Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7810–7814, December 1981
Neurobiology

Plasminogen activator secretion by granule neurons in cultures of developing cerebellum

(protease/cell migration/tetanus toxin/histogenesis/fibrin slide assay)

ALPHONSE KRYSTOSEK AND NICHOLAS W. SEEDS

Department of Biochemistry/Biophysics/Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

Communicated by David W. Talmage, August 18, 1981

ABSTRACT **Dispersed cell cultures were established from 7- to 9-day postnatal mouse cerebellum. The fibrin slide method was used to obtain a localization of plasminogen activator production to specific cells. Fibrinolytically active cells were small (5- to 8-μm diameter), round, and occurred singly or in aggregates. Fibrinolysis was both plasminogen and time dependent, inhibitable by ε-aminocaproic acid and soybean trypsin inhibitor and did not occur when cells were fixed in formalin prior to the fibrin overlay. Strong fibrin degradation occurred only when granule neurons were abundant in the cultures. These plasminogen activator secreting cells were identified as granule neurons by cell separation methods, nuclear morphology, and their ability to bind tetanus toxin and rabbit antiserum against mouse cerebellum (anti-Cbl-1 antiserum). Plasminogen activator also could be quantified in fractionated tissue homogenates or in cell culture medium by the ^{125}I-labeled fibrin plate assay. Fibrinolysis in cerebellar extracts was 95% dependent on the presence of added plasminogen; furthermore, the activity was greater in cerebellar extracts as compared to cerebral cortex of the same age. At the age examined, the cerebellum contains many migratory neurons, and plasminogen activator production may be involved in the process of cell movement.**

Many events in neurogenesis that have been described microanatomically are not yet understood at the biochemical level. The mouse cerebellum has been a favorable brain region for study because it develops largely in the first 3 postnatal weeks, yielding a highly ordered structure in which different neuron types occupy discrete layers (1, 2). Granule neurons, which account for approximately 95% of the cells in the mature cerebellum, are generated as postmitotic progeny of proliferating stem cells in the external granule layer. Radiolabeling experiments and microscopic examination of tissue sections has shown that the perikaryon of these neurons subsequently migrates inward to reside in the internal granular layer beneath the Purkinje cells, while a T-shaped axonal process is left behind in the molecular layer where synaptogenesis on Purkinje cell dendritic spines will eventually occur (3, 4). This developmental process is similar in many species (3–6). In the monkey cerebellum, the migratory granule cell soma has been shown by electron microscopy to be in direct contact with the vertically oriented Bergmann glial fibers throughout its transit of the molecular layer (6). Very little else is known about the mechanics or biochemistry of the migratory phase.

Recent research in other laboratories has demonstrated the production of the specific proteolytic enzyme plasminogen activator (PA) by cell types that are involved in migration or tissue destruction, or both. These have included activated macrophages (7), stimulated ovarian granulosa cells (8), trophoblast (9), parietal endoderm (9), and many tumor cell lines (10–13). We examined whether PA is found in developing cerebellum during this active stage of histogenesis. For this purpose we utilized the fibrin slide histochemical method to localize PA production at the single-cell level (14, 15) in dispersed cell cultures, which yield a high recovery of neurons and allow the expression of several stages of neural differentiation (16). Preliminary reports of our results have appeared (17, 18).

MATERIALS AND METHODS

Preparation of Cell Cultures. Cerebella were removed from 7- to 9-day postnatal C57B/6J mice and finely diced. The tissue was dissociated mechanically (see below) or enzymatically in 0.17% trypsin (GIBCO) in saline 1 (0.138 M NaCl/5.4 mM KCl/1.1 mM Na_2HPO_4/1.1 mM KH_2PO_4) containing 0.4% glucose, 0.01% $CaCl_2$, and 0.002% DNAse and incubated at 37°C with constant rotation for 12 min. The dissociation medium was replaced by basal Eagle's medium containing 10% (vol/vol) fetal calf serum, and the tissue was dispersed by gentle pipetting (three times) and was passed through a nylon screen to complete the dissociation to single cells. Cells were collected by centrifugation at 200 × *g* for 7 min. Single-cell suspensions (1–2 × 10^6 cells) were plated on 25-mm collagen or poly(D-lysine)-coated glass coverslips in 35-mm Corning tissue culture dishes containing 2 ml of basal Eagle's medium with 10% acid-treated fetal calf serum (19). Cultures were usually maintained for 3 days in a 37°C incubator with 10% CO_2 to allow for attachment of most cells, extension of nerve fibers, and degradation of residual trypsin. Viability of attached cells was greater than 95%. For cultures kept longer, the medium was changed every 2 days. In many experiments and for control purposes, the meninges and superficial vasculature were removed under a dissecting microscope, and the diced tissue was dissociated mechanically (without trypsin) by trituration through small-bore capillary pipettes. Although such preparations had a lower cell yield, they gave cultures of similar appearance to the enzymatic procedure.

Cell Separation. Campbell *et al.* (20) described the preparation by density gradient centrifugation of a glial-depleted, neuron-enriched population of cerebellar cells. This cell fraction C contains approximately 95% granule neurons. We found that the remaining adherent cells in this fraction interfere with its usefulness for unambiguous cell identification in the fibrin slide method. Therefore, we further fractionated mechanically dissociated cerebellar cells as follows. Fraction C cells were suspended in basal Eagle's medium with 10% fetal calf serum and incubated for 1 hr at 37°C at 5–10 × 10^6 cells per 60-mm glass Petri dish to allow for attachment of residual strongly ad-

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Abbreviations: ^{125}I-fibrin, ^{125}I-labeled fibrin; anti-Cbl-1 antiserum, rabbit antiserum against mouse cerebellum; PA, plasminogen activator; anti-GFAP, rabbit antiserum against glial fibrillary acidic protein.

herent (nonneuronal) cells. The nonadherent and loosely adherent cells were recovered, pipetted to break up aggregates, and recentrifuged on the serum albumin density gradient of the Campbell procedure (20). Fraction C cells were plated on polylysine- or collagen-coated coverslips. This highly purified cell population appeared homogeneous in size, and ≈99% of them bound tetanus toxin. The cells readily attached to polylysine coverslips and, after 3 hr in culture, proved suitable for the fibrin slide assay.

Histochemical Fibrin Slide Method. A crude plasminogen-containing fibrinogen was prepared from bovine plasma (Colorado Serum, Denver, CO) as described (21). A highly purified fibrinogen, free of plasminogen, was prepared from bovine fibrinogen (Sigma, fractional type IV) by two cycles of fractional precipitation as described (22). Crude fibrinogen was shown to be a good substrate for cell-mediated fibrinolysis in the fibrin/agar overlay assay (19) with colonies of mouse G26 glioma, whereas the purified fibrinogen was not (unpublished results).

The fibrin slide method was similar to that described by Todd (14). Coverslips with attached cerebellar cells were washed three times with phosphate-buffered saline, a drop (≈25 μl) of bovine thrombin (Sigma, grade III; 50 units/ml) was applied, allowed to coat the surface by rotating the coverslip, and drained, followed by application of a drop (≈40 μl) of crude fibrinogen (10 mg/ml). After the coverslip was drained, a thin fibrin clot was formed within 5–15 sec. Purified fibrinogen was used in a similar manner as the control experiment to test for plasminogen dependence of fibrinolysis. The coverslips were incubated for the indicated time in a moist chamber at room temperature, fixed in 10% (vol/vol) formal/saline, stained with 2.5% (wt/vol) Coomassie blue or with hematoxylin, dehydrated, and mounted on slides for microscopic observation.

^{125}I-Fibrin Plate Assay. Purified fibrinogen was iodinated with Na^{125}I (New England Nuclear, 17 Ci/mg; 1 Ci = 3.7×10^{10} becquerels) as described (23). The dialized ^{125}I-labeled fibrin (^{125}I-fibrin) was applied and dried onto 35-mm Corning dishes as described by Unkeless *et al.* (24). The plates were sterilized by a 30-min exposure to UV light, and fibrinogen was clotted as fibrin by a 1-hr incubation with 0.4 units of bovine thrombin (12), and the plates were washed free of unbound radioactivity by a 1-day incubation with phosphate-buffered saline. Various batches of plates contained 40,000–100,000 cpm of trypsin-releasable radioactivity.

Cerebellar and cortical extracts were prepared for assay as follows. The tissue was freed of meninges and superficial vasculature and minced. Homogenization and Triton X-100 extraction of the membrane fraction was as described (25). Briefly, the minced tissue was incubated for 10 min in a hypotonic homogenization buffer (0.25 M sucrose/0.001 M EDTA/0.01 M Tris·HCl, pH 8.1) at 4°C and homogenized. The homogenate was centrifuged at 600 × *g* for 10 min, and the supernatant was recentrifuged at 100,000 × *g* for 60 min to provide a soluble fraction and a particulate membrane fraction. This latter fraction was extracted for 10 min at 30°C with 0.5% Triton X-100. To collect medium for assay from 3-day-old cultures, the plates were washed three times with basal Eagle's medium and incubated for 4 hr with that medium at 37°C. The collected medium was centrifuged and used directly for assay. The standard ^{125}I-fibrin plate assay contained in 1 ml 4 μg of porcine plasminogen (Sigma), 0.1 M Tris·HCl (pH 8.1), and aliquots of culture medium or cell extract. The assay was performed in triplicate at 37°C. Aliquots (0.3 ml) were removed at various times, and solubilized radioactivity was determined by scintillation counting. Plasminogen-dependent release of ^{125}I was calculated by correction for release occurring with plasminogen alone and with cell extract or culture medium alone.

Indirect Immunofluorescence. After incubation for fibrinolysis, coverslips containing clots were overlaid with 0.1 ml of tetanus toxin (Department of Biologics, Food and Drug Administration, Washington, DC) and kept at room temperature for 20 min. All incubations were for 20 min, and coverslips were incubated with mouse anti-tetanus toxin or rabbit antiserum against mouse cerebellum (anti-Cbl-1 antiserum), followed by fluorescein-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG, respectively. Coverslips were fixed in 4% (wt/vol) paraformaldehyde, mounted in Gelvatol (Monsanto, St. Louis, MO), and observed with a Zeiss microscope with epifluorescence. Fibrin clots treated with rabbit anti-glial fibrillary acidic protein (anti-GFAP antiserum; gift of Doris Dahl) were prefixed in paraformaldehyde or acetone to permit intracellular penetration of the antibody.

RESULTS

Plasminogen Activator Activity in Developing Cerebellum. The ^{125}I-fibrin plate assay (24, 25) was used to measure PA activity in cerebellum at a developmental stage characterized by active cell proliferation and migration *in vivo*. Initial experiments with aliquots of homogenates from meningeal-free cerebella showed low activity. When subcellular fractions were tested, the Triton X-100-extracted membrane fraction had fibrinolytic activity that was dependent on time, temperature, and the amount of added protein (Fig. 1*A*). The soluble fraction was severalfold less active and the activity did not show a linear dependence on the amount of added protein. These data are

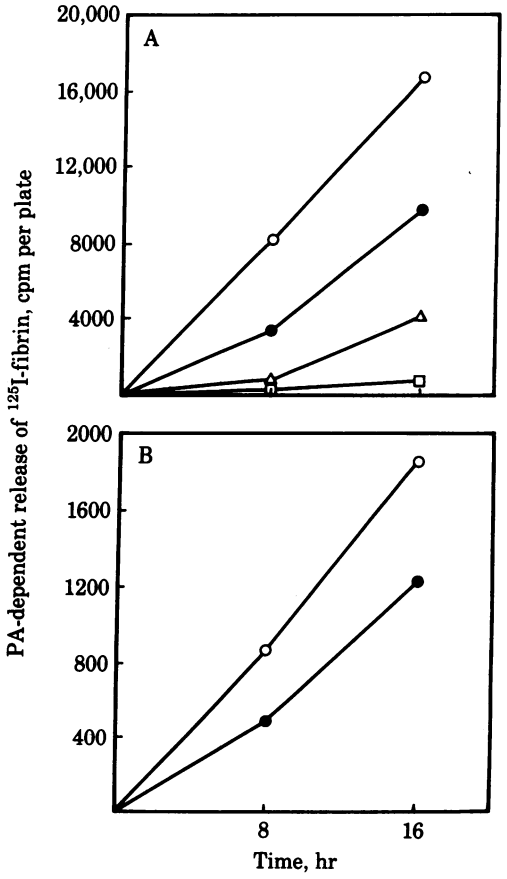

FIG. 1. PA activity in cell extracts and culture medium. (*A*) Triton X-100-extracted membrane fraction of 10-day brain: ●, 5 μg of cerebellum; ○, 10 μg of cerebellum; △, 5 μg of cerebral cortex; □, 10 μg of cerebellum at 5°C. (*B*) Aliquots of 4-hr culture media from cerebellar cultures maintained 3 days *in vitro*: ●, 0.25 ml; ○, 0.50 ml.

consistent with the presence of a soluble proteolytic inhibitor and a selective enrichment of PA in the membrane fraction. In the experiment shown in Fig. 1A, 16,800 cpm were released at 16 hr by 10 μg of cerebellar protein in the presence of 4 μg of plasminogen as compared with 940 cpm by the cerebellar extract alone. This 95% dependence on added plasminogen suggests that the assay was measuring a cellular activator of plasminogen and that this PA is a major proteolytic activity of cerebellum. Furthermore, the activity seen in 10-day cerebellum was twice that seen in the developmentally more mature cerebral cortex when equal amounts of extract protein were assayed (Fig. 1A). This difference was maintained when the extracts were passed over a benzamidine-Sepharose 4B affinity column (26) to separate PA from its inhibitors prior to assay (data not shown), suggesting that the difference is due to the amount of PA rather than to inhibitor differences.

To examine whether PA was released by viable cerebellar cells, cultures were set up as described. These cultures had the characteristic appearance already described (16). Small neurons became attached to the substratum as single cells and, to a greater extent, as cell aggregates that displayed extensive fiber outgrowth after 3 days in culture.There was also an attached population of glia and fibroblasts. Culture medium collected by a 4-hr incubation of 3-day cultures also showed PA activity (Fig. 1B), suggesting that this enzyme is produced and released *in vitro* as well as occurring *in vivo*.

Characterization of *in Vitro* PA Production. When 3-day cultures were overlaid with a crude fibrin clot for visualization of fibrinolytic activity, lytic zones were seen around aggregates of presumptive small neurons (Fig. 2*a*) but not around adherent cells with large surface area. Approximately 35% of small (30- to 40-μm diameter) aggregates (*i.e.*, 15–40 cells) possessed lytic zones, whereas 80–100% of the larger (50–200 μm) aggregates were positive for fibrinolysis. A few (<1%) individual small cells of neuronal appearance also show fibrinolytic zones (Fig. 2*b*). When overlays were performed with highly purified fibrinogen, free of plasminogen, no lytic zones were seen (Fig. 2*c*), suggesting that fibrinolysis is plasminogen dependent and, therefore, due to a cellular activator of this proenzyme. Fibrinolysis was time dependent, and lytic zones were not demonstrable by 1 min (Fig. 2*d*); very small partial zones were formed by 15 min (Fig. 2*e*) and were greatly increased in size by 60 (Fig. 2*a*) and 120 min (not shown). The involvement of proteolysis in the generation of clear zones is suggested by the inhibition of fibrin removal when the proteolytic inhibitor ε-aminocaproic acid (Fig. 2*f*) or soybean trypsin inhibitor were present. Furthermore, lytic zones were not seen when the coverslips were fixed in formalin before assay (Fig. 2*g*).

The above control experiments showed that fibrin degradation has all the expected characteristics of a PA-mediated proteolysis and also ruled out several possible artifacts in the fibrin slide method. The clear zones seen around cell aggregates was not due to tearing of the fibrin film as the clot was formed around the raised surface because zones were not seen under the conditions where proteolytic activity was inhibited (Fig. 2 *c*, *f*, and *g*). Furthermore, partial degradation of fibrin rather than large lytic zones occurred at early times of incubation (Fig. 2*e*) and around small aggregates. Small zones also could be visualized at the single-cell level (Fig. 2*b*). Fibrinolysis was not due to a carryover of trypsin from the dissociation procedure because lytic zones were seen when cerebella were mechanically dissociated (Figs. 2*h* and 3). The possibility that fibrinolysis was due to various nonneuronal contaminating cells was investigated. Fibrinolysis still was seen when the meninges and superficial vasculature were removed prior to cell dissociation (Fig. 2*h*). Furthermore, when fibrinolysis was visualized at the single-cell level, only small round cells were active, suggesting that proteolysis is not due to macrophages or other adherent cells of similar size.

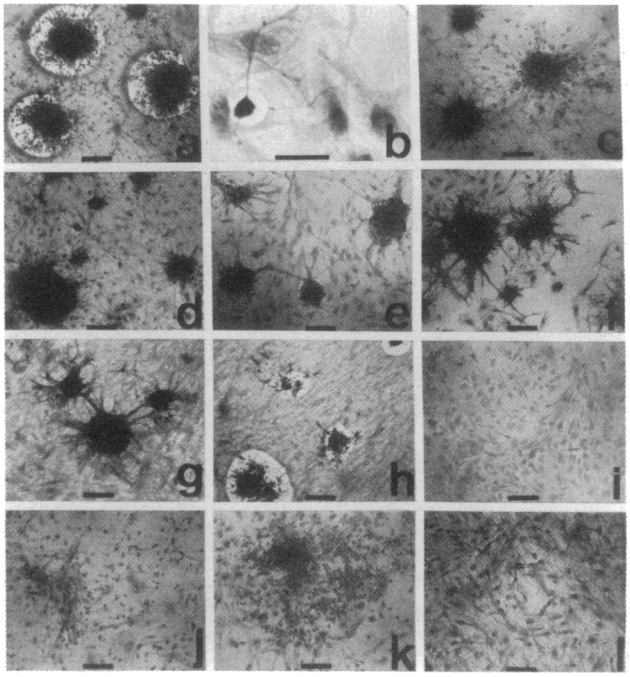

FIG. 2. Fibrin slide histochemical assay for PA. Cerebellar cells were cultured and used in the fibrin slide assay. The photomicrographs show representative fields. Unless otherwise noted the cells from 7- to 9-day cerebella were cultured for 3 days. The assay was for 1 hr with crude fibrin. The different culture and assay conditions shown are: *a* and *b*, assay with crude fibrin; *c*, purified fibrin; *d*, 1 min; *e*, 15 min; *f*, coverslip covered with ε-aminocaproic acid (1 mg/ml) after clot formation and before assay; *g*, coverslip fixed for 1 hr in 10% formalin in phosphate-buffered saline overlay; *h*, cells prepared for culture by dissociation without trypsin and with the meninges and vasculature removed; *i*, cells cultured for 7 days; *j*, cells prepared from 3-day cerebella; *k*, cells prepared from 3-day cerebral cortex; *l*, cells from mice treated *in vivo* with 5-fluorodeoxyuridine. (Bar = 100 μm except for *b*, in which bar = 25 μm.)

Neuronal Character of the Fibrinolytically Active Cells. Further experiments suggested that fibrinolysis was due to a specific class of cerebellar neurons. Fibrinolysis was not seen in cultures maintained for 7 days or longer *in vitro* (Fig. 2*i*); most small neurons also are lost from cultures by this time unless special culture methods are used (27). Fibrinolysis also was not seen under four conditions that resulted in poor neuron survival: when less than 1×10^6 cells were plated; when coverslips containing only the cells that adhered to the substratum within the first 8 hr of culture were assayed; when cultures were subcultured twice by trypsinization and replating of one-fifth of the cells; and when cultures were prepared from the few cells that survived when developmentally more mature 13-day cerebella were dissociated (data not shown).

Fibrinolysis was not seen to as great an extent when cultures were prepared from 3-day cerebella—a time before the major generation of granule neurons (Fig. 2*j*). Cultures of 3-day cerebral cortex, which is developmentally more mature than the cerebellum at this age, were also fibrinolytically inactive (Fig. 2*k*). When the external granule layer was largely eliminated by injection of mice with 5-fluorodeoxyuridine at days 2 and 3 (28), followed by culture preparation at day 8, these cultures were greatly reduced in both small neurons and fibrinolysis (Fig. 2*l*). Similar results were obtained with methylazoxymethanol (29) as the *in vivo* antimitotic agent. However, fibrinolysis was still

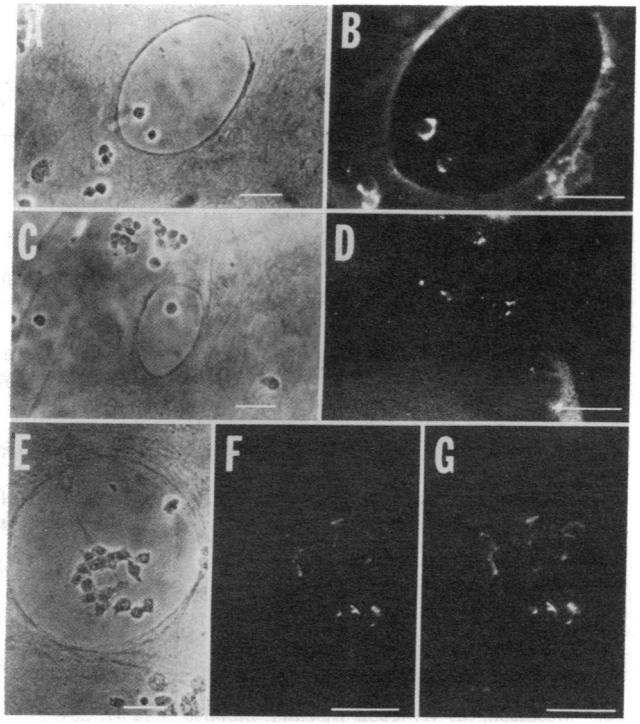

FIG. 3. Fibrinolysis, tetanus toxin, and anti-Cbl-1 antiserum binding by purified granule neurons. Granule neurons prepared without trypsin and purified by gradient centrifugation were plated on poly(D-lysine)-coated coverslips and used 3 hr later for the fibrin slide assay. The slides were incubated for 4 hr for fibrinolysis and then reacted sequentially with tetanus toxin, mouse anti-tetanus toxin, and fluorescein goat anti-mouse IgG (*B*, *D*, and *F*), and with rabbit anti-Cbl-1 antiserum and rhodamine goat anti-rabbit IgG (*G*). (*A*, *C*, and *E*) Phase-contrast micrographs. (*B*, *D*, and *F*) Fluorescein fluorescence. (*G*) Rhodamine fluorescence. (Bar = 50 μm.)

seen under conditions where cytosine arabinoside (10 μM) treatment of cultures inhibited all cell proliferation *in vitro* (not shown).

More definitive cell localization and identification was achieved by the use of the cell separation methods to obtain a highly purified granule neuron population, which was homogeneous in size and depleted of cells that adhere to collagen and proliferate in culture. Although these cells still had a propensity to aggregate when placed on poly(D-lysine)-coated coverslips, many single cells and some that are fibrinolytically active were observable (Fig. 3 *A* and *C*). Table 1 shows fibrinolytic zones are more common around cell aggregates than around single cells.

Phase-contrast microscopy (Fig. 3*E*) shows the "clockface" distribution of condensed chromatin typical of granule cells (2). More convincing evidence for these fibrinolytic cells being granule neurons is shown in Fig. 3 *B*, *D*, *F*, and *G*. Tetanus toxin, which interacts with cell surface G_{D1b} and G_{T1} gangliosides and binds to all classes of neurons (30–32), reacted with

Table 1. Fibrinolysis by granule cell neurons

Cells per cluster	Fibrinolytic clusters, %
1	2.6 (n = 115)
2	3.5 (n = 58)
3–7	13.9 (n = 93)
8–12	70.3 (n = 37)

Gradient-purified granule neurons were cultured on poly(D-lysine)-coated coverslips for 3 hr at 37°C. The cultures were overlaid with a fibrin clot, kept at room temperature for 4 hr, fixed, and stained.

virtually all cells found in the fibrinolytic zones (Fig. 3 *B*, *D*, and *F*). An antigen, Cbl-1, that is found primarily on cerebellar neurons (33), also was found on the surface of these same plasminogen activator-secreting cells (Fig. 3*G*). In contrast, these cells did not react with anti-GFAP antiserum, a marker for astrocytic cell types (34), whereas cells in the glial-enriched gradient fraction B were positive for GFAP and fibrinolytically inactive (not shown). Furthermore, in the absence of the primary antiserum, there was no fluorescence associated with the fibrinolytically active cells, thus ruling out microglia (a small phagocytic cell type that would readily bind and engulf the secondary antiserum) as the active population.

DISCUSSION

Early studies (35, 36) directed at understanding cellular metabolism and tissue contributions to blood homeostasis have shown that brain contains both fibrinolytic activators and proteolytic inhibitors. A possible role of such components in neural development has not previously been considered. The production of PA by developing cerebellum has now been demonstrated by two methods and localized to a specific neuron type. By using the ^{125}I-fibrin plate method, PA activity could be detected in the membrane fraction of tissue homogenates and in conditioned medium from cerebellar cell cultures. These studies (Fig. 1) showed that PA represented >95% of the fibrinolytic activity in cerebellar homogenates. The kinetics of the activity in homogenates suggested that there was also a proteolytic inhibitor present, as is known to occur in brain (36) and also in some cultured cells (25), and this complicated studies on the developmental regulation of the enzyme. The ^{125}I-fibrin plate assay is of widespread use in quantifying enzyme release from clonal cell lines and actively secreting macrophages. However, the cellular complexity of cerebellar cultures with five types of neurons, glia, and other supportive cells, coupled with the low level of activity (Fig. 1*B*), limited the usefulness of this assay.

The low level of PA secretion into the cell culture medium suggested that a limited population of cells may be responsible for the activity. Therefore, the fibrin slide technique of Todd (14) was used to localize the enzyme at the level of individual cells. Fibrin clot overlays of cerebellar cells showed fibrinolytic zones around aggregates consisting largely of small neurons and, in favorable cases, around individual small neurons as well. Possible artifacts such as tearing of the fibrin film were ruled out. The granule neuron is suggested as the primary active cell type based on the cell size, shape, nuclear appearance, relative abundance, and survival properties in culture, and external granule layer origin is shared by both granule neurons and the cells present most often in fibrinolytic zones. This suggestion is further strongly supported by the demonstration of PA in a population greatly enriched in granule neurons by cell separation methods. The neuronal character of the fibrinolytic cells is established by their binding of tetanus toxin and anti-Cbl-1 antiserum together with their lack of interaction with anti-GFAP antiserum, thus ruling out meningeal and endothelial cells, microglia, astrocytes, and oligodendroglia as the active cell type seen in these experiments (30–34). However, the possibility that other specific cerebellar cells secrete PA has not been studied in detail. With the purified granule neuron preparation, fibrinolytic zones were best visualized around clusters of cells (Table 1), suggesting either that the enzyme level per cell is low or that cell–cell contact, as might be expected if PA secretion is important in cell migration, is stimulatory to enzyme release or activity.

Other workers have demonstrated PA production by migratory cells (7–9), and Kalderon (37) has suggested that migratory

Schwann cells may secrete PA in spinal cord cultures. Therefore, it is interesting to consider the possibility that cerebellar PA is due to migratory granule neurons, which are prominent in histological sections of developing cerebellum. Because granule cell transit time from the external granule layer to the internal granule layer has been estimated at 3 days (38), it is likely that many of the small neurons observed in cultures were at this stage of development at the time of preparation of the cultures. Furthermore, our recent quantitative studies have shown that cerebellar extracts from 2-day-old mice (when granule cell migration is just beginning) and 20-day-old animals (when granule cell migration has been completed) are only 80% and 64% as active as extracts from 10-day-old mice (a time of extensive cell migration). However, even in the purified granule neuron fraction of the Campbell procedure (20), it has not yet been possible to evaluate the percentage of migratory, postmigratory, and neural stem cells present. Preliminary [^{3}H]thymidine birthdating experiments suggest that postmigratory granule cells do not secrete PA, in contrast to those in the external granule layer or molecular layer. Also the failure of *in vitro* administered cytosine arabinoside to diminish PA secretion suggests that the neural stem cells are not a likely candidate.

Based on differential adhesion of trypsinized neural retina cells, Thomas *et al.* (39) have suggested that extracellular proteases may temporally and spatially modulate cell–cell interactions and cell movements during development. In another study (40), we have demonstrated PA secretion at the growth cone of peripheral neurons. Therefore, these findings have suggested a possible function of PA in cell movement that is being examined by a cell culture assay for neuron migration, in which the extracellular environment may be controlled. Although plasminogen is present in brain and cerebral spinal fluid, further studies also will be needed to identify the cerebellar substrates and physiological regulation of plasminogen activator.

We express our gratitude to Drs. C. Hardigree, W. Habig, and D. Dahl for their generous gifts of tetanus toxin and anti-GFAP antiserum. The technical assistance of Mr. Jeffrey Cross is greatly appreciated. This work was supported in part by National Institutes of Health Grants NS-09818 and F-32-CA-05949 and by a grant from the Milheim Foundation.

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