Biochemical Pharmacology, 1964, Vol. 13, pp. 95–103. Pergamon Press Ltd., Printed in Great Britain.

RADIOAUTOGRAPHIC STUDIES OF BINDING OF γ-AMINOBUTYRIC ACID TO THE ABDOMINAL STRETCH RECEPTORS OF THE CRAYFISH*

BETTY SISKEN and EUGENE ROBERTS

Department of Biochemistry, Medical Research Institute, City of Hope Medical Center, Duarte, Calif., U.S.A.

(*Received* 24 *June* 1963; *accepted* 7 *August* 1963)

Abstract—A radioautographic technique was developed by which it was possible to visualize γ-aminobutyric acid (γABA) that was bound to isolated abdominal stretch receptors of the crayfish during brief incubation in a suitable salt solution containing γABA^{3}H of high specific activity. The binding, which takes place even at 0 to 4°, was enhanced at room temperature. The pattern of labeling was found to be consistent with a greater degree of localization than elsewhere at the axodendritic endings between branches of the inhibitory axon and the dendrites of the sensory neuron. Neither thymidine-^{3}H nor DL-leucine-^{3}H showed a similar pattern. The characteristic binding of γABA by the stretch receptors was disrupted by distilled water, formalin, or by employing a Na^+-free saline.

IN RECENT years increasing attention has been given to the study of inhibitory phenomena at various levels of the nervous systems of different species, and two symposia have been devoted to this subject.[1, 2] Our own interest in these phenomena largely has been centered around the attempt to define how γ-aminobutyric acid (γABA), a substance that has among the various tissues studied a unique occurrence in the vertebrate central nervous system, may be related at a molecular level to the inhibitory process which "is accompanied by a stabilization of membrane polarization in a manner which blocks the depolarizing action of excitatory impulses acting upon the same postsynaptic membrane. This may be called repolarization or hyperpolarization, but under certain circumstances it may even be accompanied by some depolarization (in a previously hyperpolarized membrane). The essential fact is that the inhibitory process in all nerve cells counteracts the depolarizing action of excitatory processes to maintain the polarization of a cell at an equilibrium level near that of its resting value."[3] Work with intracellular microelectrodes has indicated that the membrane stabilization process necessary for this inhibition is related to specific increases in the permeability of the postsynaptic membrane to potassium and/or chloride ions. Although electrical transmission has been shown to occur at some synapses,[4, 5] postsynaptic membranes are generally considered to be electrically unexcitable. Both excitatory and inhibitory effects may be transmitted by chemical mediators having effects on specialized postsynaptic membranes.

* This investigation was supported in part by Grants B-1615 and B-2655 from the National Institute of Neurological Diseases and Blindness and a grant from the National Association for Mental Health.

The crayfish stretch receptor organs offer, perhaps, the most favorable preparation in which to attempt to begin to analyze the mechanism of γABA action at a molecular level. This preparation has been studied extensively from histological,[6] electron microscopic,[7, 8] and physiological[9–11] points of view. The easy accessibility of these organs for manipulation and for insertion of measuring electrodes has led to a good understanding of their function. The body of the sensory neurons lies near the receptor muscle strand and portions of the dendrites are embedded in the strand. An inhibitory axon forms synapses extensively on the dendrites and some on the nerve cell itself, and there is also a motor-nerve supply to the muscle strand. Several studies have shown that the synaptic input to the sensory neuron is purely inhibitory. The sensory neuron can be activated by passive stretch or by contraction set up by the motor nerves[11] and can be supressed even in the presence of stretch by stimulation of the inhibitory nerve.[12] It was found that low concentrations of γABA have effects on the crayfish stretch receptor which are remarkably similar to those found upon stimulation of the inhibitory nerve.[13] γABA concentrations 1,000 times stronger than those blocking afferent discharge do not block conduction in the sensory axons. γABA action, therefore, appears to be confined to the dendrites and cell body. An analysis of the mechanisms of inhibition of the crayfish stretch receptor system has suggested that stimulation of the inhibitory nerve causes liberation of a substance (or substances) that produces a specific effect on the postsynaptic membrane of the sensory cell which is believed to be largely attributable to increased conductance of potassium and possibly chloride ions and to membrane potential changes. Actually at the equilibrium level of the inhibitory process no significant membrane potential change is set up by inhibitory impulses, although the inhibitory process is fully active. The action of γABA is not localized necessarily on specific subsynaptic receptor sites, since γABA also was found to block the stretch discharge of the median receptor in the seventh thoracic segment, a structure possessing no demonstrable inhibitory synapses. Recent electron microscopic studies of the crayfish stretch receptor showed the inhibitory axodendritic endings to consist of vesicle-packed boutons.[7, 8] Presumably the particles in these vesicles contain the crayfish inhibitory transmitter, and isolation of these vesicles and identification of the inhibitory transmitter should be feasible. γABA has been found in both the peripheral and central nervous system in crabs and lobsters, and L-glutamic decarboxylase, the enzyme that forms γABA, has been demonstrated in a particulate fraction isolated from lobster peripheral and central nervous tissue.[14, 15] Whether or not γABA should eventually prove in specific instances to be the transmitter itself, or metabolically related to it, or merely a substance that facilitates the presynaptic release or postsynaptic action of the actual transmitter, it appears likely that this substance plays some role in the inhibitory process.

Certainly the first step in any action of a substance upon cellular structures must be through some physical or chemical association of the substance with those structures. It was found that the binding of γABA-1-^{14}C took place with various mouse brain preparations even at 0° to 4°, but not in similar preparations of other tissues.[16] All the binding activity, which was found to be nonenzymatic and not to require energy, was found in the sedimentable fractions of sucrose homogenates of mouse brain, largely in the 'mitochondrial' and 'microsomal' fractions. A striking property of the binding process was an absolute requirement for Na^+ ions. Other aspects of the binding by particles from mammalian brain have been studied in some detail.[17] None of the

above experiments, however, could give precise localization of the accumulated γABA at the cellular level, nor is it at present possible to relate the results obtained in such binding studies directly to some physiologically meaningful parameter. In general, radioautographic methods have not been applied successfully to visualization in cells of water-soluble substances which are not bonded covalently in polymeric structures. However, the binding of γABA by brain particulates and the availability of tritiated γABA made it feasible to attempt to localize loosely bound γABA by radioautography in structures of the crayfish stretch receptor.

METHODS

Biological procedures and radioautography

Crayfish (*Procambarus clarkii*) were obtained from a commercial source and kept in an aerated tank at room temperature. At the time of operation the animal was placed in a plastic clamp, dorsal side up, in a vessel surrounded by an ice bath and filled with Van Harreveld's solution,[18] which was used throughout the experiments. A dorsal dissection on one segment at a time was performed as described by Eckert[19] while the crayfish was kept in this chilled, anesthetized condition, and the stretch receptors were removed to a depression on a black spot plate containing the Van Harreveld's solution. Heating by the microscope lamp was prevented by interposing a water bath between the light source and the specimen. Incubation of the receptors in solutions containing tritiated γABA, leucine, or thymidine, all at 50 μc/ml of crayfish Ringer's were carried out on this spot plate either at 0° to 4° or at room temperature (approximately 25°). After incubation in the isotopic solution, the receptors were transferred to another depression in the spot plate and rinsed with five to ten changes of nonisotopic solution. The receptor then was placed in a small drop of solution on a previously iced large-size black rubber stopper. The muscles and axons were spread out with fine forceps to approximate their original position *in vivo*, and the small amount of solution surrounding the receptors was carefully absorbed away, leaving only a moist preparation on the stopper. This was quickly taken into the darkroom and pressed upon a slide containing AR 10 stripping film prepared with the emulsion side up. After drying, exposure of the slides was carried out at 0° to 5° in a light-tight box containing Drierite for periods ranging from 3 to 5 days.

Development of the film was carried out directly by conventional methods. This provided a good fixation of the specimen, avoiding the necessity of employing the classical fixation methods which were found either to bleach the grains or damage the film. After development, azure B (0·5 mg/ml in McIlwain's buffer, pH 6) was placed for 3 min on the specimen, which was then rinsed in distilled water, air dried, and mounted in immersion oil.

Electrophoresis of extracts of labeled receptors

Receptors were isolated and incubated in tritiated γABA either at 0° to 4° or at room temperature for 10 min and rinsed ten times. In some instances the receptors were pressed directly onto Whatman 1 paper, while in others aliquots of 80% ethanolic extracts of homogenates of receptors were applied to the paper. Electrophoresis then was performed for approximately 45 min at 3,000 Volts in 8% formic acid, approximately pH 2, using Varsol as the nonconducting solvent. For reference, a standard solution was spotted containing 15 μg each of taurine, glutamic acid, alanine, and

γABA adjacent to the experimental sample on every paper. After electrophoresis and drying, the strip with the known compounds was cut away and sprayed with ninhydrin. With these spots as a guide, the paper containing the experimental sample was cut into smaller contiguous segments approximately 2 inches long and eluted with water directly into counting vials. The vials were then dried at 40° and 1 ml of 1 M hydroxide of Hyamine 10-X in methanol was added while the vials were still warm. After the addition of 15 ml of counting fluid [toluene (15 ml), containing 0·3% 2,5-diphenyloxazole and 0·01% 1,4-*bis*-2(5-phenyloxazolyl)-benzene in toluene] the vials were placed in a Packard Tri-Carb model 34 liquid scintillation counter at Tap 8 with a window setting of 10-80, 10-100 and counted for at least five periods of 10 min each.

Isotopically labeled compounds

One sample of γABA-^{3}H was obtained from U. S. Nuclear Corp., Burbank, Calif. The minimal purity was 99 per cent and the specific activity was 3·5 c/mmole. The second sample with approximately the same specific activity was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif. Identical results were obtained with both samples. DL-Leucine-4,5-^{3}H, with a specific activity of 5·4 c/mmole was purchased from New England Nuclear Corp., Boston, Mass., and thymidine-^{3}H, specific activity 1·9 c/mmole, from Schwarz Bio Research Inc., Orangeburg, N.Y.

RESULTS AND DISCUSSION

The tritium label remains largely associated with γABA throughout the experimental procedure

To ensure validity of the proposed procedure for visualizing γABA by radioautography it must be ascertained that most of the grains seen on the developed autographs

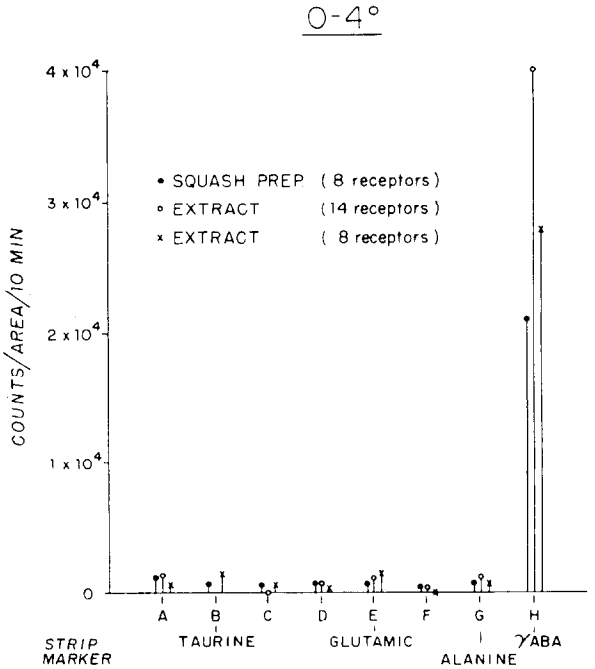

FIG. 1. Distribution of radioactivity in stretch receptors incubated at 0° to 4°.

are attributable to radioactivity actually associated with γABA itself. This is particularly important in the case of γABA since γABA is known to undergo metabolic transformation.[20] In Fig. 1 are shown the results obtained when a squash preparation and two extracts of receptors labeled at 0° to 4° were examined after electrophoresis. In each instance most of the radioactivity was found in the γABA fraction. The small amounts of radioactivity found in the other fractions were more or less evenly distributed. Preliminary experiments had shown a considerably increased uptake of label by receptors incubated at room temperature. The results in Fig. 2 show that by far the

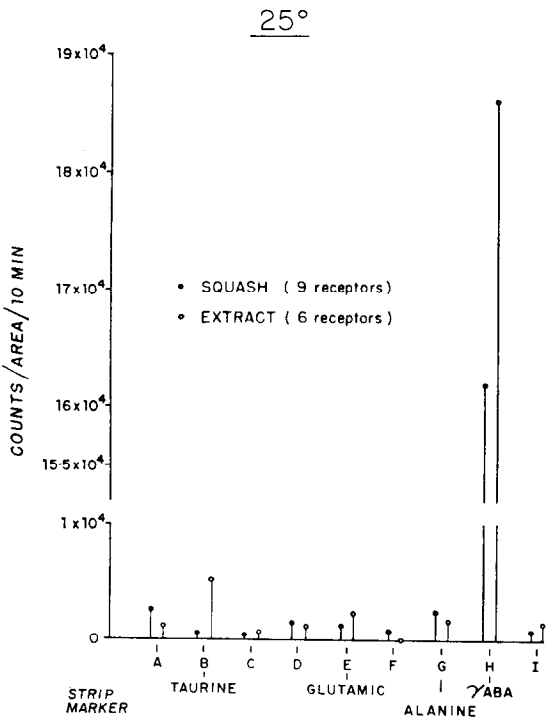

FIG. 2. Distribution of radioactivity in stretch receptors incubated at 25°.

largest proportion of the isotope probably is associated with γABA also at this temperature. It may be estimated from the results of the above experiments that at least 90 to 95 of every 100 grains seen on the autographs to be shown subsequently have resulted from the radioactivity in γABA, itself.

Localization of tritiated γABA on the crayfish stretch receptor

For purposes of orientation in evaluating the radioautographs to be presented subsequently, a schematic diagram and a stained preparation of the crayfish stretch receptors are shown in Fig. 3a and 3b. Figure 3a (Fig. 1[8]) and the following descriptions are from Ref. 8: "Fig. 1 illustrates the main structures of the paired receptors. Each receptor consists of several muscle fibers (RM) and a large sensory neuron (SN). The dendrites of the sensory neuron come off the cell body in three to five main trunks which then divide into branches and finally end in numerous, very small twig-like

processes (not shown). These dendritic processes are heavily covered with connective tissue which in turn is embedded between the muscle fibers. The axons innervating the receptors provide inhibitory innervation to the sensory neuron cell body and dendrites, inhibitory innervation to the muscle fibers, and motor innervation to the muscle fibers. Also illustrated are two sensory axons which run centrally from the sensory neurons.

The paired receptors differ from each other both physiologically and morphologically. As indicated in Fig. 1, one receptor organ has fewer muscle fibers which are smaller and of greater sarcomere length, and are physiologically 'slow' (RM_1). The sensory neuron (SN_1) associated with RM_1 is slow-adapting, and its dendritic branches show a definite orientation parallel to the muscle fibers. The other receptor organ has more muscle fibers (RM_2) which are larger in diameter and more finely striated. These muscle fibers give a fast or more 'twitch-like' response to stimulation. The dendritic processes of the sensory neuron (SN_2) associated with RM_2 do not show the well-defined orientation parallel to the muscle fibers. SN_2 adapts rapidly to stimulation.

Muscle stretch or contraction produces a mechanical distortion of the dendritic branches which in turn sets up a generator potential."

The generator potentials spread electrotonically and a propagated impulse is initiated in the axon several hundred microns from the cell body. Many physiological investigations have been consistent with the interpretation that the synaptic input to the sensory neuron is purely inhibitory.

The figures below are shown in duplicate. The first one of each set shows both the morphological features revealed by staining with azure B *and* the radioautographic image produced by the labeled structures. The second shows the grains of the autographs primarily, the stain being filtered out in photography as much as possible by the use of suitable filters (blue or blue and red together). Fig. 4a shows the stained radioautograph of the slow-adapting muscle (RM_1) and its sensory neuron (SN_1) after incubation in tritiated γABA for 5 min at room temperature. The chromidial neuroplasm surrounds the centrally placed nucleus. The inhibitory fiber (I) enters the sensory area of the muscle. In Fig. 4b is shown the radioautographic image produced by the tritiated γABA still adhering to the receptor after a number of washings with nonisotopic Van Harreveld's solution, as described under Methods. The isotope was found in varying concentrations over the axon, perikaryon, inhibitory fiber, and muscle. The dense lines of grains on the right side of the neuron are assumed to be associated with branches and endings of the inhibitory axon located within the connective tissue surrounding the sensory neuron and dendrites. The parallel distribution of grains in RM_1 corresponds to the parallel arrangements of the dendritic branches of SN_1 and of the muscle fibers themselves (see Fig. 3a).

In Figs. 5a and 5b are shown results similar to those above obtained with the fast-adapting receptor muscle (RM_2) and its neuron (SN_2) upon incubation in tritiated γABA at 0° to 4° for 10 min. The chromidial neuroplasm appears at one side of the "filamentous neuroplasm", which is continuous from dendritoplasm to axoplasm. An inhibitory fiber runs alongside SN_2 and enters the dendritic area. The dendrites of SN_2 branch in all directions upon entering the muscle fibers in RM_2, in contrast to the parallel arrangements of dendrites and muscle fibers in RM_1. There is a complicated intermeshing of dendrites and inhibitory fibers in RM_2 (see Fig. 3a). If the labeled γABA were to be associated with the axodendritic connections, an image would be

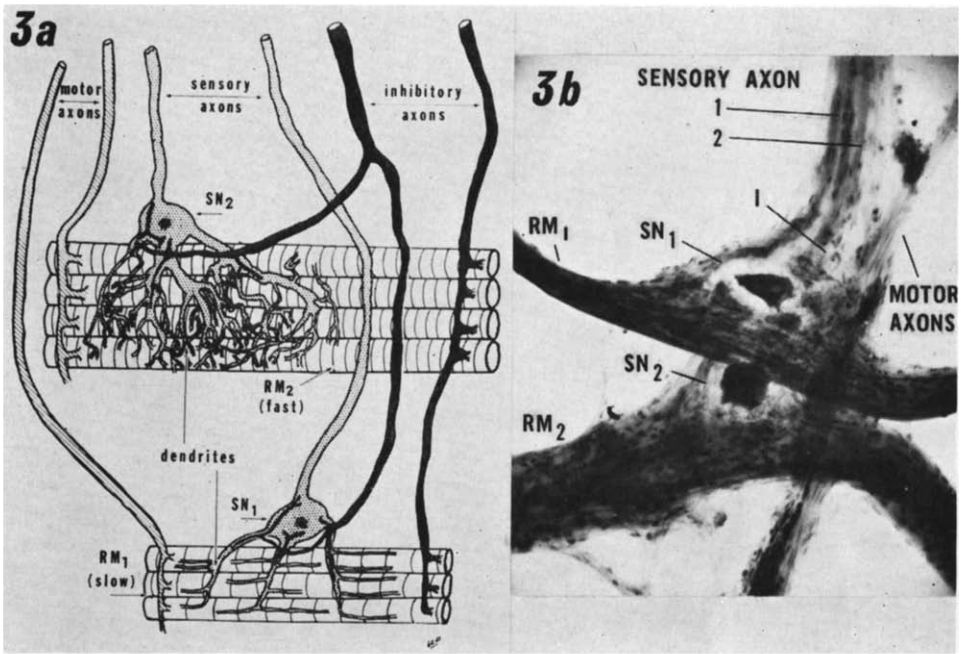

FIG. 3. a: Diagram of anatomical relationships of crayfish stretch receptors, RM_1 and RM_2. b: A preparation of stretch receptors fixed in D19 developer, rinsed in water, stained with azure B, rinsed, and dried. I points to the region of the inhibitory nerves which were difficult to demonstrate in low-power photographs.

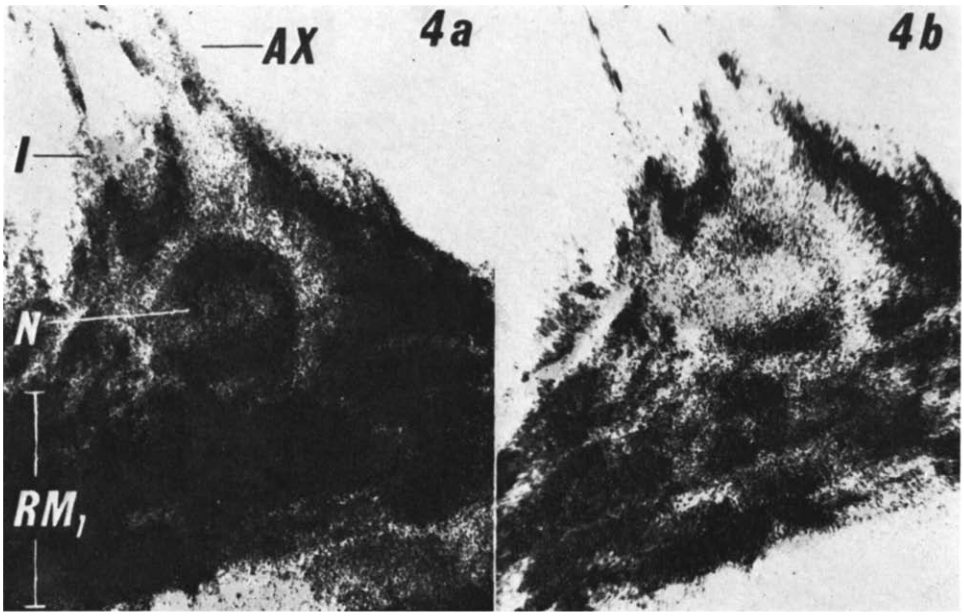

FIG. 4. a: Stained radioautograph of RM_1 complex after incubation for 5 min at 25° with γABA-^{3}H. b: Same as a after photography with filters. I, inhibitory axon; N, nucleus of SN_1; AX, sensory axon. Approximately × 252.

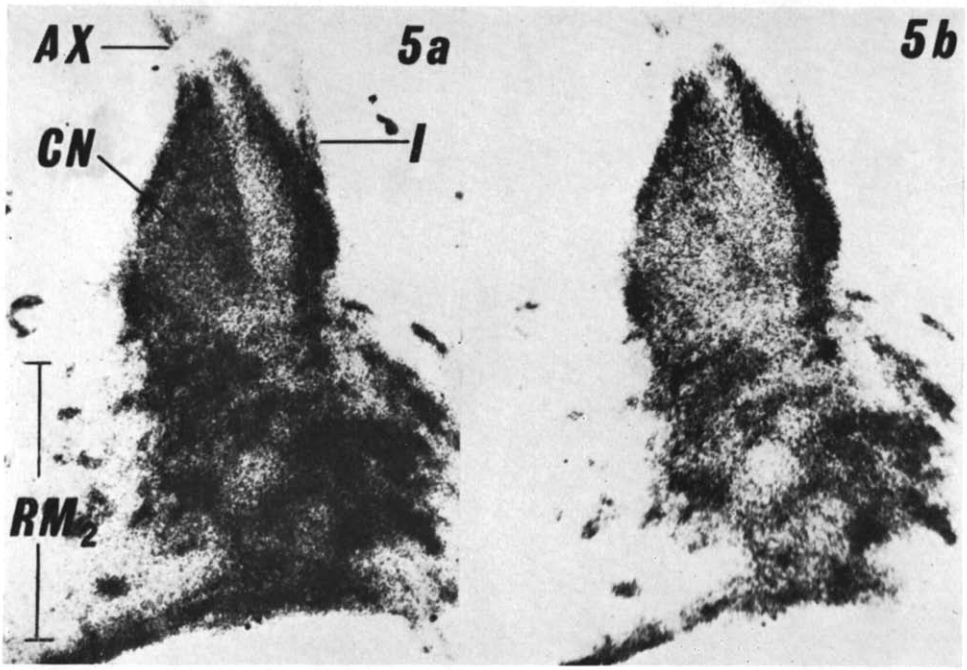

FIG. 5. a: Stained radioautograph of RM_2 complex after incubation for 10 min at 0° to 4° with γABA-^{3}H. b: Same as a after photography with filters. CN, chromidial neuroplasm. Approximately × 256.

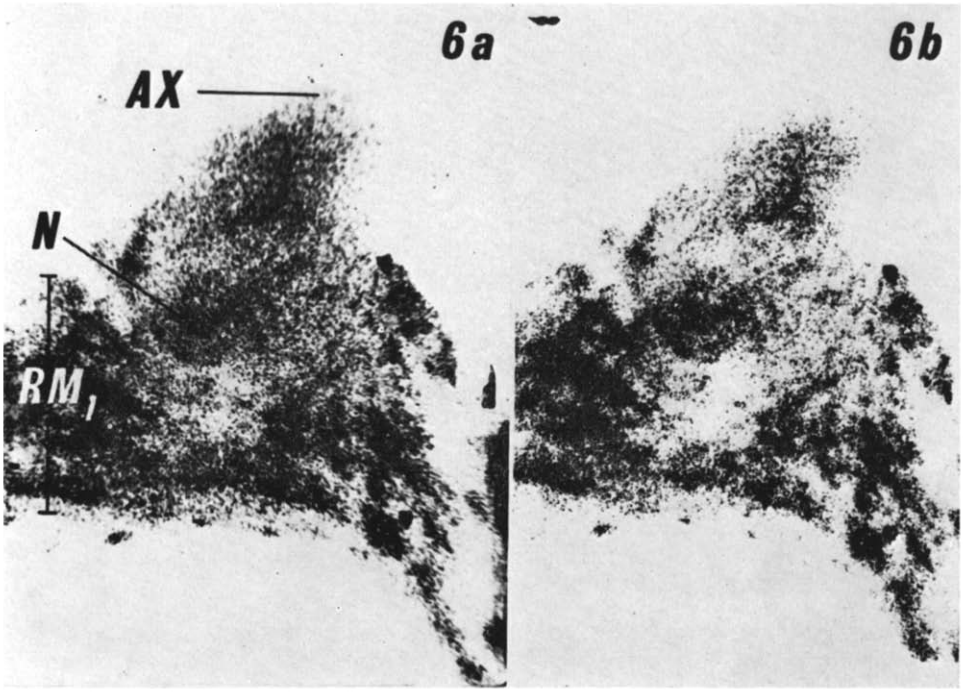

FIG. 6. Control experiment on RM_1 complex for results in Fig. 7–9. Comparable to that of Fig. 4. a: unfiltered photograph; b: filtered. Approximately ×277.

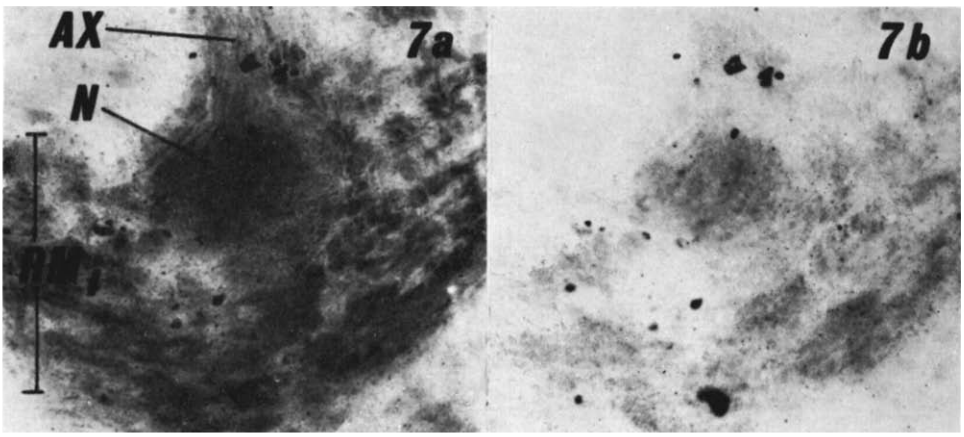

FIG. 7. Same as experiment in Fig. 6, but receptor (RM_1) incubated in sodium-free Van Harreveld's solution. a: unfiltered photograph; b: filtered. Approximately ×277.

facing page 100

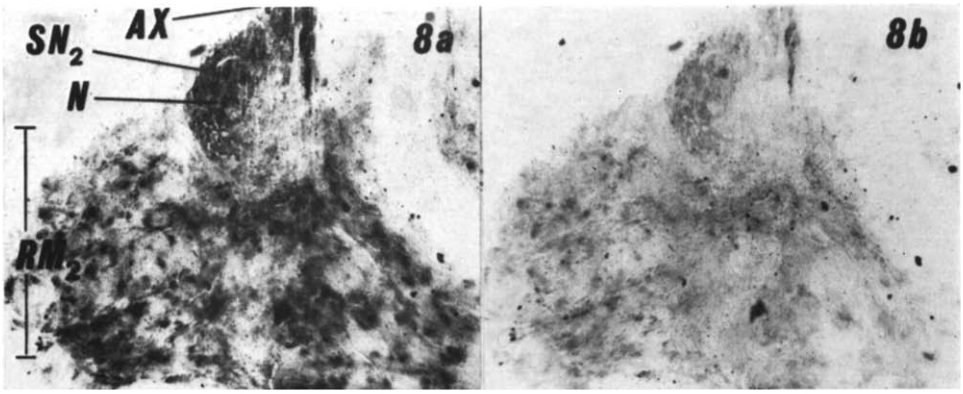

FIG. 8. Same as experiment in Fig. 6, but thymidine-^{3}H substituted for γABA-^{3}H. a: unfiltered photograph; b: filtered. Approximately $\times$ 255.

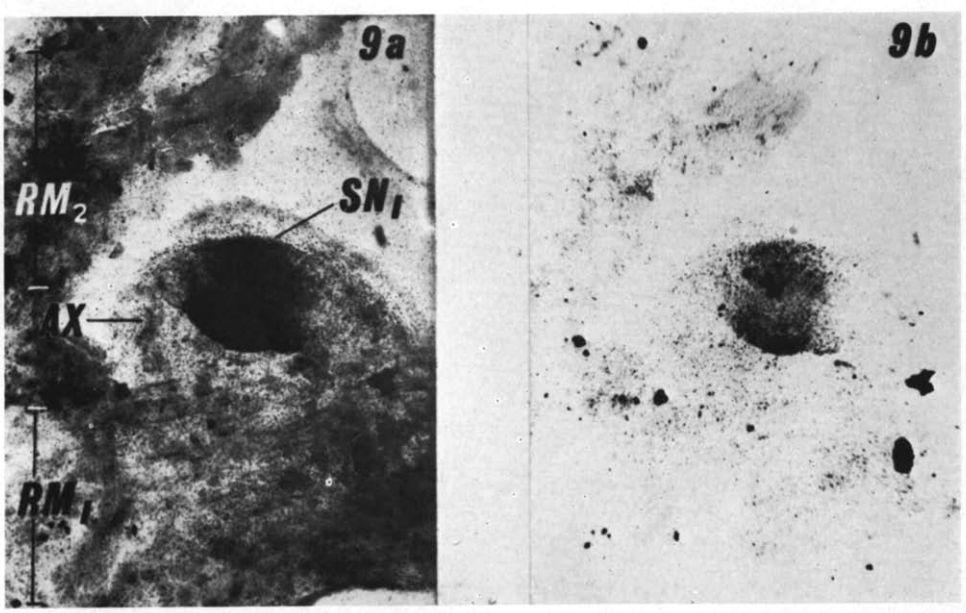

FIG. 9. Same as experiment in Fig. 6, but DL-leucine-^{3}H substituted for γABA-^{3}H. a: unfiltered photograph; b: filtered. Approximately $\times$ 255.

produced only by those endings which are close to the surface of the muscle next to the photographic emulsion. The results seen in Fig. 4b and 5b are consistent with the occurrence of relatively large amounts of bound γABA in the region of such connections, since the silver deposits of the radioautographs correspond to the histologically visualized structures. The above results are typical of many obtained with crayfish receptor organs.

Experiments designed to test some aspects of the specificity of the binding

As in the case of mouse brain particles,[17] the binding of γABA by the stretch receptor seems to be osmotically sensitive. When water instead of Van Harreveld's solution was used to rinse the receptor after exposure to labeled γABA in the saline there was a great loss of isotope from the preparation with only a diffuse, nonspecific distribution of the small amount of remaining isotope being noted on the radioautographs. Fixation in formol–saline for 3 min destroyed all localization of the isotope and caused leakage of the tritiated γABA out of the receptor organ. Similar results were observed when the preparations incubated in the usual fashion were just allowed to dry and then were washed with Van Harreveld's solution.

It was of particular interest to determine whether or not Na^+, which is obligatory for binding exogenously added γABA to brain preparations,[17] also is required for binding γABA to the stretch receptor. Experiments were performed in which incubations with labeled γABA were made for 5 min at room temperature in normal Van Harreveld's solution or in a solution identical in all respects with the exception that all of the sodium salts were replaced by isomolar concentrations of potassium salts, the anions remaining the same. The results of a typical control experiment in this series are shown in Fig. 6a and 6b, the localization of the isotope being similar to that noted previously. In the absence of Na^+ there was a remarkable decrease in binding of γABA by the stretch receptor, only a few random grains appearing on the autographs, and there was no indication of the typical pattern observed in the Na^+-containing solution (Fig. 7a and 7b). In addition, immersion in Na^+-free solution resulted in distortion of a number of the structural features of the receptor organ. Results similar to those in Fig. 6 and 7 were observed in a number of other preparations of RM_1 and RM_2.

Since it appeared possible that the areas binding the tritiated γABA might bind many other substances as well, in a nonspecific manner, it appeared necessary to test the specificity of the binding by comparing the results with γABA and those obtained with other available suitably labeled materials. A series of experiments was performed identical in all respects with those using γABA with the exception that equal concentrations of radioactivity (50 μc/ml) of thymidine-^{3}H and DL-leucine-^{3}H were included in the initial incubation solution. The radioautographs prepared after incubation with these two compounds showed a relatively small, diffuse uptake with no resemblance to the patterns seen after incubation in γABA. A direct comparison of results obtained in simultaneously performed experiments in γABA-^{3}H, thymidine-^{3}H, and leucine-^{3}H can be made by examining the results in Fig. 6, 8, and 9 respectively. In the future it will be necessary to check many other substances in addition to those above in similar experiments.

DISCUSSION

These experiments open the way to a possible means of direct determination in the crayfish stretch receptor system of the sites to which exogenously applied γABA

binds while producing its well-known inhibitory physiological effect. The resolution of the visualization of deposition sites of the silver grains will have to be improved considerably, perhaps with the aid of electron microscopy, before it will be feasible to determine with certainty whether γABA really concentrates at the axodendritic synapses and whether, if such a concentration occurs, it is greater at presynaptic or postsynaptic sites. It has not been determined definitively in any instance studied to date whether γABA exerts its action in the stretch receptor directly on the presynaptic or postsynaptic membranes or by facilitating the release at the presynaptic ending of the natural inhibitory transmitter, which in turn produces the conductance change in the postsynaptic membrane. Evidence has been adduced for the belief that some, as yet unidentified, substance which is much more potent than γABA on a molar basis is the inhibitor that occurs naturally in the crayfish,[21] although γABA now has been found in the tissue of both the peripheral and central nervous systems in crabs and lobsters, and L-glutamic acid decarboxylase has been shown to be present in a particulate fraction from lobster peripheral and central nervous tissue.[14, 15] The finding that the inhibitory effect of γABA on the normal contraction of the crayfish claw muscle disappeared after section of the inhibitory nerve alone or after total denervation, but not after section of the motor nerves with retention of the inhibitory fibers,[22] supports the point of view that γABA may not exert its action directly on the postsynaptic membrane at this neuromuscular junction. Another action of exogenously applied γABA or of the stimulation of the inhibitory nerve at a crayfish neuromuscular junction is to inhibit the presynaptic excitatory axon, presumably by decreasing release of excitatory transmitter.[23] One of the possibilities suggested by the above data is that γABA might be a substance affecting the stability of presynaptic membranes, decreasing the stability of vesicles at inhibitory endings and increasing the stability of those at excitatory endings. Thus, in the crayfish stretch receptor sensory neuron, which has only inhibitory endings, exogenously applied γABA could mimic the action of the inhibitory transmitter by decreasing the stability of the vesicles and facilitating liberation of some inhibitor-containing granules. Such a role would be consistent with the failure of γABA to have any effect after section and degeneration of the inhibitory nerve and with electron microscopic studies of the crayfish stretch receptor, which showed that axodendritic endings consist of vesicle-packed boutons.

It is obvious, however, that a number of important questions must yet be answered at the chemomorphological level before it will be possible to make a direct approach to the study of the molecular mechanism of the action of γABA.

REFERENCES

1. *Inhibition in the Nervous System and γ-Aminobutyric Acid*, E. ROBERTS, C. F. BAXTER, A. VAN HARREVELD, C. A. G. WIERSMA, W. R. ADEY and K. F. KILLAM, Eds. Pergamon Press, Oxford (1960).
2. *Nervous Inhibition*, E. FLOREY, Ed. Pergamon Press, Oxford (1961).
3. H. H. JASPER, in *Inhibition in the Nervous System*, E. ROBERTS, *et al.*, Ed., p. 12. Pergamon Press, Oxford (1960).
4. E. J. FURSHPAN and D. D. POTTER, *J. Physiol. (Lond.)* **145**, 289 (1959).
5. S. HAGIWARA and H. MORITA, *J. Neurophysiol.* **25**, 721 (1962).
6. E. FLOREY and E. FLOREY, *J. gen. Physiol.* **39**, 69 (1955).
7. D. BODIAN and A. R. BERGMAN, *Bull. Johns Hopk. Hosp.* **110**, 78 (1962).
8. R. P. PETERSON and F. A. PEPE, *J. biophys. biochem. Cytol.* **11**, 157 (1961).

9. C. EDWARDS, in *Inhibition in the Nervous System*, E. ROBERTS, *et al.*, Ed., p. 386. Pergamon Press, Oxford (1960).
10. C. EYZAGUIRRE, in *Nervous Inhibition*, E. FLOREY, Ed. Pergamon Press, Oxford (1961).
11. C. A. G. WIERSMA, E. J. FURSHPAN and E. FLOREY, *J. exp. Biol.* **30**, 136 (1953).
12. S. W. KUFFLER and C. EYZAGUIRRE, *J. gen. Physiol.* **39**, 155 (1955).
13. S. W. KUFFLER and C. EDWARDS, *J. Neurophysiol.* **21**, 589 (1958).
14. E. A. KRAVITZ, D. D. POTTER and N. M. VAN GELDER, *Nature* (*Lond.*) **194**, 382 (1962).
15. E. A. KRAVITZ, *J. Neurochem.* **9**, 363 (1962).
16. K. SANO and E. ROBERTS, *Biochem. biophys. Res. Commun.* **4**, 358 (1961).
17. K. SANO and E. ROBERTS, *Biochem. Pharmacol.* **12**, 489 (1963).
18. A. VAN HARREVELD, *Proc. Soc. exp. Biol.* (*N.Y.*) **34**, 428 (1936).
19. R. O. ECKERT, *J. cell. comp. Physiol.* **57**, 149 (1961).
20. E. ROBERTS and E. EIDELBERG, *Int. Rev. Neurobiol.* **2**, 279 (1960).
21. E. FLOREY, *J. gen. Physiol.* **43**, 518 (1960).
22. M. YA KUNTSOVA, *Bull. exp. Biol. Med.* **12**, 8 (1961).
23. J. DUDEL and S. W. KUFFLER, *J. Physiol.* (*Lond.*) **155**, 543 (1961).