Peripheral Neurons and Schwann Cells Secrete Plasminogen Activator

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ABSTRACT The secretion of the protease plasminogen activator (PA) by cells of developing peripheral nerve was demonstrated. Fetal and early postnatal dorsal root ganglia were established in culture as explants or as individual neurons and Schwann cells. A fibrin overlay assay was used to visualize the locations of PA secretion. Fibrinolytic zones formed around the somata of explants and were skewed in the direction of maximal fiber outgrowth. Individual growth cones at the tips of long fasiculated fiber bundles also released PA. Approximately 50% of individual neurons showed PA secretion; especially pronounced release occurred at some growth cones. Culture of nerve growth factor-independent adult neurons showed that PA expression was independent of effects of this growth hormone. A subpopulation of Schwann cells was also active in PA secretion, which could be detected at the soma, at the bipolar processes, or along the entire cell length. Possible functions of neural PA in development and regeneration are discussed.

Embryonic cell migrations from the neural crest figure prominently in generating the spinal ganglia of the vertebrate peripheral nervous system (1, 2). Neural maturation includes axonal innervation of target tissues and postnatal ensheathment of axons with the Schwann cell myelin membrane. Furthermore, peripheral nerve in adult mice retains the ability to respond to injury by an initial degenerative phase (chromatolysis), followed by axonal re-extension and an appropriate degree of Schwann cell proliferation, migration, and reensheathment (3). Elucidating biochemical mechanisms underlying both developmental and regenerative cell positioning, axonal growth, and tissue remodeling are of obvious interest. Localized protease secretion may play a role in these events by modifying cell surfaces and the extracellular environment to facilitate cell movements and interactions. We have now demonstrated plasminogen activator (PA)[1] secretion by both neurons and Schwann cells in cultures prepared from developing mouse sensory ganglia.

MATERIALS AND METHODS

Cell Culture: Dissected mouse dorsal root ganglia were collected into a HEPES buffered dissociation medium (4) treated with 0.25% *Clostridium histolyticum* collagenase (Worthington CLS 50S; Worthington Biochemical Corp., Freehold, NJ) at 37°C for 5 min (2-d ganglia) or 1 min (fetal ganglia). Ganglia were removed from the collagenase solution, washed once with 10% fetal calf serum in PBS, collected by settling, and washed twice in PBS alone. Ganglia were explanted onto poly-D-lysine coated coverslips in 200 μl of serum-

free N2 medium (5, 6) supplemented with 25 ng/ml 2.5S nerve growth factor (NGF) (Collaborative Research, Inc., Lexington, MA). Extensive radial nerve fiber outgrowth occurred with minimal Schwann cell migration in serum-free medium, or in association with migrating Schwann cells when serum containing medium was used. Explants established without collagenase treatment did not become firmly enough attached to the substrata to allow processing for fibrin overlay assays.

Dissociated cell cultures containing a high percentage of individual neurons, as identified by nuclear morphology and tetanus toxin binding, were obtained by trituration of collagenase treated ganglia. Incubation time with collagenase was adjusted to give maximum neuron viability, and longer incubation was required for older ganglia as described by Scott (7). Ganglia were treated with 0.25% collagenase for 5 min (2-d ganglia), 15–20 min (16–17-d ganglia), or 30–60 min (30–90-d ganglia) and washed as described above. Following the addition of 0.5 ml of serum-free N2 culture medium containing 10 μg DNAse, the ganglia were triturated in Sigma Cote-d narrow bore Pasteur pipettes (Sigma Chemical Co., St. Louis, MO), and the remaining fragments discarded. Fresh medium (4.5 ml) was added to the cell suspension in a polyethylene tube and single cells were collected by low speed centrifugation. Cells were plated in 200 μl of N2 medium on polylysine-coated coverslips. 25 ng/ml 2.5S NGF was added to the early postnatal cultures only.

Schwann cell enriched cultures were established by dissociation of nerve roots excised from dissected ganglia. Treatment with collagenase, washing, and cell dispersion was as described above, only the trituration was more vigorus. 86% of the plated cells had the bipolar spindle shape characteristic of Schwann cells. Of these, 82% bound monoclonal antibody recognizing a Schwann and oligodendroglial cell surface component (8) at 1 d in vitro. The specificity of this antibody for Schwann cells in these cultures was confirmed by double-labeled immunofluorescence with rabbit antibody to galactocerebroside as described by Mirsky (9).

Fibrin overlay assay: The secretion of PA was detected by fibrin overlay assays as previously described (10, 11), modified to include 0.4% glucose in the fibrin clot to ensure neuron viability, and 10 U/ml penicillin and 10 μg/ml streptomycin, as antibacterial prophylaxis. Appropriate controls for plasminogen dependence, time dependence, and sensitivity of the fibrinolysis to

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[1] Abbreviations used in this paper: DRG, dorsal root ganglia; NGF, nerve growth factor; PA, plasminogen activator.

protease inhibitors were performed. For all cells examined no fibrinolysis was observed in the absence of plasminogen, showing that fibrin degradation occurring in plasminogen-containing clots is due to generation of plasmin by a cellular PA.

Explant and neuronal cultures were assayed at 1–3 d in vitro, when maximal nerve fiber outgrowth was occurring. Neurons remained viable during the 4–6 h assays at room temperature as judged by the retention of a phase bright soma and maintenance or increase of the neurite length.

RESULTS AND DISCUSSION

A fibrin overlay assay was used to detect plasminogen activator secretion by explanted dorsal root ganglia. Large fibrinolytic zones were routinely detected, spreading with time, from the somata of explants. The shape of the fibrinolytic zones was skewed in the direction of maximal nerve fiber outgrowth (Fig. 1A). Fibrinolytic degradation included the
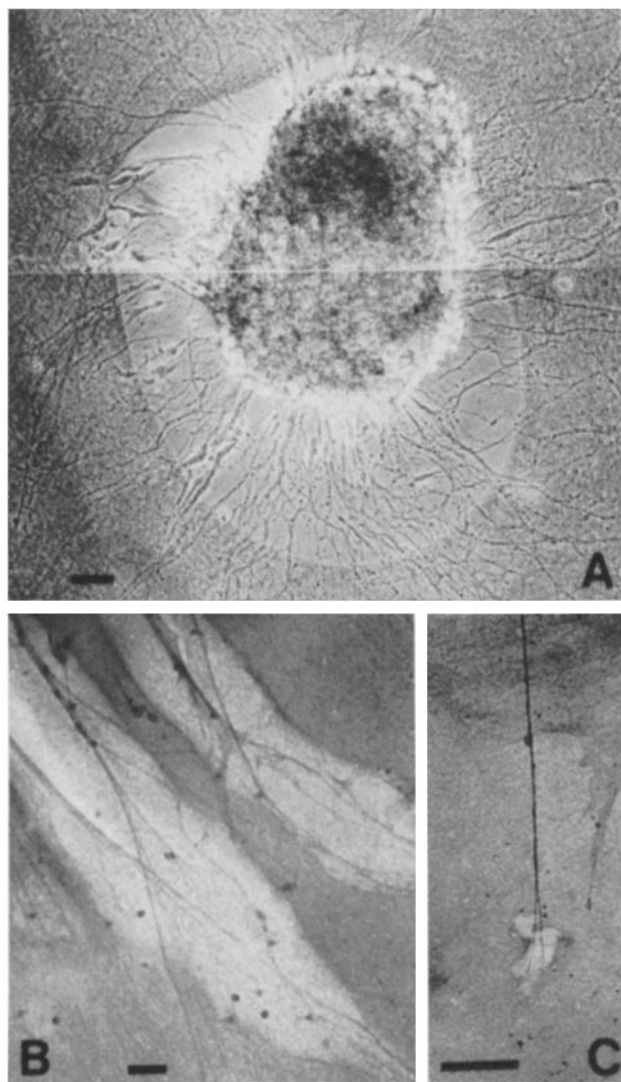

Figure 1   Plasminogen activator secretion by explanted dorsal root ganglia. Ganglia explanted as described in Materials and Methods were assayed for PA secretion by the fibrin overlay assay. Plasmin-mediated degradation of fibrin is visualized as areas of clearing in the clot and indicates sites of release of a plasminogen activator by the underlying cells. PA secretion was seen around the somata (A) of an unfixed explant in this phase contrast visualization of an area of fibrin degradation. PA secretion was also seen along the path (B) of growing neurites and at growth cones (C), when cultures were fixed and stained with Coomassie Brilliant Blue to enhance the fibrin background. Bars, 50 μm. × 100 (A and B); × 200 (C).

pathways of neurite outgrowth (Fig. 1B) and frequently separate areas of PA release were seen at the distant (500–1,200 μm) growth cones of fasiculated fiber bundles (Fig. 1C).

Since the ganglia contain both neurons and Schwann cells, the source of the PA was not obvious. To examine neurons at the single cell level ganglia were dispersed as described in Materials and Methods. A synthetic medium (N2) proved the most useful for obtaining individual neurons and extensive neurite outgrowth free of Schwann cells (6). Fibrin overlay assays were performed after 1–3 d of culture in 25 ng/ml 2.5S NGF, which lacks endogenous protease activity (12). Mean percentage of neurons showing plasminogen-dependent fibrinolytic zones was 50% (range of 41 to 61 for five experiments). Neither neurite outgrowth nor fibrinolysis was enhanced if the 7S form of NGF was used. Since Scott (7) reported that viable neurons can be obtained from older ganglia and that neurite outgrowth was independent of added NGF, we modified his protocol (Materials and Methods) to obtain NGF-independent neurite outgrowth from 16–17 d postnatal sensory neurons. A similar extent of fibrinolysis was again seen (49% active cells). These results suggest that the assay is detecting a cellular plasminogen activator and not the esteropeptidase activity of the γ subunit of NGF or the weak PA activity reported for the 116 kdalton form (13). In other experiments, 17-d ganglia dissociates were fractionated on glass beads to remove adherent non-neuronal cells (14). Fibrin overlay assays of the recovered neurons, cultured in the absence of added NGF, showed that neuronal PA expression could be independent of NGF-like or other trophic factors secreted by non-neuronal cells (15).

Localization of PA release was scored in relation to neuronal membrane specializations and results similar to those reported with clonal cell lines (10) were obtained. Neurons with and without neurites were represented among the cells secreting PA (PA$^+$ cells). PA secretion was most pronounced in cells with neurites, which represent 43% of the neuronal population. In this population PA secretion was restricted to the growth cone in 5% of the cells (Fig. 2A); these were neurons with large spread growth cones and prominent filopodia. For most cells (58%), PA secretion occurred both at the soma and the growth cone, or the cell produced a fibrinolytic zone that encompassed both soma and the entire neuritic extension (Fig. 2C). The latter fibrinolytic pattern may result from the deposition of active PA on the substratum during growth cone movement (16). In addition, some neurons that had regenerated neurites showed PA secretion only around the soma (13%) (Fig. 2B), or were PA$^-$ (24%).

The examples shown in Fig. 2 were neurons cultured in serum-free N2 medium. Neurons seeded onto either polylysine or collagen coated coverslips in basal Eagle's medium with 10% fetal calf serum also regenerated neurites and appeared PA$^+$ but were always associated with Schwann cells. Diameters of PA$^+$ neurons ranged from 16 to 32 μm for 2-d-postnatal-ganglia dissociates cultured in synthetic medium. These sizes were somewhat larger for neurons cultured on collagen in the presence of serum. PA$^+$ neurons from 90-d ganglia had a range of diameters of 25–40 μm, reflecting maturational growth. This size range should include both small and large DRG neurons as PA$^+$ cell types.

Many of the Schwann cells present in the dissociated ganglia preparation were also PA$^+$. To investigate these cells in more detail, cultures containing predominantly Schwann cells were prepared by collagenase treatment of ganglionic nerve roots.
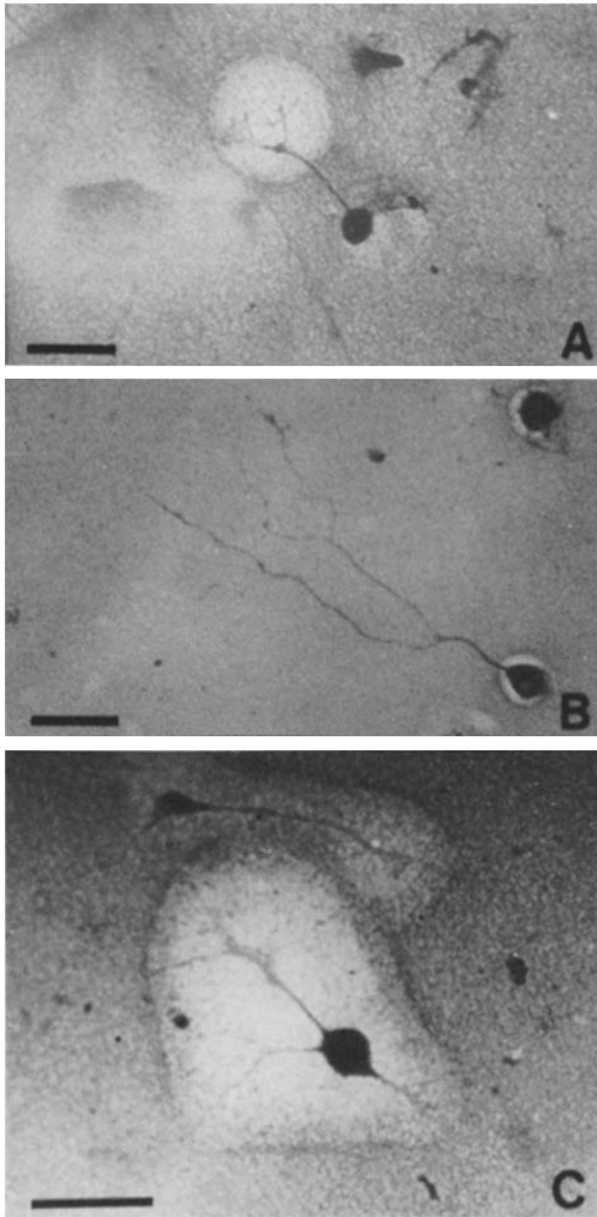
Figure 2   Localization of neuronal plasminogen activator secretion. Establishment of dissociated ganglia cultures and fibrin overlay assay was as described in Materials and Methods. The micrographs illustrate PA secretion at the neuronal growth cone (A), the soma (B), and at both locations (C). For the photomicrographic examples shown, absence of a covert Schwann cell along any part of the neurons was verified by microscopic viewing × 1,000. A–C were fixed and stained with Coomassie Brilliant Blue. A PA⁺ Schwann cell is also seen in the upper portion of C. Bars, 50 μm. × 240 (A and B); × 320 (C).

Approximately half of the Schwann cells were detected as PA secretors when cultured in N2 medium (Table I). Contaminating fibroblasts in these preparations were uniformly negative for PA secretion by the overlay assay under several culture conditions. Schwann cells were also heterogenous for the areas along the cell length at which PA secretion was detected (Fig. 3). An examination of 592 Schwann cells (from different growth conditions) showed that 32% of the cells exhibited fibrinolytic zones with the soma as foci (Fig. 3A). Whereas for 22% of the cases, release appeared to be along the entire length of the cell (Fig. 3B). However, the greatest number of cases (35%) were of fibrinolytic zones about the soma, but skewed to include a portion of one of the processes (Figs. 3, C and D). A minority of cells (10%) showed pronounced release along one or both processes but excluding the soma (Fig. 3E). Sites of PA release did not appear to depend on developmental age, substratum, medium or the presence or absence of neurons.

Although PA secretion by Schwann cells grown on polyly-sine substrata in serum-free medium was relatively constant during in vitro culture (Table I), other experiments showed that the fibrinolytic activity of Schwann cells could be modulated by the culture conditions. When Schwann cells were grown on collagen in the presence of fetal calf serum, 42% of the cells were PA⁺ at 1 d in vitro but only 2.5% were positive after 3 d in vitro. Furthermore, when Schwann cells were grown on polylysine in the presence of serum rather than N2 medium, only 2% of the cells were PA⁺ even at 1 d of culture. Reasons for the ability of different substrata and media to influence PA expression is currently under investigation. However, other studies (17, 18) have shown that serum components can modulate expression of both functional and secretory properties of Schwann cells.

In mixed cultures of dissociated neurons and Schwann cells, ~50% of Schwann cells were again PA⁺; however, expression of enzyme secretion did not appear to be influenced by Schwann cell proximity to neurons. Although PA⁺ neuronal growth cones with a Schwann cell attached along the neuritic length were observed, it was not possible by the present methodology to learn if such cell-cell interactions can modulate PA secretion.

In other experiments, Schwann cells were established in culture by allowing migratory outgrowth to occur from minced fragments of sciatic nerve. Fibrinolysis could also be observed in these cases showing that we are not merely detecting a spurious carryover of protease from the dissociation procedure. Kalderon previously suggested PA secretion by Schwann cells based on experiments detecting fibrinolysis during culture of dissociated spinal cord on ¹²⁵I-fibrin plates (19). The present experiments clearly distinguish, at the single cell level, Schwann cells from two other sources, as capable of PA secretion.

The finding that both neurons and Schwann cells are heterogenous for PA production, both in terms of percent secretory cells and ultrastructural localization of enzyme release, is intriguing. However, ganglia neurons are heterogeneous for other properties as well, such as neurotransmitter phenotype,
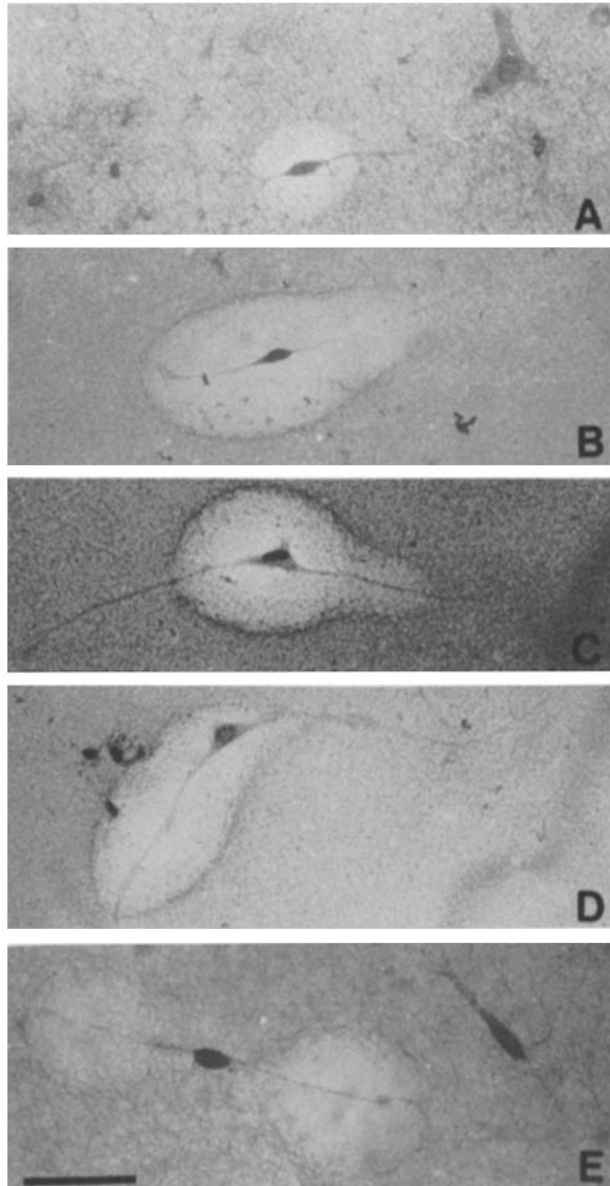
FIGURE 3 Localization of Schwann cell plasminogen activator secretion. Schwann cells prepared from DRG nerve trunks as described in Materials and Methods were evaluated for the location of PA secretion as visualized by the fibrin overlay assay. PA release appeared along the entire length of bipolar cells (B) or was restricted to the soma (A) or soma and one process (C and D), or just to the processes (E). A quantitative comparison of these localizations is presented in the text. Note the presence of a PA⁻ fibroblast-like cell and a PA⁻ Schwann cell in A and E, respectively. Bar, 50 μm. × 320.

size, and myelination (20, 21) and Schwann cells are known to vary in cell surface antigen composition (22) and proliferation (23) in culture. Neural PA secretion may have a role in cell migration and neurite extension. PA secretion by cerebellar migratory granule neurons has previously been demonstrated (11). Variability of PA expression in vitro could reflect any number of subtleties, such as rate or direction of Schwann cell movement or rate of neurite extension and growth cone microspiking. These possibilities are currently

being explored with time lapse video recording techniques. Alternately, PA expression could reflect an aspect of the cell's in vivo interactions at the time the ganglia were dissected. Neurons and Schwann cells produced PA over a broad developmental range; fetal day 13 to 3-mo-old adults were tested. This raises the possibility that the protease could be involved in several successive events in neural maturation. In preliminary experiments, we have also identified purified oligodendroglia (24) of the central nervous system as PA secretory cells. This cell type shares with the peripheral Schwann cell the property of ensheathment of the axonal membrane. A possible role for PA secretion in normal and degenerative myelin membrane metabolism remains to be investigated.

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