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γ-Aminobutyric acid uptake by a bacterial system with neurotransmitter binding characteristics

(muscimol/type A γ-aminobutyric acid receptor/*Pseudomonas fluorescens*)

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ABSTRACT **γ-Aminobutyric acid (GABA), an amino acid, has been found in every class of living organisms. In higher organisms, GABA is a neurotransmitter and binds with high affinity and specificity to GABA receptors on neurons in a sodium-independent reaction that is saturable. The role of GABA in organisms lacking nervous tissue is not known. This report describes, in a strain of *Pseudomonas fluorescens*, a GABA uptake system with binding characteristics like those of the GABA (type A) brain receptor. The binding was saturable and specific for GABA, was sodium-independent, was of high affinity (K_m = 65 nM), and was inhibited competitively by muscimol, a potent GABA analogue. The bacterial GABA system included a homogeneous binding site, and no cooperative interaction was found between sites. To our knowledge, such a system for GABA, or other neurotransmitters, in a bacterium has not been reported.**

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of invertebrates and vertebrates and causes depressant actions in the brain (1–5). *In vivo*, GABA exerts its effect on neurons by binding to a GABA type A ($GABA_A$) receptor and regulating nerve transmission through a chloride ion channel mechanism (1–5). *In vitro*, a standard assay for measuring GABA receptors involved in transmission of the nerve impulse in higher organisms consists of direct labeling of receptors in brain membrane fractions by using high specific activity [^{3}H]GABA in a sodium-free solution (6).

By using a modification of the standard GABA assay for brain membranes, uptake of GABA by a strain of *Pseudomonas fluorescens* was studied. We report the finding of a high-affinity GABA system in this bacterium with binding properties similar to those of a brain receptor (1, 6, 7).

MATERIALS AND METHODS

Organism, Growth, and Preparation. The bacterium was isolated by one of us (C.S.N.-G.) and identified as a strain of *P. fluorescens* by the Indiana State Board of Health. Growth and preparation of cells were done at room temperature (23–25°C). Cells were grown with vigorous stirring, in a medium of high salts (8) plus 9 mM NH_4Cl, 34 mM succinic acid, Difco yeast extract (0.2 g/liter), and 58 mM Tris buffered to pH 7.0 with NaOH, to an OD_{540} of 1.0–1.7 (1-cm path length). The cells were harvested by centrifugation (3000 × *g*, 10 min), washed twice with Tris citrate buffer (47.5 mM Tris/8.3 mM K_2HPO_4/5.3 mM KH_2PO_4 adjusted to pH 7.4 with citric acid), resuspended to an OD of 1.0, and soaked for 21–27 hr. While nonsoaked cells showed high-affinity GABA activity, the overnight soak reduced scatter, and data were more reproducible. Cells were not damaged by the overnight soak as judged by their stable OD and retention of motility. Ten milliliters of cells was centrifuged at 1200 × *g* for about 3 sec to remove debris; 5 ml of cell suspension was removed, centrifuged (3000 × *g*, 10 min), washed twice with Tris citrate buffer, resuspended to an OD of 0.15, and resoaked for 1 hr.

GABA Assay. Preliminary chase experiments with unlabeled GABA showed that the radioactivity remained bound after a chase. Hence, direct measurement of GABA binding to determine a dissociation constant (K_d) was not possible. Consequently, the GABA assay was designed to measure the Michaelis constant (K_m) from which characteristics of the affinity and saturation of the GABA binding site of the bacterial system could be inferred.

For the standard [^{3}H]GABA assay, 100 μl of the cell suspension (OD = 0.15) was added to triplicate 900-μl samples of 10 mM KPO_4 buffer (pH 7.3, 25°C) containing GABA solutions, and the samples were incubated with shaking for 5 min. Under these conditions, initial velocities could be determined by direct measurement because the rate of uptake of GABA was constant and maximal incorporation was ≤5% of the total available GABA in the assay solution. Nonspecific activity (background) was determined by including 1 mM unlabeled GABA in samples. The reaction was terminated by vacuum filtration of samples and collection of the cells on membrane filters (0.45-μm Millipore HA) presoaked in ultrapure water. Each filter with retained cells was immediately washed three times with 3 ml of ice-cold 10 mM KPO_4 (pH 7.3), oven dried (100°C), and dissolved in 2 ml of ethyl acetate. Ten milliliters of toluene Spectrafluor was added, and the radioactivity was determined by liquid scintillation spectrometry.

Protein Assay. Protein was measured by the method of Lowry *et al.* (9).

RESULTS

Characteristics of the GABA System. Specific GABA uptake was saturable and was ≥94% of the total activity (Fig. 1). A Lineweaver–Burk analysis (see Fig. 3, control line) showed that the GABA system included a single, high-affinity binding site with a K_m of 65 ± 3 nM (based on five experiments). Hill plots (data not shown) gave an average coefficient of 1.14, suggesting no positive or negative cooperative interaction between GABA binding sites.

Muscimol Effects on GABA Activity. Muscimol, a compound isolated from mushrooms and one of the most potent GABA agonists (binds to the receptor and enhances effects) in invertebrates and vertebrates, is widely used to study GABA binding in brain membranes (1, 6, 10, 11). It binds specifically to $GABA_A$ sites on nerves and causes neurological effects in higher organisms (1, 6, 11).

Fig. 2 shows that unlabeled muscimol and GABA competed with [^{3}H]GABA in a similar manner, demonstrating

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Abbreviation: GABA, γ-aminobutyric acid.
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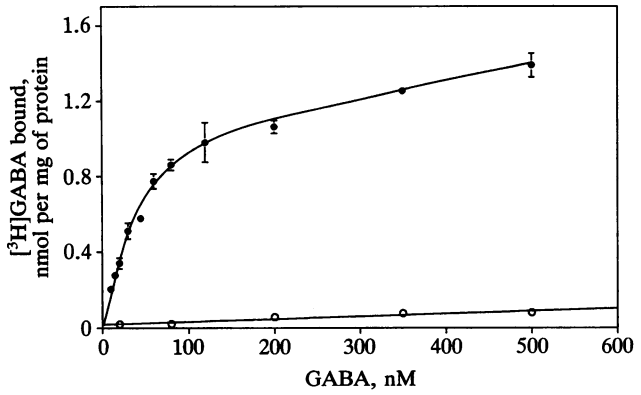


FIG. 1. Saturation of GABA uptake in *P. fluorescens*. [^{3}H]GABA bound is the amount of [^{3}H]GABA solution either specifically taken up by the cells or nonspecifically associated with the cells or filter after the standard 5-min incubation. The [^{3}H]GABA solutions were one part [^{3}H]GABA (50–71.5 Ci/mmol) and nine parts unlabeled GABA (recrystallized twice from ethanol). Total (●) = specific + nonspecific (○) GABA. Standard deviations, if measurable, are shown by bars. Similar results were obtained when the experiment was repeated four times.

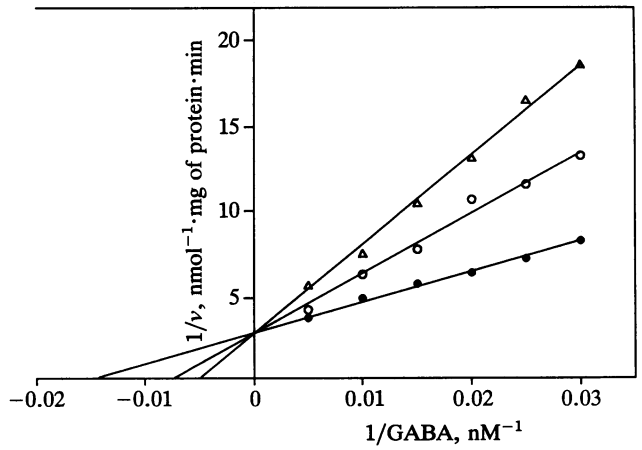


FIG. 3. Lineweaver–Burk plot showing competitive inhibition of GABA by muscimol in *P. fluorescens*. Cells were incubated with radiolabeled GABA solutions (as described in the legend to Fig. 1) and 0 (●), 40 (○), or 80 (△) nM unlabeled muscimol. Initial velocities (v_0) were calculated from the incorporation measured after the standard 5-min incubation. A typical curve from four separate experiments is shown.

that muscimol was interacting directly with the GABA system. As reported for brain membranes (12), muscimol was a competitive inhibitor of GABA (Fig. 3), further supporting its direct interaction. Since the GABA uptake system was saturable and competitively inhibited by muscimol, a specific GABA binding site was indicated. Furthermore, since muscimol replaced 100% of the GABA molecules, it was interacting with all exposed GABA binding sites (Fig. 2). The K_i for muscimol estimated from Fig. 3 was 34 nM. Although muscimol proved to inhibit better than unlabeled GABA (Table 1), the IC_{50} values of both compounds were within the range for those reported for brain membranes (not treated with detergent) (6).

GABA Competition by Agonists, Antagonists, and Analogues. To gain a better understanding of the bacterial GABA system and its binding site, compounds known to interact with the GABA receptor in the central nervous system of higher organisms were tested for their activity as GABA competitors (Table 1). Isoguvacine, 3-amino-propanesulfonic acid, kojic amine, β-alanine, and imidazole-4-acetic acid are $GABA_A$ agonists in brain (4, 14). Bicuculline is a strong $GABA_A$ competitive antagonist (binds to the receptor and inhibits the effect) in vertebrates, but is inactive or a weak,

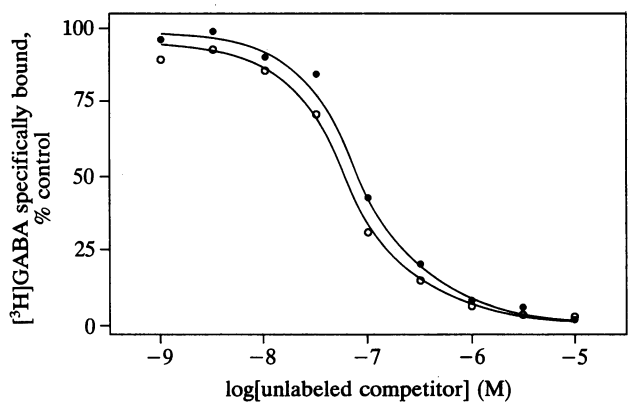


FIG. 2. Competition with [^{3}H]GABA by unlabeled GABA (●) or muscimol (○) in *P. fluorescens*. Cells were incubated with 5 nM [^{3}H]GABA and 1 nM–10 μM unlabeled GABA or muscimol. The control (100%) was specific [^{3}H]GABA incorporation (total minus nonspecific) in the absence of unlabeled compounds. A typical curve from five separate experiments is shown.

noncompetitive inhibitor in invertebrate neurons (4, 10). Picrotoxin, a potent $GABA_A$ antagonist, interacts with the chloride ion channel of the GABA receptor complex in invertebrates and vertebrates but not with the site where GABA binds (4, 10, 14). Baclofen is a potent, selective agonist of another GABA receptor, type B ($GABA_B$), but is inactive at the $GABA_A$ site; $GABA_B$, found only in vertebrates, acts by modifying the entry of calcium into the neuron (4, 6, 10). Of these compounds, only the $GABA_A$ agonists showed any activity in *Pseudomonas* as GABA competitors.

In higher organisms, GABA is removed from the extracellular synaptic region to inside the neuron by a low-affinity, sodium-dependent uptake system, which is inhibited by nipecotic acid but not by muscimol (3, 15, 16). Nipecotic acid, which is inactive on both $GABA_A$ and $GABA_B$ binding sites (6), was not effective in inhibiting [^{3}H]GABA uptake in the bacterium.

To determine if GABA was interacting with a nonspecific rather than a specific GABA site, amino acids that have a structural resemblance to GABA and have well-defined bacterial transport systems (17–19) were tested as competitors. Except for a minimal amount of competition by glutamic acid, none of the others (glycine, aspartic acid, asparagine, glutamine, or proline) demonstrated activity.

GABA analogues with a longer carbon chain were more active than shorter ones in *Pseudomonas* (Table 1), as is found in abalone (20). The most potent analogue was 5-aminovaleric acid whose activity was eliminated, however, by substituting its carboxyl with an alcohol group (see 4-amino-1-butanol).

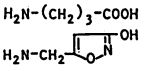
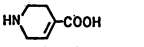
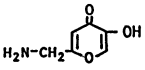
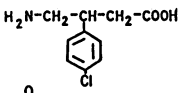
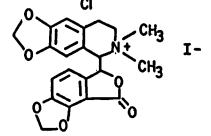
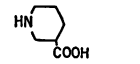
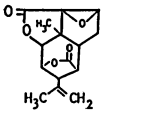
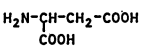
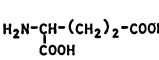
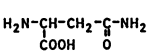
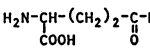
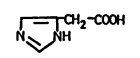
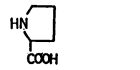
In summary, the competition studies showed the bacterial system to be very specific and have a high affinity for GABA. Its binding characteristics were similar to those found in higher organisms for a $GABA_A$ receptor rather than a $GABA_B$ site or the GABA transport system in neurons.

In invertebrate neurons, 3-amino-1-propanesulfonic acid is a very weak $GABA_A$ agonist and bicuculline is weak or not active, whereas in vertebrates both are potent (4–6, 10). Based on the competitive activities of these compounds, the binding characteristics of the bacterial system were more similar to those described in invertebrates (10).

DISCUSSION

Our data showed a K_m by the Michaelis–Menten/Briggs–Haldane analysis. Since a ligand binding to a specific site is

Table 1. Inhibition of specific [^{3}H]GABA activity by agonists, antagonists, and analogues of GABA

Compound	Structure	IC_{50}, μM
GABA	$H_2N-(CH_2)_3-COOH$	0.078
Muscimol		0.052
Isoguvacine		90
3-Amino-1-propane-sulfonic acid	$H_2N-(CH_2)_3-SO_3H$	550
Kojic amine		160
(±) Baclofen		>1000
(-) Bicuculline methiodide		>1000
(±) Nipecotic acid		960
Picrotoxin		>1000
Glycine	H_2N-CH_2-COOH	>1000
β-Alanine	$H_2N-(CH_2)_2-COOH$	120
5-Aminovaleric acid	$H_2N-(CH_2)_4-COOH$	1
ε-Aminocaproic acid	$H_2N-(CH_2)_5-COOH$	350
4-Amino-1-butanol	$H_2N-(CH_2)_4-OH$	>1000
L-Aspartic acid		>1000
L-Glutamic acid		470
L-Asparagine		>1000
L-Glutamine		>1000
Imidazole-4-acetic acid		310
L-Proline		>1000

P. fluorescens cells were incubated with 5 nM [^{3}H]GABA and unlabeled compounds. For each compound, a minimum of five different concentrations, up to at least 8 mM, was tested, except for GABA and muscimol (10 μM) and isoguvacine (4 mM). Compounds were made up fresh in 10 mM KPO_4 buffer (pH 7.3) on the day of experiment (the pH was readjusted as needed with KOH or HCl) and stored on ice. Baclofen was a gift of Ciba-Geigy. The structure shown for picrotoxin is the toxic component, picrotoxinin. The IC_{50} value, determined by probit analysis (13), was the mean concentration required to reduce specific [^{3}H]GABA incorporation by 50%, using at least two experiments, with each concentration done in duplicate or triplicate. SEMs were <4% for GABA and muscimol and <10% for other compounds.

a basic assumption of that analysis, the high-affinity GABA uptake system in *Pseudomonas* is assumed to have a binding site.

The bacterial GABA system was probably not induced but was already present, since the cells were not exposed to GABA until the time of assay. The question arises as to why a bacterium takes up GABA, an uncommon amino acid, by a high-affinity, specific system. Commonly occurring amino acids, in general, have lower binding affinities for transport in bacteria (17–19). Even for GABA, a much lower affinity (K_m = 12,000 nM) has been described in an *Escherichia coli* mutant (21) than the one reported here for a strain of *P. fluorescens* (K_m = 65 nM).

It is unlikely that the primary role of a high-affinity system for GABA is as a nutrient scavenger since GABA is not found naturally free or in proteins; thus, it would not be available as a source of carbon or nitrogen. Although *Pseudomonas* has enzymes for GABA metabolism (22) in a shunt off the tricarboxylic acid cycle, the pathway is not energy beneficial for the organism because it bypasses the formation of a high-energy phosphate. Therefore, GABA and its high-affinity uptake system may have a function other than a nutritional one.

Besides GABA, other neurotransmitters (catecholamines, serotonin, and acetylcholine) and hormones, normally associated with nervous and endocrine systems in higher organisms, are found in unicellular organisms (23). Roth *et al.* (23) have suggested that the biochemical elements used by the nervous, endocrine, and other systems of intercellular communication in vertebrates probably originated in unicellular organisms and were conserved. As a biochemical element, a high-affinity GABA site may have been conserved. If two systems in different species have similar GABA binding properties, they could have a high degree of structural (but not necessarily functional) homology in their binding sites. Thus, the high-affinity GABA system in bacteria could have evolutionary significance by having a primitive site that evolved to the one in higher organisms. It will be interesting to compare the molecular structure of the GABA site in *Pseudomonas* and higher organisms for homology.

Koshland (24, 25) has proposed that cells from brain to bacteria, in a highly similar manner, receive information (signals) through receptors and then process and integrate it to end in a response, such as the release of electrical voltage in a neuron or change in flagella rotation in a bacterium. The function of GABA in this unicellular organism may be as a signal molecule for intra- or intercellular communication.

The GABA bacterial system may prove to be only a highly efficient GABA uptake mechanism. Whatever its function, however, the system might be useful as a model to study GABA binding, to help develop and evaluate drugs proposed as GABA agonists or antagonists, or as a reproducible, sensitive, inexpensive, simple assay for GABA.

Infection of patients with GABA-producing bacteria, including *Pseudomonas*, is suggested to be associated with central nervous system disturbances because of increased brain GABA levels (26). Since a decrease in GABA or its receptors in brain causes seizures or convulsions (2, 4, 27), it is conceivable that infections with bacteria able to take up GABA with high efficiency may indirectly be involved with these brain disorders. The roles of GABA and bacteria and their possible connection with central nervous system disorders need to be further explored.

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