On the Benzodiazepine Binding Pocket in GABA \textsubscript{A} Receptors*

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Benzodiazepines are used for their sedative/hypnotic, anxiolytic, muscle relaxant, and anticonvulsive effects. They exert their actions through a specific high affinity binding site on the major inhibitory neurotransmitter receptor, the \( \gamma \)-aminobutyric acid, type A (GABA\textsubscript{A}) receptor channel, where they act as positive allosteric modulators. To start to elucidate the relative positioning of benzodiazepine binding site ligands in their binding pocket, GABA\textsubscript{A} receptor residues thought to reside in the site were individually mutated to cysteine and combined with benzodiazepine analogs carrying substituents reactive to cysteine. Direct apposition of such reactive partners is expected to lead to an irreversible site-directed reaction. We describe here the covalent interaction of \( \alpha_1 \)H101C with a reactive group attached to the C-7 position of diazepam. This interaction was studied at the level of radioactive ligand binding and at the functional level using electrophysiological methods. Covalent reaction occurs concomitantly with occupancy of the binding pocket. It stabilizes the receptor in its allosterically stimulated conformation. Covalent modification is not observed in wild type receptors or when using mutated \( \alpha_1 \)H101C-containing receptors in combination with the reactive ligand pre-reacted with a sulfhydril group, and the modification rate is reduced by the binding site ligand Ro15-1788. We present in addition evidence that \( \gamma_2 \)Ala-79 is probably located in the access pathway of the ligand to its binding pocket.

The GABA\textsubscript{A} receptors are the major inhibitory neurotransmitter receptors in the mammalian brain. They are heteromeric protein complexes consisting of five subunits, which are structurally related compounds. Derived models for the binding site for the channel agonist GABA or are located pseudo-symmetric structures (29, 43). The abovementioned residues lining either the benzodiazepine binding site or the GABA binding site are all homologous to residues suggested to form the binding site of acetylcholine on the nicotinic acetylcholine receptor (43). The recently crystallized acetylcholine-binding protein, which shows a weak homology to the extracellular part of the GABA\textsubscript{A} receptor (44), allowed a first structural insight into the ligand binding domain through homology modeling (45).

Many studies (e.g., Refs. 46–49) have been undertaken with the aim to characterize spatial properties of the benzodiazepine binding pocket. These studies used either in vivo effects or chloride flux experiments in combination with radioligand binding studies on brain membranes of a large number of structurally related compounds. Derived models for the binding pocket are complex and suggest distinct but partially overlapping binding sites for ligands differing in their allosteric effect, but a consensus view failed to emerge. A drawback of brain studies is the heterogeneity of GABA\textsubscript{A} receptors.

It is obviously important to map all the amino acid residues participating in the formation of the benzodiazepine pocket relative to the ligands of this site in a recombinant receptor. Initial approaches have indicated that the pending phenyl residue of classic benzodiazepines may be located close to \( \gamma_2 \)Phe-77 (50) and \( \alpha_1 \)His-101 (51).

In pioneering work Karlin and Akabas (for review see Ref. 52) introduced site specific mutation to cysteine in combination
with nonspecific cysteine-reactive agents for the study of proteins. Recently, a novel technique to elucidate relative position of a ligand in its binding pocket has been successfully applied in several cases (53–59). The technique has been described in detail (60). In this approach, receptors in which residues thought to reside in the binding pocket are individually mutated to cysteine and then combined with binding site ligands carrying substituents reactive to cysteine. Direct apposition of such reactive substituents with a cysteine residue is expected to lead to a covalent reaction. Given a series of controls, such engineered site-directed reactions provide reliable information on the orientation of a ligand within its binding site.

We applied here this novel approach to the benzodiazepine binding site. We show that this strategy works in the present case and describe the covalent interaction of \( \alpha_{1} \)H1101C with a reactive group attached to the C-atom in diazepam normally subcloned into the SmaI site of the polylinker by standard techniques.

**EXPERIMENTAL PROCEDURES**

**Synthesis of the Reactive Substance**

7-Isothiocyanato-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one— Thiophosgene (63 \( \mu \)l, 0.828 mmol, 2 eq.) was slowly added to a stirred solution of 7-amino-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (110 mg, 0.414 mmol) and sodium hydroxycarbonate (70 mg, 0.828 mmol, 2 eq.) in 10 ml of an aqueous solution of tetrahydrofuran (50%). The resulting mixture was stirred 15 min at room temperature. 40 ml of 5% aqueous solution of NaHCO\(_{3}\) was added to the reaction mixture, and after evaporation of the tetrahydrofuran, the aqueous layer was extracted with CH\(_2\)Cl\(_2\). The organic layer was washed with 5% aqueous solution of NaHCO\(_{3}\), dried over Na\(_{2}\)SO\(_{4}\), and evaporated under reduced pressure. The residue was purified by chromatography on silica gel eluted by heptane/ethyl acetate (1/1). NMR (300 MHz-CDCl\(_{3}\)): \( 3.41 \) (s, 3H), 3.75 (d, \( \delta = 7.60 \) Hz, 1H), 7.14 (d, \( \delta = 2.4 \) Hz, 1H), 7.26–7.60 (m, 7H). IR (KBr): \( 2104 \text{ cm}^{-1} \) (s) (NCS).

7-Nitro-5-phenyl-3-chloro-1,3-dihydro-2H-1,4-benzodiazepin-2-one—N-Chlorosuccinimide (2.5 g, 187 mmol, 55 eq.) in portions of 500 mg was added over 2 days to a solution of nitrazepam (1 g, 3.39 mmol) in CCl\(_4\). A catalytic amount of azoisobutyronitrile was added, and the solution was heated to reflux (2 days). The solvent was removed under reduced pressure. The resulting residue was purified by chromatography on silica gel eluted by heptane/AcOEt 1/1. NMR (100 MHz-CDCl\(_{3}\)): \( 5.83 \) (s, 1H), 6.70 (s, 1H), 7.35–8.51 (m, 8H).

**Construction of Receptor Subunits**

The cDNAs coding for the \( \alpha_{1}, \beta_{2}, \) and \( \gamma_{2} \) subunits of the rat GABA\(_{A}\) receptor channel have been described elsewhere (61–63). The mutant subunits \( \alpha_{1} \)H1101C, \( \gamma_{2} \)T73C, \( \gamma_{2} \)D75C, \( \gamma_{2} \)A79C, and \( \gamma_{2} \)T81C were prepared using the QuikChangeTM mutagenesis kit (Stratagene). For cell transfection, the cDNAs were subcloned into the polylinker of pBC/H9251 vector and used at most for 2 months. Cl\(_{2}\)O\(_{2}\) solution (10 mM HEPES, pH 7.4) was used at most for 2 months. Cl\(_{2}\)O\(_{2}\) was added in modified Barth solution (10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\(_{3}\) 0.82 mM MgSO\(_{4}\) 0.34 mM Ca(NO\(_{3}\))\(_{2}\) 0.41 mM CaCl\(_{2}\) 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin by standard cell culture techniques.

**Binding Assays**

Membranes were resuspended in the buffer mentioned above using a tip sonifier. Resuspended cell membranes were incubated in a total volume of 0.1–0.4 ml on ice in the presence of [\(^{3}H\)Ro15-1788 (78.6 Ci/mmol, PerkinElmer Life Sciences) or [\(^{3}H\)flunitrazepam (71–84 Ci/mmol, PerkinElmer Life Sciences) and various concentrations of competing ligands. In the case of displacement studies using NCS compound this compound was present for 20–30 min. Membranes (5–80 \( \mu \)g of protein/filter) were collected by rapid filtration on GF/C filters presoaked in 0.3% polyethyleneimine. After three washing steps with 5 ml of buffer, the filter-retained radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 100 \( \mu \)M Ro15-1788 or 100 \( \mu \)M flunitrazepam, respectively. Data were fitted by using a nonlinear least-squares method to the equations, 

\[
B(c) = B_{\text{max}}/1 + (K_{c}c)^{n}
\]

for binding curves, and

\[
B(c) = B_{\text{max}}/1 + (c/c_{\text{IC50}})^{n}
\]

for displacement curves with a single component, where \( c \) is the concentration of ligand, \( B \) is binding, \( B_{\text{max}} \) is maximal binding, \( K_{c} \) is the dissociation constant, and \( n \) is the Hill coefficient. \( IC_{50} \) values were converted to \( K_{c} \) values according to the Cheng-Prusoff equation (66). Protein concentration was determined with the BCA protein assay kit (Pierce) with bovine serum albumin as standard.

**Wash-out Procedure for Reactive Substances**

NCS compound was dissolved in dioxane at a concentration of 20 mM. A stock solution was kept at \(-20^\circ\)C and used at most for 2 months. CI compound was freshly dissolved in Me\(_{2}\)SO at a concentration of 5 mM. Final dilutions were prepared immediately before the experiment. We estimated that 

\[
\text{Approximately 60 h after transfection the cells were harvested by washing with ice-cold phosphate-buffered saline, pH 7.4, and centrifuged at 560 \times g. The buffer was removed 10 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, pH 7.4. Cells were homogenized by sonication in the presence of 10 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. Membranes were collected by three centrifugation-resuspension cycles (100,000 \times g for 20 min) and then used for ligand binding at \(-20^\circ\)C.}

**Expression in Xenopus Oocytes**

Capped cRNAs were synthesized (Ambion, Austin, TX) from the linearized pCMV vectors containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA and knowledge of concentrations of RNA in the final standard and on the same gel. cRNA combinations were precipitated in ethanol/sodium-acetate/coloh 1:1 and stored at \(-20^\circ\)C. For injection, the alcohol was removed and the cRNAs were dissolved in water. Oocytes were injected with 50 nl of the cRNA solution. The combination of wild type or mutated \( \alpha_{1}, \beta_{2}, \) and \( \gamma_{2} \) subunits was expressed at 10 min:10 min:50 min (67). The injected oocytes were injected in modified Barth’s solution (10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\(_{3}\) 0.82 mM MgSO\(_{4}\) 0.34 mM Ca(NO\(_{3}\))\(_{2}\) 0.41 mM CaCl\(_{2}\) 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin) in \(-20^\circ\)C for at least 24 h before the measurements. Xenopus laevis oocytes were prepared, injected, and depolarized as described previously (14, 68).

**Two-electrode Voltage Clamp**

Electrophysiological experiments were performed by the two-electrode voltage clamp method at a holding potential of \(-80 \text{ mV. The perfusion medium contained 90 mM NaCl}, 1 \text{ mM KCl}, 1 \text{ mM MgCl}_{2}, 1 \text{ mM CaCl}_{2}, \text{ and 5 mM Na-HEPES (pH 7.4). To quantify GABA sensitivity, agonist concentrations between 0.1 and 10,000 mM were applied for 20 s.}

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and a wash-out period of 4–20 min was allowed to ensure full recovery from desensitization. Current responses were fitted to the Hill equation: 
\[ I = \frac{I_{\text{max}}}{1 + (EC_{50}/A)^n} \]
where \( I \) is the current amplitude at a given concentration of GABA (A), \( I_{\text{max}} \) is the maximum current amplitude, \( EC_{50} \) is the concentration of agonist eliciting half maximal current amplitudes, and \( n \) is the Hill coefficient. AllostERIC potentiation via the benzodiazepine site and covalent reaction were measured at a GABA concentration eliciting 8–12% of the maximal GABA current amplitude by coapplication of GABA and the drugs acting at the benzodiazepine binding site. Unless mentioned otherwise, oocytes were only exposed to a single drug in addition to GABA, to avoid contamination, and the perfusion system was cleaned by washing with Me2SO for the same reason.

**Receptor Modification by NCS Compound**

Modification by NCS compound was measured as follows. GABA was applied several times until a reproducible response was obtained. Oocytes were then superfused during 1 min with NCS compound freshly diluted to 20 \( \mu \)M in perfusion medium. Maximal final dioxane concentration was 0.1%. This concentration of dioxane did not affect the response to GABA in control experiments. Treatment was followed by several GABA applications in intervals of 4 min to reach a steady level. The irreversible stimulation was then calculated as Stimulation = \((I_{\text{after NCS}}/I_{\text{before NCS}}) - 1 \) × 100%. Where indicated, NCS compound was inactivated by preincubating 20 \( \mu \)M NCS compound in medium containing 10 mM cysteine for either 2 or 90 min. Control experiments showed that 10 mM cysteine did not significantly alter the response to GABA. The rate of modification was determined by repeatedly exposing an oocyte to 1 \( \mu \)M NCS compound in medium for 5 s every 4 min. The current amplitude elicited by GABA was always determined 3 min after NCS exposure. In control experiments the NCS solution also contained 1 \( \mu \)M Ro15-1788. To determine the reaction rate, relative increases in current amplitudes (stimulation) were plotted against cumulative application time of the NCS compound. Data were fitted to the equation, 
\[ y = A^*(1 - \exp(-kt)) \]
where \( A \) is the final current amplitude, \( k \) the pseudo first order rate constant, and \( t \) is the time (KaleidaGraph, Synergy Software).

**RESULTS**

**Approach**—Our aim was to work out a strategy to reveal a possible position of a benzodiazepine ligand in its binding pocket on GABA \( \alpha \) receptors. In a first step, the diazepam or the flunitrazepam molecule was chemically modified such as to become reactive with a cysteine residue. Two molecules, an isothiocyanato and an alpha-chloro amido derivative respectively, were synthesized (Fig. 1). In a second step, it had to be shown that these modified molecules still retained affinity for the benzodiazepine binding site. In a third step, several of the residues in the binding pocket were individually mutated to cysteine, and it had to be demonstrated that the mutated receptors still bound benzodiazepine ligands. In a fourth step, functional mutated receptors were exposed to the modified, reactive ligand, and a covalent reaction was taken as evidence that the mutated residue and the reactive atom of the engineered ligand were directly apposed.

**Binding and Functional Properties of the Cysteine-reactive Ligands**—Fig. 2 documents that a molecule in which the CI group in diazepam is replaced with a NCS group still is able to displace \([H]funitrazepam\) from wild type \( \alpha_1\beta_2\gamma_2 \) receptors. Thus, the reactive molecule retained affinity for the benzodiazepine binding site. The \( K_i \) value for displacement of \([H]funitrazepam\) was 3170 ± 1081 nM (mean ± S.D., \( n = 3 \)). The CI compound retained a higher affinity. Its \( K_i \) value for displacement of \([H]funitrazepam\) was 262 ± 25 nM (mean ± S.D., \( n = 3 \)). At the functional level, we only tested the NCS compound. It stimulated wild type \( \alpha_1\beta_2\gamma_2 \) receptors 115 ± 7% with an \( EC_{50} \) of 0.65 ± 0.12 \( \mu \)M (\( n = 4 \)), showing that chemical modification left the positive allosteric properties intact. This was determined using concentrations of NCS compound up to 10 \( \mu \)M. At higher concentrations further stimulation, which was insensitive to Ro15-1788, was noted, possibly due to action at the low affinity binding site for benzodiazeepines (69).

**Binding Properties of GABA \( \alpha \) Receptors Carrying a Cysteine Point Mutation**—Several amino acid residues putatively located in or near the benzodiazepine binding pocket were individually mutated to cysteine. We started with an investigation of \( \alpha_1\beta_1\gamma_2\), \( \alpha_1\beta_2\gamma_2\), \( \alpha_1\beta_2\gamma_2\gamma_3\), \( \alpha_1\beta_2\gamma_2\gamma_7\), \( \alpha_1\beta_2\gamma_2\gamma_7\gamma_9\), and \( \alpha_1\beta_2\gamma_2\gamma_7\gamma_8\). Fig. 3 documents that the mutation to cysteine of histidine 101 of the \( \alpha_1 \) subunit does not compromise \([H]Ro15-1788\) binding. Similarly, all mutated receptors investigated here retained at least some affinity for ligands of the benzodiazepine binding site. Table I summarizes these binding data. \( \alpha_1\beta_2\gamma_2\gamma_7\gamma_7\) was not investigated, because it has been reported to be unable to recognize ligands of the benzodiazepine binding site (34).

**Irreversible Reaction of Reactive Compounds with Mutated Receptors**—Mutated receptors were exposed to the reactive compound, and subsequently this compound was removed by extensive washing. A substantial proportion of protein was lost during the washing steps. Therefore, each experiment comprised controls with no reactive compound included. Residual binding in this sample was assumed 100%. In some cases, treatment with a reactive compound led to a loss in recovered binding. This loss was prevented by previous inactivation of the reactive compound. 100% minus the recovered binding corresponded to the amount of covalently reacted binding sites. Fig. 4 illustrates these experiments. Fig. 4A documents residual binding of wild type \( \alpha_1\beta_2\gamma_2 \) receptors with the NCS compound. In no case loss of binding sites was observed. Fig. 4B shows similar experiments with \( \alpha_1\beta_1\gamma_2\). In this case, the NCS compound led to disappearance of more than 80% of the binding sites. This loss was prevented when the NCS compound was previously reacted with excess cysteine (i-NCS). Fig. 4C shows the same data as percent covalently reacted receptor. Table II summarizes experiments carried out with the two reactive compounds and the investigated five mutant receptors compared with wild type receptors. In no case did CI
compound lead to a covalent reaction, when assayed at 50 μM. In contrast, 100 μM NCS compound reacted with α1H101Cβ2γ2 and α1β2γ2,γ2A79C receptors, but not with wild type α1β2γ2 or mutant α1β2γ2,T73C, α1β2γ2,D75C, and α1β2γ2,T81C receptors. Prior inactivation of the NCS compound prevented reaction with α1H101Cβ2γ2 and α1β2γ2,γ2A79C.

Concentration Dependence of the NCS Compound on α1H101Cβ2γ2 and α1β2γ2,γ2A79C Receptors—Fig. 5 illustrates this concentration dependence. Please note that in this case we do not deal with a purely reversible reaction. But, if we assume so, the curve obtained with α1H101Cβ2γ2 receptors is fitted with a Ks of 2.7 ± 0.5 μM and a Hill coefficient of 1.2 ± 0.3, indicating a covalent reaction upon occupancy of the binding site. The curve obtained with α1β2γ2,γ2A79C receptors is fitted with a Ks of 294 ± 164 μM and a Hill coefficient of 0.45 ± 0.12, indicating a more complex situation.

Protection from Covalent Modification by Ro15-1788 or Flunitrazepam—Covalent modification of α1H101Cβ2γ2 and α1β2γ2,γ2A79C receptors was performed in the presence of the benzodiazepine antagonist Ro15-1788 or of the positive allosteric modulator flunitrazepam. Modification of α1H101Cβ2γ2 receptors by the 5 μM NCS compound was almost completely blocked by 25 μM Ro15-1788 (Fig. 6A). Presumably due to the low affinity of flunitrazepam, covalent modification of α1H101Cβ2γ2 receptors was only partially prevented by flunitrazepam. Averaged destruction of the binding sites in α1β2γ2,γ2A79C receptors amounted only to 37% at a concentration of 30 μM NCS compound (Fig 6B). The data obtained for α1β2γ2,γ2A79C receptors were quite variable. Therefore, even if average data indicate quite efficient protection by Ro15-1788 and flunitrazepam, all data are statistically not significant.

Covalent Modification of α1H101Cβ2γ2 Receptors by NCS Compound Leads to a Current Increase—To obtain functional evidence for a covalent modification, we expressed wild type α1β2γ2 receptors and α1H101Cβ2γ2 receptors in Xenopus oocytes. The concentration response curve for GABA for mutant receptors was characterized by an EC50 of 76 ± 19 μM and a Hill coefficient of 1.3 ± 0.2 (n = 3). These values should be compared with the corresponding values of wild type receptors with an EC50 of 41 ± 9 μM and a Hill coefficient of 1.3 ± 0.1 (n = 4) (e.g. Ref. 70). This documents that the mutation α1H101C does not strongly affect the GABA response properties of the receptor. A concentration response curve for diazepam, up to concentrations of 10 μM, indicated an EC50 of 3.5 ± 0.7 μM with a maximal stimulation of 46 ± 6%. At higher concentrations of diazepam, further stimulation was observed that was not sensitive to Ro15-1788. This further stimulation is possibly due to the low affinity binding site for benzodiazepines (69).

Wild type receptors, exposed for 1 min to 20 μM NCS compound, showed a transient increase in the current amplitude elicited by GABA. Several applications of GABA elicited successively smaller responses until the amplitude before exposure to NCS was reached (Fig. 7A) within about 12 min. This transient increase is presumably due to a nonspecific stimulation, because it could not be inhibited by the benzodiazepine antagonist Ro15-1788. In four experiments the mean change in current amplitude was 2 ± 3%. In contrast exposure of α1H101Cβ2γ2 receptors resulted in a large increase in the current amplitude (Fig. 7B) that was irreversible. In four experiments the mean change in current amplitude was 107 ± 15%. If the NCS compound was inactivated by previous exposure to 10 mM cysteine, the current amplitude elicited by GABA increased only to a very small extent (Fig. 7C). In four experiments following exposure to cysteine of the NCS compound for 2 min, the mean change in current amplitude was 36 ± 6%, if exposure was for 90 min the mean change in current amplitude decreased to 4 ± 2% (four experiments; Fig. 7D). The half-life of the NCS compound in 10 mM cysteine may be estimated to less than 2 min. The lack of an effect by cysteine reacted NCS compound indicated that the reaction product has either lost affinity for the receptor or the ability to modulate it.

Analogous experiments with α1β2γ2,γ2A79C receptors were not
Receptor was exposed to NCS compound for 30 min, which was subsequently washed away. NCS represents 100 μM NCS compound, and Cl represents 50 μM Cl compound. Residual binding was determined. Loss of binding was observed by subtracting residual binding after exposure to reagent. Three experiments each are shown for inactivated NCS compound (i-NCS), and Cl compound. Percentage of binding sites covalently reacted.

**TABLE II**

| Receptor type       | Reactive compound | NCS % | Inactivated NCS% | Cl % |
|---------------------|-------------------|-------|------------------|------|
| α1β2γ2 (wt)         | 4.7 ± 8.8 (6)     | 21.5 ± 16.5 (4) | -0.5 ± 8.4 (4)  |
| α1H101Cβ2γ2         | 84.3 ± 2.6 (6)    | 7.2 ± 18.4 (6) | 8.7 ± 17.0 (4)  |
| α1β2γ2T73C          | 5.2 ± 8.4 (4)     | ND    | 11.0 ± 16.0 (4)  |
| α1β2γ2D75C          | -5.3 ± 18.5 (3)   | ND    | 7.3 ± 15.9 (4)   |
| α1β2γ2A79C          | 36.7 ± 2.8 (3)    | -16.5 ± 27.4 (3) | -18.0 ± 20.2 (3) |
| α1β2γ2T81C          | 2.2 ± 20.5 (4)    | ND    | 2.0 ± 22.5 (3)   |

The NCS compound was inactivated using cysteine as indicated under “Experimental Procedures.”

DISCUSSION

We use here a novel approach to establish the relative position of benzodiazepine ligands in their binding pocket. Receptors in which residues thought to reside in the binding pocket were individually mutated to cysteine and were combined with binding site ligands carrying substituents reactive to cysteine. Direct apposition of such reactive substituents with a cysteine residue was expected to lead to a covalent reaction. It would be interesting to study about 10–20 amino acid residues in combination with numerous different positions in benzodiazepine ligands. A complete study clearly exceeds practical resources, and thus we started with 5 residues and 2 positions within reactive ligands. To test the method, the following 5 residues were chosen for an initial study, because different approaches have indicated that α1His-101 is part of the binding pocket (24, 25) and γ2Thr-73, γ2Asp-75, γ2Ala-79, and γ2Thr-81 cluster around γ2Phe-77, which has been proposed to be part of the binding pocket (27), are water-exposed and either part of or at least close to the binding pocket (34). In preliminary binding experiments we used 100 μM of a reactive compound exposed for 1 h to the mutated receptors studied here. Only the combinations of the NCS compound with α1H101C and γ2A79C indicated a covalent interaction (Table II). In many cases no
For the mutation dissociation constant, \( t \)epam and diazepam, whereas the mutation /H9253 most likely interpretation of the findings is that, in the case of /H9251 flunitrazepam. /H9251 pounds do not simply react indiscriminately. The mutation covalent interaction occurred indicating that our reactive compounds do not simply react indiscriminately. The mutation \( \alpha_1 \)H101C led to a loss in affinity of about 200-fold for flunitrazepam and diazepam, whereas the mutation \( \gamma_2 \)A79C affected this affinity maximally 4-fold. If it is assumed that the affinity of the NCS compound is affected in each case about 10-fold stronger than for the mentioned compounds, a very good fit of the concentration dependence is obtained for the mutation \( \alpha_1 \)H101C with the equation \( R^\text{tot} / R_\text{tot} = 1 - \exp(-kt/L(1 + K_D/L)) \) describing occupancy of the binding pocket followed by irreversible reaction (71), where \( R^\text{tot} \) is the modified receptor, \( R_\text{tot} \) is the total receptor, \( L \) is the ligand concentration, \( K_D \) is the dissociation constant, \( t \) is time, and \( h \) is the reaction constant. For the mutation \( \gamma_2 \)A79C, no satisfactory fit was obtained. The most likely interpretation of the findings is that, in the case of \( \alpha_1 \)H101C, covalent reaction follows occupancy of the binding pocket and the covalent reaction of \( \gamma_2 \)Ala-79 did not parallel the occupancy of the site. Instead, \( \gamma_2 \)Ala-79 might be located on the diffusion access pathway of ligands of the benzodiazepine binding site. Covalent reaction of \( \gamma_2 \)A79C required rather high concentrations of the reactive compound and increased slowly with higher concentrations. However, protection experiments using either Ro15-1788 or flunitrazepam indicated an overlap of ligands in the benzodiazepine pocket with the NCS compound reacting with \( \gamma_2 \)A79C. Interestingly, it has been proposed that the ester moiety of imidazopyridines points toward the central C-atom, which is presumably attacked by the thiolate group of the receptor cysteine, present at about 10% according to the \( pK_a \) of cysteine at physiological pH in a water environment. The NCS substituent is about 2.4 Å longer than the \( \text{Cl}^- \) substituent.

The importance of histidine 101 in the \( \alpha_1 \) subunit of rat (or homologous residues in other species or other isoforms of the \( \alpha \) subunit) for benzodiazepine binding has been recognized very early. Mutation work showed that histidine found in this position in \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), and \( \alpha_6 \) confers binding ability for classic benzodiazepines, whereas \( \alpha_4 \) and \( \alpha_5 \) carry an arginine and lack this ability (24, 72). As noted before, the mutation of this residue to cysteine leaves affinity for the antagonist Ro15-1788 almost unaltered, whereas the affinity for the positive allosteric modulator flunitrazepam is drastically reduced (73) (Table I). Residue 101 is also the major target of photoaffinity labeling by \( [\text{3H}] \)flunitrazepam (25). In contrast, the imidazobenzodiazepine and partial negative allosteric modulator \( [\text{3H}] \)Ro15-4513 that carries an azido- instead of a nitro-group labels \( \alpha_1 \)Tyr-209 (74). A primitive superposition of the two molecules assigns the two substituents the same position.

The residue located at the position 101 in the \( \alpha_1 \) subunit has been shown to control the allosteric response to ligands of the benzodiazepine binding site (75). In principle the mutation to a cysteine in position 101 of the \( \alpha_1 \) subunit could show an untypical reaction to ligands of the site as a consequence of the mutation. The differences in chemical nature and geometry do not seem to compromise entirely the affinity for the benzodiazepine binding site. Our data in Fig. 7B also indicate that the mutated receptor is locked by the NCS compound in the conformation stabilized by positive allosteric modulators.

A less specific approach to cysteine labeling was used by Teissere and Czajkowski (34), which is an adaptation of a procedure reviewed by Karlin and Akabas (52). It consists of individual mutation of amino acid residues of interest to cysteine and relies on the fact that these modified receptors retain their function. The response of the modified receptor is then determined before and after exposure to a cysteine-reactive nonspecific reagent, and alterations are taken as evidence of the exposure of the corresponding residue to the medium and of

![Graph](https://example.com/figure6.png)

**Fig. 6. Protection of covalent modification by Ro15-1788 or flunitrazepam.** \( \alpha_1 \)H101C\( \beta_2 \gamma_2 \) (A) and \( \alpha_2 \)\( \beta_2 \gamma_2 \)A79C (B) receptors were reacted with 5 \( \mu \)M and 30 \( \mu \)M NCS compound, respectively, in the absence and presence of 25 \( \mu \)M Ro15-1788 or 25 \( \mu \)M flunitrazepam.
ceptrons were functionally expressed in *Xenopus* and wild type receptors were exposed to 3A elicited by GABA were determined using electrophysiological methods.

![Figure 7](https://example.com/fig7.png)

**Figure 7.** Irreversible reaction of the NCS compound with $\alpha_2$H101C$\beta_2\gamma_2$ receptors stimulates receptor function. GABA$_A$ receptors were functionally expressed in *Xenopus* oocytes, and currents elicited by GABA were determined using electrophysiological methods. A, wild type receptors were exposed to 3 $\mu$M GABA (EC$_{50}$) before and after exposure for 1 min to 20 $\mu$M NCS compound (arrow). The responses elicited by GABA were unaltered after application of NCS compound. B, the mutated receptor was exposed to GABA before and after exposure for 1 min to 20 $\mu$M NCS compound (arrow). The responses elicited by GABA increased with application of NCS compound. C, before exposure of mutated receptor, the NCS compound was inactivated for 2 min. From all this we conclude that ligand as well as receptor retain their basic properties upon modification.

We show here that the basic approach to the relative orientation of ligands in the benzodiazepine binding pocket is feasible, taking into account exclusively positive information on covalent reaction. The absence of irreversible reaction between the tested cysteine mutant recombinant receptors and the Cl compound does not exclude a proximal positioning of the reactive molecule, because the reactive molecule is about 12 Å long. The bound ligand could also obstruct the access pathway of the reactive ligand or interfere sterically with the reaction, excluding the possibility of an allosteric effect. Steric interference could be envisaged if the side chain of residue 79 of the $\gamma_2$ subunit is located within a distance of 12 Å from the edge of the flurazepam molecule nearest to residue 79. In the crystal structure of the weakly homologous acetylcholine-binding protein, the residues corresponding to $\alpha$His-101 and $\gamma_2$Ala-79, Tyr-89 and Gln-55, have a predicted closed distance of about 14 Å. It should be noted that any consideration concerning homology of these proteins should be made with care.

Two major concerns should be addressed. The first is that cysteine-mutated receptors may be structurally altered and the second that modification of the ligands to make them reactive modifies also their mode of action. Both cases would result in wrong conclusions in the present approach. For the following reasons we think that this is not the case. The GABA concentration dependence of mutated $\alpha_2$H101C$\beta_2\gamma_2$ receptors is very similar as the one of wild type $\alpha_1$$\beta_2\gamma_2$ receptors. $\alpha_1$H101C$\beta_2\gamma_2$ receptors loose much of their affinity for diazepam, but in electrophysiological experiments 10 $\mu$M diazepam still stimulated currents elicited by GABA. NCS compound retained its properties as a positive allosteric modulator at wild type $\alpha_1$$\beta_2\gamma_2$ receptors, and covalent reaction with mutated $\alpha_1$H101C$\beta_2\gamma_2$ receptors led to irreversible stimulation of currents elicited by GABA. From all this we conclude that ligand as well as receptor retain their basic properties upon modification.

We show here that the basic approach to the relative orientation of ligands in the benzodiazepine binding pocket is feasible, taking into account exclusively positive information on covalent reaction. The absence of irreversible reaction between the tested cysteine mutant recombinant receptors and the Cl compound does not exclude a proximal positioning of the reactive carbon atom with the side chains of the tested amino acid residues. Nevertheless, we will test different cysteine mutant receptors. It is important to note here that the Cl compound does not display indiscriminate reactivity with the tested mutant receptors.

...after exposure for 1 min to 20 $\mu$M NCS compound (arrow). The responses elicited by GABA were unaltered after application of NCS compound. B, the mutated receptor was exposed to GABA before and after exposure for 1 min to 20 $\mu$M NCS compound (arrow). The responses elicited by GABA increased with application of NCS compound. C, before exposure of mutated receptor, the NCS compound was inactivated for 2 min. The responses elicited by GABA were unaltered after application of inactivated NCS compound (arrow). D, summary of four experiments each with wild type receptors (wt), $\alpha_1$H101C$\beta_2\gamma_2$ receptors treated with NCS compound ($H101C$), $\alpha_1$H101C$\beta_2\gamma_2$ receptors treated with NCS compound inactivated for 2 min ($H101C, 2 min Cys$), and $\alpha_1$H101C$\beta_2\gamma_2$ receptors treated with NCS compound inactivated for 90 min ($H101C, 90 min Cys$).
Orientation of Benzodiazepines in Their Binding Pocket

As soon as 3-amino acid residue side chains have been identified with the techniques presented here, two important feats will be possible. First it will be possible to do analogy modeling to the acetylcholine-binding protein with higher precision, because three side groups will be described in their relative distances, and second, it will allow docking of positive allosteric modulators to this receptor. Subsequently, we plan to extend our approach to antagonists and negative allosteric modulators with the final aim of finding a superposition of these three classes of ligand. Finally this approach will be extended to other isoforms of the α and γ subunits to find structural elements explaining the differential selectivities conferred by different receptor subunits. The final aim of this work is to achieve a rational approach to the design of GABA<sub>γ</sub> receptor subtype-specific allosteric modulators of the benzodiazepine binding site in the absence of a crystal structures of the receptor isoforms.

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Fig. 8. Time course of modification by NCS compound. Currents elicited by GABA were measured as indicated under Fig. 7. A, α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors were exposed to 3 μM GABA (EC<sub>50</sub>) GABA (horizontal bars) before and after repeated exposure for 5 s each to 1 μM NCS compound. B, error bars, mean ± S.D. of three experiments and additionally the results of three experiments where NCS compound was applied in the presence of Ro15-1788. The graph shows relative stimulation of the current amplitude versus cumulative application time of NCS compound and its inhibition by the benzodiazepine antagonist Ro15-1788.
On the Benzodiazepine Binding Pocket in GABA<sub>Α</sub> Receptors
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