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Glutamic Acid Decarboxylase of Embryonic Avian Retina Cells in Culture: Regulation by γ-Aminobutyric Acid (GABA)

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SUMMARY

1. Retina-cell aggregate cultures expressed glutamate decarboxylase activity (L-glutamate 1-carboxylase; EC 4.1.1.15) as a function of culture differentiation.

2. Glutamic acid decarboxylase (GAD) activity was low in the initial phases of culture and increased eight-fold until culture day 7, remaining high up to day 13 (last stage studied).

3. The addition of GABA to the culture medium 24 h after cell seeding almost totally prevented the expression of GAD activity.

4. In association with decreased enzyme activity, aggregates exposed to GABA did not display immunoreactivity for GAD, suggesting that GAD molecules were either lost from GABAergic neurons or significantly altered with GABA treatment.

5. Control, untreated aggregates showed intense GAD immunoreactivity in neurons. Positive cell bodies were characterized by a thin rim of labeled cytoplasm with thickest labeling at the emergence of the main neurite.

6. Heavily labeled patches were also observed throughout the aggregates, possibly reflecting regions enriched in neurites.

7. The GABA-mediated reduction of GAD immunoreactivity was a reversible phenomenon and could be prevented by picrotoxin.

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INTRODUCTION

Due to its histoanatomical characteristics the avian retina has been extensively used as a system to approach the basic principles of central nervous system (CNS) function and differentiation. Cells from the embryonic chick retina can be easily dissociated and cultured as dispersed or aggregated cells. Most, if not all, retina biochemical markers differentiate properly *in vitro,* making this system useful to follow the differentiation of neurochemical properties of the tissue (Akagawa *et al.,* 1987; Akagawa and Barnstable, 1987).

GABA is one of the major neurotransmitters in the CNS including the retina of most species. The synthesis of GABA in the CNS is accomplished mainly by the decarboxylation of glutamic acid catalyzed by the enzyme glutamic acid decarboxylase (GAD; L-glutamate 1-carboxylase; EC 4.1.1.15). Due to its ubiquitous distribution in the brain this enzyme has been used extensively as a marker of GABAergic neuron maturation during CNS differentiation. More recently, GAD genes have been cloned and used as a model for studies of gene expression in the developing brain (Bond *et al.,* 1988).

Although in the mature CNS most of the GABA comes from glutamic acid (Morgan, 1985), in the embryonic tissue a considerable portion of this compound can be synthesized from putrescine (Seiler and Al-Therib, 1974). Some lines of evidence indicate that even in differentiated neurons lacking GAD molecules, GABA can be detected in large amounts. It has been suggested that GABA in GAD-lacking cells comes from putrescine (Hokoç *et al.,* 1990).

In previous publications from our laboratory we have shown that GABA is present in the chick retina since very early in development when GAD activity is undetectable. Moreover we showed that a substantial amount of GABA in the early stages of retina ontogeny was synthesized from putrescine, a compound that is abundant in the embryonic tissue (De Mello *et al.,* 1976). An immunocytochemical study revealed that GABA, but not GAD, is localized exclusively in neuroblast-like cells of embryonic retina at the sixth day of development (Hokoç *et al.,* 1990). We suggested that GABA at this stage might have functions related to the control of embryological parameters rather than to classical neurotransmitter role. Trophic influences of GABA on embryonic CNS neurons have also been suggested by others (Spoerri, 1988). Further work from our laboratory showed that GABA fully prevented the expression of GAD activity using aggregate cultures of embryonic retina cells (De Mello, 1984).

In the present report, using polyclonal antibodies against GAD, we show that cultured aggregates of retina cells exposed to GABA are completely devoid of GAD immunoreactivity, indicating that GABA regulates GAD activity either by changing the level of enzyme molecules of GABAergic neurons or by altering antigenic sites of GAD important to confer the catalytic properties of the enzyme.

MATERIALS AND METHODS

γ-Aminobutyric acid, trypsin (Sigma Chemicals); fetal calf serum (Microbiologica); basal medium of Eagle (BME; GIBCO); and L-[U-^{14}C]glutamic acid

(260 mCi/mmol; Amersham/Searle) were used throughout this study. All other reagents were of analytical grade.

Fertilized white Leghorn eggs were obtained from a local hatchery.

Aggregate Cultures. Primary aggregate cultures of retina cells were prepared by modification of a method described before (Vogel *et al.*, 1976). Usually 8–10 retinae from 9-day-old embryos were dissected, transferred to 2 ml Ca^{2+}, Mg^{2+}-free medium (CMF) containing 0.05% 2× crystalline trypsin, and incubated in a water bath at 37°C for 12–13 min. The trypsinized tissues were then centrifuged at 500*g* for 30 sec. The supernatant was discarded and the tissue resuspended in BME (Earle's salts), containing 5% fetal calf serum and 2 m*M* glutamine. The cells were mechanically dissociated by aspirating the tissue 10 times with a large-bore 10-ml pipette. This procedure yielded approximately 55×10^6 cells/retina with a viability greater than 95%, as estimated by trypan blue exclusion.

After appropriate dilution, the cell suspension (10×10^6 cells/ml) was transferred to a rotary shaker operating at 80 rpm under a humidified atmosphere of 95% air and 5% CO_2.

In all experiments, after the addition of any test compound the aggregates were kept under the same conditions described above.

GAD Assay. Glutamate decarboxylase was assayed in the homogenate of aggregates by a method already published (Schrier *et al.*, 1974; De Mello *et al.*, 1976) using commercially available L-[U-^{14}C]glutamic acid previously purified by high-voltage electrophoresis. In some experiments the amount of [^{14}C]GABA formed from labeled glutamate was determined after dansylation and separation by thin-layer chromatography (TLC) (De Mello *et al.*, 1976). In these experiments the ratio between $^{14}CO_2$ production and [^{14}C]GABA formed was always close to one.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Histochemical Procedures. Aggregates at any stage of differentiation were collected by centrifugation (500*g* × 2 min) and washed three times with 2 ml of cold Hank's solution containing 12 m*M* glucose. After a final rinse in buffer the aggregates were immersed in fixative for 1 h. After several rinses in buffer, a portion of aggregates was frozen and sections 15–25 μm thick were cut following random orientation. The sections were mounted in gelatinized slides, air-dried, and incubated overnight with the primary antibody.

For GAD immunoreactivity, aggregates were fixed with 4% formaldehyde in 0.16 *M* phosphate buffer, pH 7.2. The sections were incubated overnight at 4°C in the presence of GAD antiserum produced in sheep (Oertel *et al.*, 1981) at a final dilution of 1:1000. The primary reaction was visualized by the peroxidase–antiperoxidase (PAP) method (Sternberger, 1979). Rabbit anti-sheep IgG, 1:150 (Sigma), and sheep PAP, 1:600 (Jackson), followed by diaminobenzidine (Sigma) as a chromogen, were used. Controls consisted of sections incubated with normal sheep serum, at a dilution of 1:1000, instead of the primary antisera and processed the same way, in parallel with the GAD antisera exposed sections. No immunoreactivity was detected in the controls. Triton-X (0.25%, v/v) was used in all solutions to enhance permeability of the reagents.

For GABA immunoreactivity, aggregates were fixed with 0.1% glutaraldehyde and 4% formaldehyde in 0.16 *M* phosphate buffer. In some experiments, glutaraldehyde was omitted from the fixative, which resulted in better cell-body labeling. Aggregate sections (15–25 μm) were incubated overnight at 4°C with GABA antisera produced in rabbit (Wenthold *et al.*, 1986), at a dilution of 1:100. As for the anti-GAD immunoreactivity, the primary reaction was visualized by the PAP method (Sternberger, 1979). In this case, swine anti-rabbit Fab fragments (1:40) and rabbit PAP (1:80) (DAKO), followed by the diaminobenzidine reaction, were used as the chromogen. Control sections were incubated with normal rabbit serum at the same dilution and processed in the same way as for the GABA antiserum-incubated sections. All the sections were examined, studied, and photographed using a Zeiss Axioplan optic microscope equipped with an interference contrast accessory.

RESULTS

Dissociated retina cells from 8-day-old embryos were cultured under conditions which favor the formation of aggregates and the specific activity of GAD was measured during the course of aggregate differentiation. The enzyme activity was low in the initial phases of the cultures and increased eightfold up to day 7, if the culture medium was changed every 24 hr (Fig. 1, squares). This activity remained high until culture day 13. However, if culture medium was changed at 48-hr intervals the enzyme reached an equilibrium level that was approximately 30% lower than that of aggregates whose medium had been changed daily (Fig. 1, triangles). One possibility was that aggregate products could be released into the medium and lower the equilibrium level of GAD activity of aggregates whose medium was changed every 2 days.

In a previous report we showed that GABA, added to culture medium, fully prevented the expression of GAD activity of aggregates. In Fig. 1 (circles) we confirm the GABA effect and show that when 5 m*M* GABA was added to the medium right after cell seeding and in subsequent changes of medium, a complete inhibition of the expression of GAD activity was observed. The high concentration of GABA used in these experiments was chosen to provide saturating levels of GABA throughout the period studied. However, the EC_{50} for GABA necessary to inhibit GAD activity is approximately 10 μM and maximal inhibition is obtained with an approximately 100 μM GABA concentration (De Mello, 1984).

GABA is present in the avian retina from early stages of differentiation, when the levels of its synthesizing enzyme cannot be detected (De Mello *et al.*, 1976). We reasoned that the release of GABA in the medium might regulate GAD activity of GABAergic neurons. The immunoreactivity of aggregate sections to GABA antiserum revealed that indeed aggregates are enriched in GABA-containing cells (Fig. 2). Random sections through aggregates showed abundant GABA-containing cell bodies that were detected through the whole section and preferentially along the periphery of the aggregates. Although

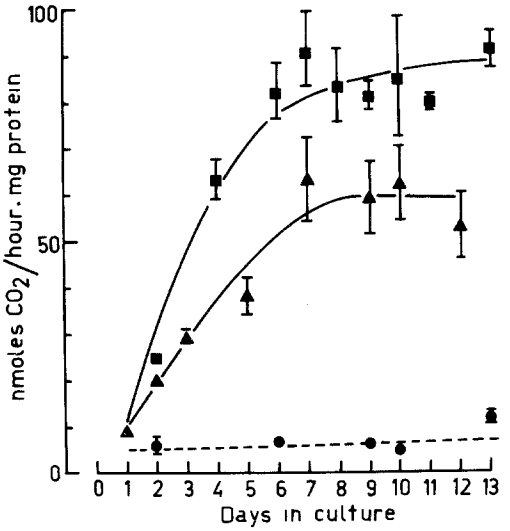

Fig. 1. Effect of GABA upon the expression of GAD activity of cultured retina cells. Aggregate cultures were prepared as described under Materials and Methods. Twenty-four hours after the onset of the cultures a group of aggregates was fed with medium containing 5 m*M* GABA, with subsequent changes of medium containing GABA at 24-hr intervals (circles). Control aggregates were fed with medium without GABA at 24- and 48-hr intervals (squares and triangles, respectively). The addition of GABA fully prevented the expression of GAD activity of aggregates. The maximal enzyme activity of aggregates that had their medium changed less frequently was 30% lower than the enzyme activity of aggregates submitted to daily changes of medium. Each point is the mean of three to five experiments ± SD. In each experiment the enzyme was assayed in triplicates. For GABA-treated cultures (circles) the values are the means of two independent experiments ± the deviation of individual values from the mean. Symbols without bars mean that the deviations are smaller than the symbols.

GABA labeling does not allow the precise definition of the retinal GABAergic cell type in aggregates, by analogy to the *in vivo* retinal tissue, it is likely that GABA immunoreactivity of aggregates may reflect the presence of horizontal and amacrine cells and, possibly, a type of GABA-containing cell that is also observed in the ganglion-cell layer of the intact tissue (Hokoç *et al.*, 1990). The data suggested the possibility that GABA released in the medium might limit the extent of GAD expression. In support of this hypothesis is the fact that aggregates do release [^{3}H]GABA taken up by cells. Figure 3 shows the result of an experiment in which aggregates (E8C8) were first incubated with [^{3}H]GABA at 5×10^{-8} *M* for 2 hr and then perfused as described previously (Do Nascimento

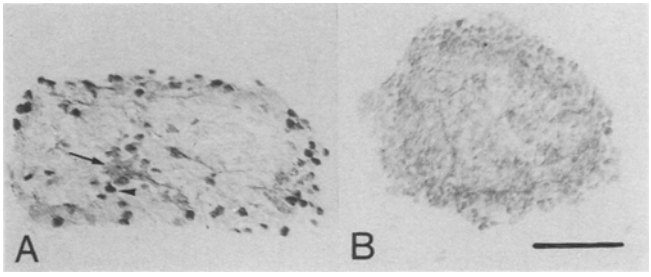

Fig. 2. (A) GABA immunoreactivity of aggregate sections from chick retina cells cultured for 6 days with medium change every other day. Abundant GABA-containing cell bodies (arrowhead) and few plexuses (arrow) are shown throughout the whole section. (B) Aggregate sections from the same culture incubated with nonimmune serum. Bar equals 50 μm.

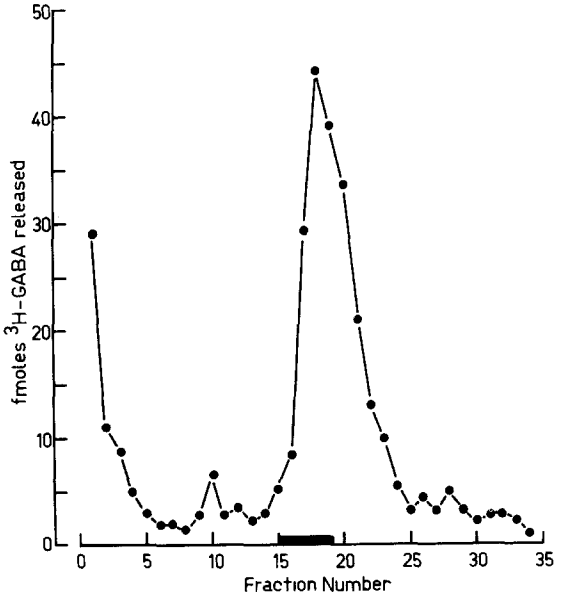

Fig. 3. [^{3}H]GABA release by retina-cell aggregate culture. Aggregates were prepared as described under Materials and Methods and cultured for 8 days with medium changes every other day. Then [^{3}H]GABA (85 Ci/mmol) was added to a sample of aggregates to a final concentration of 5×10^{-8} *M*. The aggregates were further incubated for 2 hr at 37°C under an atmosphere of 5% CO_2–95% air. The aggregates were then washed five times with 5 ml Hank's saline to remove nonincorporated GABA and superfused with the same solution as described elsewhere (Do Nascimento and De Mello, 1985). The filled bar on the abscissa indicates the exposure time of aggregates to 1 m*M* L-glutamate.

and De Mello, 1985). A steady and continuous efflux of [3H]GABA equivalent to approximately 2 fmol GABA/min is reached after 5-min perfusion of aggregates. Stimulation of the aggregates with 1 m*M* glutamate for 4 min resulted in a dramatic, 17-fold increase in the level of [^{3}H]GABA release, which returned to the basal efflux rate 6 min after stopping glutamate infusion. The characteristics of GABA release by cultured retina cells have been reported previously (De Mello *et al.*, 1988).

The kinetics of GAD inhibition and recovery of aggregates exposed to GABA suggested that changes in the levels of GAD activity might reflect changes in the number of GAD molecules found in GABAergic neurons (De Mello, 1984). In an attempt to explore this hypothesis further we carried out a series of histochemical experiments in which polyclonal antibodies against GAD, obtained by Oertel *et al.* (1981), were used to detect GAD immunoreactivity of aggregates. This antibody has been shown to react with GAD molecules of different species (Brandon, 1985) including the avian enzyme (Hokoç *et al.*, 1990). Figure 4C shows a retina section of a posthatched chicken, where the characteristic distribution of GAD immunoreactivity can be observed. Immunoreactivity is observed in cell bodies of horizontal and amacrine cells in the inner nuclear

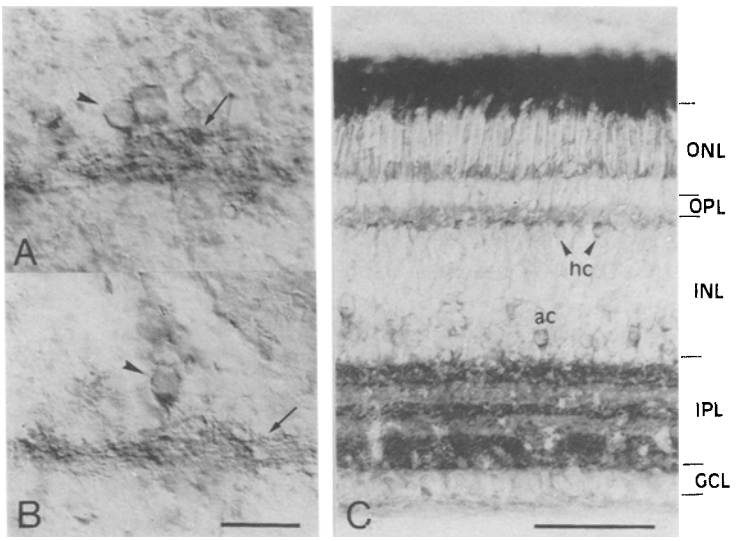

Fig. 4. Micrographs of GAD-positive cells. (A, B) High magnification of labeled cell bodies (arrowhead) and processes (arrow) of control untreated aggregates. The label consists of a thin rim of labeled cytoplasm, thickest toward the emergence of the main neurite, with no immunoreactivity in the nuclei. Immunoreactivity is detected in the presumptive neurite region where cell processes seem to arborize. Bar equals 20 μm. (C) Radial section (20 μm thick) through a mature chick retina stained for GAD immunoreactivity. Labeled horizontal (hc) and amacrine cells (ac) are found in the outer and inner portion of the inner nuclear layer (INL), respectively. Three intensively labeled bands of processes can be detected in the inner plexiform layer (IPL). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion-cell layer. Bar equals 50 μm.

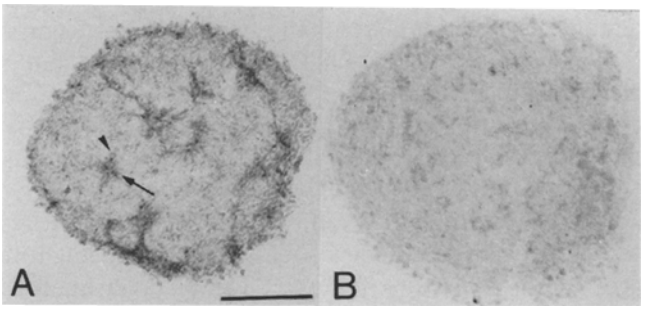

Fig. 5. Micrographs of cryostat sections (20 μm thick) of chick retina-cell aggregates incubated with GAD antiserum as described under Materials and Methods. (A) GAD-immunoreactivity of control untreated aggregate. Patches of neurites (arrow) associated with retinal-cell bodies (arrowhead) are observed. (B) Aggregate exposed to 5 m*M* GABA for 5 days. The aggregate is completely devoid of GAD immunoreactivity. Bar equals 50 μm.

layer, with labeled cytoplasm surrounding the nuclei. The inner plexiform layer is also strongly immunoreactive, with three distinct labeled bands. The outer plexiform layer is weakly immunoreactive.

The comparison of GAD immunoreactivity of control untreated and GABA-exposed aggregates showed that an intense labeling was observed in control aggregates, mostly in patches of possible neurite regions (Fig. 5A). The label is clearly visible in the cytoplasm and more intense in the region of the emergence of the main neurite (Figs. 4A and B). No immunoreactivity is observed in the nuclei.

Treated aggregates obtained from the same pool of the control group were exposed to 5 m*M* GABA for 5 days, starting on the second day after plating, and then processed for GAD immunocytochemistry. Such aggregates are completely devoid of GAD immunoreactivity (Fig. 5B). Thus the loss of enzyme activity

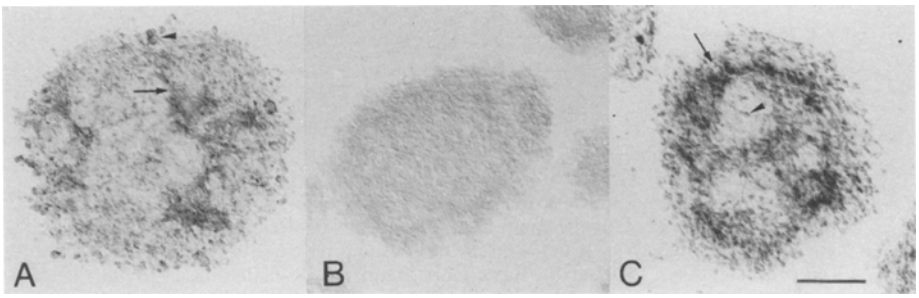

Fig. 6. Micrographs of cryostat sections (20 μm thick) of chick retina-cell aggregates incubated with GAD antiserum. (A) Control untreated aggregate. Cell bodies (arrowhead) and patches of neurites (arrow) can be noticed. (B) Aggregate exposed to 0.3 m*M* GABA for 2 days. No label is observed. (C) Aggregate cotreated for 2 days with 0.3 m*M* GABA and 0.05 m*M* picrotoxin. GAD-immunoreactive cell bodies (arrowhead) and patches of neurites (arrow) are distinguished, indicating that picrotoxin prevents the loss of GAD immunoreactivity induced by GABA. Bar equals 50 μm.

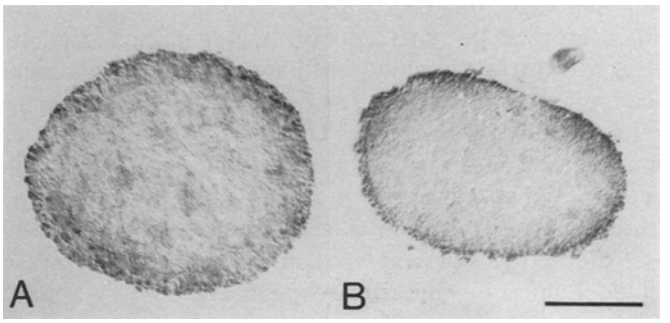

Fig. 7. GAD-containing cells in more differentiated aggregate. (A) Control untreated aggregate maintained for 15 days in regular medium. (B) Aggregate exposed to 5 m*M* GABA for 48 hr starting on culture day 13. Aggregates maintained in culture for 13 days or more are resistant to GABA treatment. GAD-containing cell bodies show the similar arrangement as in untreated aggregates. In both cases the medium was changed every other day throughout the culture period. Bar equals 50 μm.

observed with GABA treatment is accompanied by loss of GAD immunoreactivity.

GABA at concentrations as high as 10 m*M* does not interfere directly with GAD activity of retina homogenates (De Mello, 1984). This observation indicates that the GABA effect requires cells to be intact in order to respond either to GABA metabolization or to the decodification of signals carried by GABA, possibly via membrane receptors. In fact aggregates cotreated for 48 hr with GABA (0.3 m*M*) and picrotoxin (0.05 m*M*), an antagonist of GABA receptor at the level of the chloride channel, display intense GAD immunoreactivity (Fig. 6C), indicating that picrotoxin prevents the loss of GAD immunoreactivity induced by GABA. While control aggregates are heavily labeled for GAD (Fig. 6A), those treated for 48 hr with 0.3 m*M* GABA are completely nonreactive to the GAD antibody (Fig. 6B).

More differentiated aggregates, maintained in culture for longer periods of time, respond differently to GABA treatment. While younger aggregates (i.e., E9C6) exposed to GABA lose all GAD immunoreactivity, aggregates maintained in culture for 13 days or more seem to be resistant to GABA treatment (Fig. 7). In association with the decreased sensitivity of GAD to GABA, the organization of GAD immunoreactive cells within the aggregates also changed. Most of the cell bodies were arranged in the periphery of the aggregates, while patches of possible neurite regions were only faintly labeled in the center of aggregates.

DISCUSSION

Our data reveal that GAD activity is expressed in chick embryo retina cells cultured under conditions which favor the formation of aggregates. The expression of the enzyme is almost completely prevented if aggregates are incubated in culture medium containing GABA.

The immunoreactivity of aggregates to GAD antibody show that, in association with decreased enzyme activity, aggregates exposed to GABA for 48 hr or more do not display immunoreactivity for GAD, indicating either that GAD molecules are lost from the GABAergic neurons with GABA treatment or that GABA induces modifications in GAD molecules that change antigenic sites of the enzyme responsible for recognizing the antibody. This should imply that the modified sites should be important to confer catalytic activity to the enzyme. In control cultures GAD is localized in cell bodies and also in sites that mimic neurite regions. Another possible explanation for our observations would be that GABA could hinder the antigenic sites recognizable by GAD antibody. However, if we prepare control untreated aggregates in the presence of 5 *M* GABA during the histochemical procedure, the aggregates do show intense immunoreactivity for GAD. This indicates that GABA does not interfere with the interaction of the antibody with enzyme antigenic sites.

The GABA-mediated GAD inhibition is a reversible phenomenon as observed by measuring enzyme activity (De Mello, 1984). Thus the GABA effect seems not to reflect a toxic effect of this compound over GABAergic neurons. In addition, other retina neurotransmitters do not interfere with GAD activity, nor does GABA affect other decarboxylases of the retina (De Mello, 1984). Thus, the effect of GABA in controlling the level of GAD molecules of sensitive neurons seems to be selective and very specific.

A few possibilities could explain the changes in the level of GAD immunoreactivity of GABA-treated aggregates. Control of the expression of GAD genes, posttranscriptional control of GAD synthesis, degradation of GAD by regular cell metabolism, or, as mentioned above, metabolic modifications of GAD antigenic sites could also change the activity of the enzyme. The recent availabilty of genetic clones for GAD described by several groups may help to approach this problem more directly, assaying for the expression of GAD mRNAs of GABA-treated cells (Bond *et al.*, 1988; Vernier *et al.*, 1988).

As mentioned above GABA can be detected in chick embryo retinas as early as the sixth day of incubation (De Mello *et al.*, 1976) in neuroblast-like cells (Hokoç *et al.*, 1990). The source of GABA at this stage is likely to be putrescine, which is quite abundant in undifferentiated tissue (De Mello *et al.*, 1976). Since GAD activity and immunoreactivity can be eliminated from cells that are exposed to GABA at the early stages of aggregate differentiation, one attractive suggestion is that GABA synthesized from putrescine in the early stages of retina development may serve as a modulator of GAD expression during the source of GABAergic neuron differentiation. In support of this hypothesis is the fact that cultured retina neurons release GABA at early stages of development by mechanisms that depend on cell exposure to excitatory amino acids (Fig. 3) (Do Nascimento and De Mello, 1985; De Mello *et al.*, 1988). Preliminary experiments from our laboratory also indicate that GABA synthesized from putrescine can be released in the extracellular space.

What causes GAD of a subpopulation of GABAergic neurons to become resistant to inhibition by GABA in differentiated aggregates is still unknown. However, one possible explanation is that since GABA seems to decrease the

level of GAD by a mechanism that is prevented by picrotoxin treatment, it is possible that synapse formation or a built-in genetic program causes reduction of GABA receptor activity, consequently decreasing the sensitivity of these cells to GABA. Interestingly, several reports have shown that GABA can induce the appearance of a low-affinity GABA receptor in several areas of the CNS of different species including the avian retina (Meir *et al.*, 1987; Madtes, 1987). One attractive suggestion is that resistance of GAD inhibition by GABA in some cells of more differentiated aggregates may be associated with changes in the pattern of GABAergic receptor properties of resistant cells.

Although the data reported above refer to cells of embryological origin, results from our laboratory (to be published elsewhere) also reveal that GAD immunoreactivity of horizontal cells of mature avian retina changes significantly as a function of light adaptation. The change in GAD immunoreactivity of horizontal cells is inversely correlated with the amount of GABA present in these cells, as evidenced by immunocytochemistry. Thus, the mechanism of GAD control by GABA, which is quite evident in cells of embryonic origin, seems to be conserved at least in horizontal cells of differentiated retina.

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