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Binding of γ-Aminobutyric Acid to Crayfish Muscle and Its Relationship to Receptor Sites

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SUMMARY

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Binding of γ-amino[^{14}C]butyric acid (GABA) to crayfish muscle particulate fractions was measured by equilibrium dialysis at 0° in Tris-buffered Van Harreveld's solution containing β-mercaptoethanol. Binding was found to saturate at about 7 pmoles/mg of particulate protein (200 pmoles/g of wet tissue), and the concentration of GABA giving half-maximal binding was 1.3 ± 0.5 μM. A single class of binding sites exhibiting no cooperativity was apparently involved. GABA binding was insensitive to several treatments which would break cell membranes. It was partially inhibited by Na^+-free conditions, but was less sensitive in this regard than membrane transport of GABA in either unhomogenized crayfish muscle or mouse brain particles. Binding, however, like transport, was inhibited by sulfhydryl reagents. Picrotoxin did not inhibit binding at 0.3 mM, but bicuculline was a weak inhibitor (K_I = 350 μM). Chlorpromazine and imipramine were also weak inhibitors. The quantity, affinity, and tissue location of the binding sites were thus consistent with either synaptic receptor sites or transport binding sites. However, the K_m value for GABA transport showed significantly lower affinity, 22 μM. Furthermore, comparison of inhibition potency for several GABA structural analogues revealed significant differences between binding and transport; in particular D-glutamic acid and imidazoleacetic acid inhibited binding but not transport; nipecotic acid inhibited transport but not binding. Reasonably good agreement in specificity was found for compounds active on GABA synaptic receptors and in inhibition of GABA binding. Although the concentration of GABA giving half-maximal binding is lower than the concentrations usually reported to give half-maximal synaptic responses, various explanations for this discrepancy are available. We conclude that these cell-free binding studies probably measure physiological GABA receptor sites, although high-affinity binding to sites involved in membrane transport or other biological activity of GABA could also contribute. Because of the lack of totally selective blocking agents, these sites cannot be unamibiguously distinguished at this time.

INTRODUCTION

Several lines of evidence suggest that γ-aminobutyric acid is a neurotransmitter at inhibitory synapses of invertebrate neuromuscular junctions and nervous systems (1–4) and in the vertebrate central nervous

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system (1–3, 5, 6). GABA[1] synapses may be the most abundant ones in the nervous systems of most animals (7, 8). Neurotransmitters such as GABA are believed to regulate the membrane ion permeability of the postsynaptic cell through a specific combination with a membrane receptor protein, as has been well demonstrated for the acetylcholine system (9–12). Anatomical, physiological, biochemical, and pharmacological investigations of arthropod muscle preparations suggest that GABA synapses and consequently GABA receptor sites may be relatively rich in such tissues (1–4, 7). Thus it is not unreasonable to expect that these tissues might bind the neurotransmitter *in vitro*.

We have initiated a search for GABA receptors by looking for the binding of radioactive GABA to particulate fractions of homogenized crayfish abdominal and claw muscles. Binding of GABA does occur, but the conclusion that the binding activity observed represents neurotransmitter receptor sites must meet various criteria with respect to location, quantity, physical properties, and, most important, chemical specificity of action, since enzymes and transport systems might contribute to binding activity (11). This is especially important since no ligand having suitable specificity and the high affinity needed for unambiguous assay of GABA receptors *in vitro* is readily available.

The binding of GABA to crayfish muscle particulates reported here has properties which are consistent with those expected of synaptic receptor sites by the criteria mentioned. A comparison of the activities of various inhibitors of this GABA binding with those of mouse brain uptake at 0°, measured by us under similar conditions (13, 14), with GABA transport by crayfish or lobster (15) muscle and with inhibitory neuromuscular synapses in various invertebrates shows that the specificity of this binding agrees well with that of synaptic receptors and differs significantly from that of GABA transport. A preliminary report of this work has appeared (16).

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

MATERIALS AND METHODS

Chemicals. Pluronic detergents were obtained from BASF Wyandotte Corporation; Triton X-100, from Calbiochem; sodium cholate, from Schuchardt, Munich; toluene, from Mallinckrodt; and β-mercaptoethanol, from Eastman. Homohypotaurine was a gift from Dr. B. Jolles-Bergeret; *trans*-4-aminocrotonic acid, 4-aminopentanoic acid, (−)-nipecotic acid, *cis*- and *trans*-3-aminocyclopentane-1-carboxylic acid, and 3-aminopropanesulfonic acid (homotaurine) were kind gifts from Dr. G. A. R. Johnston; imipramine was a gift from Ciba-Geigy; and chlorpromazine was a gift from Smith Kline & French. Bicuculline was purchased from Pierce Chemicals (m.p. 193–196°). Bicucine methyl ester (m.p. 167–169°) was synthesized according to Johnston *et al.* (17) by diazomethane treatment of alkali-hydrolyzed bicuculline (bicucine). Bicuculline methiodide (m.p. 174–175°) was synthesized by allowing bicuculline to react with methyl iodide (17–19). All other chemicals were purchased from Sigma. Radioactive γ-amino[*U*-^{14}C]butyric acid (220–240 mCi/mmole) was obtained from Amersham.

Tissue. Crayfish (*Procambarus clarkii*) were obtained from a local lake or supplier and housed in an aquarium at room temperature, fed on fish food, for up to 5 weeks. Abdominal or claw muscles were removed and immediately homogenized in 5–10 volumes of Tris-buffered Van Harreveld's solution (20) (205 mM NaCl, 5.4 mM KCl, 13.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, 5 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 7.8) by 15–20 passes of a Teflon pestle at 600 rpm in a glass tube at 0°. This homogenate was filtered through Miracloth (Chicopee Mills, New York) and then centrifuged at 100,000 × *g* for 60 min (30,000 rpm in Spinco rotor 65). The pellet was resuspended by homogenization as before in 10 volumes of ice-cold buffer, giving a protein concentration of about 4 mg/ml as determined with the Folin reagent (Fisher Scientific), using crystalline bovine serum albumin as the standard (21). Optical measurements were made on a Beckman DB-GT spectrophotometer. In one instance

crayfish gills and hindgut, mouse liver, and mouse skeletal muscle were treated identically with the crayfish muscle for assays of GABA binding.

In later experiments, including the inhibitor studies, the total particulates were resuspended in the same buffer and centrifuged at low speed (2000 rpm in a Sorvall SS-34 rotor for 10 min). The pellet was washed once by the same procedure and then discarded. Combined supernatants were recentrifuged (30 min at 40,000 rpm in Spinco rotor 60Ti), and the high-speed pellet was resuspended in 5–10 times its volume of buffer for assay. Examination of this tissue by electron microscopy revealed mainly 200-nm-diameter vesicles and fragments of membranes.

Binding assays. All operations were carried out at 0–4°. The resuspended high-speed pellet (0.4 ml) was pipetted into small sacs of alkali- and EDTA-washed dialysis tubing (Van Waters & Rogers No. 20, 0.75 in.). Three or more closed sacs were placed in 50 ml of Tris-buffered Van Harreveld's solution containing 2 mM β-mercaptoethanol, [^{14}C]GABA, nonradioactive GABA, and inhibitors, if desired. The [^{14}C]GABA (228 mCi/mmole) was added to each assay flask at 20,000 cpm/ml, which was about 50 nM. Increasing concentrations of GABA were obtained with nonradioactive GABA (although this resulted in fewer counts bound when GABA concentrations exceeded the binding dissociation constant value). Thus in this method the maximum specific radioactivity and accuracy could be achieved at lower concentrations of ligand. At the ionic strength used, nonspecific ionic binding and Donnan membrane effects in the assay are highly unlikely (22). Sodium-free buffers contained choline chloride in place of NaCl. Experiments testing effects of detergents on binding involved incubating the tissue in normal buffer with detergent for 30 min at 0° and then assaying with detergents inside the sacs and in the dialysis buffers. Flasks were then rotated gently on an Eberbach rotator (Ann Arbor, Mich.) for 10–20 hr (22). Equilibrium was reached in about 5 hr, and no loss of activity was seen for up to 26 hr. At the end of this time the contents of the sacs were removed and 0.2-ml samples of sac contents and of dialysis buffer were collected for counting and protein assay (21). Radioactivity was determined in a Beckman CPM-100 scintillation counter, using 5 ml of a scintillation solution containing 3.0 g of 2,5-diphenyloxazole and 50 ml of Beckman Bio-Solv BBS-3 per liter of toluene. This solution dissolved the tissue (up to 300 μl) immediately, gave no fluorescence, and yielded a counting efficiency of 80%. Thin-layer chromatography was carried out on plates of silica gel G in the solvent 1-butanol–acetic acid–water (3:1:1). The plates were stained with ninhydrin spray, and the radioactivity was located by scraping 1-cm strips into scintillation vials and counting as above.

Transport assays. Thin slices (average 0.1 g) of crayfish tail muscle were incubated for 30 min at room temperature in 2 ml of Tris-HCl-buffered Van Harreveld's solution at pH 7.8 containing 2 mM β-mercaptoethanol. The buffer was then removed, and [^{14}C]GABA was added (200,000 cpm/assay; 230 mCi/mmole; final concentration, 0.4 μM) in 1.0 ml of the same buffer, at room temperature. After an appropriate time the liquid was removed, the tissue was rinsed with 5 ml of the buffer, and then the tissue sample was dissolved in 0.4 ml of 0.1 N NaOH overnight at room temperature and aliquots were taken for determination of protein content and radioactivity. The background radioactivity associated with extracellular space was subtracted from the total; this background was estimated as the radioactivity taken up in the presence of excess (10 mM) nonradioactive GABA and corresponded to approximately 9 ml for a sample of tissue of 0.1 g. This value, 9% ± 2%, also corresponded to the volume of sequestered [^{14}C]inulin (ICN chemicals, 7.7 mCi/mole) (average of six experiments).

RESULTS

GABA binding. Crayfish abdominal muscles were homogenized in Tris-buffered Van Harreveld's solution. Equilibrium dialysis experiments demonstrated the binding at 4° of [^{14}C]GABA to a 100,000 × *g* particulate fraction of this tissue. This

binding was dependent upon the homogenization conditions. The presence of the sulfhydryl-protecting reagent β-mercaptoethanol was required during the homogenization for maximum activity. Figure 1 shows that optimal conditions were obtained by homogenization in 10 mM β-mercaptoethanol followed by resuspension and assay in solution containing 2 mM β-mercaptoethanol. A concentrated (10%, w/v) suspension of particulates, containing 3–4 mg of protein per milliliter, was found to be necessary for detection of the low concentration of binding sites. By discarding a low-speed centrifugation pellet and collecting the high-speed pellet, a particulate preparation was obtained which gave binding of significant radioactivity and which was linear with protein concentration from 1 to 8 mg/ml.

Aminooxyacetic acid, which prevents metabolism of GABA (23), had no effect on GABA binding when included at concentrations up to 0.1 mM. Thin-layer chromatography of an ethanol extract of the tissue after a GABA binding experiment without aminooxyacetic acid revealed that 100% ± 3% of the radioactivity was recovered as GABA (initial radiopurity was 99%) and that no metabolism occurred. The metabolism of other compounds in the experiments was not ruled out. The activities of glutamate decarboxylase (the enzyme catalyzing the breakdown of GABA) and GABA–α-ketoglutarate transaminase (the enzyme catalyzing the breakdown of GABA) in the homogenized particulate preparation were nil.[2]

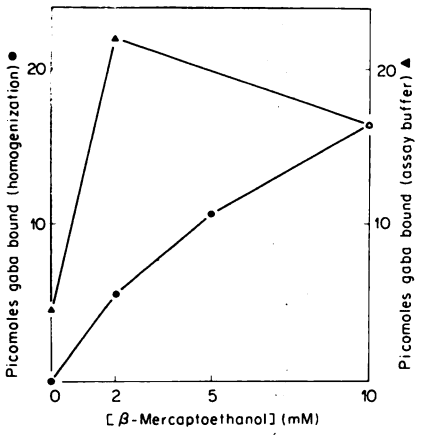

FIG. 1. *Effect of β-mercaptoethanol concentration on binding of GABA to crayfish muscle membranes, as measured by equilibrium dialysis at 0°*

The protein concentration was 3.4 mg/ml, and the GABA concentration was 1 μM; specific radioactivity, 5.7 mCi/mmole. ●, concentration of β-mercaptoethanol in homogenization buffer (Tris-buffered Van Harreveld's solution, pH 7.8) and also in assay buffer; ▲, concentration of β-mercaptoethanol in assay buffer following homogenization in buffer containing 10 mM β-mercaptoethanol. Each point represents triplicate determinations, results of four experiments. Standard error (±3%) was less than the dimensions of the point on the graph. Approximately 400 cpm were bound (in 0.2 ml).

When the concentration of GABA was varied, the binding curve depicted in Fig. 2 was obtained. Each point represents triplicate determinations, which varied by less than 2%. Data from three different tissue preparations (filtered particulate fraction) are included, to indicate the variation encountered. Binding became saturated at about 20 ± 2 pmoles/mg of protein, which is about 7 pmoles/mg of tissue protein or 0.2 nmole/g of fresh tissue. The concentration of GABA giving half-maximal saturation was 1.3 ± 0.5 μM. At least 12 experiments gave similar results. Nonradioactive GABA (1 mM) completely inhibited all [^{14}C]GABA binding. The binding was reversible, as shown by the loss of all bound radioactive GABA when samples were transferred to 100 volumes of nonradioactive buffer.

Binding of GABA to crayfish muscle was obtained with homogenized tail or claw muscle fibers. The latter bound 10 ± 5 pmoles/mg of protein (100,000 × *g* pellet) at saturation, with a half-maximal GABA concentration of 1.1 ± 0.2 μM. Dissection of deep medial extensor, superficial, or deep flexor muscles from the abdomen, or opener or closer muscles from claw, showed that all these muscles bound similar amounts of GABA at saturation. Particulate fractions of crayfish abdominal gut bound a trace of GABA; gills did not bind any GABA, nor did homogenized mouse liver or particulate fractions of mouse muscle. Preliminary experiments have revealed high-affinity binding of GABA by homogenized muscles of other crustacea

[2] R. W. Olsen, unpublished observations.

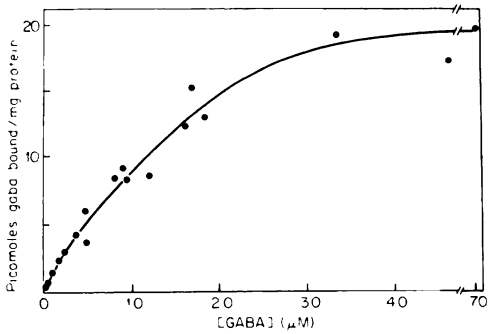

FIG. 2. *[^{14}C]GABA binding to crayfish tail muscle particulate fraction*

Tissue suspensions contained 3.0–3.6 mg of protein per milliliter, and equilibrium dialysis solutions contained 2.5 × 10^4 cpm of [^{14}C]GABA (228 mCi/mmole) per milliliter. The concentration and specific radioactivity were varied with nonradioactive GABA. Three experiments are included, and each point represents triplicate determinations with a 3% variation or less. Approximately 500 cpm were bound (in 0.2 ml).

(e.g., lobsters, crabs, and shrimp) but not with the insect *Periplaneta americana*, another type of arthropod having peripheral innervation for which GABA is probably the transmitter (2–4, 24).

GABA transport by slices of crayfish tail muscle. Thin slices of muscle were incubated at room temperature in Tris-buffered Van Harreveld's solution containing 2 mM β-mercaptoethanol and [^{14}C]GABA. The tissue was filtered and washed at the appropriate time, dissolved, and analyzed for protein content and radioactivity as described under MATERIALS AND METHODS. Blanks contained 10 mM nonradioactive GABA to block specific uptake; radioactivity remaining in the tissue sample under such conditions was considered to be in the extracellular space (see MATERIALS AND METHODS) and was subtracted from all the assays.

Variation of incubation time showed that GABA uptake was linear under these conditions for about 20 min (not shown). Initial velocity measurements were made at 10 min, at which time less than 1% of the total GABA had been taken up by the tissue, so that efflux of radioactive GABA was not likely to be significant. The dependence of initial velocity of GABA transport on GABA concentration is shown in Fig. 3. The apparent K_m for GABA was 22 ± 5 μM. Treatment of the tissue with a sulfhydryl-blocking reagent, 10 mM *N*-ethylmaleimide or 10 mM iodoacetate, for 30 min inhibited transport by 35% or 100%, respectively. 2,4-Dinitrophenol at 1 mM inhibited uptake completely. These properties agree reasonably well with those of lobster muscle (15) and mammalian nervous system (25). Uptake was inhibited by GABA analogues (data not shown) but was unaffected by 1 mM imidazoleacetic acid or DL-glutamic acid, 0.5 mM bicuculline, or 0.3 mM picrotoxin.

Properties of homogenate GABA binding. The effects of various experimental procedures on the binding of GABA to crayfish muscle homogenized particulates are summarized in Table 1.

Repeated freezing and thawing had no effect on the amount of binding to crayfish tail muscle at 0°, although uptake by mouse brain particles at 0° is severely inhibited by this treatment (13, 26). Sonication (2 × 1 min at maximum setting on a Branson Sonifier) and more vigorous homogenizations were also without effect, suggesing binding rather than transmembrane uptake of GABA.

Binding of GABA to crayfish muscle homogenates was virtually the same at 24° as at 0°, whereas uptake into mouse brain particles or crayfish tail was stimulated many fold by warming. The binding to

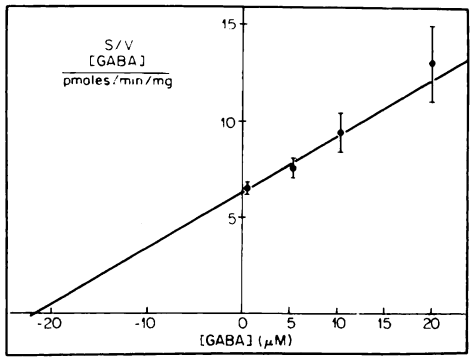

FIG. 3. *Transport of [^{14}C]GABA by slices of crayfish tail muscle*

Each assay employed 0.1 g of tissue in 1 ml of medium containing 2 × 10^5 cpm of [^{14}C]GABA, incubated for 10 min at 24°. Results are the means of three experiments ± standard errors.

TABLE 1

Percentage of control GABA binding under various conditions

Equilibrium dialysis assays of crayfish muscle were performed as described in MATERIALS AND METHODS. Results represent averages of six assays ± standard errors of the mean. GABA concentration was 0.1 μM; specific radioactivity, 100 mCi/mmole. The control binding was 450 ± 50 cpm, equal to 2 pmoles/mg of protein.

Treatment or addition	GABA binding	Treatment or addition	GABA binding
	% control		*% control*
Freezing and thawing	100 ± 10	Detergents	
Sonication	100 ± 10	Triton X-100, 0.5%	10 ± 10
Vigorous homogenization	100 ± 10	Triton X-100, 0.1%	30 ± 10
Warming to 24°	100 ± 10	Triton X-100, 0.05%	70 ± 10
Azide, cyanide, or 2,4-dinitrophenol (1 mM)	95 ± 10	Lubrol PX, 0.5%	5 ± 5
		Cholate, 0.5%	20 ± 10
Boiling	0	Tween 20/80, 0.1%	105 ± 10
Pronase (0.1 mg/ml, 30 min)	0	Brij 58, 0.1%	85 ± 10
Osmotic shock	70 ± 15	Brij 96, 0.1%	30 ± 10
Chlorpromazine (40 μM)		Picrotoxin (0.1 μM GABA)	
0.5 μM GABA	50 ± 10	300 μM	100 ± 10
0.1 μM GABA	50 ± 10	500 μM	85 ± 10
Imipramine (200 μM)		Bicuculline (0.1 μM GABA)	
0.5 μM GABA	50 ± 10	100 μM	95 ± 10
0.1 μM GABA	50 ± 10	220 μM	70 ± 10
Varied [Na^+]		350 μM	50 ± 10
205 mM	100 ± 10	350 μM (0.5 μM GABA)	50 ± 10
10 mM	100 ± 10	Bicuculline methiodide, 250 μM	50 ± 10
3 mM	60 ± 10		
None	20 ± 20		

crayfish muscle was not energy-dependent, as it was not inhibited by 1 mM cyanide, azide, or 2,4-dinitrophenol (Table 1). It was, however, inhibited by exposure to sulfhydryl-blocking groups such as 10 mM iodoacetic acid or *N*-ethylmaleimide for 30 min at 0°.

Subjection of the tissue to osmotic shock (resuspending membranes in distilled water and then assaying in normal buffer) inhibited GABA binding by 30% ± 15% (Table 1). Such treatment almost completely eliminates uptake of GABA by mouse brain particles at 0° (13, 26). Treatment with detergents also partially inhibited binding (Table 1). These concentrations of detergent destroy 100% of mouse brain GABA uptake at 0° (13, 26).[2] Retention of binding in detergent solution also supports the conclusion that the binding activity was not due to uptake into occluded space.

The effect of sodium concentration on GABA binding was analyzed, since GABA transport in rodent brain (8, 13, 25–28) and crustaceae (15) is absolutely dependent on Na^+ concentration. Reducing the Na^+ concentration from 205 to 10 mM had no effect on GABA binding, but lower concentrations of Na^+ resulted in some impairment of binding (40% inhibition at 3 mM), and removal of all Na^+ from the resuspension and dialysis buffer inhibited binding almost completely (80% ± 20% inhibition, Table 1). Thus while GABA binding was Na^+-dependent, i.e., strict removal of all Na^+ inhibited binding, it was different from the mouse brain transport at 0° in that low levels of Na^+ would support almost full activity.

Reciprocal plots of binding (Fig. 4) show that it apparently represents a homogeneous class of sites having an apparent affinity constant, K_D, of 1.3 ± 0.5 μM. Reciprocal plots emphasizing high ligand concentration (Fig. 4A) or low ligand concentration (Fig. 4B) approximated to straight lines, indicating one binding site; never-

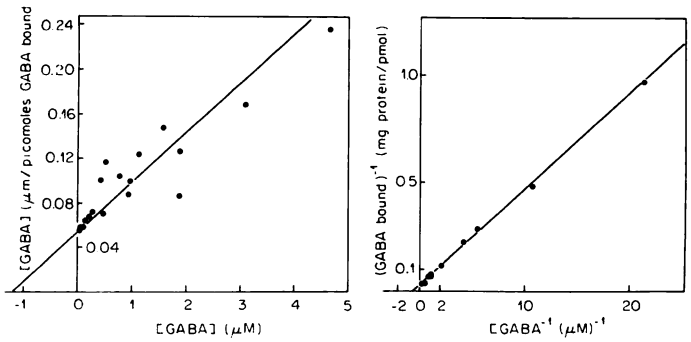

FIG. 4. *Variation of GABA binding to crayfish tail muscle particulates with free GABA concentration* A (left). Same data as Fig. 2, reciprocal plot. B (right). Lineweaver-Burk reciprocal plot. In these experiments the protein concentrations were 3.1 mg/ml; [^{14}C]GABA was the same as in Fig. 4A.

theless, these data do not rule out the possibility that two or even more sites of *similar* affinity contribute to the binding. The binding curves gave no obvious evidence of cooperativity.

Chemical specificity. Various drugs known to block GABA transport or activity at synapses, as well as structural analogues of GABA, were investigated for inhibition of GABA binding, in attempts to distinguish between transport and receptor sites on the basis of selectivity of inhibition.

Chlorpromazine and imipramine weakly inhibited GABA binding to crayfish muscle membranes (Table 1). The concentrations listed blocked 50% of GABA binding; inhibition could not be overcome by high GABA concentrations, suggesting noncompetitive inhibition (Table 1).

When assayed for its effect on crayfish muscle GABA binding, the putative GABA antagonist picrotoxin (2, 29) gave little to no inhibition at 0.5 mM. Another antagonist, bicuculline (30–32), was a weak inhibitor. Apparently noncompetitive inhibition of 50% was found at a concentration of 350 μM (Table 1). Since bicuculline is unstable at physiological pH and temperature (13, 14),[3] we also assayed some bicuculline analogues. Bicucine methyl ester was a weaker inhibitor of GABA binding to crayfish muscle, bicucine did not inhibit at 200 μM, and another potentially active analogue, bicuculline methiodide, gave 50% inhibition at a concentration of 250 μM.

Various structural analogues of GABA inhibited GABA binding competitively. Figure 5 shows a Dixon plot of inhibition by β-hydroxy-GABA, indicating a K_I of 11 ± 4 μM. Likewise, β-guanidinopropionate, DL-2,4-diaminobutyrate, (−)-nipecotic acid, *cis*- and *trans*-3-aminocyclopentane-1-carboxylic acid, *trans*-4-aminocrotonic acid, 4-aminopentanoic acid, imidazoleacetic acid, homohypotaurine, D-glutamic acid, homotaurine, L-glutamic acid, and β-alanine were inhibitors (Table 2, column 3). No inhibition was seen with 1 mM taurine, DL-α-aminobutyric acid, *cis*-4-aminocyclohexane-1-carboxylic acid, L-glutamine, or glycine. These structure-activity relationships (Table 2) seem to correlate better with receptor sites (column 5) than transport (columns 2 and 4).

DISCUSSION

Equilibrium dialysis has allowed the detection of a low level of high-affinity binding sites for GABA in particulate fractions of homogenized crustacean muscle. This binding of GABA appears to involve a single class of sites present at about 200 pmoles/g of wet tissue and having an equilibrium dissociation constant of about 1 μM. Binding occurs with a variety of mus-

[3] R. W. Olsen, M. Ban, and T. Miller, Brain Research, in press.

[4] It can readily be calculated that noncooperative binding curves having a Hill coefficient of 3 require approximately a 10th-order (not third-order) dependence on ligand concentration, and a Hill coefficient of 2 requires a fourth-order dependence.

cles that respond to GABA, but not in some tissues that do not have inhibitory GABA innervation. Assays at 0° in high ionic strength buffer make Donnan effects and nonspecific ionic binding unlikely. Binding to enzymes mediating GABA metabolism, namely, glutamic acid decarboxylase and GABA transaminase, is unlikely to be detectable, because the activity of the enzymes in these tissue fractions is very low, and the affinity of the enzymes for GABA is also low (40, 41).

The concentration of GABA binding sites is higher than that of acetylcholine receptor sites in vertebrate skeletal muscle [1–10 pmoles/g of tissue (42)], equaling the amount found in *Electrophorus* electric organ (0.1 nmole/g of tissue) but not that in *Torpedo* [1 nmole/g of tissue (10, 12, 42, 43)]. The area of postsynaptic membrane in eel electric organ has been roughly estimated as 500 ± 200 mm^2/g of tissue (43) to 2500 ± 500 mm^2/g (12), but in *Torpedo* electric organ as 7×10^4 mm^2/g (42). Using information for the number and density of synapses in crayfish muscle (44, 45), we estimate the area of the inhibitory postsynaptic membrane to be 100–1000 mm^2/g. Thus the quantity of binding sites is compatible with their being receptors.

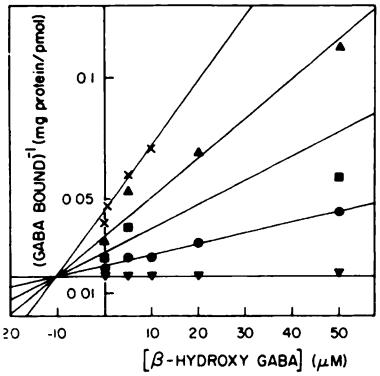

FIG. 5. *Inhibition of GABA binding to crayfish tail muscle membranes by DL-β-hydroxy-γ-aminobutyric acid*

Partially purified particulate fractions were used; [^{14}C]GABA was the same as in Figs. 2 and 4; 500–1000 cpm were bound (in 0.2 ml); and the protein concentration was 2.6 mg/ml. ×, GABA concentration, 0.153 μM; ▲, 0.26 μM; ■, 0.60 μM; ●, 1.0 μM; ▼, 5 μM.

Since a tissue rich in synapses would also be expected to be rich in neurotransmitter transport sites, one must differentiate binding to receptor sites from binding to or even actual uptake by the transport system. GABA transport in lobster muscle at room temperature has a K_m value of 60 μM (15), and our preliminary results here yielded a value of 22 μM for crayfish muscle slices (which include intact cells). Binding of GABA to homogenized muscle has a greater affinity (apparent $K_D = 1$ μM), but this does not rule out binding to the transport system, since the K_m value may not reflect simply the binding step in transport. However, several experiments suggest that the binding of GABA to crayfish muscle particulates is not actual uptake into membrane-bound space. Binding is not diminished by freezing and thawing the tissue or subjecting it to ultrasonic vibration or vigorous homogenization. It is not inhibited by metabolic blocking agents and is not sensitive to temperature. Also, binding persists in lower concentrations of Na^+ than are required for transport (15, 25, 27) and in the presence of some detergents. Binding is inhibited by imipramine and chlorpromazine, drugs known to block GABA transport (13, 15, 25–28), but, at the high concentrations which block binding, these drugs have nonspecific effects on a variety of membrane enzyme and transport activities (46). GABA binding is partially sensitive to osmotic shock, sodium-free buffer, and detergents. Both binding and transport are inhibited by sulfhydryl-alkylating agents.

In Table 2 we have compared activities for various GABA analogues with respect to their inhibition of GABA binding and their activity on GABA transport systems in mouse brain (measured at 0°) and crayfish muscle and on invertebrate GABA-mediated synapses as determined by electrophysiological studies. There is clearly a considerable discrepancy between the chemical specificity of GABA binding and that of GABA transport; on the other hand there is a reasonable agreement between GABA binding inhibition and synaptic activity. With respect to transport, most GABA analogues are better inhibitors of

TABLE 2

Potency of GABA inhibitors

1. Compound	K_I value			
	2. GABA uptake in mouse brain[a]	3. GABA binding in crayfish[b]	4. Crustacean transport[c]	5. Invertebrate GABA synapses[d]
	μM	μM	μM	μM
GABA	28 ± 5[e]	1.3 ± 0.5[f]	60,[c] 22[g]	10–100[h] (3, 4, 29–31, 33–39)
DL-2,4-Diaminobutyrate	26 ± 4	16 ± 10		1,000–10,000 (3,4 33), 100[i]
DL-β-Hydroxy-GABA	45 ± 10	11 ± 4	1000	20–200 (33, 36)
trans-4-Aminocrotonate	60 ± 20	8 ± 5		
β-Guanidinopropionate	135 ± 20	12 ± 10	500	20–200 (33, 36, 39)
(−)-Nipecotic acid	60 ± 10	>250		>400[i]
cis-3-Aminocyclopentane-1-carboxylate	65 ± 10	100 ± 50		
trans-3-Aminocyclopentane-1-carboxylate	130 ± 10	200 ± 50		
4-Aminopentanoate	200 ± 50	300 ± 50		
Imidazoleacetic acid	600 ± 100	90 ± 1	>1000[g]	50–500 (38)
D-Glutamic acid	>500	250 ± 50	>500[g]	?
Homohypotaurine	150 ± 30	500 ± 100		
Homotaurine	500 ± 100	>500		?
β-Alanine	500 ± 100	1,000	>10,000	1,000 (3, 33, 36, 38)
L-Glutamic acid	>500	550 ± 100	>1,000[g]	

[a] Crude mitochondrial fraction at 0°, filter assays (13).

[b] This study, equilibrium dialysis assays, 4°, muscle membranes.

[c] Recalculated from Iversen and Kravitz (15), estimating the concentration of inhibitor blocking GABA transport by 50%.

[d] Concentration of inhibitor blocking 50% of the conductance increase in muscle caused by 50 μM GABA application.

[e] K_m value.

[f] Apparent K_D value.

[g] This study.

[h] Concentration giving 50% of maximal conductance response, recalculated from the references given.

[i] See the text, footnote 2.

GABA binding (column 3) than of GABA transport (column 4). D-Glutamic acid and imidazoleacetic acid are good inhibitors of binding and do not inhibit GABA transport at concentrations 10 times their K_I values for blocking GABA binding. With respect to GABA uptake in mouse brain (column 2, also refs. 25–28) the chemical specificity of binding of GABA to crayfish muscle (column 3) is similar, but still significantly different.

Comparison of inhibitors of crayfish muscle binding to GABA with compounds having agonist or antagonist activities at invertebrate neuromuscular junctions revealed good agreement for β-alanine, imidazoleacetic acid, β-guanidinopropionate, β-hydroxy-GABA, and 2,4-diaminobutyrate. This last compound was found to be a poor agonist on the crayfish stretch receptor preparation (3, 33), but it appears to be an antagonist of GABA-induced membrane conductance increases.[2] Homotaurine, a possible GABA agonist in vertebrate brain (6), does not block GABA binding very potently, but this compound has not been demonstrated to act on invertebrate GABA receptors. Of particular interest are imidazoleacetic acid, which is somewhat more active than β-alanine on crayfish GABA binding and on invertebrate GABA synapses and is less active than β-alanine in inhibiting GABA transport in brain (column 2, Table 2), and (−)-nipe-

cotic acid, which is active in inhibiting GABA transport in brain (column 2, Table 2) (47), has no activity at inhibitory GABA synapses in brain (47) or insects,[2] and is a very poor inhibitor of GABA binding (column 3). Likewise, homohypotaurine is a good uptake blocker but poor synapse blocker and a poor blocker of GABA binding to crayfish muscle. This agreement in specificity suggests that the binding sites measured may be synaptic receptor sites. However, since none of the anologues is totally specific for either receptors or transport, and since activity in neurophysiological studies is difficult to quantitate, we must conclude at this time that some contribution to the binding from sites other than receptors cannot be completely ruled out.

Picrotoxin does not block GABA binding to crayfish muscle. Since inhibition of GABA-induced membrane conductance increases in crustacean muscle by picrotoxin (29) and bicuculline (30, 35) is noncompetitive with GABA, these compounds are probably not receptor antagonists, but perhaps ionophore-blocking agents. One might not then expect them to inhibit GABA binding. Bicuculline, however, somewhat resembles GABA in structure (6, 47) and does inhibit GABA binding to rat brain sites described as receptor sites (48, 49). But it also weakly inhibits GABA uptake at 0° (13, 14, 50).[3]

Bicuculline blocks GABA binding to crayfish homogenates at 0°, but only at relatively high concentrations (K_I = 350 μM), and apparently noncompetitively. Bicuculline analogues which have activity as GABA antagonists, such as bicuculline methiodide (17, 18) and bicucine methyl ester, also weakly block GABA binding. Bicucine, which is not very active as a convulsant (17, 50),[3] does not. Bicuculline is not very potent on invertebrate muscle [I_{50} ~ 20–200 μM (30, 31)[3]], although its potency may have been underesimated because of lability (14, 50)[3] or solubility (17, 18, 32).[3] While activity of bicuculline at the GABA binding sites described here (K_I = 350 μM) could explain this biological activity, some other site of action (e.g., the GABA ionophore), more sensitive to bicuculline, could be involved in bicuculline inhibition of GABA synapses and in convulsions.

Finally, it must be noted that the K_D for GABA itself in our assays (1 μM) is somewhat lower than the concentration (40 μM) giving 50% of the maximal response in synaptic transmission studies (3, 4, 29–31, 33–39). There are possible explanations for such a discrepancy. For example, neurophysiological determination of agonist activity is difficult because invertebrate neuromuscular junctions are buried deeply by infoldings of the tissue (4, 29, 31, 39, 44, 45, 51); GABA activity could be underestimated as a result of diffusion barriers or transport into the tissue. Temperature differences between experiments might give different dose-response curves (51), and of course membrane homogenates might have different properties from intact membranes.

Another possible explanation involves the mechanism of receptor action. The membrane conductance increase (to Cl^-) in arthropods, induced at inhibitory synapses by GABA, is highly sigmoidal (29, 30, 34, 35, 38, 39, 50, 52), with a Hill coefficient of 2–4 (29, 39, 52), suggesting some sort of cooperative phenomenon. The cooperativity might quite possibly be a property of the receptor itself, which is likely to be a multisubunit protein (9–12, 43). Theoretical treatments of sigmoidal dose-response curves invoke either allosteric models (43, 53, 54) or responses dependent on a ligand concentration of order higher than 1 (29, 43, 50, 52).[4] Indeed, a response dependent on a higher order of ligand concentration than 1 would predict half-maximal binding to receptors at lower concentrations of transmitter than those producing half-maximal responses of the membrane ion permeability. Such a model is feasible, with cooperativity arising in the receptor-ionophore coupling mechanism or via proteins interacting in a fluid membrane (11, 50, 54).

At this time the relationship of the GABA binding to sites in crayfish muscle reported here to the GABA "receptor" activity reported for rat brain (48, 49, 55) and shrimp muscle (56, 57) is unclear, although

they do not appear to be the same in any case.

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