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Binding of [^{3}H]α-Dihydropicrotoxinin, a γ-Aminobutyric Acid Synaptic Antagonist, to Rat Brain Membranes

MAHARAJ K. TICKU, MATT BAN, AND RICHARD W. OLSEN

Department of Biochemistry, University of California, Riverside, California 92521

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SUMMARY

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α-Dihydropicrotoxinin, which has a pharmacological activity similar to that of picrotoxin in producing convulsions and inhibiting the synaptic responses of γ-aminobutyric acid in arthropod muscle and the vertebrate central nervous system, was investigated as a possible probe for characterizing γ-aminobutyric acid receptor-ionophore function in mammalian brain. [^{3}H]α-Dihydropicrotoxinin was synthesized and found to bind rapidly, reversibly, and in a saturable fashion to particulate fractions of rat brain homogenates, with an apparent K_D of 1-2 μM. The binding sites showed a similar subcellular as well as brain regional distribution to presumed γ-aminobutyric acid receptor binding sites, consistent with a postsynaptic membrane location. The density of dihydropicrotoxinin binding sites was about 5 pmoles/mg protein, or 130 ± 20 pmoles/g of wet brain, which is within a factor of 2 of the number of γ-aminobutyric acid receptor sites. Six picrotoxin analogues showed an excellent correlation between convulsant activity and potency in inhibiting binding, and some other convulsant and anticonvulsant drugs inhibited the binding, suggesting that the binding sites may be related to the pharmacological effects. γ-Aminobutyric acid (up to 1 mM) and muscimol (0.1 mM) did not affect the binding. Since picrotoxinin has likewise been found not to inhibit γ-aminobutyric acid binding to receptor sites in brain, the results support the interpretation that picrotoxinin inhibits γ-aminobutyric acid synapses by binding at a site distinct from the γ-aminobutyric acid recognition site. The picrotoxinin binding sites are likely to be related to macromolecules that regulate chloride permeability and translate the γ-aminobutyric acid recognition site (receptor) interaction into the physiological response.

INTRODUCTION

γ-Aminobutyric acid appears to be a major neurotransmitter in the vertebrate central nervous system (1, 2) as well as in the peripheral nervous system of many invertebrates (3). In most synapses mediated by GABA,[1] the postsynaptic cell response involves an increased permeability to chloride ion, which inhibits the cell by stabilizing the membrane potential near the resting potential (1-6). GABA synapses are almost invariably inhibited by the convulsant drugs bicuculline and picrotoxin (1-5). Since certain neurological disorders appear to involve dysfunctions in GABA (7), a

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[1] The abbreviation used is: GABA, γ-aminobutyric acid.

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clearer understanding in molecular terms of the postsynaptic action of this neurotransmitter and its inhibition by convulsant drugs would be beneficial.

The inhibition by picrotoxin of GABA synapses is relatively potent and specific (1, 2). Picrotoxin does not resemble GABA in chemical structure, however, and it has been reported (4) to be a noncompetitive inhibitor of GABA-mediated chloride conductance increases in crustacean muscle. Furthermore, picrotoxin, at concentrations (500 μM) far exceeding the biologically effective concentrations [1–10 μM (1, 5, 8, 9)], does not inhibit the binding of radioactive GABA to sites having the properties of postsynaptic receptors in mammalian brain (10–13).[2] This suggests that the drug inhibits the GABA receptor-ionophore function at a site other than the recognition site (receptor) for the neurotransmitter. However, the exact mechanism of action of picrotoxin is still unknown, and warrants further study.

In an attempt to learn more about the action of picrotoxin, we have synthesized a radioactive picrotoxin analogue and investigated its binding to tissues receiving GABA innervation. Whereas numerous neurotransmitter receptor proteins have been identified and assayed by radioactive ligand binding, and some even purified (14), in general it is difficult to assay *in vitro* other macromolecules that may be involved in neurotransmitter responses, e.g., ionophores, unless one has the good fortune that all functions are contained within the receptor macromolecule. Few drugs or toxins (e.g., refs. 15–17) are known to interact with these ionophores, elements that are so far only functionally defined. Picrotoxin provides such a possibility for probing the function of GABA receptor ionophores.

Picrotoxin, a natural product isolated from plants of the Menispermaceae family, consists of the more active picrotoxinin and the less active picrotin in a 1:1 molar ratio. Structure-function studies in mice (18) indicate that α-dihydropicrotoxinin is only slightly less active as a convulsant than picrotoxinin, and we have confirmed this potency profile in several species. We report that radioactive [^{3}H]α-dihydropicrotoxinin binds to particulate fractions of rat brain in a manner consistent with the physiological action of the drug in inhibiting postsynaptic receptor ionophore sites for GABA.

A preliminary report of part of this work has appeared (19).

[2] D. V. Greenlee, P. C. Van Ness, and R. W. Olsen, manuscript submitted for publication.

MATERIALS AND METHODS

Materials

Picrotoxin was obtained from Aldrich, and picrotoxinin was separated and purified by the method of Jarboe and Porter (20). The α-dihydro derivative of picrotoxinin was obtained from picrotoxinin by catalytic hydrogenation at room temperature and under 1 atm of H_2 gas, in glacial acetic acid, using 5% platinum on charcoal as the catalyst (Fig. 1). The product was distinguished from starting material on the basis of the melting point [255°; reported, 252° (21)], whereas picrotoxinin has m.p. 210°. The dihydro derivative also had a slightly greater R_F on thin-layer chromatography in chloroform–methanol (95:5) of 0.51 (picrotoxinin, 0.45), and stained less well with iodine than the parent compound. Furthermore, the infrared and proton magnetic resonance spectra were consistent with complete conversion to product. The NMR spectrum of the product showed the disappearance of both a broad singlet at 1.94 ppm and a broad singlet at 4.98 ppm, assigned to 3 protons on the isopropylidene methyl, C_{14} (numbering system of ref. 22), and 2 protons on the vicinal C_{13}, respectively, in picrotoxinin (23). A doublet appeared at 1.10 ppm, assigned to 6 protons of C_{13} and C_{14} in α-dihydropicrotoxinin, consistent with reduction of the double bond. A single peak at 1.22 also shifted to 1.33 (assigned to 3 protons on C_{10}). By these criteria the pure α-dihydropicrotoxinin, after recrystallization from acetone–cyclohexane, was obtained in 95% yield.

[^{3}H]α-Dihydroxypicrotoxinin was synthesized by catalytic hydrogenation of picrotoxinin with $^3H_2$ by Amersham/Searle. Briefly, pure picrotoxinin (3 mg $\cong$ 10 μmoles) was dissolved in about 5 ml of glacial acetic acid, to which were added about 3 mg of 5% platinum on charcoal that had been flushed with $^3H_2$ gas. At room

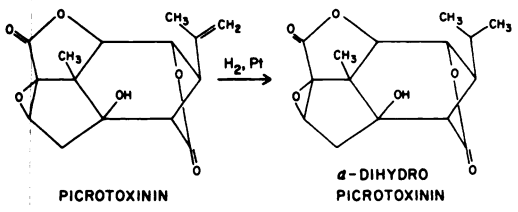

FIG. 1. *Chemical structures of picrotoxinin and α-dihydropicrotoxinin*

temperature and atmospheric pressure, the material was then exposed to 15 Ci of carrier-free $^3H_2$ for 30 min. The mixture was then filtered, and the catalyst was washed with several volumes of acetic acid and then ethanol. The solvent (presumably having removed all the readily exchangeable hydrogen atoms from the product) was evaporated, and the residue was dissolved in a small volume of ethanol for storage (yield, 90 ± 10 mCi).

In our laboratory the product was purified by thin-layer chromatography in chloroform–methanol (95:5). [^{3}H]α-Dihydropicrotoxinin moved as a single radioactive peak with the same R_F value (0.51) as pure dihydropicrotoxinin; 97.5% of the radioactivity was recovered in this peak.

The yield of the tritiated product was 90 mCi, which indicated that the specific radioactivity of the product had a minimum value of 8 Ci/mmole (assuming no loss of material but some dilution of ^{3}H isotope during the reaction and washes) and a maximum value of 58 Ci/mmole (assuming a theoretical yield of 2 ^{3}H atoms incorporated per molecule of product, with no dilution of isotope but some loss of material; such loss could be more substantial than in the nonradioactive synthesis). Since the amount of labeled material was rather small for either chemical or biological assay, the specific activity could be estimated more accurately only by dilution with approximately equivalent amounts of nonradioactive ligand and measuring the activity in binding to tissue, for example, as described below. This technique is valid only if the product is a single compound having the same structure and activity as the unlabeled dihydropicrotoxinin. The purity and nature of the labeled compound appear satisfactory, but possible isotope effects in equilibrium binding activity cannot be estimated. By plotting the amount of specific ligand binding to tissue as a function of ligand concentration, the same curve was obtained (not shown) using [^{3}H]α-dihydropicrotoxinin, either undiluted with nonradioactive dihydropicrotoxinin or diluted 2- or 8-fold with nonradioactive ligand. This indicates that the radioactive and nonradioactive ligands are chemically equivalent and gives an estimate of the specific radioactivity of the ligand. The best estimate of the specific radioactivity, based on isotope dilution in this binding assay and details of the chemical synthesis, was 12 ± 4 Ci/mmole.

γ-Aminobutyric acid was obtained from Sigma Chemical Company, and bicuculline, from Pierce Chemicals. Radioactive γ-amino[*G*-^{3}H]*n*-butyric acid (12.5 Ci/mmole) was obtained from Amersham. Picrotoxinin acetate was synthesized from picrotoxinin; m.p. 250–252° [reported, 252–254° (24)]; PMR spectrum consistent with the structure expected. Tutin was a kind gift from Dr. G. A. R. Johnston of Canberra; muscimol, from Dr. P. Krogsgaard-Larsen of Copenhagen; and isopropyl bicyclophosphate (4-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane), from Dr. J. Collins of London.

Methods

Tissue preparation. Male Sprague-Dawley rats (100–250 g) were decapitated, and their brains were removed and homogenized (14 passes at 800 rpm) in 20 volumes of ice-cold 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 2000 rpm (1000 × *g*) for 10 min in a Sorvall SS-34 rotor. The supernatant fraction was saved, and the pellet was washed once with 0.32 M sucrose and centrifuged again at 2000 rpm (1000 × *g*) for 10 min to give fraction P_1. The supernatant fractions were combined and centrifuged at 45,000 rpm (140,000 × *g*) for 45 min in a Spinco 60 Ti rotor. The pellet (microsomal plus mitochondrial fractions) was resuspended in buffer (0.2 M NaCl–5 mM sodium phosphate, pH 7.0 ± 0.1) and centrifuged at 45,000 rpm (140,000 × *g*) for 30 min in the 60 Ti rotor. The pellet (P_2 + P_3) was resus-

pended in the above buffer at a final protein concentration of 1–4 mg/ml for assay.

For subcellular fractionation studies, the method of Whittaker and Barker was used (25) to obtain the crude nuclear, mitochondrial, microsomal, myelin, and synaptosomal fractions. The crude microsomal pellet, fraction P_3, was subfractionated on a linear (0.5–1.5 M) sucrose gradient centrifuged for 5 hr at 50,000 × *g* (rotor SW-27). Fractions were collected by pumping from the bottom of the tube and analyzed for sucrose (refractive index) and protein. All fractions were pooled, diluted 2–3-fold with assay buffer, and collected by pelleting at 140,000 × *g* for 60 min, all at 0–4°.

Ligand binding assays. The binding of [^{3}H]α-dihydropicrotoxinin to membrane fragments from rat brain was measured by a centrifugation assay. Routinely, 1-ml aliquots (1–4 mg of protein) of membrane suspension (from sucrose gradient preparation or fraction P_2 + P_3 of rat brain) were incubated in plastic scintillation vials at 0° for 15 min with 63 nM [^{3}H]α-dihydropicrotoxinin (12 ± 4 Ci/mmole), with or without other drugs, in a total incubation volume of 2 ml. Following incubation, the reaction was stopped by centrifugation for 15 min at 3° at 20,000 rpm (50,000 × *g*) in a Sorvall SM-24 rotor. The supernatant fluid was decanted away, and the vials were rapidly rinsed twice with 7 ml of ice-cold buffer without disturbing the pelleted tissue. Pellets were solubilized overnight with 0.3 ml of Soluene-100 (Packard). To the solubilized material were added 5 ml of a scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene and counted at an efficiency of 29% in a Beckman CPM-100 counter as determined by the internal standard method with [^{3}H]toluene (New England Nuclear). Specific binding was obtained by subtracting from the total pelleted radioactivity the background, i.e., the amount not displaced by high concentrations (0.1 mM) of unlabeled α-dihydropicrotoxinin.

To study the effect of enzymes on picrotoxinin binding, fraction P_2 + P_3 of rat brain was treated with a given concentration of an enzyme for 30 min at 37°. This was followed by centrifugation at 140,000 × *g* for 30 min and resuspension of the pellets in the buffer for [^{3}H]picrotoxinin binding as described above. To study the effect of Cl^- on binding, Cl^- was either varied or replaced by propionate in the buffer during the binding assay.

The binding of [^{3}H]GABA was determined by a similar centrifugation assay. Subcellular fractions of rat brain (obtained as above) were assayed by a modification of the method of Zukin *et al.* (26, 27). The tissue was prepared and assayed as described elsewhere (11–13),[2] i.e., homogenization in more than 10 volumes of distilled H_2O at 0° (osmotic shock) and centrifugation, followed by resuspension of the pellet in H_2O and freezing. The samples were then thawed and centrifuged, resuspended in assay buffer (50 mM Tris-citrate buffer, pH 7.0; no sodium), pelleted, resuspended in the same buffer, frozen, thawed, and repelleted, followed by resuspension in the same buffer at about 1 mg of protein per milliliter, with [^{3}H]GABA at 10 nM (12.6 Ci/mmole). Samples were incubated without or with excess (100 μM) nonradioactive GABA (to determine background) for 5 min at 0°, followed by centrifugation and other treatment as described above for [^{3}H]α-dihydropicrotoxinin binding. The properties of GABA binding sites detected in this manner are totally consistent with their identity as receptor sites (10–13, 26, 27).

Protein was measured by the method of Lowry *et al.* (28).

RESULTS

Characteristics of [^{3}H]α-dihydropicrotoxinin binding. As measured by a centrifugation assay (see above), specific or displaceable binding of [^{3}H]α-dihydropicrotoxinin at 0° (or 22°) to rat brain particulate fractions was observed; i.e., more radioactivity was pelleted with tissue in the presence of labeled ligand alone (1–100 nM) than was pelleted with samples of the same tissue and labeled ligand that also included excess (0.1 mM) nonradioactive α-dihydropicrotoxinin (the latter would saturate all sites of reasonably high affinity). At concentrations of labeled ligand below 30 nM, very little specific binding was observed, indicating that binding sites with a disso-

ciation constant under 100 nM, if present at all, must be present in low quantities [far less than observed for GABA receptor binding sites (12, 13, 27)[2]]. At 30–100 nM labeled ligand, considerable specific binding was observed: under standard assay conditions of 63 nM [^{3}H]dihydropicrotoxinin and with the concentration of nonradioactive ligand varied (Fig. 2), the pelleted radioactivity was decreased by increasing amounts of nonradioactive ligand, leveling off at about 30 μM, whereafter higher concentrations could not decrease the value further, indicating that saturation had occurred, with 50% displacement (IC_{50} value) at 1.3 ± 0.2 μM. The residual nondisplaceable pelleted radioactivity or background, sometimes incorrectly called "nonspecific binding," involves primarily soluble ligand contained in the trapped water of the pelleted tissue, and is virtually the same volume (12 ± 2 μl/mg of protein) for [^{14}C]sucrose, [^{3}H]-GABA in the presence of 0.1 mM nonradioactive GABA, and $^{36}Cl^-$, as well as [^{3}H]-dihydropicrotoxinin.

In the experiment represented in Fig. 2, the background (arrow at lower right) contained 23,009 ± 132 cpm and the experimental point (arrow at upper left) contained 26,031 ± 342. The difference of 3022 cpm (3207 dpm/mg) is significant ($p < 0.001$) by Student's t-test. This amount of specific binding was reproduced dozens of times; the average of six such experiments (mean of means ± standard errors) was 3614 ± 303 dpm/mg. These standard conditions were subsequently employed for further characterization of dihydropicrotoxinin binding. The unfavorable ratio of specific binding to background (1:8.6) was unavoidable because of the low quantity of binding sites in the tissue and a relatively loose ligand affinity. This signal to noise ratio could be improved to 1:4 in certain brain regions and subcellular fractions.

The saturability of binding indicated in Fig. 2 could also be demonstrated by varying the concentration of labeled ligand. The background was found to increase linearly with varying amounts of [^{3}H]dihydropicrotoxinin of constant specific radioactivity (1.34 Ci/mmole). Specific binding deviated

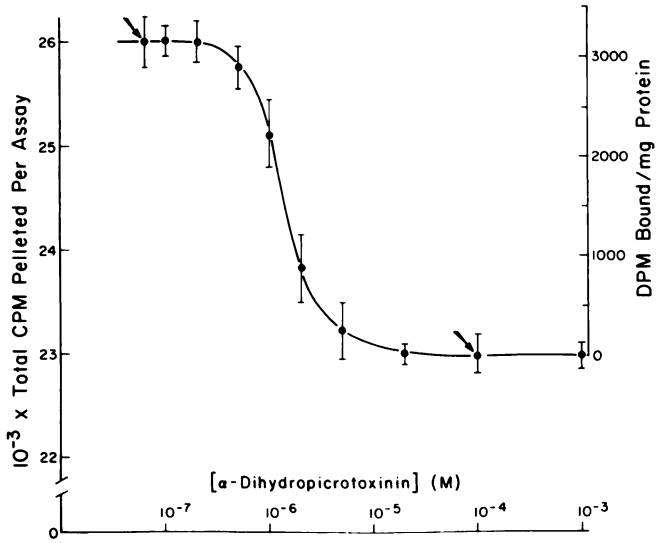

FIG. 2. *[^{3}H]Dihydropicrotoxinin binding to rat brain homogenates as a function of nonradioactive ligand concentration*

The crude mitochondrial plus microsomal fractions of fresh tissue were incubated with[^{3}H]α-dihydropicrotoxinin (63 nM, 12 Ci/mmole) and various concentrations of nonradioactive dihydropicrotoxinin for 15 min at 0° and pelleted to give the counts indicated on the left-hand scale. Each point is the mean of quadruplicates, and the experiment is typical of four. Protein was 3.25 mg/assay. Specific binding, obtained by subtracting the background counts undisplaceable at high ligand concentration, is indicated on the right-hand scale, converted to disintegrations per minute bound per milligram of protein.

from linearity at about 1 μM and tended to saturate at concentrations over 2 μM. Figure 3 represents a double-reciprocal plot of such an experiment. The linear plot obtained indicated an apparent K_D of about 2 μM and a number of binding sites at saturation of about 5 pmoles/mg of protein, corresponding to 130 ± 20 pmoles/g of wet brain.

Specific [^{3}H]α-dihydropicrotoxinin binding in rat brain was linear with protein concentration over the range 1–5 mg (Fig. 4), and all experiments were conducted in this range. At 0° the specific binding reached equilibrium within 5 min and did not change up to 60 min. Therefore assays were routinely incubated at 0° for 15 min. The reversibility of binding was established by adding excess nonradioactive ligand (0.1 mM) to vials that had reached equilibrium with radioactive ligand. This resulted in total dissociation of the bound ligand in 1–5 min (as soon as measurable) at 0°.

Tissue specificity of picrotoxinin binding. Whereas rat brain homogenates showed significant binding of [^{3}H]α-dihydropicrotoxinin, no displaceable binding could be detected with liver or lung, tissues not containing GABA synapses (Table 1). By contrast, specific binding was found with crayfish muscle (29), another tissue known to contain picrotoxin-sensitive, GABA-mediated inhibitory synapses (4). Also, binding varied with brain region, being higher in cerebellum and cerebral cortex than in brain stem (Table 1), a distribution similar to that of GABA receptor binding sites.

Inhibition by drugs. Under the standard binding conditions (63 nM dihydropicrotoxinin, 12 Ci/mmole), high concentrations of dihydropicrotoxinin analogues displaced [^{3}H]α-dihydropicrotoxinin binding down to the same background levels as nonradioactive dihydropicrotoxinin. The IC_{50} values for picrotoxinin (Fig. 5) and tutin were 0.3–0.5 μM, whereas picrotin was a much less active inhibitor, and picrotoxinin acetate and alkali-hydrolyzed picrotoxinin were inactive. The relative activities of these six analogues agreed with their biological activities as convulsants (Table 2).

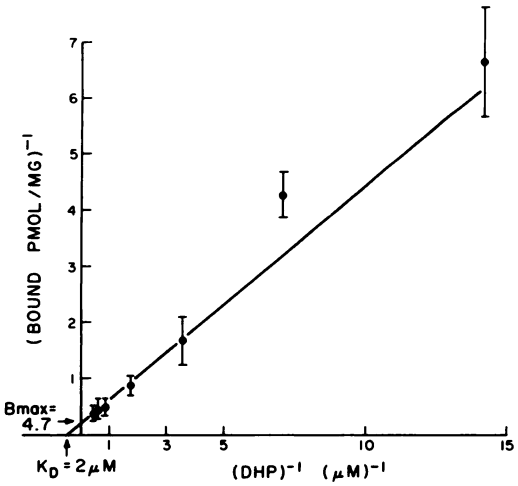

FIG. 3. *Double-reciprocal plot of specific binding vs. concentration of [^{3}H]α-dihydropicrotoxinin (DHP)*

The ligand was held at a constant specific radioactivity of 1.34 Ci/mmole (12 Ci/mmole of stock [^{3}H]dihydropicrotoxinin diluted 9-fold with nonradioactive ligand). Tissue was prepared as described in *Methods*, and binding was determined by the centrifugation assay, with 2.3 mg/ml of protein. The ratio of specific binding to background was about 1:10 (see Fig. 2). Points are means ± standard errors of quadruplicates; the experiment is typical of four.

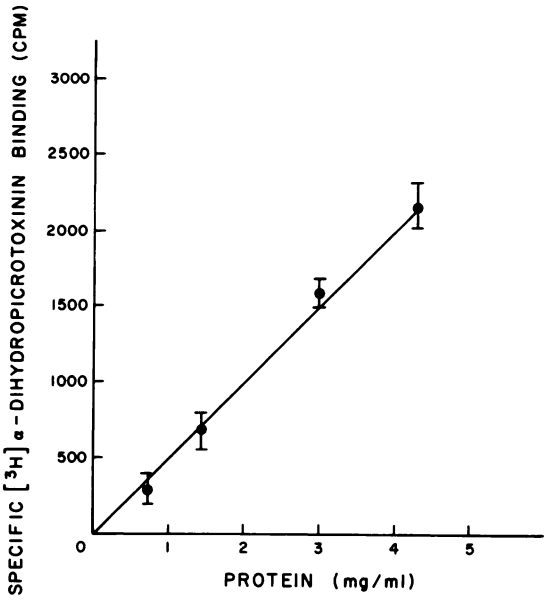

FIG. 4. *Dependence of specific binding of [^{3}H]α-dihydropicrotoxinin to rat brain homogenates on protein concentration*

Tissue preparation and binding assays are described in Fig. 2, where the arrows indicate standard conditions.

TABLE 1

Distribution of picrotoxinin and GABA binding in selected tissues

Tissue in each case was homogenized in 0.32 M sucrose and centrifuged at 1000 × *g* for 10 min, and the pellets were discarded. The supernatant fractions were pelleted at 100,000 × *g* for 1 hr, and these pellets were resuspended for assay of [^{3}H]dihydropicrotoxinin binding, or disrupted, frozen and thawed, and thoroughly washed (11–13)[2] for assay of [^{3}H]GABA binding. Optimal preparation and assay conditions for each ligand were employed. [^{3}H]Dihydropicrotoxinin, 63 nM (12 Ci/mmole), was incubated with the tissue in 0.2 M NaCl–5 mM sodium phosphate buffer, pH 7.0. [^{3}H]GABA, 10 nM (12.6 Ci/mmole), was incubated in 50 mM Tris buffered to pH 7.1 with sodium-free citric acid. In both cases backgrounds were estimated by including 0.1 mM nonradioactive ligand. The ratios of specific binding to background were 1:5 to 1:10 for picrotoxinin and 3:1 to 1:2 for GABA. Values are means ± standard errors of three experiments in quadruplicate.

Region	Specific binding	
	α-Dihydropicrotoxinin	GABA
	dpm/mg protein	
Cerebellum	5,140 ± 520	11,230 ± 620
Cerebral cortex	4,300 ± 690	7,760 ± 600
Brain stem	2,380 ± 440	2,500 ± 90
Liver	NS[a]	
Lung	NS	

[a] No specific binding.

Bicuculline and strychnine inhibited binding, but only at high concentrations ($IC_{50} \cong 100\ \mu M$, Table 2). GABA up to 1 mM and muscimol at 0.1 mM did not significantly inhibit binding, nor did a variety of other drugs and putative neurotransmitters. However, reasonably effective inhibition of binding was found with some anticonvulsant and convulsant drugs. Diphenylhydantoin ($IC_{50} = 140\ \mu M$) and pentobarbital ($IC_{50} = 50\ \mu M$) were effective (Fig. 5) at therapeutic concentrations. Another convulsant, isopropyl bicyclophosphate, inhibited dihydropicrotoxinin binding with an IC_{50} of 10 μM.

Effects of enzymes and chloride. Treatment of membrane fragments ($P_2 + P_3$) of rat brain with 2 μg/ml of trypsin resulted in 59% inhibition of specific [^{3}H]α-dihydropicrotoxinin binding; other enzymes, such as phospholipase C (up to 10 μg/ml) and neuraminidase (up to 25 μg/ml), had no effect on binding (Table 3). Increasing the chloride concentration from 200 mM (normal concentration present during binding assays) to 400 mM or replacing it completely with propionate had no significant effect on binding (Table 3). Similarly, replacing the sodium ions by potassium had no significant effect (not shown).

Subcellular localization. The binding of [^{3}H]α-dihydropicrotoxinin and [^{3}H]GABA was measured in various subcellular fractions of rat brain. GABA binding was measured under conditions such that only receptor sites are detected (12, 13, 26, 27). Both types of ligand binding were localized primarily in P_2 (crude mitochondrial) and P_3 (crude microsomal) fractions (Table 4). Within P_2, the binding sites for both ligands were highly enriched in the synaptosomal fraction. On a linear sucrose gradient of the crude microsomal fraction, protein was found in one broad peak ranging from 0.5 to 1.4 M sucrose. This was divided into three fractions, which were pooled and assayed; the majority of binding sites for each ligand were found in lighter (below 1.1 M sucrose) fractions. In all the fractions, picrotoxinin and presumptive GABA receptor binding sites showed a similar although not identical distribution, which is consistent with, but does not prove, a postsynaptic membrane location.

DISCUSSION

[^{3}H]α-Dihydropicrotoxinin, a biologically active analogue (29) of picrotoxin, binds specifically to rat brain membranes but not to non-nervous tissue. The dissociation constant for binding is similar to the biologically effective concentration of picrotoxinin in inhibiting the postsynaptic receptor-ionophore response to GABA in various organisms (29). The quantity of binding sites (130 ± 20 pmoles/g of wet brain) is roughly twice the quantity of GABA binding sites [85 ± 15 pmoles/g (12, 13)[2]]. The subcellular location of dihydropicrotoxinin binding sites in rat brain [and in crayfish muscle (28)] parallels closely, but not exactly, that of GABA receptor sites, with greatest amounts in the synaptosome and light microsomal fractions; the amount of binding varies from one brain region to another and

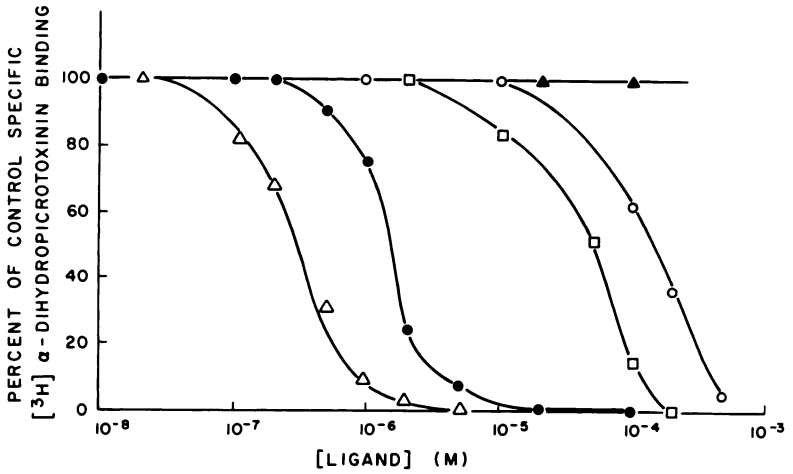

FIG. 5. *Concentration for specific [^{3}H]α-dihydropicrotoxinin binding sites by various ligands*

Specific binding was measured routinely using 63 nM [^{3}H]dihydropicrotoxinin of 12 Ci/mmole and 2 mg/ml of protein, assayed as described in *Methods*. The displacement of specific binding was determined by varying the concentrations of drugs: △, picrotoxinin, ●, dihydropicrotoxinin; □, pentobarbital; ○, diphenylhydantoin; ▲, GABA, muscimol, acetylcholine, glycine, β-alanine.

again resembles the distribution of GABA receptor sites (12, 26) and shows a distribution similar to that of binding sites for radioactive bicuculline methochloride (30).

[^{3}H]α-Dihydropicrotoxinin binding was completely inhibited by 30 μM nonradioactive ligand (IC_{50} = 1.1 μM) and also by picrotoxinin (IC_{50} = 0.4 μM) and tutin (IC_{50} = 0.35 μM); the relative potencies of six picrotoxinin analogues agreed with their relative biological activities, picrotoxinin and tutin being 2-5-fold more active than dihydropicrotoxinin in all systems, picrotin being weaker, and picrotoxinin acetate or alkali-hydrolyzed picrotoxinin inactive (cf. ref. 29).

Other inhibitors of dihydropicrotoxinin binding included the drugs diphenylhydantoin and pentobarbital. The primary action of some barbiturates appears to involve potentiation of inhibition in the nervous system (31–35); they have been reported to mimic and/or potentiate postsynaptic responses to GABA involving chloride conductance (33, 34) (which picrotoxinin blocks) and to reverse the effects of GABA antagonists such as picrotoxin (35, 36). Picrotoxin has been used in treatment of barbiturate overdose (32). Pentobarbital inhibited dihydropicrotoxinin binding (IC_{50} = 50 μM) at concentrations similar to those reportedly required in the nervous system for anesthetic or anticonvulsant activity (37, 38). These concentrations of barbiturates do not inhibit GABA receptor binding or GABA uptake or release from nerve endings *in vitro* (10–12, 39).

Similarly, diphenylhydantoin (0.1 mM) has been reported to prolong the GABA-mediated, chloride-dependent inhibitory postsynaptic potentials in crayfish stretch receptor neurons (40) and to reverse picrotoxin antagonism of GABA-mediated hyperpolarization (41). Diphenylhydantoin also inhibited dihydropicrotoxinin binding. These findings raise the interesting possibility that part of the pharmacological action of pentobarbital and diphenylhydantoin may be related to their binding to the picrotoxin-sensitive sites on the GABA receptor-ionophores.

Likewise, another series of drugs, the bicyclophosphates, appear to act as convulsants in a manner similar to bicuculline and picrotoxin but not to strychnine, and their convulsant action was alleviated by barbiturates (42). These compounds were reported to inhibit GABA responses in spinal cord (43), but, like picrotoxin and barbiturates, the bicyclophosphate convulsants do not inhibit GABA receptor binding (11, 12) [although some biologically less active an-

TABLE 2

Inhibitors of [^{3}H]α-dihydropicrotoxinin binding in rat brain

Tissue preparation and the binding assay are described in *Methods*. Samples of 2 mg/ml of protein were incubated with [^{3}H]α-dihydropicrotoxinin at 12 Ci/mmole (63 nM) and various concentrations of drugs as shown in Fig. 5. In part 1, the > sign signifies no effect up to the concentration listed.

Ligand	Binding IC_{50}[a]	Convulsant activity, CD_{50}
	μM	*mg/kg*
1. Picrotoxin analogues		
Picrotoxinin	0.4 ± 0.2	1.5
Tutin	0.35 ± 0.2	1.5
α-Dihydropicrotoxinin	1.1 ± 0.2	8
Picrotin	70 ± 15	80
Picrotoxinin acetate	>200	>272
Alkali-hydrolyzed picrotoxinin	>200	>300
2. Others		
Isopropyl bicyclophosphate	10 ± 2	
Pentobarbital	50 ± 10	
Diphenylhydantoin	140 ± 15	
Benzylpenicillin	150 ± 20	
Bicuculline	100 ± 35	
Strychnine	100 ± 50	
3. Diazepam, pentylenetetrazole	—[b]	
4. GABA, muscimol, glycine, β-alanine, acetylcholine	—[c]	

[a] Intraperitoneal injection in mice; Jarboe *et al.* (18).

[b] No inhibition up to 10 μM.

[c] No inhibition up to 100 μM.

TABLE 3

Effects of enzymes and ions on specific [^{3}H]α-dihydropicrotoxinin binding the rat brain

Treatment	[^{3}H]α-Dihydropicrotoxinin binding	
	cpm/mg protein	*% control*
Enzyme[a]		
None (control)	880 ± 80	100
Trypsin (2 μg/ml)	360 ± 35	41
Phospholipase C (10 μg/ml)	910 ± 90	103
Neuraminidase (25 μg/ml)	890 ± 85	101
Ions[b]		
200 mM NaCl (control)	985 ± 75	100
200 mM propionate	970 ± 75	98
400 mM NaCl	900 ± 40	90

[a] Membranes were treated with the indicated concentration of enzyme at room temperature for 30 min and then assayed as described in *Methods*. Controls were treated in the same manner without enzyme. Results are means of quadruplicates.

[b] Propionate replaced chloride under isoionic conditions; 400 mM NaCl was included in the phosphate assay buffer instead of 200 mM NaCl. Results are means of quadruplicates, performed twice.

alogues of this type of compound did inhibit GABA receptor binding (10)]. A potent example of this series, isopropyl bicyclophosphate (42, 43), inhibited dihydropicrotoxinin binding reasonably well and also deserves further study to determine whether these drugs and picrotoxinin might share a site of action in the nervous system.

GABA and muscimol, even at high doses, did not inhibit dihydropicrotoxinin binding, consistent with the lack of inhibition by picrotoxinin of GABA binding to receptors (10–12). Bicuculline, a classical GABA antagonist (1, 2), inhibits GABA receptor binding with a K_I value of about 5 μM (10–13); likewise GABA, but not picrotoxinin or barbiturates, inhibits binding of radioactive bicuculline methochloride to brain homogenates (30). Bicuculline inhibits dihydropicrotoxinin binding, but only at concentrations of about 100 μM, where bicuculline is known to cause numerous other nonspecific effects (2, 44). These differential effects on picrotoxinin, bicuculline, and GABA binding suggest that the picrotoxin binding sites, if related to the physiological action of inhibiting GABA synapses, are distinct from the GABA receptor sites, and radioactive dihydropicrotoxinin binding may provide a tool for analysis of some other region of the receptor-ionophore system, perhaps the ionophore. That the two sites are distinct is further demonstrated by the differential sensitivity of GABA and dihydropicrotoxinin binding to inhibition by chemical treatment (12, 45).

Dihydropicrotoxinin binding was not significantly affected by varying chloride concentrations from 0 to 400 mM. Although it was shown that picrotoxin potency in crayfish muscle was inversely related to chloride concentration (4), it is not likely that picro-

TABLE 4

Subcellular localization of [^{3}H]dihydropicrotoxinin binding and GABA receptor binding in rat brain

Assays are described in *Methods*. The binding of [^{3}H]dihydropicrotoxinin (12 Ci/mmole, 63 nM) and [^{3}H]-GABA (12.6 Ci/mmole, 10 nM) was assayed in the same fractions. Results are averages of two preparations of 10 brains each, each assayed in triplicate.

Fraction	Protein[a]	Dihydropicrotoxinin binding		GABA binding	
	mg/g wet tissue	*dpm/mg*	*% total*	*dpm/mg*	*% total*
Crude homogenate	49	1858 ± 96	100	2570 ± 205	100
P_1	13	695 ± 198	11	2387 ± 260	24
P_2	17	2489 ± 303	50	2962 ± 230	39
P_3	14	2405 ± 314	40	3328 ± 210	36
			% P_2		*% P_2*
Mitochondria	7.5	695 ± 54	23	1653 ± 150	38
Synaptosomes	4.0	3058 ± 224	55	4308 ± 310	53
Myelin	3.7	1368 ± 108	23	891 ± 97	9
			% P_3		*% P_3*
P_3-1 (<0.8 M sucrose)	4.0	4563 ± 420	74	2888 ± 150	35
P_3-2 (0.8–1.1 M)	5.0	1284 ± 150	26	2903 ± 200	45
P_3-3 (>1.1 M)	3.6	0	0	1815 ± 300	20

[a] Estimates include particulates only. Aliquots used in the binding assay contained 2–3 mg/ml. All tissue had been frozen and washed thoroughly, as required for GABA binding and satisfactory for picrotoxinin binding. Recovered activity was about 70%.

toxinin, an uncharged molecule, inhibits the chloride ionophore function by competing directly with the chloride ions. Dihydropicrotoxinin binding also was not affected by complete replacement of sodium with potassium ions. The binding was not inhibited by treatment of the tissue with phospholipase C or neuraminidase under conditions known to hydrolyze phospholipid polar head groups and sialic acid residues from glycolipids. The proteolytic enzyme trypsin did, however, inhibit binding, suggesting that a protein is involved.

In the vertebrate central nervous system, picrotoxin has been found to antagonize the action of GABA and inhibitory neurotransmission of several regions (1, 2, 8, 9, 32–34, 46–48), as well as to show convulsant action (18). This action of the drug (except for very high doses) is relatively specific for GABA postsynaptic membrane responses (1, 2, 9, 46, 47), although some reports of nonspecificity have appeared (1, 2, 48, 49). While it is possible that picrotoxin could inhibit some responses to other neurotransmitters, such as glycine (1) or 5-hydroxytryptamine (48), the neurophysiological studies of such effects generally involve polysynaptic preparations, in which the possibility of GABA mediation cannot be ruled out. It is also not certain whether picrotoxin can block all GABA responses (1, 2), but negative results might sometimes be explained by the difficulty in applying the drug to the tissue, or by receptors for other neurotransmitters, e.g., glycine, which are insensitive to picrotoxin but nevertheless could respond to experimentally applied GABA (2).

The possibility that picrotoxinin action on GABA receptor-ionophores represents a relatively nonspecific anesthetic-like action is made unlikely by the potency and specificity of the pharmacological action, and by the demonstration of a relatively low quantity of reasonably high-affinity binding sites in tissues containing GABA synapses, with no indication of any nonsaturable association of drug with the tissue.

At this time it certainly cannot be stated unequivocally that all GABA receptor-ionophores are sensitive to picrotoxinin, nor that all picrotoxinin binding sites are related to GABA receptor-ionophores. Nevertheless, by several criteria, the dihydropicrotoxinin binding sites appear to be

related to the pharmacological action of the drug. Although this ligand does not possess a really high binding affinity, it is adequate to measure significant binding of a unique nature and is the best ligand of its kind currently available. By binding to membrane proteins related to the postsynaptic action of GABA but distinct from the GABA receptor, [^{3}H]α-dihydropicrotoxinin should prove a useful tool in analyzing neurotransmitter and convulsant/anticonvulsant drug action. This site of action appears to involve a modulation of the chloride ion channels that are controlled by GABA receptor binding, or the coupling mechanism between receptor and ion channel.

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