Benzodiazepine binding to GABA_A receptors: differential effects of sulfhydryl modification

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The effects of sulfhydryl modification on benzodiazepine binding to γ-aminobutyric acid type A (GABA_A) receptors have been studied in membrane preparations from bovine cerebral cortex. After reduction of membranes with increasing concentrations of dithiothreitol, the binding of the partial inverse agonist [3H]Ro15-4513 (2.5 nM) was inhibited with an IC_50 of 4.6 mM, while the binding of the classical agonist [3H]flunitrazepam was affected only at much higher concentrations (> 30 mM). Prior desensitization of the GABA_A receptor by incubation with 10 μM muscimol had no effect on dithiothreitol inhibition of [3H]Ro15-4513 binding. In equilibrium assays, 10 mM dithiothreitol increased the K_D for [3H]Ro15-4513 binding by more than three-fold and reduced the density of binding sites by more than one-third. The binding sites for [3H]Ro15-4513 were protected from the effects of dithiothreitol by carrying out the reduction step in the presence of 10 μM Ro15-4513 or 10 μM flunitrazepam. Alkylation of brain membranes by N-ethylmaleimide inhibited the binding of both [3H]Ro15-4513 and [3H]flunitrazepam with a similar concentration dependence. Equilibrium binding assays in the presence of 10 mM N-ethylmaleimide showed that both the affinity and number of binding sites for [3H]Ro15-4513 were reduced, whereas only the K_D for [3H]flunitrazepam binding was affected. These results demonstrate that sulfhydryl modification of GABA_A receptors has different effects on the binding of a classical benzodiazepine agonist and a partial inverse agonist, suggesting differences in the modes of binding of the two ligands.

GABA_A receptor; Benzodiazepine; Ro15-4513; Flunitrazepam

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. GABA_A receptors are GABA-gated chloride channels which have been extensively studied because of both the importance of the transmitter in the central nervous system and the rich pharmacology of the receptor. In addition to binding sites for GABA and its analogues, the receptor complex carries distinct sites for many important pharmacological agents such as benzodiazepines, barbiturates, picrotoxin, steroid anesthetics, and naturally-occurring steroids (Squires, 1988; Olsen et al., 1990). Of particular interest are the benzodiazepines, since their anxiolytic, anti-convulsant and sedative properties have led to their widespread use in clinical practice (Haefely, 1990). In the presence of GABA, the clinically important benzodiazepines potentiate receptor-mediated chloride currents and thus modulate inhibitory tone throughout the central nervous system. However, despite their importance, the molecular mechanisms by which the benzodiazepines elicit their actions on the GABA_A receptor and the structural determinants with which they interact remain to be elucidated.

There exist subsets of benzodiazepine ligands which interact within a similar binding domain to modulate the GABA_A receptor-chloride channel complex; agonists, inverse agonists, and antagonists. The classical agonists potentiate, while the inverse agonists reduce, the response elicited by GABA (Squires, 1988). An imidazobenzodiazepine, Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]benzodiazepine-3-carboxylate) is a partial inverse agonist which binds not only to sites that are sensitive to classical agonists such as diazepam, but also to diazepam-insensitive sites in the cerebellum (Sieghart et al., 1987; Malminiemi and Korpi, 1989) and, to a lesser extent (< 2.5% of sites), in the cerebral cortex (Turner et al., 1991). These diazepam insensitive sites appear to correlate with the presence of an α_6 subunit in the GABA_A receptor complex and a high-affinity Ro15-4513, diazepam-insensitive binding protein has been
expressed in cells transfected with cDNAs encoding \( \alpha_6, \beta_2 \) and \( \gamma_2 \) subunits (Lüdden et al., 1990). Recent reports have shown that specific amino acids are involved in the differential binding of the classical benzodiazepine agonists, such as diazepam and flunitrazepam, and the partial inverse agonist Ro15-4513. A crucial histidine residue has been identified which is required for the binding of agonist and antagonist ligands to benzodiazepine receptors sites, but not for Ro15-4513 (Binkley and Ticku, 1991). In studies of domain-exchange and site-directed mutagenesis of recombinant receptors, it has also been demonstrated that a particular histidine residue (His-101 in \( \alpha_1 \)) is a major determinant of benzodiazepine agonist and antagonist binding (Wieland et al., 1992).

In this study, sulphhydryl modification of the GABA\(_A\) receptor(s) in membrane preparations from bovine cerebral cortex has been examined for its effect on the binding of the classical agonist flunitrazepam and the partial inverse agonist Ro15-4513. It is shown that Ro15-4513 binding, much more so than flunitrazepam binding, is sensitive to disulfide reduction but that the binding is protected by carrying out the reduction in the presence of either benzodiazepine. The binding of the two benzodiazepines can also be distinguished by their different sensitivities to the reaction of GABA\(_A\) receptors with N-ethylmaleimide.

2. Materials and Methods

2.1. Preparation of membranes from bovine cerebral cortex

Bovine brains were obtained from a local slaughterhouse. The brains were immediately frozen on solid CO\(_2\) and stored at \(-86^\circ\)C. Membranes were prepared from cerebral cortex as described elsewhere (Dunn et al., 1989) and stored at \(-86^\circ\)C. Prior to use, a membrane aliquot was thawed, diluted in the appropriate buffer, and recovered by centrifugation. Protein concentrations in the final samples were measured by the method of Bradford, 1976. Unless otherwise stated, the buffer used in all experiments was 100 mM Tris-HCl, pH 7.4, 0.02% NaN\(_3\).

2.2. Binding of radiolabelled ligands to membrane preparations

Equilibrium binding of \([\text{H}]\)flunitrazepam and \([\text{H}]\)Ro15-4513 (Du Pont Canada, 74.1 and 26.0 Ci/mmol, respectively) to membrane preparations was measured in filtration assays using a Hoefer filtration apparatus. In competition experiments, 350 \(\mu\)l aliquots of membranes were added to 350 \(\mu\)l buffer containing either \([\text{H}]\)Ro15-4513 or \([\text{H}]\)flunitrazepam (2.5 nM) and the indicated concentrations of unlabelled flunitrazepam (Sigma Chemicals) or Ro15-4513 (Hoffmann-La Roche) to give a final protein concentration of 1 mg/ml. Non-specific binding was measured in the presence of 1.4 \(\mu\)M unlabelled ligand. After incubation in the dark for 45 min at 4°C, 0.5 ml of each sample was filtered under vacuum through Whatman GF/C filters, and the filters were immediately washed with two 5-ml volumes of ice-cold buffer. The filters were dried, 5 ml of ACS scintillation fluid (Amersham Canada) added, and counted for \([\text{H}]\). 50-\(\mu\)l aliquots of the incubation mixture were also removed and counted for \([\text{H}]\) in order to provide estimates of total ligand added.

In \([\text{H}]\)Ro15-4513 and \([\text{H}]\)flunitrazepam binding assays on membranes pretreated with sulphhydryl-modifying agents, the membrane aliquots were added to various concentrations of dithiothreitol or N-ethylmaleimide (Sigma Chemicals). Following preincubation in the dark for 30 min at room temperature, 2.5 nM radiolabelled ligand was added. The mixture was further incubated in the dark for 45 min at 4°C prior to filtration. \([\text{H}]\)Ro15-4513 and \([\text{H}]\)flunitrazepam binding assays with dithiothreitol pre-treated membranes under conditions of desensitization were performed in a similar manner, except that 10 \(\mu\)M muscimol (Sigma Chemicals) was included with the membranes and dithiothreitol in the pre-incubation mixture. To assess a potential direct interaction between the sulphhydryl-modifying agents and the \([\text{H}]\)benzodiazepine ligands, thin-layer chromatography of \([\text{H}]\)Ro15-4513 and \([\text{H}]\)flunitrazepam in the absence and presence of dithiothreitol and N-ethylmaleimide was performed on Silica gel using a methylene chloride/methanol (9:1) solvent system. The mobility of the ligands was unaffected by incubation with either agent.

Equilibrium binding assays of \([\text{H}]\)Ro15-4513 and \([\text{H}]\)flunitrazepam to membrane preparations for determination of dissociation constants (\(K_d\)) and density of binding sites (\(B_{max}\)) were also measured by using a filtration assay. Membrane aliquots (350 \(\mu\)l) were added to different concentrations of radiolabelled ligand to give a final volume of 700 \(\mu\)l and a protein concentration of 1 mg/ml. Non-specific binding was measured in the presence of 1.4 \(\mu\)M unlabelled ligand. Following incubation in the dark for 45 min at 4°C, the samples were filtered and assayed as described above. When investigating the effects of dithiothreitol and N-ethylmaleimide on binding parameters for \([\text{H}]\)Ro15-4513 and \([\text{H}]\)flunitrazepam binding, the sulphhydryl-modifying agent was included at a concentration of 10 mM in the incubation mixture. The mixture was incubated in the dark for 30 min at room temperature and 45 min at 4°C, after which bound and free ligand was separated by filtration. Parallel control samples were also prepared which did not contain dithiothreitol or N-ethylmaleimide.
2.3. Protection of membranes from dithiothreitol reduction by preincubation with ligands

Four 25-ml membrane aliquots (protein concentration 1 mg/ml) were incubated in the dark for 45 min at 4°C with either (a) 10 μM unlabelled Ro15-4513, (b) 10 μM unlabelled flunitrazepam, or (c) and (d) an equal volume of buffer. Dithiothreitol was added to a final concentration of 10 mM to samples (a), (b) and (c). To sample (d), an equal volume of buffer was added. The mixtures were incubated for 30 min at room temperature, then centrifuged at 40,000 rpm in a Beckman type 70 Ti rotor for 45 min. The membranes were resuspended with a Potter S homogenizer in 25 ml of 100 mM Tris-HCl, pH 7.4, 0.02% NaN₃ containing 0.1 mM dithiothreitol. This concentration of dithiothreitol was included to maintain reducing conditions, but was present at a sufficiently low concentration so as not to interfere with subsequent binding assays. Following an incubation in the dark for 30 min at 4°C, the samples were repelleted. The membranes were subjected to this cycle of resuspension and repelleting two more times to ensure the complete removal of unlabelled ligand and excess dithiothreitol. The membranes were finally resuspended at a protein concentration of 2 mg/ml. Equilibrium measurements of [³H]Ro15-4513 binding to the membrane preparations were performed by filtration assay as described above.

2.4. Statistical analysis

The data obtained from equilibrium binding of [³H]Ro15-4513 and [³H]flunitrazepam to membrane preparations for determination of Kᵃ and Bₘₐₓ are expressed in the Results section as the mean ± S.E.M. For binding data obtained in the presence of a sulfhydryl-modifying agent, significance levels (P) were determined with the Student's t-test by comparison to the corresponding control. The results obtained from [³H]Ro15-4513 binding to membrane samples in the protection from reduction experiments were analysed by a one-way analysis of variance and Duncan's new multiple range test at 95% confidence limits (Dowdey and Weardon, 1983).

3. Results

3.1. Specificity of [³H]benzodiazepine binding to bovine brain cortical membranes

It has previously been reported that, in some brain preparations, there are diazepam-insensitive sites that bind [³H]Ro15-4513 with high affinity (see above). The specificity of the high-affinity benzodiazepine binding sites in bovine brain cortical membrane preparations has been demonstrated by the ability of unlabelled flunitrazepam and Ro15-4513 to displace bound radio-labeled ligand. Examples of such competition curves are shown in fig. 1 and illustrate that the membranes used in this study do not contain a significant population of diazepam-insensitive binding sites.

3.2. Effects of dithiothreitol on the binding of [³H]Ro15-4513 and [³H]flunitrazepam

The equilibrium binding of [³H]Ro15-4513 and [³H]flunitrazepam to bovine brain membranes is differentially affected by dithiothreitol reduction. As shown in fig. 2A, binding of the partial inverse agonist [³H]Ro15-4513 was significantly decreased by 300 μM dithiothreitol and almost completely abolished at a concentration of 100 mM, with an IC₅₀ value of 4.6 mM. However, high concentrations of dithiothreitol (> 30 mM) only moderately inhibited the binding of the classical agonist, [³H]flunitrazepam. These results indicate that there exists a structural requirement of an intact disulfide bridge(s) necessary for the high-affinity binding of Ro15-4513 but not for that of flunitrazepam. The differential effect of dithiothreitol on benzodiazepine binding was unaffected by receptor desensitization, as illustrated in fig. 2B. GABA_A receptors were desensitized by incubation of the membranes with 10 μM muscimol. In control experiments, we have shown that dithiothreitol, at concentrations up to 100 mM,
has no significant effect on [3H]muscimol binding (data not shown).

The effects of dithiothreitol (10 mM) on the parameters for [3H]benzodiazepine binding have been investigated in more detail, as shown in the Scatchard plots in fig. 3. In the case of [3H]Ro15-4513, the presence of dithiothreitol caused an increase in the K_D by more than three-fold (from 12 ± 2 nM to 40 ± 7 nM, P < 0.01, n = 4) and a reduction in binding site density from 5.8 ± 0.4 to 3.7 ± 0.4 pmol/mg (P < 0.01). Dithiothreitol (10 mM) had no significant effect on either the K_D for [3H]flunitrazepam binding (6.2 ± 0.8 nM) or the concentration of its binding sites (2.6 ± 0.3 pmol/mg) when compared to control values (K_D 5.4 ± 0.5 nM; B_max 3.0 ± 0.1 pmol/mg).

3.3. Ligand protection of membranes from the effects of dithiothreitol reduction

The involvement of an intact disulfide bridge in Ro15-4513 binding was also demonstrated by the ability of unlabelled Ro15-4513 to protect against the inhibitory effect of dithiothreitol when this ligand was included during the reduction step (fig. 4). After reduc-
Fig. 4. [3H]Ro15-4513 binding to bovine brain membranes: protection from the effects of dithiothreitol by the presence of unlabelled Ro15-4513 during reduction. Equilibrium binding data of [3H]Ro15-4513 to control membranes (■), to membranes reduced with 10 mM dithiothreitol (△), and to membranes pre-incubated with 10 μM Ro15-4513 prior to reduction with 10 mM dithiothreitol (○) are presented as Scatchard plots. Results shown are representative of data from three different experiments. Best fit values for K_D of (■), (△), and (○) are 14, 21, and 16 nM and for B_max are 4.9, 3.8, and 5.2 pmol/mg (respectively).

Fig. 5. Effects of N-ethylmaleimide (N-EM) on the binding of [3H]Ro15-4513 and [3H]flunitrazepam to bovine brain membranes. (A) Experimental procedures were as in the legend to fig. 2A except that membranes were added to different concentrations of N-ethylmaleimide. Results are expressed as the averages ± S.E.M. from 3 distinct experiments. IC_{50} values were estimated to be 13.0 mM for [3H]Ro15-4513 (○) and 20.0 mM for [3H]flunitrazepam (□). (B) Scatchard plots of [3H]Ro15-4513 binding to membranes in the absence (■) or presence (○) of 10 mM N-ethylmaleimide. Linear least squares regression gave values for K_D and B_max are 12.0 nM, 5.1 pmol/mg (■) and 17.8 nM, 2.9 pmol/mg (○), respectively. (C) Scatchard plots of [3H]flunitrazepam binding to membranes in the absence (■) or presence (○) of 10 mM N-ethylmaleimide. Best fit values for K_D and B_max are 4.0 nM, 2.8 pmol/mg (■) and 12.7 nM, 2.8 pmol/mg (○), respectively. Results shown are representative data from three experiments.
ethylenmaleimide, also has different effects on the binding of the two benzodiazepines.

4. Discussion

Investigations in recent years have identified some important structural components required for benzodiazepine modulation of GABA<sub>A</sub>-mediated responses. Although the exact subunit composition of a native GABA<sub>A</sub> remains unknown, it has been shown in expression studies that the minimal subunit requirement for recombinant receptors to exhibit the full spectrum of benzodiazepine sensitivities is an α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> combination (Pritchett et al., 1989). For GABA<sub>A</sub> receptors expressed in cells by transfection with cDNAs, point mutation of an α<sub>4</sub> amino acid (glutamate 225) to an α<sub>1</sub> glycine 225 results in a functional switch from type II to type I benzodiazepine pharmacology (Pritchett and Seeburg, 1991). It has also been shown that mutation of histidine 100 in α<sub>1</sub> to arginine 101 in α<sub>6</sub> produces a diazepam-insensitive receptor (Wieland et al., 1992). In the present study, we have investigated the importance of cysteine/cystine residues in benzodiazepine binding and demonstrate that sulphydryl modification has different effects on the binding of a classical benzodiazepine agonist and a partial inverse agonist.

The results of binding experiments to bovine brain membranes reduced with dithiothreitol demonstrate that the binding of [3H]Ro15-4513 requires an intact disulfide bridge(s) for high-affinity binding, whereas reduction of this bond(s) does not affect [3H] flunitrazepam binding. These contrasting effects of dithiothreitol cannot be explained by interaction of the benzodiazepine ligands with different GABA<sub>A</sub> receptor subtypes, since these membranes did not possess any significant population of diazepam-insensitive binding sites and flunitrazepam appeared to competitively inhibit all of the [3H]Ro15-4513 binding, as did Ro15-4513 inhibit all of the [3H]flunitrazepam binding. Although the binding of the two ligands appears to be mutually competitive, it is evident that the density of binding sites for [3H]Ro15-4513 differs from that of [3H]flunitrazepam binding. Previous studies using rat brain membranes prepared from cerebral cortex have also reported discrepancies in binding site density for these benzodiazepine ligands (Binkley and Ticku, 1991) and comparable results for the number of [3H]Ro15-4513 binding sites in bovine cortical membranes (approximately 5 pmol/mg protein) have been found by Turner et al. (1991). However, the present results demonstrating differential effects of dithiothreitol reduction cannot be explained by the discrepancy in number of sites. Not only do the sites for Ro15-4513 and flunitrazepam appear to be mutually exclusive but the results shown in fig. 2 show no evidence for heterogeneity in the effects of dithiothreitol on [3H]Ro15-4513 binding as would be expected if dithiothreitol was affecting only the 'additional' sites for this ligand. The Scatchard plots in fig. 3 also suggest that the binding of [3H]Ro15-4513 is to a homogeneous population of sites both in the presence and absence of dithiothreitol.

The effects of dithiothreitol were unaffected by desensitization of the GABA<sub>A</sub> receptor(s) induced by prolonged exposure to the agonist, muscimol (Krjevic, 1981). Thus, the intact disulfide bond(s) that is crucial for Ro15-4513 binding is sensitive to reduction in either the resting or desensitized conformation of the receptor protein. Investigation of the mechanism by which dithiothreitol inhibits [3H]Ro15-4513 binding has revealed that both the affinity and number of binding sites are significantly reduced after disulfide reduction. In contrast, the binding of [3H]flunitrazepam was affected only by higher concentrations of dithiothreitol (> 30 mM). This relative insensitivity to reduction is in agreement with an earlier report (Korneyev et al., 1985) in which 50 mM dithiothreitol caused only a slight change in the K<sub>D</sub> (<2-fold) for [3H]flunitrazepam without affecting the B<sub>max</sub> in several areas from bovine brain.

The ability of unlabelled benzodiazepines to protect the disulfide(s) that are important for Ro15-4513 binding provides further evidence for the presence of a disulfide bridge that is either in close proximity to the benzodiazepine binding domain or is involved in stabilizing the conformation of the binding site. Occupancy of benzodiazepine binding sites by Ro15-4513 during the reduction, negated the inhibitory effects of dithiothreitol on [3H]Ro15-4513 binding. This protective effect may be interpreted either by steric occlusion of a nearby disulfide(s) by the presence of the ligand, or by a mechanism whereby occupancy of the benzodiazepine binding sites induces a receptor conformation that reduces the reactivity of the disulfide(s). In photoaffinity labelling studies it has been suggested, though not proven, that the [3H]flunitrazepam binding site occurs within the segment between residues 106 and 149 of the GABA<sub>A</sub> receptor α subunit (Stephenson and Duggan, 1989; Stephenson et al., 1990; Olsen et al., 1990), a segment that is predicted to lie in the large N-terminal extracellular domain. All known subunits of the ligand-gated ion channel family, contains a highly conserved pair of cysteine residues which can form a β-structural loop (Schofield et al., 1987), that has been predicted to play a role in stabilizing the ternary structure of the receptor. While this disulfide has been implicated in α-bungarotoxin binding to the nicotinic acetylcholine receptor (Sumikawa and Gehle, 1992), it is not possible to conclude that it is the reduction of this disulfide bond which results in the loss of high-affinity binding for the partial inverse agonist. The presence of flunitrazepam during reduction also provided
some protection of the disulfide involved, suggesting commonalities in the binding sites for the two benzodiazepine ligands or in the receptor conformations that they induce.

The binding of $[^{3}H]Ro15-4513$ and $[^{3}H]flunitrazepam$ also displayed differences in their sensitivity to N-ethylmaleimide. It was previously reported (Zundel et al., 1985) that 1 mM N-ethylmaleimide slightly potentiated (by about 9%) the binding of $[^{3}H]flunitrazepam$ to rat hippocampal membranes, but higher concentrations were not investigated. In the present study, N-ethylmaleimide, at concentrations up to 1 mM had little effect on either $[^{3}H]flunitrazepam$ or $[^{3}H]Ro15-4513$ binding but the binding of both benzodiazepines was inhibited at higher concentrations (fig. 5). More detailed analysis revealed that both the affinity and number of binding sites for $[^{3}H]Ro15-4513$ were reduced, whereas only the affinity for $[^{3}H]flunitrazepam$ was affected. Thus both a reducing agent and an alkylating agent have similar effects on the binding of $[^{3}H]Ro15-4513$, suggesting that both a disulfide bond and a free sulfhydryl group may be important for stabilising the conformation of the binding site, as has been shown, for example, for the binding of agonists to the nicotinic acetylcholine receptor (Moore and Rafterty, 1979).

In conclusion, it has been demonstrated that sulfhydryl groups are involved in the benzodiazepine interaction with the GABA$_A$ receptor and that an intact disulfide bond is required for the binding of Ro15-4513, but not for flunitrazepam binding. The GABA$_A$ receptor is a complex multi-subunit protein, and this complexity is compounded by the presence of multiple variants of each subunit class (Burt and Kamatchi, 1991). It may be predicted, therefore, that the structural requirements underlying different benzodiazepine modulatory mechanisms will be equally complex. Further investigations which lead to a better understanding of these structural determinants will give further information about receptor function and this may provide a basis for rational drug design.

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References


Korneyev, A.Ya., O.B. Belongoff and V.N. Zukan, 1985, Differences in the properties of bovine brain benzodiazepine receptors in the cerebellum and hippocampus revealed after reduction of disulfide bonds, Neurosci. Lett. 61, 279.


Moore, H-P.H. and M.A. Raftery, 1979, Ligand-induced interconversion of affinity states in membrane-bound acetylcholine receptor from Torpedo californica. Effects of sulfhydryl and disulfide reagents, Biochemistry 18, 1907.


Stephenson, F.A., M.J. Duggan and S. Pollard, 1990, The $\gamma_2$ subunit is an integral component of the $\gamma$-aminobutyric acid A receptor but the $\alpha$1 polypeptide is the principle site of the agonist benzodiazepine photoaffinity labelling reaction, J. Biol. Chem. 265, 21160.

Sumikawa, K. and V.M. Gehle, 1992, Assembly of mutant subunits of the nicotinic acetylcholine receptor lacking the conserved disulfide loop structure, J. Biol. Chem. 267, 6286.
