (Duncalfe et al., 1996), and this residue is an important determinant of benzodiazepine affinity (Weiland et al., 1992; Davies et al., 1998) and efficacy (Dunn et al., 1999). Here we show that, whereas photoaffinity labeling of the receptor markedly compromises the affinity of the classical benzodiazepines, zopiclone affinity is largely unaffected. However, we also show that mutating residue histidine 101 results in similar changes in the binding profiles of both zopiclone and diazepam.

Materials and Methods

Mutagenesis. Mutant α1-subunit cDNAs were generated as described previously (Davies et al., 1998) using the Altered Sites mutagenesis kit (Promega, Madison, WI). Mutagenic oligonucleotides (described previously in Davies et al., 1998) incorporated a silent restriction site that was used to initially screen for mutants. Those that were found to contain this site were then sequenced to confirm the presence of the desired substitution(s). The mutated cDNA was then subcloned into the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA).

Transient Transfection. tsA201 cells were transfected with 10 μg of each subunit in a 1:1:1 ratio (mutant or wild-type α1:β2:γ2 or γ3) as previously described (Davies et al., 1998). After 48 h, the cells were harvested in ice-cold buffer (Tris-HCl, pH 7.5) containing protease inhibitors. The cells were homogenized with two 10-s pulses using an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany). Homogenates were washed by centrifugation and finally resuspended in ice-cold Tris-HCl buffer (pH 7.5). The homogenates were stored at −80°C until the day of the experiments.

Radioligand Binding. Radioligand binding was performed in duplicate with a cell harvester (Brandel, Gaithersburg, MD). For [^{3}H]Ro15-4513 saturation experiments, concentrations of radioligand ranging from 1 to 50 nM were used, with nonspecific binding determined in the presence of 10 μM Ro15-4513. Cell homogenates were incubated with radioligand (and with a displacing compound in the case of competition experiments) in 4°C buffer (50 mM Tris-HCl, 250 mM KCl, pH 7.4) for 1 h before filtration. After the sample was filtered, the filters were washed twice with 5 ml of ice-cold buffer. The filters were allowed to dry and were then placed in 5-ml scintillation vials. After the addition of 5 ml of scintillation fluid, the samples were counted for radioactivity.

Photoshift Experiments. For photoshift experiments, membranes were prepared from rat cerebellum. The tissue was placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and homogenized for 10 s with an Ultra Turrax homogenizer (IKA-Labortechnik). The homogenate was then centrifuged for 20 min at 40,000*g* and 4°C. Subsequently, the pellet was resuspended in the same volume of fresh buffer and centrifuged again. This step was repeated twice more for a total of three washes. After the final centrifugation, the pellet was resuspended in 20 volumes of buffer, frozen, and stored at −80°C.

To perform photoshift experiments, membranes were thawed and diluted 1:80 to give a final protein concentration of approximately 1 mg/ml. The photoshift experiments were performed essentially as described by McKernan et al. (1998). The cerebellar homogenate was incubated for 30 min at 4°C in the presence of flunitrazepam, after which the mixture was exposed to UV light for 60 min. The membranes were then washed by centrifugation and resuspension six times to remove free flunitrazepam. To control for any changes to ligand recognition that might result from exposure of the receptor to UV light, membranes were irradiated in a similar fashion but in the absence of flunitrazepam during the 60-min exposure period.

Homologous competition was used to determine the K_d value for [^{3}H]Ro15-1788. Displacement experiments were performed with five different concentrations of flunitrazepam and zopiclone. Nonspecific binding was defined with clonazepam at a concentration of 3 μM. There was no difference between the nonspecific binding in the photolabeled and the irradiated samples.

Electrophysiology. Oocytes from *Xenopus laevis* were maintained at 14°C in Barth's solution (in mM): NaCl (88), KCl, (1), $CaCl_2$ (0.5), $Ca(NO_3)_2$ (0.5), $MgSO_4$ (1), $NaHCO_3$ (2.4), HEPES (15), pH 7.4. cRNA (50 ng) was injected into oocytes for α1-, β2-, and γ2- or γ3-subunits in a ratio of 1:1:1. Approximately 48 h later, the oocytes were used in concentration-response experiments. During the experiments, the oocytes were continuously perfused with frog Ringer's solution (in mM): NaCl (120), HEPES (5), KCl (2), $CaCl_2$ (1.8), pH 7.4. The eggs were impaled with two electrodes (resistances 0.5–2.0 MΩ in frog Ringer's solution) filled with 3 M KCl and voltage clamped at −60 mV with a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). To examine potentiation of GABA-gated ion flux, the oocytes were preperfused with zopiclone or flunitrazepam for 3 min before the addition of GABA and the allosteric modulator. Potentiation experiments with $\alpha1\beta2\gamma2$-receptors were performed at the EC_{10} for GABA, which was 5 μM. For γ3-containing receptors, potentiation was studied using the EC_{15} value (5 μM).

Data and Statistical Analysis. Ligand binding and electrophysiological data were analyzed with the curve-fitting programs of GraphPad Prism (San Diego, CA). Dose-response curves for GABA-gated currents were fitted by the equation:

$$I = \frac{I_{max}[L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the measured amplitude of the evoked current, [L] is the concentration of GABA, EC_{50} is the GABA concentration that produces 50% of the maximal response (I_{max}), and n is the Hill coefficient. The modulation of the GABA response brought about by allosteric modulators was analyzed using the equation

$$I = I_o + \frac{(E_{max} - I_o)10^{Xn}}{10^{Xn} + 10^{Cn}}$$

where I is the amplitude of the observed current, X is the logarithm of the concentration of the allosteric modulator, I_0 is the current observed in the absence of modulator, E_{max} is the current observed at the maximally effective concentration, C is the logarithm of the EC_{50} for the response of the allosteric modulator, and n is the Hill coefficient.

Results

The Effects of Photolabeling on the Recognition of Flunitrazepam and Zopiclone. Flunitrazepam was used to covalently photolabel the receptor to examine the consequences on the subsequent binding of flunitrazepam and zopiclone. It was previously shown that photoaffinity labeling causes a dramatic reduction in the affinity of the labeled receptor for classical benzodiazepines (Karobath and Supavilai, 1982; Thomas and Tallman, 1983; Brown and Martin, 1984). In our studies, photoaffinity labeling of rat cerebellar membranes reduced the affinity for flunitrazepam by approximately 50-fold (Table 1). However, the affinity for zopiclone was reduced only 3-fold (Table 1). The large decrease in flunitrazepam affinity with photolabeled cerebellar receptors mirrors the results obtained by others using recombinant human $\alpha1\beta3\gamma2$-receptors (McKernan et al., 1998).

Effects on Ligand Binding of Substitutions at Histidine 101. All ligand-binding studies were carried out by competition with [^{3}H]Ro15-4513. As reported previously (Davies et al., 1998), all mutant receptors examined retained high affinity for this ligand, indicating that none of the mutations compromised the overall structure or expression of the receptors.

Wild-type $\alpha1\beta2\gamma2$-receptors recognized zopiclone with an affinity of 51.3 nM (Fig. 1; Table 2), a value that closely matches previous reports with recombinant (Faure-Halley et al., 1993) and native (Julou et al., 1985) receptors. All of the mutations introduced at position 101 of the $\alpha1$-subunit produced dramatic effects on the recognition of zopiclone when coexpressed with $\beta2$- and $\gamma2$-subunits, except for the phenylalanine mutant, which produced a relatively modest 4-fold decrease in affinity (Fig. 1). The H101Q and H101Y mutations resulted in receptors having a 15- to 17- fold lower affinity than wild-type receptors, whereas the H101A, H101K, and H101E mutations produced receptors that did not recognize zopiclone in the concentration range used in these experiments (up to 10 μM).

The effects of these mutations on diazepam recognition paralleled those of zopiclone. Diazepam showed decreased recognition for all of the mutants in this study and completely failed to recognize H101A, H101K, and H101E (Fig. 2; Table 2). Again, as with zopiclone binding, the smallest shift in affinity for diazepam was observed with the phenylalanine substitution, which produced a 10-fold decrease in affinity.

Influence of γ-Subunits on Zopiclone Recognition. The identity of the γ-subunit within the $GABA_A$ receptor oligomer can have profound effects on the pharmacology of ligands that interact with the benzodiazepine site (Lüddens et al., 1994; Tögel et al., 1994; Hadingham et al., 1995). We therefore investigated the interaction of flunitrazepam and zopiclone with recombinant $\alpha1\beta2\gamma2$- and $\alpha1\beta2\gamma3$-receptors. Binding studies revealed that the K_d values for [^{3}H]Ro15-4513 binding were not significantly different between the two subtypes (Table 2; Fig. 3). However, the inclusion of the $\gamma3$-subunit in the receptor oligomer resulted in decreased affinity for both zopiclone and flunitrazepam, with a relatively greater decrease in flunitrazepam affinity compared to zopiclone (decreases of approximately 30- and 3-fold, respectively, Fig. 3). The effects of flunitrazepam and zopiclone on GABA-gated currents mediated by $\alpha1\beta2\gamma2$- and $\alpha1\beta2\gamma3$-receptors were also examined (Fig. 4). Functionally, both zopiclone and diazepam were agonists at each receptor subtype, with flunitrazepam being the more potent of the two (Fig. 4; Table 3). The substitution of $\gamma2$ with $\gamma3$ resulted in a rightward shift (approximately 3-fold) in the concentration-response curves for both agonists. Zopiclone produced a greater potentiation of GABA-gated current at both subtypes, having a larger effect at the $\gamma3$-containing receptors.

Discussion

Zopiclone is an effective hypnotic agent that clearly produces its overt effects by interaction with the $GABA_A$ receptors in the central nervous system. Although the pharmacological spectrum of this agent is very similar to that of the classical benzodiazepines, there has been much speculation

TABLE 1

Photolabeling-induced shifts in affinity for Ro15-1788, zopiclone, and flunitrazepam

Values are means ± S.E., where n = 6 for Ro15-1788 and n = 3 for zopiclone and diazepam. Values are K_d for Ro15-1788 and K_i for zopiclone and flunitrazepam.

Ligand	Control	Irradiated	Photolabeled	PAL/Irradiated Ratio
		nM		
Ro15-1788	2.03	0.97 ± 0.09	1.48 ± 0.22*	1.53
Zopiclone	29.5	29.6 ± 2.6	83.2 ± 6.7*	2.81
Flunitrazepam	6.8	4.61 ± 1.0	235 ± 39*	51.0

PAL, photolabeled.
* $P < .01$ compared to irradiated samples.

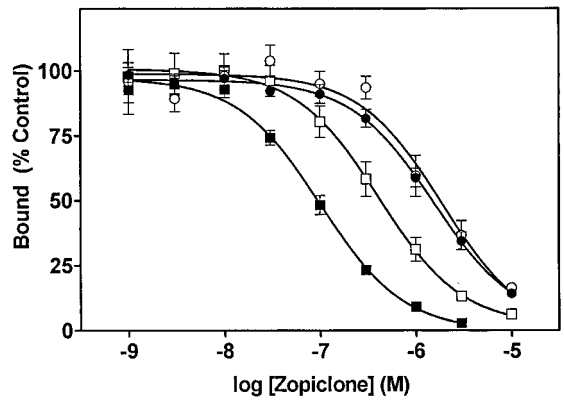

Fig. 1. Competition curves showing the displacement of [^{3}H]Ro15-4513 by increasing concentrations of zopiclone from membranes prepared from cells expressing wild-type (■), H101F (□), H101Q (●), and H101Y (○) α-subunits coexpressed with $\beta2$- and $\gamma2$-subunits. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for each receptor. Data shown represent the mean ± S.E. of at least three independent experiments performed in duplicate.

that the molecular mechanisms of action of the cyclopyrrolones and the benzodiazepines may be different.

Photoaffinity labeling of rat cerebellar membranes markedly compromised the affinity for flunitrazepam but had a much smaller effect on zopiclone binding (Table 1). These experiments were carried out with rat cerebellar membranes, but the results are consistent with those from similar studies in which rat cortex membrane preparations were used (Blanchard et al., 1983). The restricted expression of $GABA_A$ receptor subtypes in the rat cerebellum, compared with the cortex, suggest that the distinct consequences of photoaffinity labeling on the affinities of zopiclone and flunitrazepam are not due to the differential expression of $GABA_A$ receptor subtypes in these two brain regions.

Our previous studies showed that photoaffinity labeling with flunitrazepam results in covalent modification of histidine 101 (Duncalfe et al., 1996). We have, therefore, now compared the effects of point mutations of this residue on the binding characteristics of zopiclone and other benzodiazepine site ligands. The pattern that emerges from the binding studies strongly suggests that the residue at position 101 influences zopiclone binding in a similar manner to that of the classical benzodiazepines. As was previously observed with flunitrazepam and the β-carboline agonist ZK93423 (Davies et al., 1998), aromatic residues and glutamine are generally well tolerated substitutions for agonist recognition. The smallest of the amino acid substitutions, alanine, abolished recognition of both drugs, as did the glutamate and lysine substitutions. Although interpretation of these mutagenesis studies requires more detailed knowledge of receptor-ligand interactions, the data show that the recognition properties of the mutants are similar for agonist ligands with disparate structures. This suggests that all agonists interact with His-101 in a similar fashion and derive significant binding energy from this interaction.

McKernan et al. (1998) recently investigated the effects of photoaffinity labeling with flunitrazepam on the binding of various benzodiazepine site ligands. They proposed that ligands that display marked changes in affinity as a consequence of photoaffinity labeling derive binding energy through their interaction with histidine 101, specifically through the pendant 5-phenyl substituent (C-ring) of the classical benzodiazepines. In contrast, they suggested that ligands that do not interact with histidine 101 do not display reduced affinity for the photolabeled receptor. Previous modeling studies by Zhang et al. (1995) suggested that phenyl substituents in the R6 position of β-carboline agonists would occupy the same lipophilic pocket (L3) of the binding site as the C-ring of 1,4 benzodiazepine agonists (Fig. 5). The results of the study by McKernan et al. (1998) cast some doubt on the

TABLE 2

K_i values for zopiclone and diazepam binding at $GABA_A$ receptors containing wild-type and mutant α-subunits

Values are means ± S.E. for at least three experiments performed in duplicate.

α-Subunits	[^{3}H]Ro15-4513 (K_d)	Zopiclone (K_i)	Times Wild Type	Diazepam (K_i)	Times Wild Type
	nM			*nM*	
Wild type	6.2 ± 0.54[a]	51.3 ± 5.4		19.4 ± 0.3	
H101F	0.95 ± 0.07[a]	199 ± 28	4	164 ± 49	8
H101Y	7.01 ± 0.71[a]	863 ± 389	17	504 ± 51	25
H101Q	0.49 ± 0.07[a]	761 ± 216	15	1476 ± 782	74
H101E	2.13 ± 0.13[a]	>10,000		>10,000	
H101K	1.54 ± 0.11[a]	>10,000		>10,000	
H101A	8.3 ± 0.4	>10,000		>10,000	

[a] Values reported previously (Davies et al., 1998).

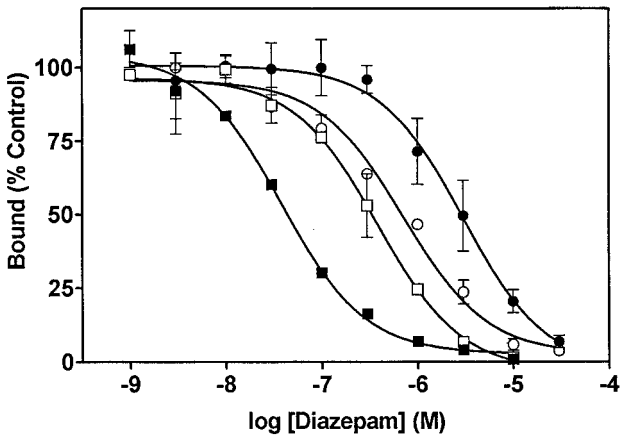

Fig. 2. Competition curves showing the displacement of [^{3}H]Ro15-4513 by increasing concentrations of diazepam from membranes prepared from cells expressing wild-type (■), H101F (□), H101Q (●), and H101Y (○) α-subunits coexpressed with β2- and γ2-subunits. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for each receptor. Data shown represent the mean ± S.E. of at least three independent experiments performed in duplicate.

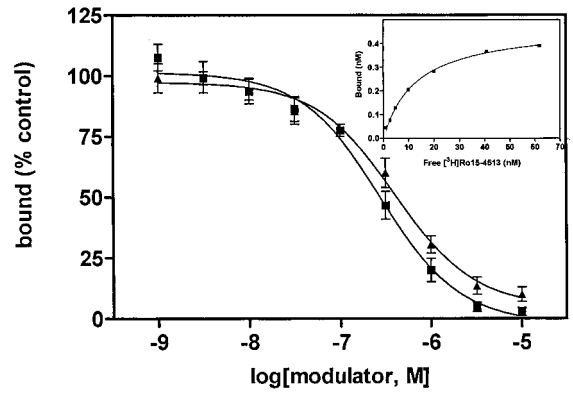

Fig. 3. The effect of the γ3-subunits on the affinities of benzodiazepine site ligands. Inset, representative data showing the saturation analysis of [^{3}H]Ro15-4513 using membranes containing α1β2γ3-receptors. The experiment was repeated three times in duplicate. The K_d value was 10.4 ± 1.4 nM. B, the displacement of [^{3}H]Ro15-4513 by zopiclone (▲) or flunitrazepam (■) from membranes containing α1β2γ3. The K_i values were 135 ± 16 and 202 ± 1.4 nM for zopiclone and flunitrazepam, respectively. Data shown represent the mean ± S.E. for at least three independent experiments performed in duplicate.

accuracy of this model; they proposed that, because photolabeling did not greatly reduce the affinity of abecarnil (a β-carboline with a phenyl substituent in the R6 position), this ligand does not interact with His-101. Additionally, they suggested that ligands that do not show a large shift in affinity for photolabeled receptors (e.g., imidazopyridazines, cyclopyrrolones, and β-carbolines) receive no binding energy from His-101 and do not occupy this part of the pocket. The present results and those from previous studies indicate that this may not be the case and tend to support the models proposed by Zhang et al. (1995), as well as those of Fryer et al. (1986) and Gardner (1992). Previous mutagenic studies have showed that the β-carboline agonist ZK93423 displays a binding profile similar to flunitrazepam with several His-101 mutants (Davies et al., 1998). The results presented here show that diazepam and zopiclone also share this profile, suggesting that agonists at this site interact with His-101 in a similar manner and derive binding energy from the interaction. However, zopiclone does not suffer a significant reduction in affinity as a consequence of receptor photolabeling, consistent with the notion that His-101 does not form part of the L3 lipophilic pocket. These seemingly disparate findings can be explained by the fact that His-101 is unlikely to be the only amino acid in the extracellular N terminus that is involved in forming the recognition domain for these compounds. Our previous studies demonstrated that photolabeling with flunitrazepam occurred at several sites within the $GABA_A$ receptor, only one of which was identified: His-101 (Duncalfe et al., 1996). Since the binding of these ligands to the receptor inevitably involves multiple points of interaction, it seems likely that the differential consequences of photoaffinity labeling on the affinities of flunitrazepam and zopiclone are a result of labeling at a site other than His-101. The identification of this site must await further experimental studies.

There is accumulating evidence to suggest that the benzodiazepine binding site is found at the interface between α- and γ-subunits (for review, see Sigel and Buhr, 1997). We have therefore also investigated the consequences of replacing the γ2-subunit with γ3 on the binding and function of zopiclone and the classical benzodiazepine flunitrazepam. In agreement with Reynolds and Maitra (1996), we found that zopiclone does potentiate GABA-gated current with recombinant receptors. Our data show that zopiclone is a full agonist at α1β2γ2- and α1β2γ3-receptors and is, in fact, more efficacious than flunitrazepam at both subtypes. Zopiclone produced a 32% greater potentiation than flunitrazepam at γ2-containing receptors, and at γ3-containing receptors, the

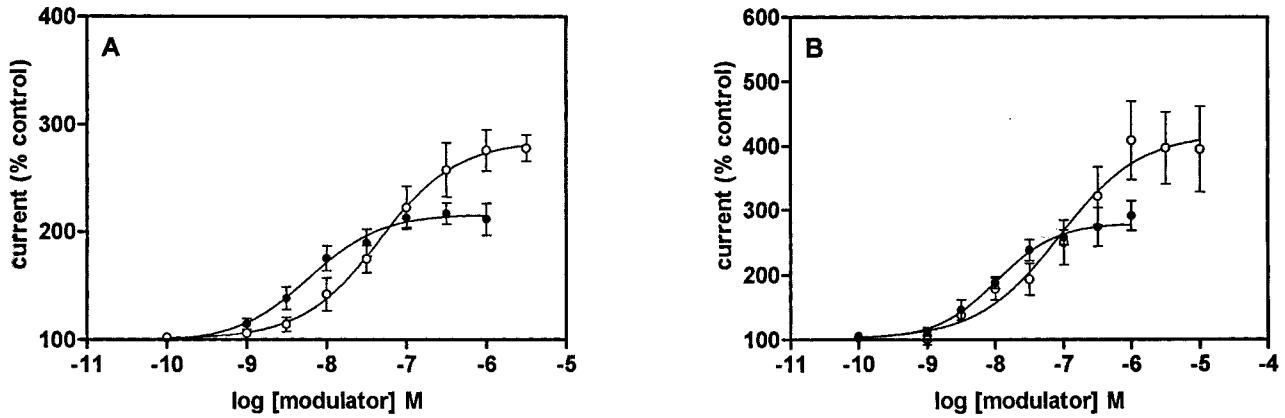

Fig. 4. Concentration-response curves illustrating the potentiation of GABA-mediated Cl^- conductance by flunitrazepam (●) and zopiclone (○) with recombinant α1β2γ2 (A) and α1β2γ3 (B) $GABA_A$ receptor expressed in *Xenopus* oocytes. Data represent the mean ± S.E. of three to four independent experiments performed in duplicate. The EC_{50} values for α1β2γ2-receptors were 5.9 ± 1.6 (●) and 42.4 ± 2.5 nM (○) for flunitrazepam and zopiclone, respectively. The potency of both modulators was shifted to the right in α1β2γ3-receptors, and the EC_{50} values were increased to 14.8 ± 2.9 (●) and 135 ± 40 nM (○), respectively. E_{max} and log (EC_{50}) values were analyzed by a two-way ANOVA, followed by a post hoc Bonferroni *t* test (Table 1) to determine the levels of significance.

TABLE 3

Concentration-response data for flunitrazepam and zopiclone on GABA-mediated current in recombinant α1β2γ2 or α1β2γ3 $GABA_A$ receptor expressed in *Xenopus* oocytes

Values (means ± S.E.) for the potency (EC_{50}), maximum potentiation (E_{max}), and Hill slope (n_H) were determined for each experiment (n) using GraphPad Prism Software. E_{max}, n_H, and log (EC_{50}) values were analyzed using a two-way ANOVA, followed by a post hoc Bonferroni test to determine the levels of significance. Flunitrazepam was more potent at α1β2γ2 and α1β2γ3[a] receptors than zopiclone. Potency decreased at γ3-containing receptors for both flunitrazepam and zopiclone.[b] Zopiclone produced a greater maximal effect at γ2- and γ3[c]-containing receptors. Both compounds were more efficacious at γ3-containing receptors than at γ2-containing receptors.[d]

Modulator	α1β2γ2			α1β2γ3		
	EC_{50}	E_{max}	n_H	EC_{50}	E_{max}	n_H
	nM	*% control*		*nM*	*% control*	
Flunitrazepam	5.9 ± 1.6 (3)	215 ± 4.0	1.00 ± 0.08	14.8 ± 2.9 (3)	280 ± 6.2	0.91 ± 0.26
Zopiclone	42.4 ± 2.5 (3)	284 ± 3.1	0.99 ± 0.06	135 ± 40 (4)	419 ± 25	0.72 ± 0.09

[a] $F = 58.68$ (1,9); $P < .0001$.
[b] $F = 15.04$ (1,9); $P < .005$.
[c] $F = 39.51$ (1,9); $P < .001$.
[d] $F = 36.53$ (1,9); $P < .001$.

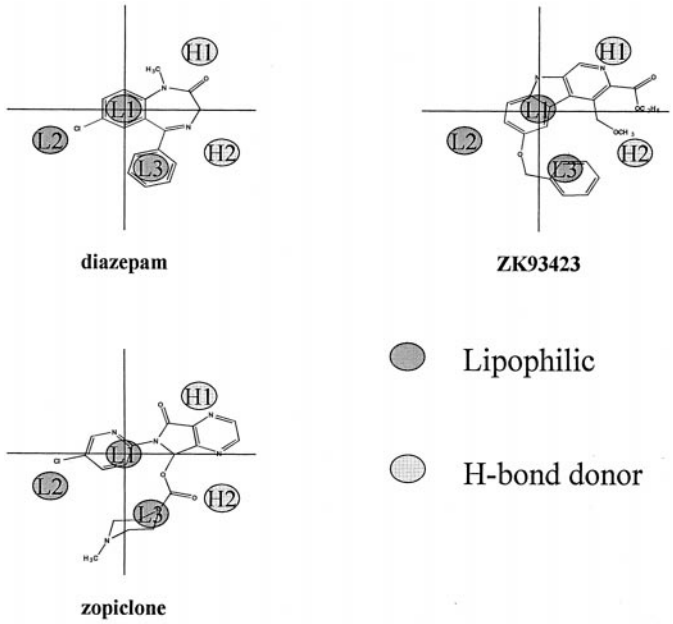

Fig. 5. Orientation of diazepam, ZK93423, and zopiclone in the benzodiazepine pharmacophore of Zhang et al. (1995). The positions of the lipophilic pockets L1, L2, and L3, together with the hydrogen-bond donor sites H1 and H2, have been used to orient both diazepam and ZK93423 as proposed in the agonist pharmacophore suggested by Zhang et al. (1995). The relative orientation of zopiclone is taken from that proposed by Borea et al. (1986).

potentiation by zopiclone was 50% greater than that of flunitrazepam. Indeed, zopiclone appears to be a "superagonist" at these receptors. Interestingly, although γ3-containing receptors showed a large reduction in affinity for flunitrazepam compared with γ2-containing receptors in radioligand-binding assays with tsA201 cells (approximately 30-fold), the same degree of shift was not reflected in the electrophysiological data with *Xenopus* oocytes (2.5-fold). The reason for this discrepancy is unclear; generally, the ligand affinity values obtained from radioligand-binding experiments tend to closely mirror the values obtained from electrophysiological experiments. However, we previously described a $GABA_A$ receptor mutant that displayed a binding constant that was 10-fold greater than the EC_{50} value obtained in functional studies (Davies et al., 1998; Dunn et al., 1999). The reason(s) underlying these instances in which binding and functional data seem not to match is currently unknown, although differences in expression systems may play some role.

There has been considerable discussion about the molecular mechanism of zopiclone interaction with $GABA_A$ receptors; indeed, here we show that the photolabeling of the receptor differentially affects zopiclone and flunitrazepam recognition. In the present study we demonstrated that zopiclone, like the classical benzodiazepines, interacts with the α1-subunit His-101 residue of the benzodiazepine binding-site domain and further that the functional effects of these ligands are comparable. Thus, the differences between zopiclone and the classical benzodiazepines that have been reported previously must be due to distinct interactions of these ligands with recognition site domains other than that represented by histidine 101.

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