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Short communication

[^{3}H]Propyl-6-azido-β-carboline-3-carboxylate: a new photoaffinity label for the GABA$_A$-benzodiazepine receptor

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Abstract

[^{3}H]Propyl-6-azido-β-carboline-3-carboxylate ([^{3}H]ACCP) exhibited a high affinity for GABA$_A$ receptors affinity purified from the brains of adult rats, and binding of this compound could be inhibited by several ligands of the benzodiazepine binding site of GABA$_A$ receptors. On irradiation with UV light, [^{3}H]ACCP, similarly to [^{3}H]flunitrazepam, irreversibly labeled a protein with an apparent molecular weight of 51 kDa in affinity-purified GABA$_A$ receptors, and this labeling could be inhibited in the presence of diazepam. These data indicate that [^{3}H]ACCP can be used as a photoaffinity label for GABA$_A$ receptors.

Keywords: GABA$_A$ receptor; β-Carboline; Photoaffinity label

1. Introduction

GABA$_A$ receptors are ligand-gated chloride ion channels which can be modulated by allosteric binding sites for the anxiolytic benzodiazepines, for some sedative-hypnotic barbiturates, some steroids, some anesthetics, the anthelmintic avermectin B_1a and some convulsant chloride channel ligands such as picrotoxinin and *t*-butylbicyclophosphorothionate (Sieghart, 1992a). Recently, a large variety of GABA$_A$ receptor subunits have been cloned and sequenced, and it has been demonstrated that GABA$_A$ receptors consist of 5 subunits and that the subunit composition determines the pharmacological properties of these receptors (Burt and Kamatchi, 1991; Sieghart, 1992b).

Several photoaffinity ligands are available which can be used to identify the GABA$_A$ receptor-associated benzodiazepine binding site. [^{3}H]Flunitrazepam, the first photoaffinity label used (Möhler et al., 1980), has been demonstrated to specifically identify several distinct proteins in membranes from different brain tissues (Sieghart and Karobath, 1980; Sieghart, 1992b). These proteins have been shown to represent different α-subunits of the GABA$_A$ receptor (Zezula et al., 1991). Proteins with a similar apparent molecular weight could be labeled by two other photoaffinity ligands, the benzodiazepines [^{3}H]clonazepam (Sieghart and Möhler, 1982) or [^{3}H]Ro 15-4513 (Sieghart et al., 1987). Recently, a structurally different type of photoaffinity label for the central benzodiazepine receptors, the ethyl ester of 6-azido-β-carboline-3-carboxylate, has been synthesized and partially characterized in the unlabeled form (Dellouve-Courillon et al., 1989). In the present study, for the first time a radiolabeled azido-β-carboline, [^{3}H]propyl-6-azido-β-carboline-3-carboxylate ([^{3}H]ACCP), was investigated as a photoaffinity ligand for GABA$_A$-benzodiazepine receptors.

2. Materials and methods

Brains from adult or 6- to 8-day-old rats were homogenized with a Potter-Elvejhem homogenizer in a solution containing 0.32 M sucrose, 10 mM Hepes, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM benzamidine, 0.3 mM phenylmethylsulfonyl fluoride, pH 7.4. The homogenate was centrifuged at $1000 \times g$ for 15 min. The supernatant was then centrifuged at $45\,000 \times g$ for 45 min, and the membrane pellets were rehomogenized in the same buffer and stored at −20°C for at

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least 18 h. For binding assays membrane fractions were washed 5 additional times by centrifugation and resuspension in 50 vols. 50 mM Tris-citrate buffer, pH 7.4.

$GABA_A$ receptors were extracted from brain membranes and purified at least 1000-fold by affinity chromatography on Ro 7-1986-coupled Affigel 15 (Bio-Rad, Richmond, CA, USA) as described previously (Item and Sieghart, 1994). Purified and dialyzed $GABA_A$ receptor (100 μl, about 3 pmol reversible [^{3}H]flunitrazepam binding sites) in a buffer containing 10 mM Hepes, 0.1% Triton X-100, 150 mM KCl and various protease inhibitors (1 mM EDTA, 1 mM benzamidin and 0.5 mM dithiothreitol, pH 7.4) was added to 900 μl of a solution containing 50 mM Tris-citrate buffer, pH 7.1, 150 mM NaCl, 500 μg γ-globulin, 15% polyethyleneglycol (PEG) and various concentrations of [^{3}H]flunitrazepam (80 Ci/mmol, DuPont-New England Nuclear, Dreieich, Germany) or [^{3}H]ACCP (25 or 19.3 Ci/mmol, generously provided by Dr. Steve Hurt, DuPont-New England Nuclear, Boston, MA, USA) in the absence or presence of various concentrations of benzodiazepine receptor ligands or of 10 μM diazepam. After incubation at 4°C for 90 min in the dark, samples were filtered under vacuum through Whatman GF/B filters and washed twice with an ice-cold solution containing 8% PEG in 50 mM Tris-citrate buffer, pH 7.4 (Item and Sieghart, 1994). Radioactivity on the filters was measured by liquid scintillation counting and unspecific binding measured in the presence of 10 μM diazepam was subtracted from total binding to give specific binding.

For the investigation of irreversible binding to proteins, affinity purified $GABA_A$ receptors were incubated with 10 nM [^{3}H]flunitrazepam or 10 nM [^{3}H]ACCP in the absence or presence of 10 μM diazepam for 90 min at 4°C in the dark as described above. The samples were then irradiated with a CAMAC de Luxe UV light (15 min at 366 nm for [^{3}H]flunitrazepam and 5 min at 254 nm for [^{3}H]ACCP) at a distance of 3 cm (Sieghart and Karobath, 1980; Sieghart et al., 1987). Diazepam (10 μM) was added, and the incubation was continued for an additional 30 min at 4°C to allow the dissociation of reversibly bound [^{3}H]flunitrazepam or [^{3}H]ACCP. Proteins in the solution were precipitated with chloroform-methanol (Wessel and Flügge, 1984) and were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously (Sieghart and Karobath, 1980; Sieghart et al., 1987).

3. Results

For the present study the propyl ester of 6-azido-β-carboline-3-carboxylate was tritium-labeled and purified by DuPont-New England Nuclear, Boston, MA, USA. Over time, two separate batches of the purified [^{3}H]ACCP were used in the experiments described. In preliminary experiments, it was demonstrated that in contrast to affinity-purified $GABA_A$ receptors from adult rats, where about 60–80% of total [^{3}H]ACCP binding could be inhibited in the presence of 10 μM diazepam or 10 μM β-carboline-3-carboxylate ethyl ester (βCCE), high unspecific but no reproducible specific binding of [^{3}H]ACCP could be obtained with brain membranes from adult rats or with affinity purified $GABA_A$ receptors from 6- to 8-day-old rats. Therefore, affinity-purified $GABA_A$ receptors from the brains of adult rats were used for all further experiments on [^{3}H]ACCP binding.

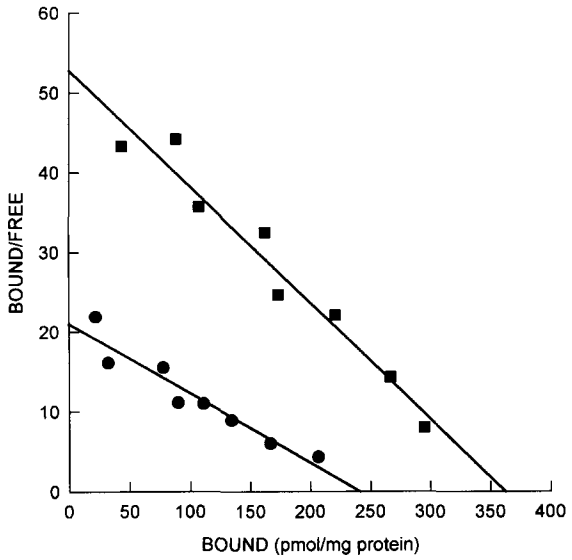

Fig. 1. Scatchard analysis of specific [^{3}H]ACCP (filled circles) or [^{3}H]flunitrazepam (filled squares) binding to $GABA_A$ receptors affinity purified from the brains of adult rats. Data are from a single experiment performed in triplicate with the same $GABA_A$ receptor preparation. The experiment was performed 3 times with similar results.

With purified $GABA_A$ receptor preparations, the binding equilibrium of [^{3}H]ACCP was reached after 60 min of incubation at 4°C. Dissociation of [^{3}H]ACCP initiated by the addition of 10 μM diazepam occurred in a monophasic manner with a $t_{1/2}$ of 298 ± 60 s (mean ± S.D., $n = 3$). From the association and dissociation rate constants a K_d of [^{3}H]ACCP for its binding site of 12.3 ± 1.2 nM (mean ± S.D., $n = 3$) could be calculated. Scatchard analysis of saturation isotherms were linear ($r > 0.97$, $n = 3$) and revealed an almost identical K_d of 12.8 ± 3.5 nM and a B_{max} of 238 ± 10 pmol/mg protein (mean ± S.D., $n = 3$; Fig. 1), whereas [^{3}H]flunitrazepam binding in the same $GABA_A$ receptor preparation exhibited a K_d of 6.6 ± 0.8 nM and a

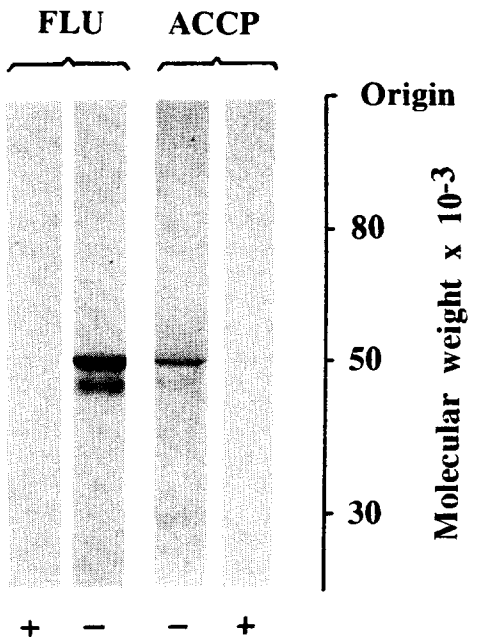

Fig. 2. Fluorography demonstrating irreversible binding of [^{3}H]flunitrazepam or [^{3}H]ACCP to proteins of $GABA_A$ receptors affinity purified from adult rat brain. Affinity-purified $GABA_A$ receptors were incubated with 10 nM [^{3}H]flunitrazepam or [^{3}H]ACCP in the absence or presence of 10 μM diazepam and were then irradiated with UV light as described in Materials and methods. Proteins were then precipitated and subjected to SDS-PAGE and fluorography. The apparent molecular weights of the labeled protein bands were determined by calibration of the gel with standard proteins of known molecular weight. The experiment was performed 3 times with similar results.

B_{max} of 357 ± 7 pmol/mg protein (mean ± S.D., $n = 3$; Fig. 1).

Binding of 5 nM [^{3}H]ACCP to purified $GABA_A$ receptors could be displaced by diazepam, by βCCE and by Ro 15-1788 with IC_{50} values of 67 ± 9.7 nM (mean ± S.D., $n = 3$), 18 ± 3 nM (mean ± S.D., $n = 3$) and 10 ± 1.1 nM (mean ± S.D., $n = 3$), respectively. GABA up to a concentration of 100 μM and chloride ions (150 mM) had no effect on [^{3}H]ACCP binding (experiments not shown).

When [^{3}H]ACCP was incubated with affinity-purified $GABA_A$ receptors and was then irradiated with UV light at 254 nm, a protein with an apparent molecular mass of 51 kDa was irreversibly labeled (Fig. 2). Under these conditions, 16% of the reversibly bound [^{3}H]ACCP became irreversibly bound to the purified receptors. In agreement with previous results (Fuchs and Sieghart, 1989) for the same receptor preparation, [^{3}H]flunitrazepam was able to photolabel a protein with the same molecular mass, and the efficiency of labeling was identical with that of [^{3}H]ACCP. Thus, 16% of reversible [^{3}H]flunitrazepam binding irreversibly bound to purified $GABA_A$ receptors (Sieghart et al., 1987). Photolabeling with both tritiated ligands could be inhibited in the presence of 10 μM diazepam (Fig. 2). The relatively weak irreversible labeling of the 51 kDa protein by [^{3}H]ACCP was due to the 4-fold lower specific radioactivity of [^{3}H]ACCP as compared to that of [^{3}H]flunitrazepam. The photolabeled protein with an apparent molecular mass of 48 kDa (Fig. 2) represents a degradation product of the major labeled protein and occurred in various quantities in different purified $GABA_A$ receptor preparations.

4. Discussion

In the present investigation, previous studies with the unlabeled ethyl ester of azido-β-carboline-3-carboxylate (Dellouve-Courillon et al., 1989) were extended by characterizing the reversible and irreversible binding properties of the radiolabeled propyl ester of this compound. Results indicate that [^{3}H]ACCP reversibly and specifically binds to a homogenous population of binding sites on $GABA_A$ receptors affinity purified from adult rat brain. The binding affinity of [^{3}H]ACCP ($K_D = 12.8$ nM) was lower than that of the previously investigated ethyl ester of azido-β-carboline-3-carboxylate ($K_i = 3.3$ nM, Dellouve-Courillon et al., 1989), and [^{3}H]ACCP binding could be inhibited by the benzodiazepine agonist diazepam, the inverse agonist βCCE and the benzodiazepine antagonist Ro 15-1788 with nanomolar potencies. This seems to indicate that [^{3}H]ACCP specifically binds to the benzodiazepine binding site of $GABA_A$ receptors. Since GABA or chloride ions were unable to stimulate [^{3}H]ACCP binding, this compound seemed to act as an antagonist or inverse agonist at the benzodiazepine binding site of the $GABA_A$ receptors (Braestrup et al., 1984).

After irradiation with UV light, [^{3}H]ACCP was able to irreversibly and specifically label a protein with an apparent molecular mass of 51 kDa in purified $GABA_A$ receptor preparations. A protein with an identical molecular weight could also be photolabeled with [^{3}H]flunitrazepam and was recently identified as the α_1-subunit of the $GABA_A$ receptor by immunological methods (Zezula et al., 1991). The B_{max} value of reversible [^{3}H]ACCP binding, however, was lower than that of [^{3}H]flunitrazepam binding measured in the same receptor preparation. Since the non-azido form of [^{3}H]ACCP, the β-carboline-3-carboxylate propyl ester, exhibits a 10-fold higher affinity for $GABA_A$ receptors associated with BZ_1-benzodiazepine binding sites (and containing α_1-subunits) than for those containing other types of benzodiazepine binding sites (and other α-subunits, Braestrup et al., 1984; Sieghart, 1992b), this could indicate that [^{3}H]ACCP might be the first BZ_1 binding site-specific photolabel. This conclusion is indirectly supported by our inability to obtain

specific [^{3}H]ACCP binding in $GABA_A$ receptor preparations affinity purified from the brains of 6- to 8-day-old rats. These preparations contain low amounts of $GABA_A$ receptors containing α_1-subunits, but relatively high amounts of receptors containing other α-subunits (Fuchs and Sieghart, 1989; Sieghart et al., 1992b). Since specific [^{3}H]ACCP binding could not be observed in $GABA_A$ receptor preparations containing significant amounts of different α-subunits, the possible selective labeling of α_1-subunits by this compound could not be further investigated.

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