*RELEASE OF GAMMA-AMINOBUTYRIC ACID FROM INHIBITORY NERVES OF LOBSTER**

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In 1957, Bazemore, Elliott, and Florey[1] showed that γ-aminobutyric acid (GABA) blocked the discharges of crayfish stretch-receptor neurons. This finding focused attention on the possible role of GABA as an inhibitory transmitter compound. At various crustacean synapses GABA was found to mimic the effects of inhibitory (I) nerve stimulation.[2–9] Furthermore, in the lobster nervous system GABA is highly concentrated in I neurons, the ratio of GABA concentrations in I and excitatory (E) axons being more than 100:1.[10–12] Although this evidence is consistent with GABA being an inhibitory transmitter compound, a crucial experiment has hitherto been lacking: the demonstration that GABA is released from I-nerve terminals in response to nerve stimulation.

The results of the present study show that stimulation of the I-nerve innervating various lobster muscles causes a release of GABA into the bathing fluid; the amount released is proportional to the number of stimuli applied.

Materials and Methods.—Saline medium used to wash nerve-muscle preparations had the following composition: 460 mM NaCl, 15.6 mM KCl, 26 mM $CaCl_2$, 8.3 mM $MgSO_4$.

Dowex-50W-X-2, H^+ resin (100–200 mesh) was obtained from Baker Chemical Co. After fine particles were removed by repeated decantation, the resin was cycled through ammonium and hydrogen forms, and was freshly washed with 5-bed vol of 2 *N* hydrochloric acid and 2-bed vol of water immediately before use.

Dowex-1-X2, Cl^- resin (100–200 mesh) was obtained from Bio Rad Laboratories. The resin was thoroughly washed with 2 *N* hydrochloric acid, and then with 2 *N* acetic acid to convert it to the acetate form; resin columns were freshly washed with 5-bed vol of 2 *N* acetic acid and 5-bed vol of water immediately before use.

3-H^3-GABA (specific activity 5 c/mmole) was synthesized from DL-3-H^3-glutamate (New England Nuclear Corp., Boston, Mass.) by decarboxylation with a bacterial glutamate decarboxylase.

Nerve-muscle preparations: Opener muscles from the crusher and cutter claws of 0.5-kg lobsters (*Homarus americanus*) were set up as illustrated in Figure 1. The single E and I axons innervating the muscle lie in separate bundles in the carpopodite segment: these were dissected and stimulated separately with suction electrodes. The closer muscle and overlying connective tissue were carefully removed to expose the opener muscle. The opener muscles remained *in situ* in the exoskeleton which formed a convenient chamber for washing with saline medium. Junctional (synaptic) potentials were recorded intracellularly from muscle fibers with a glass microelectrode filled with 2 *M* potassium citrate. Chilled, oxygenated saline medium was pumped onto the proximal end of the muscle at a rate of approximately 2 ml/min. The perfusion medium percolated through the muscle and dripped out of the cut end of the dactyl (cleaned of internal tissue). The temperature of the effluent medium was 12–14°. I or E nerves were stimulated at frequencies of 5–20 impulses/sec for the first 15 min of the collection period.

Superficial flexor muscles (M. superfic. vent. abdom.) with nerve supply and skeletal attachments intact were dissected from 1.5-kg lobsters and immersed in 3 ml of saline medium in a small chamber (2.5 × 2.5 × 0.6 cm). Chilled saline medium (7–9°) was pumped continuously through the chamber at a rate of approximately 1.5 ml/min, and 30-min fractions were collected. In most cases stimulation at 5/sec for 30–60 min was applied to the entire bundle of axons innervating the muscle and thus included E nerves; however, in three experiments the single I axon was dissected and stimulated.

Isolation and assay of GABA: GABA was isolated from samples of the saline medium by adsorption and selective elution from a cation exchange resin. Samples (50 ml) of the medium were acidified with 2 ml of 1 *N* hydrochloric acid, and 2×10^{-11} moles of H^3-GABA were added as an internal standard to estimate recovery. Samples were then passed through a column (1.8 cm × 20 cm) of Dowex-50-H^+ resin. The resin column was washed with 25 ml of water and 20 ml of IN ammonium hydroxide; GABA and other amino acids were then eluted with a further 30 ml of IN ammonium hydroxide. The eluates were taken to dryness in a rotary evaporator; the residue was dissolved in a total of 4 ml of water and passed through a column (0.4 × 3 cm) of Dowex-1-acetate resin to remove acidic amino acids and fluorescent contaminants eluted from the Dowex-50 resin. The effluent solution was taken to dryness, and the residue dissolved in 0.1 ml of 0.1 *M* Tris buffer, pH 7.9. GABA was assayed in 20-μl aliquots of this purified sample, using a micromodification of the highly specific enzymic assay of Jakoby and Scott.[11, 13] For each analysis a blank and two assay samples were run; an internal standard of 5×10^{-11} moles of GABA was added to a fourth sample. The over-all recovery of GABA was determined by measuring the amount of H^3-GABA in an aliquot of each purified sample; assay results were corrected for recoveries which ranged from 75 to 95%. The enzymic assay is sensitive to 10^{-11} moles of GABA and is highly specific; the only other substance known to react in this assay is the β-hydroxy derivative of GABA. This substance, however, is not found in the lobster nervous system.[10]

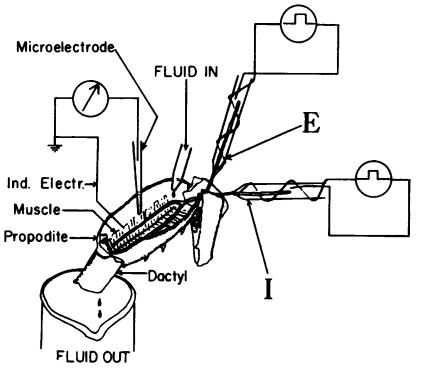

FIG. 1.—Diagrammatic view of opener muscle preparation. *E* and *I* are bundles of axons containing the single excitatory and single inhibitory axons which innervate this muscle. The E and I nerve bundles are shown in suction electrodes used for stimulation. The preparation is described in detail in the text.

Results.—Opener muscle of crusher claw: GABA was released in response to I-nerve stimulation in each of 12 experiments with this preparation. It proved necessary to wash the muscles for 3–4 hr before starting an experiment in order to reduce the spontaneous efflux of GABA to a low and stable level. The results of a single experiment are illustrated in Figure 2, which shows that GABA was liberated during I-nerve stimulation but not during E-nerve stimulation. Both periods of I-nerve stimulation released GABA but the amount liberated declined in the course of the experiment. In this particular experiment, stimulation at a frequency of 10 impulses/sec released less than stimulation for the same period at a frequency of 5/sec (75 min earlier). There was a considerable variation in the amount of

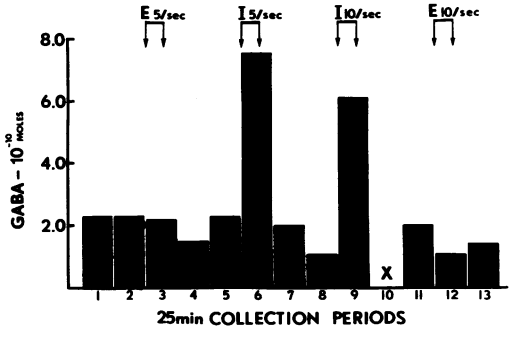

FIG. 2.—Results of a single experiment demonstrating the release of GABA in response to I-nerve stimulation in the opener muscle from the crusher claw. The preparation was washed with saline medium for 4 hr prior to the first resting collection (no. 1). GABA was assayed in 25-min collection samples during rest and during E- and I-nerve stimulation. The values obtained in this experiment for 5/sec are higher than generally observed. In most experiments the assay failed on one or more samples, indicated by *X* in this figure.

TABLE 1

SUMMARIZED RESULTS FOR GABA RELEASE IN LOBSTER NERVE-MUSCLE PREPARATIONS

Preparation	Duration of collection period (min)	Average GABA Content of Collection Periods—10^{-10} moles				
			I Stimulation		Post I stimulation	E stimulation
		Rest	(5/sec)	(10/sec)	(rest)	(5–10/sec)
Opener of crusher claw	25	1.7 ± 0.12 (46)	3.2 ± 0.76* (8)	4.9 ± 0.61† (5)	2.1 ± 0.28 (10)	1.7 ± 0.26 (10)
Opener of cutter claw	25	1.2 ± 0.24 (15)	—	2.2 ± 0.30* (10)	1.7 ± 0.26 (10)	0.80 ± 0.09 (6)
Flexor muscle						
(*a*) "Positive"	30	2.0 ± 0.24 (19)	5.0 ± 0.66* (13)	—	3.4 ± 0.64‡ (6)	—
(*b*) "Negative"	30	1.8 ± 0.32 (9)	2.0 ± 0.08 (9)	—	2.2 ± 0.38 (5)	—

Average amounts of GABA in rest and stimulation periods in three lobster nerve-muscle preparations. Values are means ± standard errors of means; number of samples indicated in parentheses.
* $P < 0.01$.
† $P < 0.001$ when compared with control rest periods.
‡ $P < 0.05$.

GABA released during I-nerve stimulation. Average values and statistical analyses of the results for the 12 experiments are given in Table 1. During eight I-nerve stimulation periods for 15 min at frequencies of 5/sec, the average amount of GABA released in excess of the spontaneous resting efflux (i.e., net release) was 1.5×10^{-10} moles; during five similar periods of stimulation at a frequency of 10/sec, the average net release was 3.2×10^{-10} moles. The amount of GABA released was thus clearly dependent on the frequency of stimulation, although further experiments will be necessary to define this relationship more precisely. During ten periods of E-nerve stimulation at frequencies of 5/sec or 10/sec for 15 min, there was no detectable release of GABA in excess of the resting levels (Table 1, Fig. 2).

Nerve stimulation was discontinued 10 min before the end of each collection period (e.g., Fig. 2), since studies with C^{14}-sucrose or H^3-GABA demonstrated that the extracellular space of the tissue equilibrated slowly with the external medium under our experimental conditions (half time for washout of extracellular space 13–18 min).

Since at other chemically transmitting synapses calcium ions are known to be necessary for transmitter release,[14–17] we determined the effects of a low-calcium medium on the liberation of GABA. A series of muscles was set up as described above. Intracellular recordings were made of junctional potentials from muscle fibers, and extracellular recordings with a monopolar electrode monitored the action potentials from the E and I nerves on the muscle surface (Fig. 1). Muscles were washed with normal medium for 4 hr before collections were started; after two control collection periods the I nerve was stimulated at 7.5/sec for 15 min; action potentials, inhibitory junctional potentials, and GABA output were recorded (Fig. 3*A*, *1–4*). The muscle was then washed with a medium containing only 10 per cent of the normal calcium content. The low-calcium medium caused a brief period (5–10 min) of high frequency firing in I and E nerves before the preparation became quiescent. This initial burst of spontaneous nerve activity may account for the high GABA content of the first collection period after changing to the low-calcium medium, which is particularly evident in the experiment illustrated in Figure 3*B*, *5*. After being washed for 1 hr in the low-calcium medium, the I axon was stimulated once more at 7.5/sec. I stimulation now failed to produce inhibitory

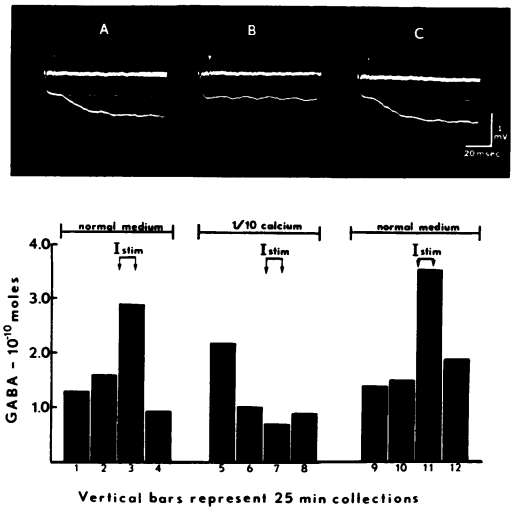

FIG. 3.—Effects of low-calcium medium on inhibitory neuromuscular transmission and GABA release in the opener muscle from the crusher claw. The oscilloscope records in the upper part of the figure show extracellularly recorded action potentials in the nerve (upper trace, *arrows*) and the first part of inhibitory junctional potentials simultaneously recorded intracellularly from a muscle fiber (lower trace). In normal medium (*A*) stimulation of the I nerve evoked inhibitory junctional potentials in the muscle and was accompanied by a release of GABA (lower part of figure). After exposure of the preparation to a medium containing only 10% of the normal calcium (*B*), I stimulation no longer produced inhibitory junctional potentials or GABA release, although action potentials were still recorded (upper trace of *B*). This effect was reversed on return to normal medium (*C*).

junctional potentials in the muscle fibers and no release of GABA was detected, although action potentials were still recorded from the nerve (Fig. 3*B*, 7). On returning to a normal calcium medium, inhibitory junctional potentials and GABA release could again be demonstrated in response to I-nerve stimulation (Fig. 3*C*, *11*). Average results for four similar experiments are presented in Table 2.

The opener muscle of the crusher claw was the most satisfactory of the three nerve-muscle preparations used in these studies, presumably because it is several

TABLE 2

EFFECT OF LOW-CALCIUM MEDIUM ON GABA RELEASE FROM CRUSHER OPENER DURING I-NERVE STIMULATION

Collection period	No. samples	GABA—10^{-10} moles/25 min
A. Normal medium		
Resting output	11	1.7 ± 0.26
I-nerve stimulation	3	3.2 ± 0.61*
B. Low-calcium medium (2.6 mM)		
Resting output†	7	1.4 ± 0.23
I-nerve stimulation	4	1.7 ± 0.35
C. Normal medium		
Resting output	12	1.9 ± 0.17
I-nerve stimulation	3	3.9 ± 0.61‡

Average results for four expts. with low-calcium medium, similar to that illustrated in Fig. 2. Values are means ± standard errors of the means. Variable number of samples in each group is due to failure of assay in some samples in each experiment.

* $P < 0.05$.

† Resting output for low-calcium medium does not include data obtained in first rest period after exposure to this medium (see Fig. 3); average GABA output for this period was 1.8×10^{-10} moles.

‡ $P < 0.001$ when compared with resting output.

times larger than the other preparations (average wet weight in gm: crusher opener = 1.0; cutter opener = 0.5; superficial flexor = 0.3). The average GABA content of eight nerve-muscle preparations from the crusher claw was 2.2×10^{-7} moles. The amounts of GABA collected in response to I-nerve stimulation in these experiments thus represent only a small proportion of the total GABA of the preparations.

Other muscles: The results obtained from other nerve-muscle preparations were more variable. In 11 experiments with the superficial flexor muscle, a release of GABA in response to nerve stimulation could be demonstrated in only six ("positive") experiments, three with combined E- and I-nerve stimulation, and three with single I-axon stimulation (Table 1). In these experiments stimulation at a frequency of 5/sec was administered during the entire 30-min collection period. Since equilibration of the extracellular space was again slow (half time for washout approximately 15 min), there was a significant release of GABA in the rest collection immediately following I-stimulation periods (Table 1). In some experiments nerve stimulation was continued for up to 60 min. The average net release of GABA (including GABA collected during subsequent 30-min rest period in each case) in 12 separate periods of stimulation for 30 min (9,000 stimuli) was 4.4×10^{-10} moles, and the average net release in four periods of stimulation for 60 min (18,000 stimuli) was 8.5×10^{-10} moles, indicating that GABA release was related to the number of stimuli.

Opener muscles from the cutter claw proved less satisfactory than the larger preparations from the crusher claw. Nevertheless, the average results for six experiments with this preparation demonstrated a significant release of GABA in response to I-nerve stimulation, while no release of GABA was observed during E-nerve stimulation (Table 1).

Discussion.—These results provide the first demonstration of a release of GABA in response to stimulation of an identified I nerve. The amounts of GABA released increased with the number of I impulses, depending on frequency and/or duration of stimulation. No GABA was released by E axons, a result which is consistent with previous findings that E axons contain only small amounts of GABA.[11] In conjunction with findings in previous studies, these results add strong support to the view that GABA is the transmitter substance at inhibitory neuromuscular synapses in the lobster.

In the experiments with a low-calcium medium, nerve conduction along the axons was unimpaired. No recording was made from the terminal region, but in analogy with detailed studies at the frog neuromuscular junction,[17] one may assume that conduction remained intact in the terminals while transmitter release was prevented. The effect of calcium on inhibitory neuromuscular transmission in the lobster thus appears to be similar to that observed at other chemically transmitting synapses.

It should be emphasized that the amounts of GABA collected in the present experiments represent minimum estimates of the actual amounts of GABA released by I nerves during stimulation. A specific transport process accumulates extracellular GABA in lobster nerve-muscle preparations,[18] and this process may serve to inactivate the inhibitory transmitter by removing it from the site of action. The GABA uptake process functions under our experimental conditions and would certainly have removed some of the released GABA from the extracellular space of the tissue. In an attempt to reduce the rate of removal of released GABA by up-

take by some other unknown enzymic mechanism, experiments were performed at the lowest practicable temperatures.

In a recent study, Takeuchi and Takeuchi[6] estimated that the amount of GABA required to simulate an inhibitory junctional potential when applied electrophoretically to GABA-sensitive areas of crayfish muscle was 4×10^{-15} moles. In the present experiments the net amount of GABA released per stimulus from the various nerve-muscle preparations was $1\text{–}4 \times 10^{-14}$ moles. In the absence of information concerning the number of inhibitory terminals in the preparations used in the present studies, and since an unknown amount of the released GABA was inactivated in the tissues, it is impossible to make any precise estimate of the amount of GABA released at each nerve terminal.

Summary.—A release of γ-aminobutyric acid (GABA) in response to inhibitory nerve stimulation has been demonstrated at three different neuromuscular junctions of the lobster. The amounts of GABA released were dependent on the frequency of stimulation and the duration of the stimulation period. The average amount of GABA recovered per stimulus ranged from 1 to 4×10^{-14} moles. GABA was not released by stimulation of excitatory nerves, or in response to inhibitory nerve stimulation when neuromuscular transmission was blocked by exposing the tissue to a low-calcium medium.

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