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Dihydropicrotoxinin Binding to Crayfish Muscle Sites Possibly Related to γ-Aminobutyric Acid Receptor-Ionophores

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SUMMARY

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[^{3}H]α-Dihydropicrotoxinin, a biologically active analogue of the γ-aminobutyric acid synaptic antagonist picrotoxin, was found to bind to a small quantity of saturable, high-affinity (K_D, 1 μM) sites in crayfish muscle homogenates. Dihydropicrotoxinin binding was rapid, reversible, and proportional to protein concentration. This binding, like that of γ-aminobutyric acid, was enriched in sarcolemma fractions, being present at 6–8 pmoles/mg of protein or per gram of wet muscle. The quantity of binding sites varied with muscle types in a manner consistent with varying degrees of inhibitory innervation. The binding was inhibited by picrotoxinin analogues in the same order of potency shown by these compounds in convulsant activity or in inhibition of γ-aminobutyric acid synaptic responses: picrotoxinin and tutin were slightly more active than dihydropicrotoxinin in all systems, whereas picrotin was less active and picrotoxinin acetate and alkali-hydrolyzed picrotoxinin were inactive. γ-Aminobutyric acid up to 0.2 mM did not inhibit dihydropicrotoxinin binding. α-Dihydropicrotoxinin binding sites appear to be related to the physiological action of the drug, and this radioactive toxin may provide a useful probe for postsynaptic membrane macromolecules that regulate the inhibitory chloride ionophore at sites distinct from the γ-aminobutyric acid receptor.

INTRODUCTION

γ-Aminobutyric acid is generally accepted as the neurotransmitter at inhibitory neuromuscular junctions in arthropods (1–4) and is a strong candidate for the major inhibitory transmitter in the central nervous system of all phyla (4–6). In general the postsynaptic responses to GABA[3] involve a rapid increase in chloride ion permeability (3–8), which can be blocked by the plant toxin picrotoxin (3–6, 8). In arthropod muscle, picrotoxin is a rather potent and specific antagonist for GABA synapses (3, 4) and inhibits GABA-mediated chloride conductance in crayfish muscle at 1–5 μM (8). Crustacean muscle preparations have been useful for numerous physiological studies of GABA action and pharmacology (e.g., ref. 4).

The action of picrotoxin has been pro-

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

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posed (9) to involve a blockade of GABA-regulated chloride permeability without direct competition for GABA receptor sites. This is consistent with the lack of effect of picrotoxin on GABA binding to sites having the properties of postsynaptic receptors in mammalian brain (10–12) or crayfish muscle (13). The site of action of picrotoxin might therefore involve some other region of the postsynaptic GABA receptor-ionophore complex; radioactive picrotoxin might label these sites. In order to characterize such sites and study the mechanism of action of picrotoxin, we synthesized a radioactive analogue, [^{3}H]α-dihydropicrotoxinin, which is biologically almost as potent in arthropods as picrotoxinin (the active ingredient in picrotoxin). This radioactive ligand was found to bind specifically to sarcolemma-enriched fractions of crayfish muscle in a manner consistent with its pharmacological activity of blocking GABA synapses.

A preliminary report of part of this work has appeared (14).

MATERIALS AND METHODS

[^{3}H]α-Dihydropicrotoxinin was prepared as described in the accompanying paper (15); tutin was a gift from Dr. G. A. R. Johnston of Canberra, and muscimol was a gift from Dr. P. Krogsgaard-Larsen of Copenhagen. Other chemicals, unless noted, were obtained from Sigma Chemical Company.

Convulsant activity of dihydropicrotoxinin. As previously described (16, 17), the activity of housefly (*Musca domestica*) flight motor neurons was measured by recording with wire electrodes postsynaptic potentials at the dorsolongitudinal muscle fibers. The typical activity of a right and left pair of muscles was recorded before, during, and after application of drugs to the exposed thoracic ganglion in saline (154 mM NaCl, 2.7 mM KCl, and 1.8 mM $CaCl_2$, unbuffered). Convulsant drugs have been demonstrated to increase the rate of discharge of these motor neurons and to cause a loss of synchrony between right and left motor units (16).

Intracellular recording of inhibitory postjunctional potentials. Spontaneous or evoked inhibitory postsynaptic potentials of 1–5-mV amplitude were recorded with a glass microelectrode filled with 1 M potassium citrate from the metathoracic coxal muscle (No. 177 or 182) of the cockroach (*Periplaneta americana*) as previously described (17, 18). The physiological saline consisted of 200 mM NaCl, 10.7 mM KCl, 3.4 mM $CaCl_2$, 0.99 mM $MgSO_4$, 2.14 mM $NaHCO_3$, and 0.1 mM NaH_2PO_4, adjusted to pH 6.9. Drugs were added to the saline bathing the neuromuscular junction, and inhibitory postjunctional potentials were monitored for 20 min.

$^{36}Cl^-$ uptake in crayfish muscle. This assay has been described in detail elsewhere (8) and is briefly summarized as follows. Small segments (0.5 cm long, less than 0.1 cm thick) of abdominal muscle of crayfish (*Procambarus clarkii*) were equilibrated in oxygenated Van Harreveld's solution (19) for 60 min. Following equilibration, the muscle strips were exposed for exactly 15 sec to $^{36}Cl^-$ (0.1 μCi/ml, 0.4 μCi/mmole) in Van Harreveld's solution with or without GABA (200 μM). To ascertain the effect of dihydropicrotoxinin and other picrotoxinin analogues on GABA-induced $^{36}Cl^-$ uptake, the muscle strips were previously incubated for 5 min with a given concentration of these antagonists, and the same concentration of the antagonist was also present in the $^{36}Cl^-$ medium (with or without GABA) during the measurement of uptake. The muscle strips were then removed, blotted, weighed, solubilized, and counted (efficiency, 98%) as described earlier (8). The $^{36}Cl^-$ uptake was expressed as $^{36}Cl^-$ space (milliliters per kilogram of wet tissue).

Crayfish tissue preparation. Crayfish (*Procambarus clarkii*) were obtained from local suppliers and housed for up to 1 month in an aerated aquarium. Abdominal muscles were removed, minced, and homogenized in a glass homogenizer fitted with a Teflon pestle in 5–7 volumes of buffered Van Harreveld's solution (205 mM NaCl, 5.4 mM KCl, 13.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.7) by 10–20 passes at 0°. The homogenate was centrifuged at 34,000 rpm (140,000 × *g*) in a Spinco type 35 rotor for 30 min at 0–4°. The pellet was rehomogenized (10 passes)

with Van Harreveld's solution and centrifuged at 2000 rpm (1000 × *g*) in a Sorvall SS-34 rotor for 10 min. The supernatant fraction was saved, and the pellet was washed once with Van Harreveld's solution and recentrifuged as before at 2000 rpm (1000 × *g*) for 10 min in the Sorvall SS-34 rotor. The combined supernatants were centrifuged at 35,000 rpm (140,000 × *g*) for 30 min in the type 35 rotor. The pellet so obtained is the microsomal fraction and is referred to as P_3. Picrotoxinin binding studies were carried out either with the resuspended P_3 fraction or with the enriched plasma membrane fragments obtained by layering P_3 on a linear sucrose gradient as described below.

Linear sucrose gradient centrifugations. P_3 membrane suspension (18 mg of protein in 6 ml) was applied to a linear sucrose gradient (0.3–1.6 M sucrose, 0.1 M NaCl, and 5 mM Tris-HCl, pH 7.7) in a 37-ml nitrocellulose centrifuge tube. Gradients were centrifuged in the Spinco SW-27 rotor at 25,000 rpm (50,000 × *g*) for 2 hr. Fractions were collected by pumping from the bottom of the tube and analyzed for sucrose concentration (by refractive index), protein, and ligand binding.

Binding assays. Binding of [^{3}H]α-dihydropicrotoxinin to the crayfish microsomal fraction (P_3) or the membrane fragments obtained by linear sucrose gradient was studied by centrifugation assay as described for rat brain (15). Routinely, quadruplicate 1-ml samples of membrane fragments (1–3 mg of protein) were incubated in plastic scintillation vials for 15 min at 0° with 63 nM [^{3}H]dihydropicrotoxinin (12 Ci/mmole) in a total incubation medium of 2 ml of 0.2 M NaCl–5 mM sodium phosphate, pH 7.0. To determine the background, excess nonradioactive ligand was included in parallel quadruplicate samples. The vials were briefly mixed (vortex), and the reaction was terminated by centrifuging the vials for 15 min at 0–4° at 20,000 rpm (50,000 × *g*). The supernatant was discarded, and the pellet was gently and rapidly rinsed twice with ice-cold buffer to remove radioactivity adhering to the sides of the vials. The pellet was solubilized overnight with 0.3 ml of Soluene-100 (Packard). To the solubilized material were added 5 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene, and radioactivity was determined in a Beckman CPM-100 counter. Specific binding was determined by subtracting the background from the total bound radioactivity in the pellet. The efficiency of counting was 29% as determined by the internal standard method with [^{3}H]toluene (New England Nuclear).

The binding of [^{3}H]GABA acid to crayfish membrane fragments was measured by a centrifugation assay as described elsewhere (13),[4] which is strictly analogous to that employed for picrotoxinin. Samples of 1–2 mg of particulate protein were incubated at 0° in 1 ml of Van Harreveld's solution containing 44 nM [^{3}H]GABA (12.5 Ci/mmole) for 5 min, followed by centrifugation at 50,000 × *g* for 15 min and superficial rinsing. The vials were prepared for counting as described for picrotoxinin. Under these conditions, GABA binding to crayfish muscle has been found to involve a mixture of sites, including the membrane transport system and synaptic receptors, as judged by numerous criteria (13).[4] That fraction of binding which is inhibited by low concentrations (10 μM or less) of the receptor-specific ligand, muscimol, is taken to represent likely receptor sites. Such a criterion can also be applied to GABA binding in mammalian brain (11).[5]

RESULTS

Blockade of inhibitory synaptic transmission by dihydropicrotoxinin. Jarboe *et al.* (20) compared the convulsant activity of picrotoxinin analogues and reported that intraperitoneal injections of either picrotoxinin or dihydropicrotoxinin produced convulsions in mice, the former being approximately 5 times more potent than the latter. We have confirmed this in our laboratory. Likewise, the potencies of dihydropicrotoxinin and other analogues (tutin, picrotin, and picrotoxinin acetate) were compared with the ability of picrotoxinin to cause convulsions in an insect (housefly)

[4] B. M. Meiners, P. Kehoe, D. M. Shaner, and R. W. Olsen, manuscript in preparation.

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

and to block inhibitory transmission in insect (cockroach) nerve-muscle preparations, measured with intracellular electrodes as previously described (17). Dihydropicrotoxin and tutin were approximately equipotent to picrotoxinin in producing convulsions and in blocking inhibitory postsynaptic potentials at 1 μM, while picrotin, alkali-hydrolyzed picrotoxinin, and picrotoxinin acetate were inactive up to 10 μM (Table 1, column A).

Blockade of GABA-induced ^{36}Cl uptake by dihydropicrotoxinin. Picrotoxinin and dihydropicrotoxinin were compared with respect to their ability to inhibit GABA-induced $^{36}Cl^-$ uptake in crayfish muscle. This assay has been shown to measure GABA receptor-ionophore-mediated events (8). Figure 1 shows that previous incubation of crayfish abdominal muscle with dihydropicrotoxinin for 5 min at room temperature produced concentration-dependent inhibition of GABA-induced $^{36}Cl^-$ uptake. Dihydropicrotoxinin at 5 μM produced 50% inhibition of the increment in $^{36}Cl^-$ uptake induced by just-maximal GABA (200 μM), while picrotin up to 10 μM and alkali-hydrolyzed picrotoxinin up to 100 μM had no effect. Picrotoxinin under identical conditions was found previously (8) to inhibit (IC_{50} = 2–3 μM) GABA-mediated $^{36}Cl^-$ uptake in this muscle. Thus, in this and other assays, dihydropicrotoxinin shows a pharmacological activity similar to

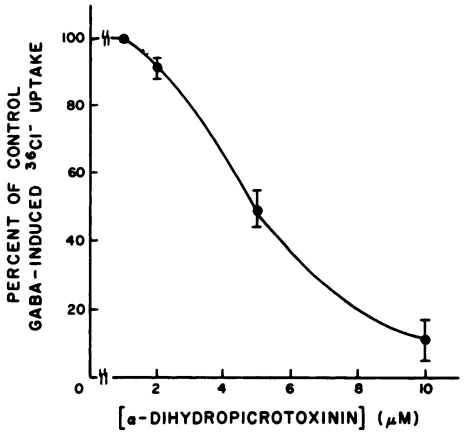

FIG. 1. *Dose dependence of α-dihydropicrotoxinin inhibition of GABA-stimulated chloride permeability in crayfish muscle*

Uptake of $^{36}Cl^-$ by small strips of crayfish abdominal muscle was assayed as previously described (8), with 15-sec incubations with and without just-maximal (200 μM) GABA. The muscles were incubated for 5 min at 23° in Van Harreveld's solution with the concentrations of α-dihydropicrotoxinin indicated prior to $^{36}Cl^-$ uptake measurements. Results are means ± standard errors of five fibers, measured twice.

TABLE 1

Comparison of picrotoxin analogue biological activity and inhibition of binding of [^{3}H]dihydropicrotoxinin

Compound	Invertebrate neurophysiology		Binding IC_{50}		E. Mammalian convulsions (CD_{50} in mice)[e]
	A. Effective concentration in insects[a]	B. IC_{50} in crayfish[b]	C. Crayfish muscle[c]	D. Rat brain[d]	
	μM	μM	μM	μM	mg/kg
Picrotoxinin	1	3	0.6 ± 0.2	0.4 ± 0.2	1.5
Tutin	1	—	0.5 ± 0.2	0.35 ± 0.2	1.5
α-Dihydropicrotoxinin	1	5	1.1 ± 0.2	1.1 ± 0.2	8
Picrotin	>10	>10	45 ± 15	70 ± 15	80
Picrotoxinin acetate	>10	—	>200	>200	>272
Alkali-hydrolyzed picrotoxinin	>10	>100	>200	>200	>300

[a] Causes convulsions when applied to the exposed insect nervous system and also at the same concentration inhibits GABA-mediated inhibitory postsynaptic potentials in insect muscle, assayed according to Olsen *et al.* (17). The > sign in all columns signifies no effect up to the concentration given.

[b] GABA stimulated $^{36}Cl^-$ flux assayed according to Ticku and Olsen (8). Dashes signify that the compound was not tested.

[c] Binding of [^{3}H]dihydropicrotoxinin was measured as described in MATERIALS AND METHODS, and the IC_{50} value (concentration of drug inhibiting 50% of the specific binding) was determined as in Fig. 5.

[d] Ticku *et al.* (15).

[e] Jarboe *et al.* (20), by intraperitoneal injection.

that of picrotoxinin but is 2-5-fold less potent, while picrotin and alkali-hydrolyzed picrotoxinin are relatively inactive (Table 1, column B). Dihydropicrotoxinin was also found to be roughly equal in activity to picrotoxin in inhibiting GABA responses of increased chloride conductance in crayfish stretch receptor.[6]

[^{3}H]α-dihydropicrotoxinin binding. The binding of [^{3}H]α-dihydropicrotoxinin to the particulate fraction of crayfish tail muscle was studied by a centrifugation assay. Routinely, binding was studied at 0° for 15 min with 63 nM [^{3}H]α-dihydropicrotoxinin (12 Ci/mmole) (see MATERIALS AND METHODS). These conditions allow maximum radioactive ligand binding and an optimal ratio of specific binding to background, as discussed regarding Fig. 2 of the accompanying paper (15). Specific, i.e., displaceable, binding was obtained by subtracting from the total radioactivity in the pellet the background, which is the amount of radioactivity that could not be inhibited by excess (0.1 mM) nonradioactive dihydropicrotoxinin. This background appears to be due primarily to trapped solvent in the pellet, since the same value was obtained for radioactive ligands of diverse chemical structure (11, 15).

Under the standard assay conditions, the microsomal fraction bound dihydropicrotoxinin to the extent of 1128 ± 148 dpm/mg of protein (mean ± standard error of five experiments). The specific binding reached equilibrium at 0° in 5 min (Fig. 2); the specific binding was reversible, as shown by an immediate drop to background levels when nonradioactive ligand was introduced. The radioactivity in the background was invariant with time from the earliest point possible to measure. Specific binding was proportional to protein in the 1-5 mg/ml concentration range (not shown).

While binding to the microsomal fraction was displaceable by 0.1 mM nonradioactive ligand, the dissociation constant was difficult to measure accurately, partly because of the low density of the binding sites in this fraction and partly because of high background and large variation within the replicates at high concentrations of ligand (0.8 μM or more). To overcome this problem, the subcellular distribution of [^{3}H]α-dihydropicrotoxinin binding was studied, and an enriched fraction was obtained.

Subcellular distribution of ligand binding. The subcellular distribution of [^{3}H]α-dihydropicrotoxinin binding was determined in various fractions obtained from a linear sucrose gradient (Fig. 3) and compared with GABA binding (Table 2). Assuming that a similar binding function is present in different fractions or tissues, the measurement of binding under standard conditions can be taken as an estimate of the specific activity of binding sites. Since GABA binding to putative receptor sites in crayfish muscle has not been unambiguously characterized, that fraction of radioactive GABA bound to the tissue which can be inhibited by low concentrations (10 μM) of the receptor-specific ligand muscimol is taken to represent likely GABA receptor sites (13).[4] Those fractions (3 + 4) banding

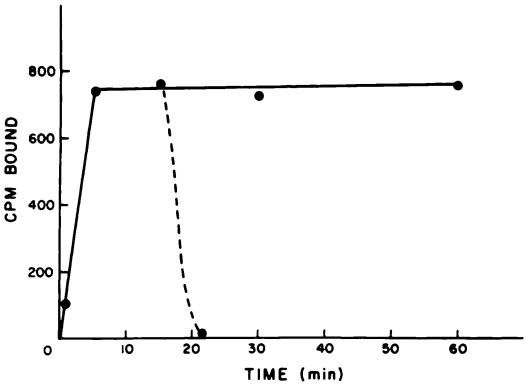

FIG. 2. *Time course of α-dihydropicrotoxinin binding to crayfish muscle homogenate*

Microsomal fraction P_3, 1.7 mg/ml of protein, was incubated for the times indicated at 0° in 0.2 M NaCl-5 mM sodium phosphate buffer, pH 7.0, with [^{3}H]α-dihydropicrotoxinin (12 Ci/mmole, 63 nM) prior to centrifugation. Specific binding was determined by subtracting the radioactivity pelleted with tissue in the presence of 0.1 mM nonradioactive ligand from that pelleted in the absence of nonradioactive ligand. Results are means of triplicates and are typical of three experiments, with variability of less than 15%. After the 15-min point was taken, an aliquot of the reaction mixture containing radioactive ligand only (equilibrium binding obtained) was made 0.1 mM in nonradioactive dihydropicrotoxinin and centrifuged (- - -); this resulted in a return to the baseline (22-min point), which was 7600 ± 100 cpm.

[6] N. Hino, K. Ikeda and E. Roberts, personal communication.

in the 0.8–1.1 M sucrose concentration range gave a 4-fold enrichment of picrotoxinin binding over the applied material (P_3). This pooled fraction (bands 3 + 4) gave 4720 ± 540 dpm/mg bound (mean ± standard error of four experiments) under the standard assay conditions. For example, in a typical experiment, the background was measured to be 20,418 ± 412 cpm and the radioactivity pelleted in the absence of excess nonradioactive ligand was 24,208 ± 287 cpm. The difference was 3790 cpm (signal to noise ratio of 1:5.5 and $p < 0.005$), which is equal to 13,075 dpm bound per 2.5 mg of protein, or 5230 dpm/mg.

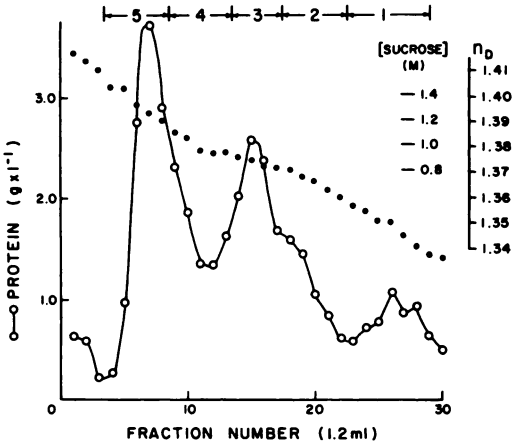

FIG. 3. *Fractionation of crayfish muscle microsomal fraction by sucrose gradient centrifugation*

Aliquots of 33 mg of microsomal protein were applied in 6 ml of Van Harreveld's solution to a 30-ml linear sucrose gradient (0–1.8 M) and centrifuged for 2 hr at 26,000 rpm in rotor SW-27. Fractions were collected and assayed for protein and sucrose concentration. The numbers across the top indicate the fractions that were pooled and assayed for binding of radioactive α-dihydropicrotoxinin and GABA. The profile is typical of over 50 gradients, of which three were analyzed for protein and sucrose.

Most of the picrotoxinin and GABA binding was localized in the same region (bands 3 + 4) (Fig. 3 and Table 2). As in rat brain (11, 15), ligand binding sites for picrotoxinin and GABA showed a similar although not identical distribution. The subcellular fractions in crayfish that bound these ligands have been shown by marker enzyme studies and electron microscopy (13)[4] to be enriched in vesicular plasma membrane and perhaps endoplasmic reticulum, but to be relatively low in actomyosin, mitochondria, and sarcoplasmic reticulum. This distribution of picrotoxinin binding sites is consistent with a postsynaptic (sarcolemma) membrane localization.

Binding of dihydropicrotoxinin to plasma membrane fragments. To characterize [^{3}H]α-dihydropicrotoxinin binding to plasma membrane fragments, the specific radioactivity (12 Ci/mmole) was diluted approximately 9-fold with nonradioactive ligand, to 1.34 Ci/mmole. Figure 4 shows the binding curve obtained by varying the concentration of radioactive ligand. The specific binding (Fig. 4A) deviated from linearity at concentrations over 0.6 μM, tending toward saturation at about 2.4 μM. The background increased linearly over the same concentration range (Fig. 4B). The ratio of specific binding to background was 1:5 at low concentrations and about 1:10 at concentrations over 1 μM. The differences between background and total pelleted li-

TABLE 2

Comparison of dihydropicrotoxinin and GABA binding: subcellular localization in crayfish muscle

Ligand binding was measured as discussed in MATERIALS AND METHODS, with protein at 2.0 mg/ml and [^{3}H]dihydropicrotoxinin at 63 nM (12 Ci/mmole) or [^{3}H]GABA at 44 nM (12.6 Ci/mmole). Background was estimated in the presence of 0.1 mM nonradioactive dihydropicrotoxinin, or 10 μM muscimol for GABA binding.

Tissue fraction[a]	Protein	[^{3}H]Dihydropicrotoxinin binding		[^{3}H]GABA binding inhibited by 10 μM muscimol	
	mg/60 g tissue	*dpm/mg protein*	*% total*	*dpm/mg protein*	*% total*
P_3 (microsomes)	200 ± 20	1200 ± 60	100	1400 ± 200	100
1	21	0	0	0	0
2	24	430 ± 200	3.5	865 ± 150	7
3	43	2150 ± 600	32	3320 ± 1220	48
4	42	4030 ± 990	57	2350 ± 580	32
5	52	340 ± 150	7	630 ± 300	13

[a] Sucrose gradient centrifugation, pooled fractions as described in Fig. 3.

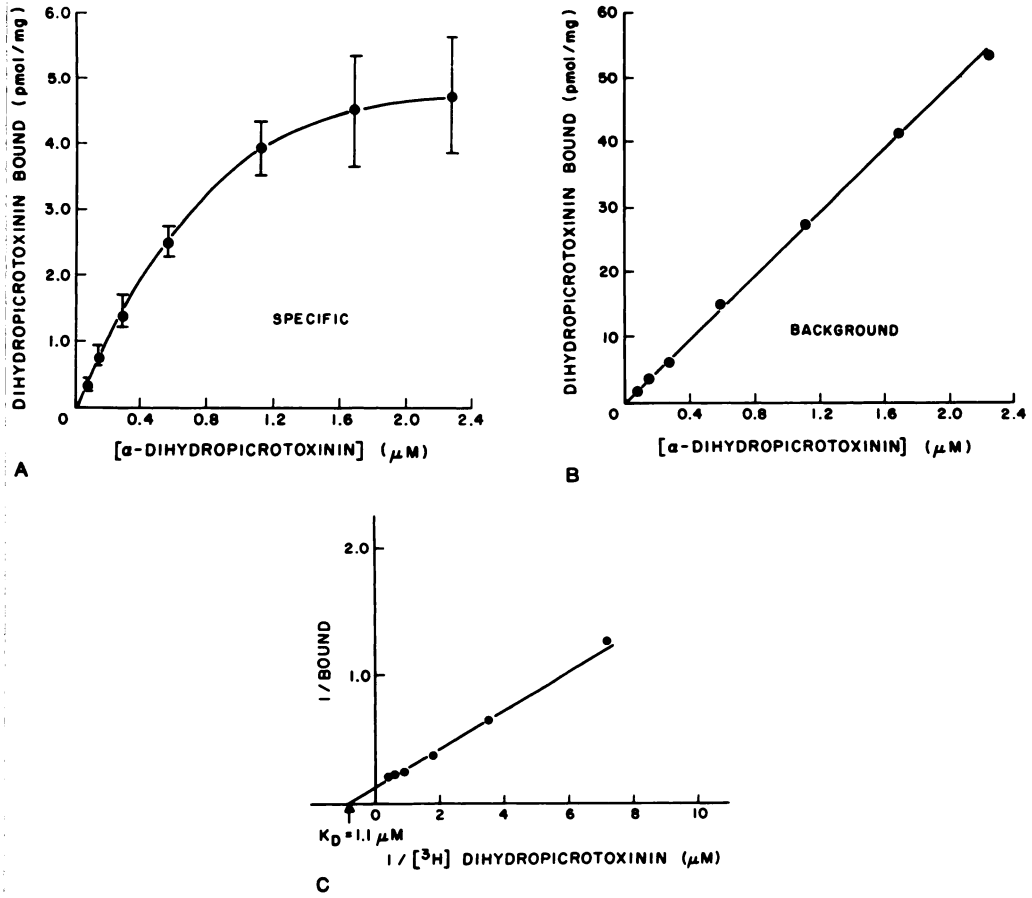

FIG. 4. *[^{3}H]α-Dihydropicrotoxinin binding to crayfish muscle as a function of concentration of ligand*
A. Specific binding vs. concentration. B. Background vs. concentration. C. Double-reciprocal plot of data in Fig. 4A. Crayfish muscle sarcolemma fraction was prepared as described in MATERIALS AND METHODS and assayed by centrifugation. [^{3}H]α-Dihydropicrotoxinin (1.34 Ci/mmole) was varied, with background determined at each point (B), with excess 0.1 mM nonradioactive ligand. Specific binding (A) was obtained by subtracting the background at each point from the total radioactivity pelleted with the tissue. Points are means ± standard errors of two experiments in quadruplicate.

gand at each point were significant ($p < 0.025$). A double-reciprocal plot of specific binding data was linear (Fig. 4C). The apparent K_D was 1.1 μM, and the quantity of binding sites was 6.5 ± 1.5 pmoles/mg of protein, which corresponds to 8 ± 1 pmoles of sites recovered in this fraction per gram of wet muscle.

[^{3}H]α-Dihydropicrotoxinin binding to crayfish membrane fragments was not significantly affected by varying the chloride ion concentration from 0 to 400 mM. Although a slight but insignificant (10–15%) decrease in specific binding was observed under standard assay conditions (200 mM NaCl) compared with zero chloride concentration (using 200 mM sodium propionate), higher chloride concentrations (up to 400 mM) did not cause a further decrease in binding.

Displacement of bound [^{3}H]α-dihydropicrotoxinin. Figure 5 shows some examples of the displacement of bound [^{3}H]α-dihydropicrotoxinin by various ligands. All assays were performed under standard conditions, in which optimal binding was obtained (4000–5000 dpm/mg) in the absence of displacing ligands. Nonradioactive α-di-

hydropicrotoxinin at 30 μM completely inhibited the specifically bound ligand, displacing 50% at 1.1 μM. High concentrations (more than 10 μM) of picrotoxinin inhibited the specific binding down to the same background levels. Picrotoxinin was slightly more potent than dihydropicrotoxinin (as it also is pharmacologically), with an IC_{50} value of 0.6 μM. The GABA antagonist bicuculline weakly inhibited [^{3}H]α-dihydropicrotoxinin binding (IC_{50} = 200 μM), whereas GABA (up to 0.2 mM), sucrose, glutamate, and muscimol (0.1 mM) had no effect on binding.

Table 1, column C, indicates that tutin (IC_{50} = 0.5 μM) and picrotoxinin were the most potent inhibitors of binding, being 2–3 times more active than α-dihydropicrotoxinin. Picrotin (IC_{50} = 45 μM) was a much weaker inhibitor of binding, and picrotoxinin acetate and alkali-hydrolyzed picrotoxinin were inactive. Likewise, alkali-hydrolyzed samples of labeled ligand did not bind. This order of potency agreed perfectly with the pharmacological activity of these six compounds, compared for convulsant dose in mice (20), convulsant concentration in insects, concentration inhibiting GABA-mediated inhibitory postsynaptic potentials in insect muscle, and concentration inhibiting GABA-stimulated increase in chloride permeability in crayfish muscle (this study). Virtually identical values were also observed for inhibition of [^{3}H]α-dihydropicrotoxinin binding to rat brain membranes (11, 15).

[^{3}H]α-Dihydropicrotoxinin binding to different muscle fibers. The amount of muscimol-sensitive GABA binding and dihydropicrotoxinin binding was determined in four different types of crayfish muscle fibers. Several grams of each fiber type were carefully dissected and subjected to the same tissue fractionation scheme outlined above for total tail, and the plasma membrane-rich fractions were obtained for assay. The three abdominal muscles gave a pattern identical with that seen in Fig. 3. Claw muscle was more difficult to dissect cleanly, and also the sucrose gradient centrifugation pattern for this muscle was more complex than that of the abdominal muscles, containing the same tissue bands seen in Fig. 3 plus some additional bands. The tissue corresponding to the sarcolemma (bands 3 + 4 in Fig. 3) was taken in all cases. Under the standard assay conditions, both dihydropicrotoxinin binding and muscimol-sensitive GABA binding were enriched in these purified fractions (ratio of specific binding to background was about 1:3), and the two showed a similar variation in the four muscles studied (Table 3). Furthermore, variation from muscle to muscle showed a correlation with the degree of inhibitory GABA-ergic innervation. High-

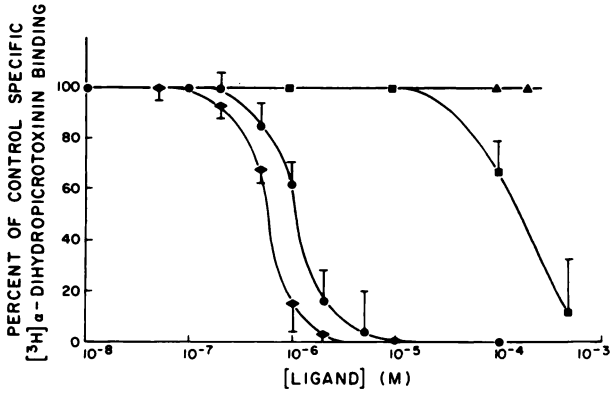

FIG. 5. *Displacement of specific [^{3}H]α-dihydropicrotoxinin binding to crayfish muscle by analogues*

Sarcolemma fraction (2–4 mg/ml) was prepared and assayed with 63 nM (12 Ci/mmole) radioactive ligand as described in MATERIALS AND METHODS. Backgrounds were determined with 0.1 mM nonradioactive dihydropicrotoxinin. Specific binding was measured in the presence of varied concentrations of the following compounds: ◆, picrotoxinin; ●, α-dihydropicrotoxinin; ■, bicuculline; ▲, GABA, muscimol, sucrose, glutamate. Points are means of two or more experiments done in quadruplicate.

TABLE 3

Comparison of [^{3}H]α-dihydropicrotoxinin and [^{3}H]GABA binding in different crayfish muscle types

Each muscle type (30 g) was fractionated as described in Fig. 3 on two 37-ml sucrose gradients to give pooled bands 3 + 4 (about 40 mg of protein); each pool was then divided in half for the two types of assays, given as the means ± standard errors of quadruplicates. Binding of radioactive ligands was measured as described in MATERIALS AND METHODS, using the standard assay conditions of 63 nM [^{3}H]dihydropicrotoxinin (12 Ci/mmole) or 44 nM [^{3}H]GABA (12.5 Ci/mmole) and buffers optimal for each. Dihydropicrotoxinin assays were done without and with excess 0.1 mM nonradioactive ligand to measure background; GABA assays were done without and with 10 μM muscimol.

Muscle	[^{3}H]Dihydropicrotoxinin	Relative binding	[^{3}H]GABA	Relative binding
	dpm/mg		*dpm/mg*	
Abdominal				
Fast flexor	10,900 ± 1,680	1.0	8,700 ± 1,170	1.0
Superficial tonic flexor	9,710 ± 2,510	0.89	6,450 ± 1,420	0.74
Deep medial extensor	16,510 ± 2,360	1.51	14,520 ± 2,490	1.67
Claw	6,650 ± 2,150	0.61	4,830 ± 580	0.56

est binding activity was observed with abdominal deep medial extensor fibers; these are known to have considerable GABAergic innervation (21). Less binding was found with claw muscles (although the fraction assayed was perhaps less pure) and tonic abdominal flexor fibers. The latter were reported to contain less inhibitory innervation than the fast flexor or extensor fibers (21).

DISCUSSION

The binding of dihydropicrotoxinin specifically to tissues containing synapses for GABA with a dissociation constant similar to its biologically effective concentration (1 μM) argues that such binding sites are related to the site of action of picrotoxin in inhibiting the postsynaptic receptor-ionophore response to GABA. The regional and subcellular location of dihydropicrotoxinin binding sites in crayfish muscle [as well as rat brain (15)] parallels closely, but not exactly, that of GABA binding sites, with the greatest amounts found in the sarcolemma fractions. [^{3}H]α-Dihydropicrotoxinin binding was saturable (6.5 pmoles/mg of protein) with increasing concentrations of labeled ligand, and also was inhibited by high concentrations of nonradioactive dihydropicrotoxinin and some analogues in parallel displacement curves and down to the same baseline level. Half-maximal displacement with nonradioactive dihydropicrotoxinin (IC_{50}) occurred at 1.1 μM, and for tutin and picrotoxinin, IC_{50} = 0.6 μM; the relative potency of these three agreed with their relative biological activities, picrotoxinin and tutin being 2–5 times more active in all systems. Likewise, biologically inactive picrotoxin analogues were poor inhibitors of binding.

GABA, even at high doses, did not inhibit dihydropicrotoxinin binding, consistent with the lack of inhibition by picrotoxinin of GABA binding to receptors (10–12). This suggests that the picrotoxinin binding site involves some region of the receptor-ionophore system other than the GABA recognition site, perhaps the chloride channel coupled to GABA receptors. Bicuculline, a classical GABA antagonist (5), inhibits GABA receptor binding with a K_I value of about 1–10 μM (10–12); it inhibited dihydropicrotoxinin binding also, but only at concentrations of 100 μM, where bicuculline is known to cause numerous other nonspecific effects (17, 22). This differential action of bicuculline on picrotoxinin and GABA binding further supports the idea that the two sites are distinct.

Dihydropicrotoxinin binding was not significantly affected by varying the chloride concentrations from 0 to 400 mM. Picrotoxin action was reported (9) to be more potent in inhibiting GABA responses in crayfish muscle in a chloride-deficient medium, but this observation was not confirmed in the stretch receptor neuron (23)[6]. Picrotoxinin, an uncharged molecule, is not likely to inhibit the chloride ionophore function by competing directly with the

chloride ions.

Picrotoxin produces consistent, specific inhibition of peripheral inhibitory neuromuscular junctions in arthropods (2–4, 8, 9, 24–28); this action in crayfish muscle has been reported to be noncompetitive (9) or competitive (23)[6] with GABA. No effects of picrotoxin on other types of synapses or on nerve conduction have been reported, except for blockade of axonal conduction at high doses (25).

Thus picrotoxin action is directed quite uniquely at the postsynaptic GABA receptor-ionophore complex, and at a site distinct from the GABA receptor (recognition) site. However, it is not clear whether picrotoxinin acts directly on the ion channel site, at an allosteric site affecting the ion channel, or at a membrane location near to and perturbing the ion channel for chloride translocation. Radioactive α-dihydropicrotoxinin binds to crayfish muscle sites that appear, on the basis of binding affinity, quantity, tissue specificity, and chemical specificity, to be related to the drug action. This ligand may thus provide a useful probe of GABA and picrotoxin action.

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