RELEASE OF [³H]γ-AMINOBUTYRIC ACID FROM GLIAL (MÜLLER) CELLS OF THE RAT RETINA: EFFECTS OF K⁺, VERATRIDINE, AND ETHYLENEDIAMINE¹

P. VIJAY SARTHY

Department of Ophthalmology, University of Washington School of Medicine, Seattle, Washington 98195

Received September 28, 1982; Revised July 5, 1983; Accepted July 6, 1983

Abstract

In several neural systems, glial cells appear to take up and release γ-aminobutyric acid (GABA) upon depolarization. We have studied the release of [³H]GABA from Müller (glial) cells in the rat retina by a double isotope-labeling technique in which Müller cells are preloaded with ³H-GABA while a population of neurons is prelabeled with [¹⁴C]glycine. By autoradiography, we have confirmed that [³H]GABA is taken up by the radially oriented Müller cells, whereas [³H]glycine is accumulated by a subset of amacrine cells (neurons). Using the double-labeling procedure, we have examined the effects of two depolarizing agents, high K⁺ and veratridine, and the GABA mimetic, ethylenediamine, on transmitter release from glial cells and neurons simultaneously. We found the following. (1) Depolarization with 56 mM K⁺ released both [³H]GABA and [¹⁴C]glycine. About 70 to 80% of this release was blocked in Ca²⁺-free medium. (2) Veratridine (10 μM) also released both of the transmitters. This release was strongly inhibited by 100 nM tetrodotoxin or 1 mM procaine. Under Ca²⁺-free conditions, < 20% isotope release was observed. (3) Ethylenediamine released [³H]GABA readily, whereas little [¹⁴C]glycine release was observed. Removal of Ca²⁺ had no significant effect on transmitter release. Furthermore, in Na⁺-free medium ethylenediamine failed to induce [³H]GABA or [¹⁴C]glycine release. These results suggest that high K⁺ and veratridine release [³H]GABA from Müller cells by a Ca²⁺-dependent process. Ethylenediamine, on the other hand, appears to induce [³H]GABA release by a Ca²⁺-independent, carrier-mediated exchange mechanism.

Like neurons, glial cells appear to take up and release γ-aminobutyric acid (GABA) upon depolarization (Varon and Somjen, 1979). This observation has led to the question as to whether glial cells and neurons release the transmitter by analogous mechanisms. In several neural systems, glia have been reported to release preloaded GABA upon stimulation with high K⁺ or veratridine (Bowery and Brown, 1972; Voaden and Starr, 1972; Minchin and Iversen, 1974; Roberts, 1974; Schrier and Thompson, 1974; Minchin, 1975; Hertz, 1977; Sellstrom and Hamberger, 1977; Bowery et al., 1979; Neal and Bowery, 1979; Hertz and Schousboe, 1980; Jaffe and Cuello, 1981; Pearce et al., 1981). However, there has been disagreement as to whether this release is Ca²⁺-dependent or not. Recently, a GABA mimetic, ethylenediamine, has been shown to release GABA from rat

striatal slices by a Ca²⁺-independent mechanism (Lloyd et al., 1982). The effect of this chemical on GABA release from retina has not been reported.

We have examined release of GABA from glial cells in the vertebrate retina. In the rat retina, [³H]GABA is taken up by a sodium-dependent, high affinity uptake system. Autoradiographic localization shows that the sites of uptake are the glial (Müller) cells (Neal and Iverson, 1972). In an earlier study, [³H]GABA release was not detected upon exposure of preloaded rat retina to high K⁺ or veratridine (Neal and Bowery, 1979). During a reinvestigation of this question, we observed that preloaded [³H]GABA could be released by depolarization of the retina. However, the amount of release was small and appeared to depend on the physiological condition of the tissue.

In order to pursue this observation more rigorously, we used a double isotope-labeling technique in which glial cells were preloaded with [³H]GABA while a population of neurons was prelabeled with [¹⁴C]glycine. Autoradiographic studies have shown that, unlike GABA, which is taken up by glial cells, glycine is accumulated by a subset of amacrine cells (neurons) located in the

¹ This study was supported in part by National Institutes of Health Research Grants EY-03523, EY-03664, and EY-01730 from the National Eye Institute, and in part by an unrestricted grant from Research to Prevent Blindness, Inc. I thank Bill Catterall for suggesting the use of α-latrotoxin, Brad Clifton and Jim Foltz for photographic help, and Julie Seng for secretarial assistance.

inner nuclear layer (Bruun and Ehinger, 1974; Marshall and Voaden, 1974). This double-labeling procedure provided us with an opportunity to examine glial and neuronal release concurrently.

Using this approach, we have demonstrated that $[^3\text{H}]$ GABA can be released from glial cells by depolarization with high K^+ or veratridine. Furthermore, this release was suppressed by removal of external Ca^{2+} . We have also shown that $[^3\text{H}]$ GABA can be selectively released from glial cells by exposure to ethylenediamine and that this release occurs by a Ca^{2+} -dependent mechanism.

Materials and Methods

Sprague-Dawley rats (~200 to 250 gm body weight) were anesthetized with ether and decapitated. After enucleation, the lens was cut off and the retina was gently isolated in mouse bicarbonate Ringer (MBR). The composition of this Ringer is (in mM): NaCl, 120; KCl, 4.8; NaHCO_3 , 25; CaCl_2 , 1.9; MgCl_2 , 0.49; dextrose, 5.0; and HEPES, 2.8. The medium was adjusted to pH 7.55 and bubbled with a 95% O_2 and 5% CO_2 mixture to yield a final pH of 7.45.

Autoradiography. After preincubation for 5 min at 37°C, retinas were incubated in 0.2 ml of MBR containing either 100 $\mu\text{Ci/ml}$ of $[^3\text{H}]$ GABA (28.2 Ci/mmol) or 50 $\mu\text{Ci/ml}$ of $[^3\text{H}]$ glycine (12.3 Ci/mmol). The incubation medium also contained 100 μM amino-oxyacetic acid (AOAA) and a mixture of alanine, proline, and serine, each at 5 mM concentrations (APS mixture). These amino acids have been reported to block the leucine-preferring (L) neutral amino acid uptake system by which glycine enters cells, without significantly affecting the high affinity system present in glycinergic neurons (Oxender and Christensen, 1963; Sepulveda and Smith, 1978; Chin and Lam, 1980). Incubations with $[^3\text{H}]$ GABA were carried out in the presence of 4 μM glycine, and $[^3\text{H}]$ glycine incubations were carried out in the presence of 3.5 μM GABA. Following a 20-min incubation at 37°C, the retinas were washed for 5 min in MBR and fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 4 hr at room temperature, and overnight at 4°C.

In order to examine the effect of the presynaptic toxin, latrotoxin, retinas preincubated with $[^3\text{H}]$ GABA or $[^3\text{H}]$ glycine were rinsed and further incubated for 20 min in 0.2 ml of bicarbonate Ringer containing 20 μl of the toxin solution and AOAA and APS. Subsequently, retinas were washed twice in 5 ml of Ringer for 5 min and fixed in glutaraldehyde. Spider venom toxin was prepared by homogenizing 10 black widow spider venom glands (Sigma Chemical Co., St. Louis, MO) in 0.25 ml of cold 0.05 M Tris-HCl buffer, pH 8.2. The homogenate was spun at $15,000 \times g$, and the supernatant was used (Frontali et al., 1976).

The fixed tissue was washed twice in phosphate buffer (5 min each), and treated with 1% OsO_4 for 2 hr. After three 5-min washes in the buffer, the tissue was dehydrated in an alcohol series consisting of 25, 75, 90, and 100% (15 min each). Following dehydration, the tissue was treated with propylene oxide two times, for 15 min each, and left overnight in a 1:1 mixture of propylene oxide and 2, 4, 6-tri(dimethylaminomethyl)phenol

(DMP-30). After a 3-hr treatment with resin and DMP-30 mixture, the tissue was transferred to fresh Epon in flat embedding molds and carefully oriented for radial sectioning. Polymerization was carried out at 65°C for 48 hr.

About 1- μm -thick sections were cut on a Reichardt microtome and placed on acid-cleaned glass slides. Slides were dipped in the dark, in 50% Kodak NTB2 nuclear tract emulsion at 40°C. After exposure for 1 to 4 weeks at 4°C and 0% relative humidity, slides were developed in 50% Dektol for 2 min at 15°C. After a brief rinse in water, the slides were fixed in Kodak fixer for 15 min at 15°C. After three washes in water, the sections were stained with diluted Richardson's stain. Autoradiograms were examined under transmitted and darkfield illumination to assess the distribution of silver grains.

Release experiments. After preincubation, retinas were transferred to 0.2 ml of MBR containing 100 μM AOAA, 5 mM APS mixture, and 1 $\mu\text{Ci/ml}$ each of $[^3\text{H}]$ GABA and $[^{14}\text{C}]$ glycine (112 mCi/mmol). Following 20 min incubation, retinas were rinsed and then washed three times, for 5 min each, in 20 ml of MBR containing AOAA (100 μM). Next, a piece of retina was introduced into a 15-ml conical tube containing 2 ml of MBR. At 2-min intervals, 1.9-ml aliquots were withdrawn and replaced by the same amount of fresh medium. At 10 min, MBR contained 56 mM K^+ or the test chemical, such as veratridine, was added. After recovering three 2-min samples, the medium was changed back to MBR, and isotope release was followed for an additional 12 min. At the end of the experiment, the tissue was homogenized in 1 ml of 10 mM HCl. Aliquots (1 ml) of the different fractions and the homogenate were mixed with 10 ml of Aquasol II (New England Nuclear Corp., Boston, MA), and the radioactivity was determined in a Beckman scintillation counter. AOAA was present throughout the course of the experiments.

Radioactive counts were calculated on the basis of single or double-isotope internal standards. ^3H activity was counted at an efficiency of 12%, whereas ^{14}C efficiency was about 40%. The counting windows were set such that about 15% of the ^{14}C counts spilled into the ^3H channel. Release is defined as:

$$\frac{(\text{cpm under peak of release} - \text{basal level efflux})}{(\text{cpm in all fractions} + \text{homogenate})}$$

where basal level efflux = (average cpm per prestimulation fraction $\times$ number of peak fractions). In several control experiments, the basal level efflux values were found to be similar to those of the nonstimulated controls. Furthermore, the nonstimulated controls never showed transmitter release, either in the presence or in the absence of Ca^{2+} . This method of estimating the percentage of release is limited by the subjective nature of the basal level and peak cpm estimates. However, since the same criteria are used throughout the study, results from different experiments can be compared.

Veratridine was used at a concentration of 10 μM in all experiments. In order to test for the specificity of veratridine action, Na^+ channel blockers, tetrodotoxin or procaine were included along with veratridine, at concentrations of 100 nM and 1 mM, respectively. In

addition, retinas were pretreated with the inhibitors for 2 min before exposure to veratridine.

Uptake experiments. Three-millimeter discs of retina were incubated in 0.5 ml of MBR, containing 5 μ M [^{3} H] GABA, 1 μ M [^{14} C]glycine, 100 μ M AOAA, and 5 mM APS mixture for 15 min at 37°C. In addition, 1 mM nipecotic acid or 5 mM ethylenediamine (EDA) were included when required. After incubation, retinas were washed for 5 min to get rid of extracellular radioactivity, homogenized in 10 mM HCl, and counted for radioactivity. ^{14} C counts accumulated in the different conditions were close but not identical, and the ^{3} H radioactivity was normalized to

the ^{14} C uptake in the different retinas.

Product identification. The identity of ^{3} H and ^{14} C compounds accumulated in the tissue was determined as follows. Retinas were incubated with [^{3} H]GABA and [^{14} C]glycine as described earlier and homogenized in 250 μ l of 0.1 N HCl containing GABA and glycine (2 mg/ml) each. Fifty microliters of this mixture were spotted on Whatman 3 MM paper and electrophoresed at 6000 V for 1 hr at pH 1.9. The paper was air dried and later stained with ninhydrin. The spots corresponding to GABA and glycine were cut out and eluted with 1 ml of 10 mM HCl, and their radioactivity was determined.

Figure 1. Autoradiograms of retinas incubated with [^{3} H]GABA (A) and [^{14} C]glycine (D). Retinas were incubated with 100 μ Ci/ml of [^{3} H]GABA or [^{3} H]glycine in the presence of AOAA (0.1 mM) and 5 mM each of alanine, proline, and serine (APS). Glycine was included at a concentration of 4 μ M in [^{3} H]GABA incubations, and 3.5 μ M GABA was added to [^{14} C]glycine incubations. After 20 min at 37°C, the retinas were rinsed, fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and processed for autoradiography. Alternatively, preloaded retinas were washed five times for 5 min in Ringer containing AOAA (C) and stimulated with veratridine for 6 min before fixation (D). In some experiments, retinas prelabeled with [^{3} H]GABA (E) or [^{3} H]glycine (F) were incubated with spider toxin for 20 min at 37°C in the presence of AOAA and APS. Subsequently, the retinas were washed three times for 5 min, fixed, and processed. *on*, outer nuclear layer; *in*, inner nuclear layer; *ip*, inner plexiform layer. Scale bar = 60 μ m.

More than 99% of ^3H and $\sim 95\%$ ^{14}C activity in the tissue co-migrated with GABA and glycine spots, respectively. These results established that little GABA and glycine metabolism occurred under our experimental conditions.

Solutions. High potassium (56 mM) Ringer was prepared by replacing an equivalent amount of Na^+ from MBR with K^+ . Na^+ -free medium was made up by replacing NaCl and NaHCO_3 with choline chloride and adding 10 mM Tris-HCl to the medium.

Ca^{2+} -free medium was prepared by eliminating CaCl_2 and including 10 mM MgCl_2 or 5 mM CoCl_2 in the medium.

Materials. All chemicals used were AnalR grade. GABA and AOAA were purchased from Sigma Chemical Co., St. Louis, MO. The radiochemicals used were purchased from New England Nuclear Corp., Boston, MA. Tetrodotoxin and procaine were kindly given to us by Dr. Bertil Hille, and Dr. William Catterall provided us with veratridine.

Results

Localization of uptake sites. Before we began experiments on transmitter release, we examined the pattern of GABA and glycine uptake under our experimental conditions. Retinas were incubated with $[^3\text{H}]\text{GABA}$ or $[^3\text{H}]\text{glycine}$ for 20 min at 37°C , rinsed briefly, fixed in glutaraldehyde, and processed for autoradiography. Results of these experiments presented in Figure 1 show that, with $[^3\text{H}]\text{GABA}$, silver grains were located radially throughout the retina with intensely labeled cell bodies distributed in the inner nuclear layer (INL). Silver grains were also dispersed at a high density along the external limiting membrane and throughout the inner retina (Fig. 1A). The disposition of the silver grains suggests that most of the labeled cells were the radially oriented glial (Müller) cells which have cell bodies in the INL and send out processes into both the outer and inner retina (Ramon y Cajal, 1982). In the case of $[^3\text{H}]\text{glycine}$, labeling was confined essentially to the inner retina. Labeled cell bodies were seen in the INL, and the inner plexiform layer (IPL) was also heavily labeled, presumably due to labeling of glycinergic processes (Fig. 1D). We infer that these labeled cells are a subset of amacrine cells which are glycinergic neurons. Our observations on GABA labeling of glial cells and glycine uptake by amacrine cells are in agreement with previous reports (Neal and Iversen, 1972; Brunn and Ehinger, 1974; Marshall and Voaden, 1974; Voaden, 1976).

It has been reported that a rapid redistribution of ^3H activity occurs with prolonged washing of rabbit retinas preloaded with $[^3\text{H}]\text{GABA}$ (Ehinger, 1977). Since some of the newly labeled cells appear to be a population of amacrine cells, it has been suggested that postincubation washing may lead to transfer of ^3H label from glial cells to neurons. To examine this possibility, we incubated rat retinas with $[^3\text{H}]\text{GABA}$ for 15 min at 37°C in the presence of AOAA and APS mixture. After washing five times for 5 min at 37°C in normal Ringer, the retinas were fixed in glutaraldehyde and processed for autoradiography. Alternatively, washed retinas were exposed to 10 μM veratridine for 6 min and processed. Results of the experiment presented in Figure 1B show that the

distribution of silver grains was essentially the same as that in unwashed controls (Fig. 1A), which suggests that little redistribution of ^3H activity occurred during postincubation of the retina. Similarly, the distribution of $[^3\text{H}]\text{glycine}$ label was not changed in veratridine-treated retinas (Fig. 1E).

K^+ -evoked release. Because glial cells are considered to be highly sensitive to external K^+ (Kuffler and Nicholls, 1966), we examined the effect of elevated K^+ on the release of $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$. Retinas were incubated with the labeled transmitters, washed to eliminate extracellular radioactivity, and exposed to a 6-min pulse of 56 mM K^+ -Ringer. When Ca^{2+} was eliminated from the high K^+ -Ringer, 5 mM Co^{2+} or 10 mM Mg^{2+} was included. The effect of high K^+ stimulation on neurotransmitter release is presented in Figure 2. Upon exposure to high K^+ , a marked increase in the release of both ^3H and ^{14}C isotope was observed. This high release rate was reduced, upon returning the tissue to normal Ringer. When Ca^{2+} was eliminated from the high K^+ medium, only a small increase in isotope efflux was observed. Exposure to high K^+ released about 2.1% of $[^3\text{H}]\text{GABA}$ and 10.5% $[^{14}\text{C}]\text{glycine}$ from the retina. In the absence of Ca^{2+} , the amount was reduced to 0.63% and 2.2% for GABA and glycine, respectively (Table I).

Figure 2. Effect of high K^+ on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ release. Retinas were incubated with $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ for 15 min at 37°C . After a series of washes, they were exposed to bicarbonate Ringer containing 56 mM K^+ for 6 min. Radioactivity in samples collected during the experiment was determined by standard double-isotope scintillation counting methods. The Ca^{2+} -free Ringer was devoid of Ca^{2+} and contained 5 mM Co^{2+} .

TABLE I

Amount of transmitter released under various conditions

Transmitter release is expressed as percentage of input cpm which ranged from 10 to 12×10^4 cpm for [^3H]GABA and 18 to 20×10^3 for [^{14}C]glycine. All values are mean $\pm$ SEM.

	K ⁺		Veratridine			EDA			GABA		NH ₄ ⁺ (3)
	+Ca ²⁺ (6) ^a	-Ca ²⁺ (6)	+Ca ²⁺ (6)	+TTX (3)	+Procaine (3)	-Ca ²⁺ (4)	+Ca ²⁺ (3)	-Ca ²⁺ (3)	+Ca ²⁺ (4)	-Ca ²⁺ (4)	
[^3H]GABA	2.1 ± 1.0	0.63 ± 0.2	0.93 ± 0.03	<0.06	<0.17	0.26 ± 0.1	1.8 ± 0.2	1.3 ± 0.3	11.0 ± 1.6	6.5 ± 2.1	0.18 ± 0.1
[^{14}C]Glycine	10.5 ± 2.4	2.2 ± 0.8	16.4 ± 1.5	1.1 ± 0.6	0.90 ± 0.2	3.2 ± 0.7	<0.1	<0.1	1.1 ± 0.5	0.4 ± 0.3	1.6 ± 0.4

^a Numbers in parentheses, number of separate experiments.

Release from spider toxin-treated retina. Although our autoradiographic data show that [^3H]GABA is accumulated predominantly by the radially oriented Müller cells, the data cannot rule out labeling of some GABAergic neurons additionally. Furthermore, the K⁺-evoked, Ca²⁺-dependent release of [^3H]GABA could be adduced as evidence for the presence of [^3H]GABA-labeled neurons in the retina.

In order to examine this possibility, we have studied transmitter release from retinas treated with the presynaptic toxin, α -latrotoxin. This compound, which is present in the venom glands of the black widow spider (*Latrodectus mactans tredecimguttatus*), is a protein of $M_r = 130,000$, which has been demonstrated to cause massive release of acetylcholine, noradrenaline, dopamine, and GABA from cortical slices (Tzeng and Siekevitz, 1978, 1979; Tzeng et al., 1978) and synaptosomes (Grasso and Senni, 1979; Baba and Cooper, 1980; Meldolesi, 1982; Nicholls et al., 1982).

Retinas were prelabeled with [^3H]GABA and [^{14}C]glycine, rinsed, and incubated with the toxin for 20 min at 37°C. After three 5-min washes in 5 ml of Ringer containing AOAA, K⁺-evoked transmitter release was examined. Results of the experiment are presented in Figure 3. Treatment with high K⁺ resulted in a large increase in [^3H]GABA release from both toxin-treated and control retinas. In contrast, only a small increase in [^{14}C]glycine release occurred from toxin-treated retinas, although substantial glycine release was observed in untreated control retinas. In two separate experiments, the average GABA release was 2.3 and 2.1% for the control and the toxin-treated retinas, respectively. With glycine, release amounted to 9.5% for the control and 1.5% for the toxin-treated retinas. Since toxin treatment leads to a loss of >80% of [^{14}C]glycine release, it may be concluded that most of the [^3H]GABA release occurred from Müller cells.

To further explore the effect of spider toxin, we have examined the distribution of [^3H]GABA and [^3H]glycine in toxin-treated retinas using autoradiography. Results of the study are shown in Figure 1, C and F. Whereas toxin treatment did not appear to alter prelabeled [^3H]GABA distribution (Fig. 1C), the pattern of [^3H]glycine uptake was markedly affected. In these retinas, most of the [^3H]glycine had been depleted from cell bodies and the IPL. However, many heavily labeled cells were still present after toxin treatment (Fig. 1F). These autoradiographic data are in agreement with the lack of [^{14}C]glycine release from toxin-treated retinas.

Figure 3. High K⁺-evoked release of [^3H]GABA and [^{14}C]glycine from spider toxin-treated retina. Retinas prelabeled with [^3H]GABA and [^{14}C]glycine were rinsed and incubated in 0.2 ml of Ringer containing 20 μl of spider venom gland extract (ST) and AOAA and APS. After 20 min at 37°C, the retinas were washed three times for 5 min and exposed to high K⁺-Ringer. Radioactivity in different fractions was determined by scintillation counting.

Veratridine-induced release. The alkaloid, veratridine, depolarizes neurons by selectively increasing the permeability of resting membrane to external Na⁺ by activation of the voltage-dependent Na⁺ channel (Ulbricht, 1969; Narahashi, 1974). In order to examine the presence of such channels on glial cell membrane, we examined the effect of 10 μM veratridine on the efflux of [^3H]GABA and [^{14}C]glycine from preloaded retinas. Results of the experiments presented in Figure 4 show that exposure to veratridine resulted in a significant increase in the release of both ^3H and ^{14}C radioactivity. This elevated release was diminished when veratridine-containing me-

dium was replaced by normal Ringer. Although less effective than high K^+ , veratridine still released about 0.93% of $[^3\text{H}]\text{GABA}$. $[^{14}\text{C}]\text{Glycine}$ release was enhanced to 16.4% (Table I).

The specificity of veratridine action was established by carrying out stimulation with the drug in the presence of 100 nM Tetrodotoxin (TTX), a specific inhibitor of the Na^+ -channel (Narahashi et al., 1964). As shown in Figure 4, veratridine-stimulated release was markedly suppressed in the presence of the toxin. Only about 0.06% of ^3H and 1.1% of ^{14}C radioactivity was released under these conditions (Table I). In analogous experiments, procaine, another inhibitor of voltage-sensitive Na^+ channel, which acts by a mechanism different from that of TTX (Hille, 1977), also blocked veratridine-stimulated release of $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ (Table I).

It is generally accepted that transmitter release by veratridine involves an influx of Ca^{2+} following activation of the Na^+ channel at the nerve terminal (Blaustein, 1975). Since veratridine stimulated $[^3\text{H}]\text{GABA}$ release from glial cells, we examined the Ca^{2+} dependency of the release mechanism. In these experiments, preloaded retinas were exposed to Ca^{2+} -free Ringer containing 10 mM Mg^{2+} and 10 μM veratridine. Results of the experiment presented in Figure 5 show that removal of Ca^{2+} leads to

Figure 4. Effect of veratridine on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ release. Retinas were incubated for 20 min at 37°C in bicarbonate Ringer containing $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$. After initial washes, the retinas were exposed to 10 μM veratridine for 6 min. Radioactivity in the different fractions was determined by scintillation counting. When stimulation with veratridine was carried out in the presence of TTX, the retinas were preincubated with TTX in normal Ringer for 2 min.

Figure 5. Influence of Ca^{2+} on veratridine-stimulated release of $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$. Retinas preloaded with $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ were exposed to 10 μM veratridine in normal Ringer or Ca^{2+} -free Ringer supplemented with 10 μM Mg^{2+} .

a reduction in the amount of GABA (0.26%) and glycine (3.2%) released.

Effect of ethylenediamine. EDA has been observed to be a strong inhibitor of neuronal firing in the cerebral cortex with a potency comparable to that of GABA. Moreover, this action of EDA could be blocked by bicuculline, a GABA receptor antagonist (Perkins et al., 1981). EDA has also been reported to block uptake of $[^{14}\text{C}]\text{GABA}$ by brain slices, stimulate $[^3\text{H}]\text{diazepam}$ binding, block $[^3\text{H}]\beta\text{-alanine}$ uptake into cortical slices (glial uptake), and selectively release preloaded GABA from cortical slices or synaptosomes by a Ca^{2+} -independent mechanism (Forster et al., 1981; Davis et al., 1982; Lloyd et al., 1982).

We have examined the effect of EDA on the efflux of GABA from Müller cells both in the presence and in the absence of external Ca^{2+} . Retinas preloaded with $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ were exposed to 5 mM EDA in normal Ringer or in Ca^{2+} -free Ringer containing 10 mM Mg^{2+} . Results of the experiments presented in Figure 6 show that, upon exposure to EDA, there was a significant release of $[^3\text{H}]\text{GABA}$, whereas little $[^{14}\text{C}]\text{glycine}$ release occurred. When Ca^{2+} was eliminated from the Ringer solution containing EDA, a large induction of $[^3\text{H}]\text{GABA}$ release still occurred, whereas $[^{14}\text{C}]\text{glycine}$ release remained unaffected. EDA stimulated about 1.8% GABA and < 0.1% glycine release in normal Ringer. In the absence of Ca^{2+} , EDA induced about 1.3% GABA, whereas no change in glycine release was noted.

Figure 6. Effect of EDA on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ release. Retinas preloaded with $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ were exposed to 5 mM EDA in normal or Ca^{2+} -free Ringer containing 10 mM Mg^{2+} .

Figure 7. Effect of EDA on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ release in Na^+ -free medium. Retinas preloaded with $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ were washed and exposed to 5 mM EDA in Na^+ -free medium either containing Ca^{2+} or without Ca^{2+} .

In order to examine the role of Na^+ in EDA-stimulated release of $[^3\text{H}]\text{GABA}$, experiments were performed in Na^+ -free medium with and without added Ca^{2+} . Although the basal rates of ^3H and ^{14}C isotope efflux were 10-fold higher in this medium than in normal Ringer, no measurable stimulation of $[^3\text{H}]\text{GABA}$ or $[^{14}\text{C}]\text{glycine}$ was observed upon exposure to EDA. No differences in release were noted in Ca^{2+} -containing or Ca^{2+} -free medium (Fig. 7).

Effect of GABA. Since EDA is a GABA mimetic, a direct mechanism for the selective release of $[^3\text{H}]\text{GABA}$ would be by homoechange of intracellular $[^3\text{H}]\text{GABA}$ with exogenous EDA. In order to examine this possibility, we studied the release of $[^3\text{H}]\text{GABA}$ in the presence of 1 mM GABA. Results of the experiment presented in Figure 8 show that, in the presence of GABA, about 11% of $[^3\text{H}]\text{GABA}$ was released from the tissue, whereas only 1.1% $[^{14}\text{C}]\text{glycine}$ was released concurrently. Furthermore, in the absence of Ca^{2+} , about 6.5% $[^3\text{H}]\text{GABA}$ was still released compared to 0.4% for $[^{14}\text{C}]\text{glycine}$.

Effect of NH_4^+ . Yet another mechanism by which EDA could release $[^3\text{H}]\text{GABA}$ is by entering cells as an uncharged molecule. Once inside, it could get protonated, and thus elevate the intracellular pH, which in turn could lead to release of GABA. It is well established that treatment of cells with NH_4^+ leads to an increase in

Figure 8. Effect of GABA on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{glycine}$ release. Retinas preloaded with the transmitters were exposed to 1 mM GABA in normal or Ca^{2+} -free Ringer containing 10 mM Mg^{2+} . The dotted bar indicates duration of exposure to GABA.

Figure 9. Effect of NH_4^+ on [³H]GABA and [¹⁴C]glycine release. Retinas preloaded with the transmitters were exposed to 5 mM NH_4Cl in normal Ringer.

Figure 10. Inhibition of [³H]GABA uptake by EDA and nipeicotic acid (NA). Retinas were incubated with [³H]GABA and [¹⁴C]glycine for 15 min at 37°C. EDA and NA were present at a concentration of 5 mM and 1 mM, respectively. After incubation, the retinas were washed and homogenized in 0.5 ml of 10 mM HCl, and the radioactivity was determined by double-isotope scintillation counting. ³H counts were normalized to the ¹⁴C counts taken up. Values are mean $\pm$ SEM for three separate experiments.

intracellular pH (Thomas, 1974). Hence, we examined the effect of 5 mM NH_4^+ on the efflux of [³H]GABA and [¹⁴C]glycine. We found that NH_4^+ ions stimulated release of both [³H]GABA (0.18%) and [¹⁴C]glycine (1.6%) from the retina (Fig. 9).

Discussion

Both K^+ - and veratridine-evoked release of GABA have been examined in glial cells from the CNS and PNS with varying results. A partial suppression of GABA release was noted in some cases under Ca^{2+} -free conditions, whereas others have reported release to be completely Ca^{2+} -independent (Bowery and Brown, 1972;

Voaden and Starr, 1972; Minchin and Iversen, 1974; Roberts, 1974; Schrier and Thompson, 1974; Minchin, 1975; Hertz, 1977; Sellstrom and Hamberger, 1977; Bowery et al., 1979; Neal and Bowery, 1979; Hertz and Schousboe, 1980; Jaffe and Cuello, 1981; Pearce et al., 1981). In our experiments using 56 mM K^+ stimulation, about 70% of the GABA release was blocked by removal of external Ca^{2+} from the medium. We do not know whether the remaining (30%) [³H]GABA release is due to a Ca^{2+} -independent component or some other factor, since [¹⁴C]glycine release from neurons was only reduced by about 80% under the same conditions.

With veratridine, glia from central nervous tissue have been reported to release significant amounts of preloaded GABA, although peripheral glia from sensory, sympathetic, or trigeminal ganglion cells responded poorly (Minchin, 1975; Neal and Bowery, 1979; Jaffe and Cuello, 1981). In a different system, glial cells in culture were found to accumulate exogenous ²²Na upon stimulation with veratridine (Munson et al., 1979; see also Catterall and Nirenberg, 1973; Stallcup and Cohen, 1976; Villegas et al., 1976). These observations have been put forward as evidence for the presence of voltage-sensitive Na^+ channels on glial cells. Our experimental results also suggest that Müller cells in the retina may respond to veratridine stimulation by activation of TTX-inhibited, voltage-sensitive Na^+ channels on their membrane.

It is intriguing that, although the K^+ -evoked release of both GABA and glycine was Ca^{2+} dependent, the effect of α -latrotoxin on release was markedly different for the two transmitters. Whereas K^+ -evoked [³H]GABA release was unaffected, [¹⁴C]glycine release was strongly suppressed in toxin-treated retinas. α -Latrotoxin appears to act by increasing the Na^+ and Ca^{2+} conductance of pre-synaptic membranes by a mechanism which is independent of the voltage-sensitive Na^+ and Ca^{2+} channels. This in turn results in loss of the ion electrochemical potential and subsequent release of transmitter (Nicholls et al., 1982). If such a mechanism accounts for the [¹⁴C]glycine efflux from toxin-treated retinas, our results with [³H]GABA release suggest that α -latrotoxin-binding sites are absent on the Müller (glial) cells. Our autoradiographic data are in accord with this inference.

When examining the effect of veratridine and EDA on GABA release, we did not employ toxin-treated retinas because concurrent release of the second radiolabel, [¹⁴C]glycine, could not be monitored in such experiments. In such a protocol, the advantage of double-label studies would be lost.

A basic limitation of our studies is the assumption that the Müller cells alone take up [³H]GABA under our experimental conditions. Although this observation is in general agreement with findings of others, we have not performed any independent tests to confirm the validity of our assumption. Indeed, even if a small number of GABAergic neurons were labeled in addition to the glial cells, the [³H]GABA release observed may preferentially occur from neurons and not glial cells. However, our experiments with the presynaptic toxin, α -latrotoxin, indicate that most of the [³H]GABA released probably did not occur from GABAergic neurons. Due to its limited

availability and the difficult purification procedure, we have used an aqueous extract of the venom glands instead of the toxin in our studies. It has been demonstrated (Frontali et al., 1976) that the physiological effect of the extract on vertebrate neurons is mediated primarily by the presynaptic toxin, α -latrotoxin, present in this preparation.

Neuronal labeling may also occur by redistribution of [^{3} H]GABA during the postincubation washing procedures, as reported for the rabbit retina (Ehinger, 1977). However, our autoradiographic data from retinas fixed after postincubation wash and veratridine stimulation show that little redistribution of [^{3} H]GABA occurred under these conditions. The presence of 100 μ M AOAA in all of our media might have retarded such a redistribution process (Ehinger, 1977).

Yet another mechanism for [^{3} H]GABA release is that endogenous retinal transmitters such as GABA and glutamate, released by veratridine stimulation of neurons, may act on Müller cells directly or indirectly to release preloaded GABA (Hosli et al., 1980). For example, endogenous GABA released into the synaptic cleft may release preloaded [^{3} H]GABA by a homoeexchange process; alternatively, glutamate or aspartate released from neurons may depolarize Müller cells to release preloaded GABA. Since veratridine-stimulated release of [^{3} H]GABA was Ca^{2+} dependent, we could not distinguish between these possibilities.

Unlike K^+ - and veratridine-stimulated release of GABA, which were suppressed by Ca^{2+} removal from the medium, EDA-stimulated release was largely Ca^{2+} independent. Moreover, little glycine release was observed under these conditions. These results, taken together with other studies on neurons, show that EDA can release preloaded GABA from both neurons and glial cells by a Ca^{2+} -independent process. An obvious mechanism for this phenomenon would be the homoeexchange of intracellular [^{3} H]GABA with exogenous EDA. The observed homoeexchange of [^{3} H]GABA with exogenous GABA (Fig. 8) and the reduction of [^{3} H]GABA uptake by EDA (Fig. 10) are compatible with this interpretation. A lack of Ca^{2+} dependency of EDA-stimulated GABA efflux also supports a carrier-mediate exchange rather than a vesicular release mechanism.

References

- Baba, A., and J. R. Cooper (1980) The action of black widow spider venom on cholinergic mechanisms in synaptosomes. *J. Neurochem.* 34: 1369-1379.
- Blaustein, M. P. (1975) Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. *J. Physiol. (Lond.)* 247: 617-655.
- Bowery, N. G., D. A. Brown (1972) γ -Aminobutyric acid uptake by sympathetic ganglia. *Nature New Biol* 238: 89-91.
- Bowery, N. G., D. A. Brown, and S. Marsh (1979) γ -Aminobutyric acid efflux from sympathetic glial cells. Effect of depolarizing agents. *J. Physiol. (Lond.)* 293: 75-101.
- Bruun, A., and B. Ehinger (1974) Uptake of certain possible neurotransmitters into retinal neurones of some mammals. *Exp. Eye Res.* 19: 435-447.
- Catterall, W. A., and M. Nirenberg (1973) Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 70: 3759-3763.
- Chin, C. A., and D. M. K. Lam (1980) The uptake and release of [^{3} H]glycine in the goldfish retina. *J. Physiol. (Lond.)* 308: 185-195.
- Davies, L. P., J. W. Hambley, and G. A. R. Johnston (1982) Ethylenediamine as a GABA agonist: Enhancement of diazepam binding and interaction with GABA receptors and uptake sites. *Neurosci. Lett.* 29: 57-61.
- Ehinger, B. (1977) Glial and neuronal uptake of GABA, glutamic acid, glutamine and glutathione in the rabbit retina. *Exp. Eye Res.* 25: 221-234.
- Forster, P., H. G. E. Lloyd, P. F. Morgan, M. Parker, M. N. Perkins, and T. W. Stone (1981) Ethylenediamine acts at GABA receptor and uptake sites. *Br. J. Pharmacol.* 74: 274P.
- Frontali, N., B. Ceccarelli, A. Gorio, A. Mauro, P. Siekevitz, M. C. Tzeng, and W. P. Hurlburt (1976) Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. *J. Cell Biol.* 68: 462-479.
- Grasso, A., and M. I. Senni (1979) A toxin purified from the venom of black widow spider venom affects uptake and release of radioactive γ -aminobutyrate and *N*-epinephrine from rat brain synaptosomes. *Eur. J. Biochem.* 102: 337-344.
- Hertz, L. (1977) Biochemistry of glial cells. In *Cells, Tissue and Organ Cultures in Neurobiology*, S. Federoff and L. Hertz, eds., pp. 39-71, Academic Press, Inc., New York.
- Hertz, L., and A. Schousboe (1980) Interactions between neurons and astrocytes in the turnover of GABA. *Brain Res. Bull.* 5: 389-396.
- Hille, B. (1977) Local anesthetics: Hydrophilic and hydrophobic pathways for the drug receptor interaction. *J. Gen. Physiol.* 69: 497-515.
- Hosli, L., P. F. Anders, and E. Hosli (1980) Neurone-glia interaction: Indirect effects of amino acid transmitters on cultured glial cells. In *Tissue Culture in Neurobiology*, E. Giacobini, A. Veruadakis, and A. Shahan, eds., pp. 373-383, Raven Press, New York.
- Jaffe, E. H. and A. C. Cuellar (1981) Neuronal and glial release of [^{3} H]GABA from the rat olfactory bulb. *J. Neurochem.* 37: 1457-1466.
- Kuffler, S. W., and J. G. Nicholls (1966) The physiology of neuroglial cells. *Ergeb. Physiol.* 57: 1-90.
- Lloyd, H. G. E., M. N. Perkins, and T. W. Stone (1982) Ethylenediamine as a specific releasing agent of γ -aminobutyric acid in rat striatal slices. *J. Neurochem.* 38: 1168-1169.
- Marshall, J., and M. Voaden (1974) An investigation of the cells incorporating [^{3} H]GABA and [^{3} H]glycine in the isolated retina of the rat. *Exp. Eye Res.* 18: 367-370.
- Meldolesi, J. (1982) Studies on α -latrotoxin receptors in rat brain synaptosomes: Correlation between toxin-binding and stimulation of transmitter release. *J. Neurochem.* 38: 1559-1569.
- Minchin, M. C. W. (1979) Factors influencing the efflux of [^{3} H] gamma-aminobutyric acid from satellite glial cells in rat sensory ganglia. *J. Neurochem.* 24: 571-577.
- Minchin, M. C. W., and L. L. Iversen (1974) Release of [^{3} H] gamma-aminobutyric acid from glial cells in rat dorsal root ganglia. *J. Neurochem.* 23: 533-540.
- Munson, R., Jr., B. Westermarck, B. and L. Glaser (1979) Tetrodotoxin-sensitive sodium channels in normal human fibroblasts and normal human glia-like cells. *Proc. Natl. Acad. Sci. U. S. A.* 76: 6425-6429.

- Narahashi, T. (1974) Chemicals as tools in the study of excitable membranes. *Physiol. Rev.* 54: 813–889.
- Narahashi, T., J. W. Moore, and W. R. Scott (1964) Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* 47: 965–974.
- Neal, M. J., and N. G. Bowery (1979) Differential effects of veratridine and potassium depolarization on neuronal and glial GABA release. *Brain Res.* 167: 337–343.
- Neal, M. J., and L. L. Iversen (1972) Autoradiographic localization of [³H]GABA in rat retina. *Nature New Biol.* 235: 217–218.
- Nicholls, D. G., M. Rugolo, I. G. Scott, and J. Meldolesi (1982) α -Latrotoxin of black widow spider venom depolarizes the plasma membrane, induces massive calcium influx, and stimulates transmitter release in guinea pig brain synaptosome. *Proc. Natl. Acad. Sci. U. S. A.* 79: 7924–7928.
- Oxender, D. L., and H. N. Christensen (1963) Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* 238: 3686–3699.
- Pearce, B. R., D. N. Currie, R. Beale, and G. R. Dutton (1981) Potassium-stimulated, calcium-dependent release of [³H]GABA from neuron and glia-enriched cultures of cells dissociated from rat cerebellum. *Brain Res.* 206: 485–489.
- Perkins, M. N., N. G. Bowery, D. R. Hill, and T. W. Stone (1981) Neuronal responses to ethylenediamine: Preferential blockade by bicuculline. *Neurosci. Lett.* 23: 325–327.
- Ramon y Cajal, S. (1892) *The Structure of the Retina*, S. A. Thorpe and M. Glickstein, transl. (1972), Charles C Thomas, Springfield, IL.
- Roberts, P. J. (1974) Amino acid release from isolated rat dorsal root ganglia. *Brain Res.* 74: 327–332.
- Schrier, B. K., and E. J. Thompson (1974) On the role of glial cells in the mammalian nervous system. Uptake, excretion, and metabolism of putative neurotransmitters by cultured glial tumor cells. *J. Biol. Chem.* 249: 1769–1780.
- Sellstrom, A., and A. Hamberger (1977) Potassium-stimulated γ -aminobutyric acid release from neurons and glia. *Brain Res.* 119: 189–198.
- Sepulveda, F. V., and M. W. Smith (1978) Discrimination between different entry mechanisms for neutral amino acids in the rabbit ileal mucosa. *J. Physiol. (Lond.)* 282: 73–90.
- Stallcup, W. B., and M. Cohen (1976) Correlation of surface antigens and cell type in cloned cell lines from the rat central nervous system. *Exp. Cell Res.* 98: 285–294.
- Thomas, R. C. (1974) Intracellular pH of snail neurons measured with a new pH-sensitive glass microelectrode. *J. Physiol. (Lond.)* 238: 159–180.
- Tzeng, M. C., and P. Siekevitz (1978) The effect of the purified major protein factor (α -latrotoxin) of black widow spider venom on the release of acetylcholine and norepinephrine from mouse cerebral cortex slices. *Brain Res.* 139: 190–196.
- Tzeng, M. C. and P. Siekevitz (1979) The binding interaction between α -latrotoxin from black widow spider venom and a dry cerebral cortex synaptosomal membrane preparation. *J. Neurochem.* 33: 363–274.
- Tzeng, M. C., R. S. Cohen, and P. Siekevitz (1978) Release of neurotransmitters and depletion of synaptic vesicles in cerebral cortex slices by α -latrotoxin from black widow spider venom. *Proc. Natl. Acad. Sci. U. S. A.* 75: 4016–4020.
- Ulbricht, W. (1969) The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol.* 61: 18–71.
- Varon, S. S., and G. G. Somjen (1979) Neuron-glia interactions. *Neurosci. Res. Program Bull.* 17: 1–239.
- Villegas, J., C. Sevcik, F. V. Barnola, and R. Villegas (1976) Grayanotoxin, veratridine and Tetratoxin-sensitive sodium pathways in the Schwann cell membrane of squid nerve fibers. *J. Gen. Physiol.* 67: 369–380.
- Voaden, M. J. (1976) Gamma-aminobutyric acid and glycine as retinal neurotransmitters. In *Transmitters in the Visual Progress*, S. L. Bonting, Ed., pp. 107–125, Pergamon Press, New York.
- Voaden, M. J., and M. S. Starr (1972) The efflux of radioactive GABA from rat retina *in vitro*. *Vision Res.* 12: 559–566.