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Mapping of $GABA_A$ receptor sites that are photoaffinity-labelled by [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513

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Abstract

The $GABA_A$ receptor in brain membranes prepared from bovine cerebral cortex and cerebellum has been photoaffinity-labelled by the classical benzodiazepine agonist, [^{3}H]flunitrazepam, or by the partial inverse agonist [^{3}H]Ro 15-4513. Following solubilization and precipitation with trichloroacetic acid, the photoaffinity-labelled receptor preparations were subjected to specific chemical cleavage using hydroxylamine, a reagent which cleaves specifically at a relatively rare Asn-Gly bond. The resulting peptides were resolved by denaturing polyacrylamide gel electrophoresis and mapping of these peptides to the known amino acid sequences of the $GABA_A$ receptor subunits has localized the photoaffinity-labelling sites for these two ligands to distinct portions of the α subunits. It is shown that the site for [^{3}H]flunitrazepam photoaffinity-labelling in the receptor populations of both the cerebral cortex and cerebellum occurs within residues 1–103 of the bovine α_1 subunit sequence (or within analogous segments of homologous α subunits). In contrast, the site of photoaffinity-labelling by [^{3}H]Ro 15-4513 in the cerebral cortex and in the diazepam-sensitive $GABA_A$ receptor population of the cerebellum lies between residues 104 and the carboxy-terminus of the bovine α_1 or homologous α subunits. However, the [^{3}H]Ro 15-4513 photoaffinity-labelling site for the diazepam-insensitive receptors of the cerebellum is shown to occur within residues 1–101 (α_6 subunit numbering). These results demonstrate that the photoaffinity-labelling sites for [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 on the $GABA_A$ receptor are localized to distinct domains of the α_1 subunit and that [^{3}H]Ro 15-4513 photoaffinity labels a site on the α_6 subunit that is unique from its site of labelling on the α_1 subunit.

Keywords: $GABA_A$ receptor; Benzodiazepine; Flunitrazepam; Ro 15-4513; Hydroxylamine

1. Introduction

The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily (Schofield et al., 1987) and is composed of several, probably five, homologous subunits which assemble to form an integral chloride ion channel (Barnard, 1992). To date, six α, four β, four γ, one δ and two ρ subunit subtypes of the $GABA_A$ receptor have been identified, in addition to splice variants of several of the subunit isoforms. The $GABA_A$ receptor contains multiple allosteric binding sites for many important pharmacological agents (Sieghart, 1992), including the clinically useful benzodiazepines which interact with the receptor to potentiate GABA-mediated inhibitory tone throughout the mammalian brain. The benzodiazepine ligands which interact with the $GABA_A$ receptor can be grouped into three categories; agonists, inverse agonists and antagonists. Ro 15-4513 (an imidazobenzodiazepine, ethyl-7-chloro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) is a partial inverse agonist that binds competitively to sites that are sensitive to classical agonists and antagonists, as well as to a sub-population of diazepam-insensitive $GABA_A$ receptors found mainly in the cerebellum (< 20% of sites) and to a minor extent (< 2.5% of sites) in the cerebral cortex (Sieghart et al., 1987b; Turner et al., 1991).

Although the binding domains for the benzodiazepines on the $GABA_A$ receptor have yet to be determined, several groups have identified structural features that are important for ligand recognition and modulatory action (reviewed by Smith and Olsen, 1995). The classical benzodiazepine agonist, [^{3}H]flunitrazepam, has been shown to specifically and irreversibly photoaffinity-label several α subunit isoforms of the $GABA_A$ receptor (Stephenson et al., 1990; Fuchs et al., 1988) and the site of covalent attachment of the ligand has been shown to lie within the large extracellular amino-terminal domain (Czajkowski and Farb, 1986; Schmitz et al., 1989). Partial sequencing of proteolytic

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fragments from [^{3}H]flunitrazepam photoaffinity-labelled receptors has indicated that the binding site most likely occurs within amino acid residues 8–297 of the α_1 sequence (Olsen et al., 1991). In addition, peptide fragments from photoaffinity-labelled receptors have been probed with sequence-specific antibodies to show that the [^{3}H]flunitrazepam label does not lie within the amino acids 1–58 or 149–429 of the α_1 subunit (Stephenson and Duggan, 1989). Molecular biological approaches utilizing site-directed mutagenesis have identified that replacement of α_3 Glu 225 with α_1 Gly 225 results in altered benzodiazepine pharmacology (Pritchett and Seeburg, 1991) and a mutation of α_1 His 102 to α_6 Arg 100 results in a loss of classical agonist sensitivity (Wieland et al., 1992). Biochemical approaches first suggested the interaction of benzodiazepines with a histidine residue by investigating the effect of histidine modification on the binding of agonists and antagonists (Lambolez and Rossier, 1987; Lambolez et al., 1989), and later with the partial inverse agonist [^{3}H]Ro 15-4513 (Binkley and Ticku, 1991; Maksay, 1992; Uusi-Oukari, 1992a, b). Sulfhydryl modification of $GABA_A$ receptors (Duncalfe and Dunn, 1993; Otero de Bengtsson et al., 1993) has also been shown to have differential effects on the binding of different benzodiazepines to the $GABA_A$ receptor.

In the present study, we have subjected [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 photoaffinity-labelled $GABA_A$ receptor preparations from the cerebral cortex and cerebellum to specific chemical cleavage with hydroxylamine. Hydroxylamine cleaves specifically at an Asn-Gly bond, a peptide bond that occurs with a probability of the order of 1/400 for most proteins (Bornstein and Balian, 1977). The resulting labelled peptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight determination. The hydroxylamine cleavage patterns demonstrate differences in the sites of photoaffinity-labelling by the classical agonist, [^{3}H]-flunitrazepam, and the partial inverse agonist, [^{3}H]Ro 15-4513.

2. Materials and methods

2.1. Preparation and photoaffinity-labelling of bovine brain membranes

Brain membranes were prepared from bovine cerebral cortex or cerebellum as described previously (Dunn et al., 1989) and resuspended in 20 mM Tris citrate pH 7.5 containing 1 mM EDTA, 1 mM benzamidine, 0.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml bacitracin, 0.02% NaN_3 to a final protein concentration of 1 mg/ml, determined by the Biorad method. [^{3}H]Flunitrazepam (5 nM final) or 10 nM [^{3}H]Ro 15-4513 (Dupont Canada, 85.8 and 27.4 Ci/mmol, respectively) was added to the brain membranes and the mixture was incubated on ice for 45 min prior to ultraviolet irradiation (Spectroline ENF 260C lamp, long wavelength) at a distance of 6 cm for 45 min on ice with constant stirring. Non-specific photoaffinity-labelling was determined in the presence of excess unlabelled ligand. For [^{3}H]Ro 15-4513 photoaffinity-labelling of the cerebellar diazepam-insensitive population of $GABA_A$ receptor sites, 5 μM unlabelled flunitrazepam (Sigma Chemicals) was included in the incubation mixture. After photoaffinity-labelling, the preparations were subjected to repeated cycles of centrifugation (150 000 $\times$ g, 45 min) and resuspension to remove free ligand. The labelled membranes were resuspended at a final concentration of 15 mg/ml in the above buffer.

2.2. Solubilization of photoaffinity-labelled membranes and protein precipitation

An equal volume of solubilization buffer, 20 mM Tris citrate pH 7.5, 0.5 M KCl, 3% 3-[-(cholimidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.3% asolectin and protease inhibitors as noted above was added dropwize to the photoaffinity-labelled membranes followed by stirring on ice for 60 min prior to centrifugation at 100 000 $\times$ g for 60 min. The supernatant containing the solubilized protein was added to an equal volume of 24% (w/v) trichloroacetic acid, incubated on ice for 15 min and centrifuged (10 000 $\times$ g, 15 min). The protein pellets were washed twice with acetone before resuspension in the solution for the hydroxylamine cleavage reaction.

2.3. Cleavage of photoaffinity-labelled receptor preparations

Hydroxylamine cleavage of the labelled receptor was performed as described (Bornstein and Balian, 1977). The labelled protein (3–5 mg/ml final) was added to a solution of 2 M hydroxylamine, 6 M guanidine, 4.5 M lithium hydroxide (Fisher Scientific) and incubated for 4 h at 45°C, maintaining the pH at 9 with lithium hydroxide as titrant. The reaction was terminated by acidification to pH less than 3 using concentrated formic acid. The mixture was desalted on a Sephadex G-25 superfine column (1.5 $\times$ 25 cm) in 9% formic acid. The fractions containing radioactivity that eluted in the void volume were pooled, freeze-dried and resuspended in buffer for subsequent resolution by SDS-PAGE. The hydroxylamine cleavage reaction was repeated at least twice for all photoaffinity-labelled receptor preparations studied.

2.4. SDS-PAGE and scintillation counting

The photoaffinity-labelled receptor preparations and peptides generated from chemical cleavage by hydroxylamine were analyzed by SDS-PAGE under reducing conditions using a Tricine buffer system (described by Schägger

and Von Jagow, 1987) for superior resolution of molecular weight species in the 1–100 kDa range. The gels utilized for electrophoretic separations consisted of a resolving slab of 10%T, 3%C, a spacer gel of 7%T, 3%C and a stacking gel of 4%T, 3%C, where %T = (gram acrylamide + bis-acrylamide) per 100 ml and %C = gram bis-acrylamide per (gram acrylamide + bis-acrylamide). Immediately following electrophoresis, sample lanes were cut into 0.25 cm slices, eluted in 0.5 ml 2% SDS at 50°C overnight and radioactivity was determined by scintillation counting after addition of 5 ml Ecolite scintillant (Fisher Scientific). Molecular weight values of peptides were estimated by the migration profiles of standard proteins run in parallel lanes on each gel, using both high and low molecular weight rainbow-colored standards (Amersham Canada). The photoaffinity-labelled receptor preparations and hydroxylamine-generated peptides were resolved by electrophoresis on two to three separate gels for molecular weight estimations.

2.5. pH dependency of radioligand binding

Brain membranes prepared from bovine cerebral cortex were diluted to a final protein concentration of 0.4 mg/ml in 50 mM Tris, 25 mM acetic acid, 25 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES), 100 mM NaCl, 0.02% NaN_3 buffer titrated with either 1 N HCl or 1 N NaOH to pH values ranging from 5 to 9. Over this pH range, the ionic strength of this buffer mixture remains essentially constant (Ellis and Morrison, 1982). [^{3}H]Flunitrazepam or [^{3}H]Ro 15-4513 (15 nM final) was added to the membranes prior to incubation for 60 min at 4°C. Non-specific binding was determined in the presence of 10 μM cold ligand added to parallel sample sets. Duplicate aliquots of each sample were filtered under vacuum through Whatman GF/C filters using a Hoeffer filtration apparatus, and the filters were immediately washed with two 5-ml volumes of ice cold pH 7 buffer. The filters were dried, 5 ml of scintillation fluid added and samples were counted for ^{3}H.

3. Results

3.1. Photoaffinity-labelled receptor preparations from bovine cerebral cortex

Photoaffinity-labelling of bovine brain membranes from cerebral cortex with [^{3}H]flunitrazepam led to covalent incorporation of the ligand which, by SDS-PAGE, was shown to be associated with a major protein band of 53 kDa (Fig. 1A), corresponding to $GABA_A$ receptor(s) α subunits (Fuchs et al., 1988). The major α subunit isoform found in cortical $GABA_A$ receptors is the α_1 subunit, although other less abundant isoforms have been identified

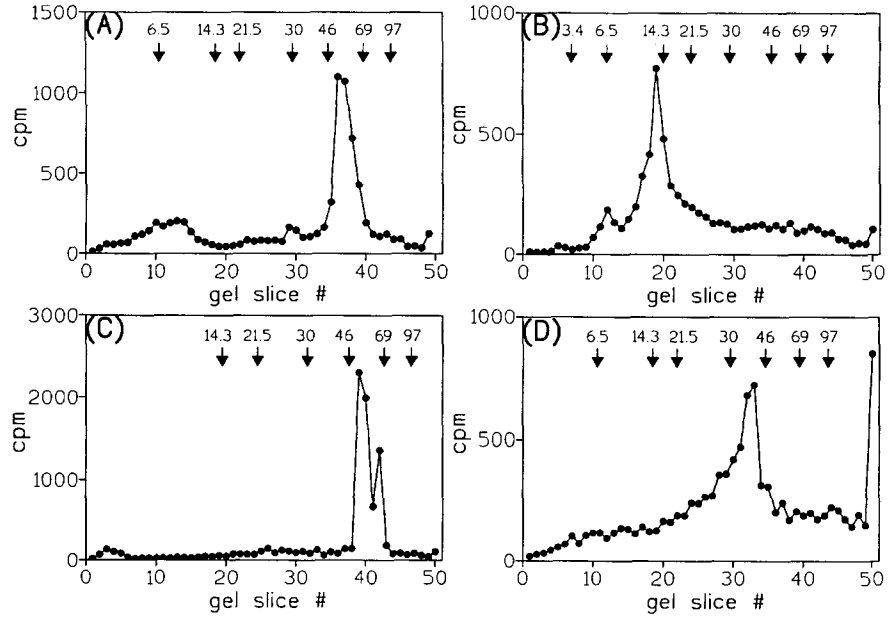

Fig. 1. Photoaffinity-labelled $GABA_A$ receptors of brain membranes from bovine cerebral cortex and hydroxylamine-generated peptides of the labelled receptor preparations. Representative radioactivity profiles of labelled species resolved by Tricine SDS-PAGE are shown. The numerals above each arrow indicate the relative position of the molecular mass standards (expressed in kDa). (A) [^{3}H]Flunitrazepam photoaffinity-labelled receptor resolves to one major peak that corresponds to a 53 kDa protein band. (B) [^{3}H]Flunitrazepam-labelled peptides generated by hydroxylamine cleavage yield a major radioactive peak which corresponds to a peptide with an apparent mass of 12 kDa. (C) [^{3}H]Ro 15-4513-labelled receptor resolves as a double peak with a main 55 kDa protein band and a less abundant 65 kDa species. (D) Hydroxylamine-cleaved [^{3}H]Ro 15-4513 preparations yield a broad protein band which peaks at an apparent mass of 40 kDa. Some labelled protein does not enter into the resolving gel and results in a radioactive peak at the interface (gel slice #50). Results shown are representative data from at least two distinct receptor preparations that have each been resolved by gel electrophoresis, a minimum of 2 times for molecular weight determination.

(Duggan and Stephenson, 1990). As shown in Fig. 1B, SDS-PAGE analysis of the hydroxylamine cleavage pattern of the [^{3}H]flunitrazepam-labelled receptor preparation indicated that a major radiolabelled peptide of approximate mass of 12 kDa is generated. Since no residual 53 kDa radiolabelled species from the receptor preparation is evident following hydroxylamine treatment, the extent of specific chemical cleavage is presumed to be complete. The primary amino acid sequences of the α_1, α_2 and α_5 subunits are known to possess only one asparagine-glycine bond, located at residues 103/104 (α1 subunit numbering), that is susceptible to specific cleavage by hydroxylamine. Cleavage of the subunit at this peptide bond would generate only two major products differing greatly in size; 1–103 and 104–430 of approximate 10 kDa and 43 kDa molecular mass, respectively. The α_3, α_4 and α_6 subunits also contain the Asn-Gly bond between residues 103/104, plus an additional hydroxylamine cleavage site located 21 residues carboxy-terminal to the first site. The β, γ and δ subunits of the $GABA_A$ receptor do not contain an Asn-Gly peptide bond, so hydroxylamine treatment will not specifically cleave these subunits. Therefore, the 12 kDa peptide generated from hydroxylamine cleavage of [^{3}H]-flunitrazepam photoaffinity-labelled receptors demonstrates that the major site of labelling lies prior to Asn residue 103 of the $GABA_A$ receptor α subunit(s).

[^{3}H]Ro 15-4513 photoaffinity-labelling of bovine cerebral cortical membranes generated a covalently labelled 55 kDa protein and a secondary component of 65 kDa, as resolved by gel electrophoresis (Fig. 1C). As seen in Fig. 1D, hydroxylamine treatment of the [^{3}H]Ro 15-4513 photoaffinity-labelled receptor preparation cleaved both the 55 and 65 kDa radiolabelled components to generate a peptide with an apparent mass of 40 kDa. These results indicate that the major site of [^{3}H]Ro 15-4513 labelling occurs on the α subunit(s), between the Gly residue at position 104 and the carboxy-terminus. The higher molecular weight species observed at the top of the gel in Fig. 1D is due to the poor solubility of the hydroxylamine-cleaved peptides which results in some precipitation at the spacer-resolving gel interface (see below).

3.2. *Photoaffinity-labelled receptor preparations from bovine cerebellum*

$GABA_A$ receptors located in the cerebellum can be classified into two distinct populations; diazepam-sensitive and diazepam-insensitive. The diazepam-insensitive binding sites correlate with the presence of an α_6 subunit in the $GABA_A$ receptor (Lüddens et al., 1990). In Fig. 2A, the total population of cerebellar binding sites for [^{3}H]Ro 15-4513 have been photoaffinity-labelled and resolved by SDS-PAGE to a 58 kDa protein band. The peptides resulting from hydroxylamine treatment of this labelled receptor

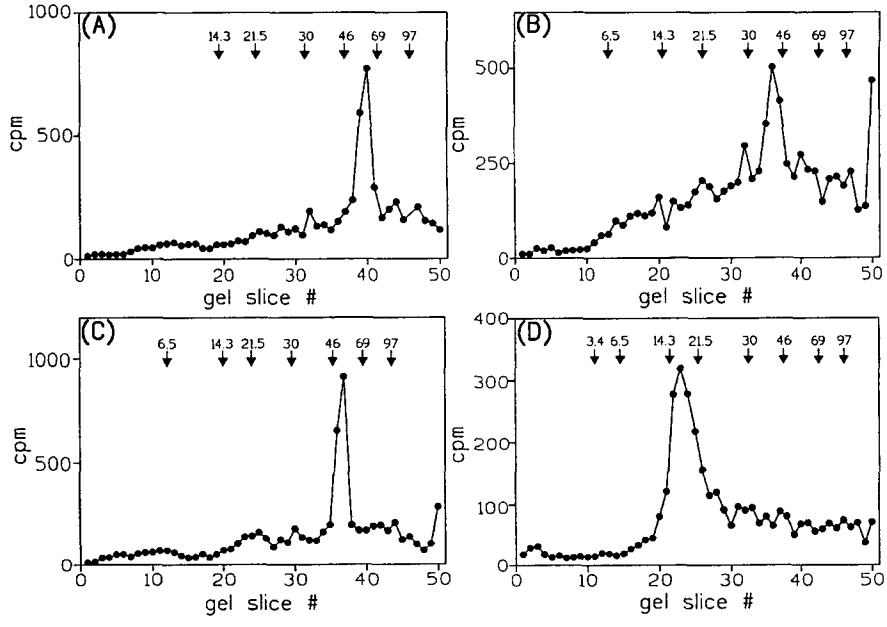

Fig. 2. Representative radioactivity profiles of [^{3}H]Ro 15-4513 photoaffinity-labelled $GABA_A$ receptors and hydroxylamine-generated peptides of bovine cerebellar total sites and diazepam-insensitive sites resolved by SDS-PAGE. (A) The total population of cerebellar sites that are photoaffinity-labelled by [^{3}H]Ro 15-4513 resolve to a single protein band with an apparent mass of 58 kDa. (B) The hydroxylamine peptides generated from chemical cleavage of the total receptor population resolve to major peak of 43 kDa. Some components of the hydroxylamine reaction of this receptor preparation remain at the top of the resolving gel (peak at gel slice #50). (C) [^{3}H]Ro 15-4513 photoaffinity-labelled diazepam-insensitive receptor population of the cerebellum resolves to a main 57 kDa band. (D) Photoaffinity-labelled peptides generated by hydroxylamine cleavage of the diazepam-insensitive $GABA_A$ receptors resolve to a band with an apparent mass of 14 kDa. Results shown are representative data from at least two preparations that were resolved by SDS-PAGE at least twice.

preparation (Fig. 2B) resolve to a major 43 kDa component. These results demonstrate that, as in cortex, the site of [^{3}H]Ro 15-4513 photoaffinity-labelling occurs between residue Gly 104 and the carboxy-terminus (α_1 subunit numbering) in the cerebellar population of diazepam-sensitive $GABA_A$ receptors. Photoaffinity-labelling of cerebellar preparations with [^{3}H]Ro 15-4513 in the presence of excess unlabelled flunitrazepam will lead to selective incorporation of the label into the diazepam-insensitive receptor population. This covalently labelled protein resolves as a 57 kDa species by SDS-PAGE (Fig. 2C) and the peptides generated by hydroxylamine cleavage of this receptor preparation resolve to 14 kDa (Fig. 2D). Therefore, the [^{3}H]Ro 15-4513 photoaffinity-labelling site for the diazepam-insensitive receptors occurs within residues 1–101 (α_6 subunit numbering). Since the diazepam-insensitive population of [^{3}H]Ro 15-4513 binding sites comprise less than 20% of the $GABA_A$ receptors in the cerebellum, the 14 kDa hydroxylamine peptides resulting from these receptors are not discernible in the preparation generated from the total cerebellar sites due to the background noise present in the radioactivity profile (Fig. 2B).

[^{3}H]Flunitrazepam photoaffinity-labelling to bovine cerebellar $GABA_A$ receptors and hydroxylamine peptides generated from the labelled receptor preparations parallels the cleavage patterns obtained for the receptor population of the cerebral cortex, i.e. the covalently attached [^{3}H]flunitrazepam label was shown to be associated with a major protein band of 55 kDa (Fig. 3A) and the peptides

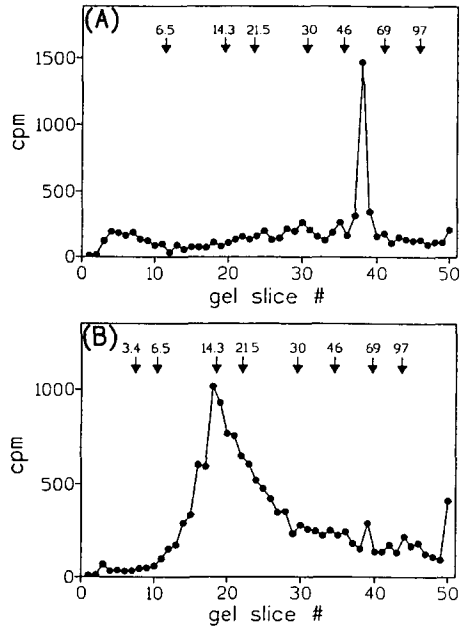

Fig. 3. [^{3}H]Flunitrazepam photoaffinity-labelled receptor of cerebellum and hydroxylamine-generated peptides. (A) [^{3}H]Flunitrazepam-labelled receptor resolves to major radioactive peak of apparent mass of 55 kDa. (B) The hydroxylamine peptides of this preparation resolve to a broad peak with an approximate mass of 13 kDa. Data shown are representative and have been repeated with similar results.

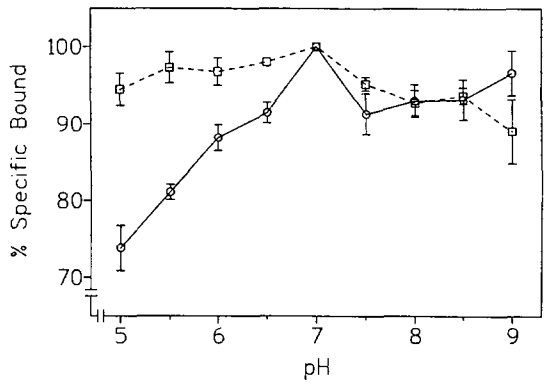

Fig. 4. Effect of pH on the binding of [^{3}H]FNZ and [^{3}H]Ro 15-4513 to brain membranes from cerebral cortex. Binding assays were performed as described in Methods. Data are expressed as the means ± S.E.M. of five separate experiments performed in duplicate. Specific bound for each ligand is normalized to pH 7 of 100%. The binding of [^{3}H]flunitrazepam to brain membranes (○) is significantly reduced below pH 7 compared to control, while [^{3}H]Ro 15-4513 binding (□) displays no significant differences across the pH range tested.

resulting from hydroxylamine treatment resolved by SDS-PAGE as a somewhat broad band with the major peak of 13 kDa (Fig. 3B). These data demonstrate that the site of photoaffinity-labelling for [^{3}H]flunitrazepam in the cerebellum also occurs prior to residue Asn 103 of the $GABA_A$ receptor α subunit.

Non-specific photoaffinity-labelling by [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 in both cortical and cerebellar receptor preparations represented a minor fraction of radioactivity, such that no ^{3}H-peaks above background levels were detectable in the gel profiles.

3.3. pH dependency of benzodiazepine radioligand binding

The binding of [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 to brain membranes prepared from bovine cerebral cortex under equilibrium conditions was investigated over a pH range from 5 to 9 in a buffer system which controls for ionic strength (see Methods). Data are expressed as means ± S.E.M. ($n = 5$) and were calculated to percent specific bound by subtracting non-specific from total bound (non-specific accounted for less than 5% of the total bound) and normalizing to pH 7 as 100%. As seen in Fig. 4, the binding of [^{3}H]flunitrazepam is significantly reduced in the pH range from 5 to 7, while the binding of [^{3}H]Ro 15-4513 does not significantly deviate from control values over the pH range investigated.

4. Discussion

Previous studies have investigated the proteins from rat cerebellar and hippocampal membranes that are photoaffinity-labelled by [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 and the peptide products that are generated from prote-

olytic cleavage reactions. A differential degradation of the photoaffinity-labelled proteins was demonstrated from proteolytic digestion with papain, trypsin and chymotrypsin (Sieghart et al., 1987a; Sieghart, 1988). However, these reports were unable to conclude if the different cleavage patterns were due to [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 photoaffinity-labelling sites on different subtypes of the $GABA_A$ receptor or were due to labelling sites on different domains of the same receptor protein(s). To our knowledge, although there is strong evidence for the α subunit(s) being the major site of labelling by [^{3}H]flunitrazepam (see Introduction), similar evidence for the location of the sites of labelling by [^{3}H]Ro 15-4513 has not been reported. The present study has demonstrated; (1) that the α subunit(s) of the $GABA_A$ receptor is the major site for photoaffinity-labelling by both [^{3}H]flunitrazepam and [^{3}H]Ro 15-4513 and (2) that the different peptide products generated by hydroxylamine cleavage is due to labelling of distinct domains of this receptor subunit(s).

The hydroxylamine cleavage of [^{3}H]flunitrazepam photoaffinity-labelled receptors from cerebral cortex and cerebellum results in a peptide that could only be generated from an α subunit, because the β, γ and δ subunits do not contain hydroxylamine-specific cleavage sites. Since the α_1 subunit possesses only one potential Asn-Gly peptide bond for cleavage by hydroxylamine at 103/104, the site of the [^{3}H]flunitrazepam photoaffinity label can be further localized to lie within amino acid residues 1–103 (α_1 subunit numbering). Previous reports have shown that the [^{3}H]flunitrazepam label occurs on the α subunit and its position is most likely limited to lie within residues 59–149 of the α subunit (see Introduction). The information gained from the hydroxylamine cleavage pattern extends these predictions and further limits the site of [^{3}H]flunitrazepam photoaffinity label to within α_1 subunit residues 59 and 103. In addition, our laboratory has obtained preliminary results from microsequencing of [^{3}H]flunitrazepam photoaffinity-labelled peptides generated by cyanogen bromide cleavage of bovine $GABA_A$ receptors. These results indicate that the photolabel is likely to be associated with His 102 of the α_1 subunit (manuscript in preparation; see also Olsen and Smith, 1994). In the present study, the involvement of a histidine residue in the binding of the agonist to bovine brain membranes is supported by the decrease in [^{3}H]-flunitrazepam binding in the pH range from 5 to 7. The only amino acid with a pK_a in solution over this range is a histidine residue. The binding of [^{3}H]Ro 15-4513 over the pH range from 5 to 7 is not significantly altered. Although previous reports show that varying the buffer pH has differential effects on the binding of radiolabelled benzodiazepines (see above), the results cannot be unequivocally attributed to pH, since in earlier investigations changes in ionic strength were not controlled and may have contributed to the observed effects.

[^{3}H]Ro 15-4513 has also been shown to label $GABA_A$ receptor α subunits in cerebral cortex and cerebellum. In the diazepam-sensitive population, this ligand does not label the same fragment as [^{3}H]flunitrazepam, but instead labels the larger Gly 104-carboxy terminal peptide generated by hydroxylamine cleavage. It is noteworthy that in these hydroxylamine-cleaved preparations, there was a small but reproducible portion of radiolabelled product that aggregated at the top of the resolving gel when attempting to resolve by SDS-PAGE. Although it is not possible to make a conclusive explanation for this lack of resolution, it is possible that the removal of a large portion of the amino-terminal domain from the receptor subunit adversely affected the solubility properties of the peptide.

Photoaffinity-labelling of the diazepam-insensitive receptors in the cerebellum by [^{3}H]Ro 15-4513 occurs in the amino-terminal domain, 1–101 (α_6 subunit numbering). Previous reports have presented data that suggested the interaction of [^{3}H]Ro 15-4513 with the diazepam-insensitive receptors was unique compared to the agonist-sensitive population. For example, GABA has been shown to enhance [^{3}H]Ro 15-4513 binding to the diazepam-insensitive sites in cultured cerebellar granule cells (Malminiemi and Korpi, 1989), whereas GABA has an inhibitory effect on [^{3}H]Ro 15-4513 binding to diazepam-sensitive sites. It is known that the α_6 subunit possesses an arginine in position 100 instead of the histidine residue that is present in the α subunits sensitive to classical benzodiazepine agonists (Wieland et al., 1992). It is tempting to speculate that it is the lack of this histidine residue and/or the presence of an arginine that facilitates [^{3}H]Ro 15-4513 binding to this domain of the α_6 subunit.

The present study has shown that the sites for photoaffinity-labelling by the classical agonist and the partial inverse agonist are located within distinct domains of the α subunit(s). The specific chemical cleavage by hydroxylamine was presumably complete, since intact photolabelled subunits were not evident in the cleavage products from either the [^{3}H]flunitrazepam or [^{3}H]Ro 15-4513 receptor preparations. However, residual intact subunits in low abundance may not have been detectable amongst the background radioactivity in the SDS-PAGE profiles. Therefore, while the α subunit(s) have been established as the major site for photoincorporation by these ligands, the possibility for a minor extent of photolabelling occuring on other non-α subunits cannot be dismissed. Inevitably, the conclusive characterization of the photoaffinity-labelling sites of these benzodiazepines on the $GABA_A$ receptor will require complete sequencing of the peptide segments to which the labels are covalently attached.

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