Molecular sizes of photolabeled GABA and benzodiazepine receptor proteins are identical

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Received 22 November 1982

[^{3}H]Muscimol was irreversibly incorporated into rat cerebellar membranes upon irradiation with ultraviolet light. GABA agonists and antagonists inhibited this incorporation. The reversible muscimol-binding decreased after photoaffinity-labeling of the membranes with muscimol. These results indicated that this irreversible incorporation is to the GABA receptor. Photolabeled GABA receptor protein showed its M_r of 50000 ± 1000 in SDS–polyacrylamide gel electrophoresis. This molecular size is identical to that of the benzodiazepine receptor which was photolabeled with ^{3}H-flunitrazepam.

GABA receptor *Photoaffinity label* *Muscimol* *Benzodiazepine receptor* *Flunitrazepam* *Rat cerebellum*

1. INTRODUCTION

The benzodiazepine receptor appears to couple to the γ-aminobutyric acid (GABA) receptor, as suggested by allosteric activation by GABA of benzodiazepine binding [1], by protection of both binding sites from thermal inactivation either by GABA analogues or by benzodiazepines [2], by co-purification of both binding sites [3–5], and by the similarity of barbiturate effects on the binding of both GABA and benzodiazepines to their receptors [6,7]. A subunit of the benzodiazepine was shown to be of M_r 50000 by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis of photolabeled benzodiazepine receptor molecules [8]. However, it is unknown whether GABA and benzodiazepine binding sites are on the same subunit. Therefore, irreversible labeling of the GABA receptor protein is also necessary to resolve this problem. Here, we demonstrate photoaffinity labeling of the GABA receptor protein with GABA agonist, [^{3}H]muscimol. The photolabeled GABA receptor protein has exactly the same M_r as the benzodiazepine receptor in SDS–polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

Crude synaptosomal fractions (P_2) from rat cerebellum [9] were frozen at −20°C until use. After thawing, P_2 (2 mg protein/ml) was incubated with 0.05% Triton X-100 in Buffer A (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride) for 30 min at 37°C and centrifuged for 20 min at 20000 × g. The pellet was washed twice with Buffer A, and then resuspended in 50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and 5% polyethyleneglycol. This procedure removes endogenous inhibitors of the GABA receptor. In photoaffinity labeling experiments, 1 ml of Triton X-100-treated P_2 (about 1 mg protein) was incubated with [^{3}H]muscimol (15.5 Ci/mmol, New England Nuclear) for 20 min at 0°C in the dark, and the sample was then irradiated routinely for 20 min at 0°C with an ultraviolet (UV) lamp (Tokyo Kogaku PUV-1, 250–400 nm) at a distance of 4.5 cm. Membranes were then washed repeatedly by centrifugation in Buffer A containing 100 μM GABA until the radioactivity in the supernatant fell to background level. The radioactivity in the tissue was measured.

Published by Elsevier Biomedical Press
00145793/83/0000—0000/$3.00

The amount of radioactivity retained after irradiation in the presence of 500 μM GABA was termed 'non-specific incorporation' and was subtracted from that incorporated by irradiation in the absence of GABA ('total incorporation') to obtain the 'specific incorporation' of [^{3}H]muscimol.

Photoaffinity-labeling of the benzodiazepine receptors in Triton X-100-treated P_2 was performed with [^{3}H]flunitrazepam (86 Ci/mmol, Amersham), as in [8]. The photolabeled P_2 pellet was subjected to SDS–polyacrylamide gel electrophoresis, and radioactive bands were visualized by fluorography as in [10]. Protein was determined as in [11] with bovine serum albumin as a standard.

3. RESULTS

When the P_2 fraction was incubated with [^{3}H]muscimol and exposed with UV light, specific incorporation into P_2 increased with the time of irradiation and reached a plateau after 30 min (fig.1). To determine whether the [^{3}H]muscimol that was incorporated into P_2 upon irradiation was irreversibly attached, we solubilized photolabeled P_2 by boiling for 5 min in 50 mM Tris–HCl (pH 7.4) containing 2.5% SDS and 2.5% 2-mercaptoethanol. On a Sephadex G-25 column, the radioactivity appeared with the protein in the void fractions. When the void fraction was precipitated with ethanol, suspended in Tris–HCl (pH 7.4), treated with pronase, and again applied to a column of Sephadex G-25, the radioactivity appeared in the fractions with low M_r. Furthermore, in a separate experiment, the incorporated radioactivity was not removed by dialysis after the photolabeled P_2 was solubilized in deoxycholate. These results indicate that [^{3}H]muscimol irreversibly binds to a protein or to a macromolecule containing a protein. Muscimol itself is stable in UV light in the absence of brain tissue as judged by two kinds of thin-layer chromatography.

Scatchard analysis of specifically incorporated [^{3}H]muscimol yielded curvilinear plots, and the apparent affinity constants were calculated by analyzing the data according to the two binding site models as in [12] (fig.2). The results are as follows: K_{d1} = 1.7 ± 0.5 nM, I_{max1} (maximal

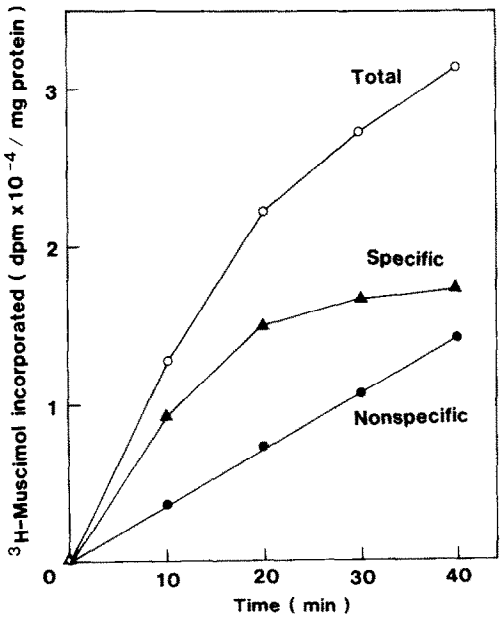

Fig.1. Time-dependent of [^{3}H]muscimol incorporation. P_2 was photolabeled for different times, with 50 nM [^{3}H]muscimol with (non-specific incorporation), and without (total incorporation) 500 μM GABA. Subtracting non-specific from total incorporation gives specific incorporation.

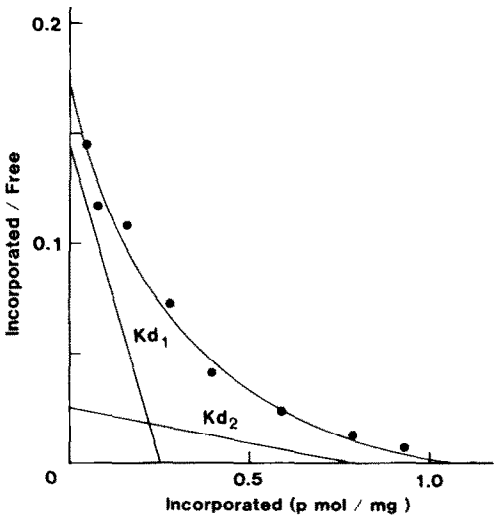

Fig.2. Scatchard analysis of specifically incorporated [^{3}H]muscimol to P_2. Binding constants were calculated as in [10]: K_{d1} = 1.7 ± 0.5 nM, I_{max1} = 0.26 × 0.04 pmol/mg protein; K_{d2} = 31 ± 8 nM, I_{max2} = 0.8 ± 0.04 pmol/mg protein. Values are means ± SEM from 3 expts.

amount specifically incorporated) = 0.26 ± 0.04 pmol/mg protein; K_{d2} = 31 ± 8 nM, I_{max2} = 0.80 ± 0.04 pmol/mg protein. On the other hand, Scatchard analysis of reversible [^{3}H]muscimol binding to Triton X-100-treated P_2 also showed two binding sites (K_{d1} = 1.4 ± 0.2 nM, B_{max1} = 3.2 ± 0.3 pmol/mg protein; K_{d2} = 17 ± 3 nM, B_{max2} = 2.5 ± 0.2 pmol/mg protein). These affinity constants were similar to those of [^{3}H]muscimol incorporation. In the absence of muscimol, the apparent affinities of reversible muscimol binding in the Triton X-100-treated P_2 were not affected by 20-min UV irradiation (K_{d1} = 1.5 ± 0.2 nM, K_{d2} = 20 ± 1 nM). However, the maximal binding of muscimol for high affinity sites was reduced by prior irradiation of the P_2 fraction (B_{max1} = 2.5 ± 0.3 pmol/mg protein), while that for low affinity sites was not affected (B_{max2} = 2.7 ± 0.3 pmol/mg protein). In comparison with this reversible [^{3}H]muscimol binding to irradiated membranes, about 10% of the high affinity sites and 30% of the low affinity sites were photolabeled with muscimol.

When Triton X-100-treated P_2 was irradiated by UV light, with or without cold muscimol (50 nM), washed repeatedly, and subjected to the reversible [^{3}H]muscimol binding assay, the maximal bindings in the membrane irradiated with muscimol were 0.12 ± 0.04 pmol/mg protein (B_{max1}) and 0.57 ± 0.07 pmol/mg protein (B_{max2}) less than those without muscimol. These decreases are quite small, but fairly comparable to the irreversible binding of muscimol. Small decrease of B_{max1} may be due to the protection by muscimol itself from an inactivation of binding sites by UV light, because GABA slightly protected binding sites from it.

If specific incorporation of [^{3}H]muscimol represents its irreversible binding to the GABA receptor, then other GABA agonists and antagonists should prevent specific incorporation of [^{3}H]muscimol with a potency corresponding to their affinities for the GABA receptor. GABA, imidazoleacetic acid (a GABA agonist) and (+)-bicuculline (an antagonist) prevent incorporation of [^{3}H]muscimol. Their IC_{50}-values (the concentration causing 50% inhibition) were 0.13, 1.9, and 6.1 μM, respectively, when 20 nM [^{3}H]muscimol was used for photolabeling. These values are in good agreement with their IC_{50}-values for the inhibition of reversible [^{3}H]GABA binding to the GABA receptor [3]. These results suggest that [^{3}H]muscimol is specifically incorporated into the GABA receptor.

In order to determine the M_r of the GABA receptor protein, after photolabeling with [^{3}H]muscimol, SDS–gel electrophoresis was performed with photolabeled membranes. Labeling was performed with and without excess GABA, and only the samples labeled in the absence of GABA showed a prominent peak of radioactivity (fig.3). This peak corresponded to a protein of M_r of 50000 ± 1000. When the benzodiazepine receptors in the same membrane fraction were photolabeled with [^{3}H]flunitrazepam as in [8], and also

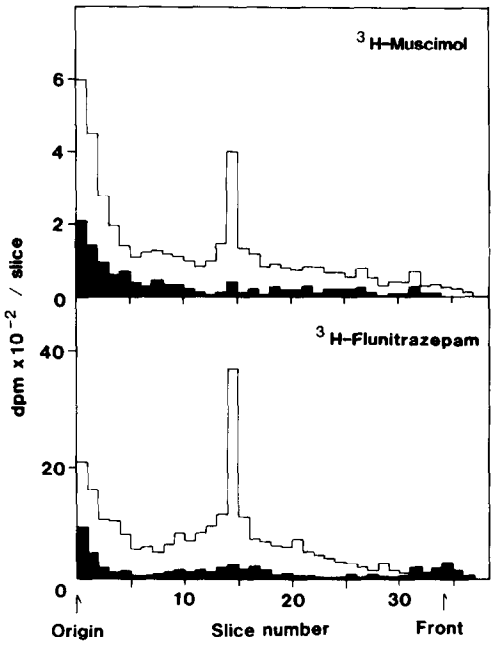

Fig.3. Distribution of radioactivity on SDS–polyacrylamide gel electrophoresis of photolabeled membranes. Triton X-100-treated P_2 were photolabeled with 50 nM [^{3}H]muscimol or 5 nM [^{3}H]flunitrazepam, washed, dissolved in buffer containing 3% SDS and 5% mercaptoethanol, and subjected to 7.5% SDS–polyacrylamide gel electrophoresis. Gel lanes were cut into 2-mm slices and each slice was incubated for 20 h in 5 ml of scintillation cocktail containing 8% Protosol. Using this procedure it was possible to recover more than 80% of the radioactivity applied to a gel lane. Open column: total incorporation; closed column: non-specific incorporation (photolabeled in the presence of 500 μM GABA or 5 μM diazepam).

subjected to SDS–gel electrophoresis, the major radioactive peak showed the M_r of 50000 ± 1000 (fig.3), which is in good agreement with the result in [8]. Therefore, the molecular sizes of GABA and benzodiazepine receptor proteins are identical.

4. DISCUSSION

Various experiments suggested that the benzodiazepine receptor may couple to the GABA receptor, but it was unknown whether GABA and benzodiazepine binding sites are on the same subunit. Here, we have shown that GABA receptors in rat cerebellum were photolabeled with [^{3}H]muscimol, and the M_r of photolabeled GABA receptor was identical to that of the labeled protein of the benzodiazepine receptor. This result suggests that benzodiazepine binding sites may be on the GABA receptor protein. Although both high and low affinity binding sites were irreversibly labeled, low affinity sites were labeled much more than high affinity sites. Since [^{3}H]muscimol incorporated into P_2 showed only one peak on SDS–polyacrylamide gel electrophoresis, it is possible that both high and low affinity binding sites are on a single protein. However, it is also possible that the M_r 50000 polypeptide represents only the low affinity sites. It was recently demonstrated [13] that benzodiazepines increase GABA receptor binding and that this stimulation is attributed only to an increase in the affinity of the lower affinity component of GABA receptors. Thus, the higher affinity component of muscimol binding may represent a separate protein that is not coupled to the benzodiazepine receptor. This material may be accounted for by the 30% of bound [^{3}H]muscimol that did not enter to SDS gel in fig.3.

Since muscimol can be irreversibly incorporated to P_2 up to only 20% of its total reversible binding and, furthermore, the specific radioactivity of [^{3}H]muscimol from commercial source is disadvantageously low (5–20 Ci/mmol) in comparison with [^{3}H]flunitrazepam (60–85 Ci/mmol), the radioactivity incorporated to P_2 is fairly low. Therefore, higher specific radioactivity of [^{3}H]muscimol is needed to use this photolabeling for purification of GABA receptors or to detect the possible minor components from other brain regions such as hippocampus and cortex in which heterogenous benzodiazepine receptor molecules have been demonstrated [10].

ACKNOWLEDGEMENTS

We thank Dr E.M. Ross for his critical reading of the manuscript. This work was supported in part by research grant No. 56770567 from the Ministry of Education, Science and Culture of Japan.

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