The EMBO Journal vol.9 no.8 pp.2471 - 2477, 1990

A plasminogen activator is induced during goldfish optic nerve regeneration

Fernando J.Sallés, Nisson Schechter[1] and Sidney Strickland

Departments of Molecular and Cellular Pharmacology, [1]Psychiatry and Behavioral Sciences, [1]Biochemistry, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

Communicated by E.Reich

The use of purified piscine plasminogen in a chromogenic solution assay enabled us to detect plasminogen activator (PA) activity in crude homogenates of goldfish optic nerve following nerve injury. In contrast, no activity was detected in the homogenates of uninjured nerve. Under conditions allowing regeneration of the optic axons (optic nerve crush), PA activity peaked 8 days after crush, and decreased to undetectable levels by 60 days. Under conditions allowing only degeneration of the axons (enucleation), the activity peaked at 8 days but decreased more rapidly. Casein zymography of samples after fractionation in SDS-PAGE showed that PA activity migrated as a doublet at M_r = 60-65 kd. Using this assay, activity was also observed in uninjured control nerves. This plasminogen-dependent activity migrated as three bands of higher molecular weight (M_r = 75, 95 and 120 kd) and was undetectable in solution assays of unfractionated extracts, suggesting complex formation with an inhibitor(s). Fibrin overlay assay of retinal explants and isolated primary cells in culture suggest that the goldfish PA is associated with the glial cells of the goldfish visual pathway.

Key words: nerve degeneration/nerve growth/piscine plasminogen/proteases

Introduction

Innervation, both during development and regeneration, requires axonal extension through intervening tissues. It has been proposed that a specific class of serine proteases, plasminogen activators (PA), might be involved in degradation of the extracellular matrix during axonal growth (Reich, 1978). An increase in PA activity has been correlated with many invasive processes including macrophage migration (Unkeless *et al.*, 1974), metastasis (Ossowski *et al.*, 1973; Danø *et al.*, 1985), and embryo implantation (Strickland *et al.*, 1976; Sappino *et al.*, 1989). Plasminogen activator activity is, in fact, associated with some cells of neuronal origin. Several neuroblastoma cell lines (Krystosek and Seeds, 1981a; Soreq *et al.*, 1983), dorsal root ganglia neurons (Krystosek and Seeds, 1984; Baron-Van Evercooren *et al.*, 1987), migrating cephalic neural crest cells (Valinsky and Le Douarin, 1985), and cerebellar granule cells (Krystosek and Seeds, 1981b; Soreq and Miskin, 1983), have been shown to display PA activity. In addition, PAs are associated with certain glial cell types. Peripheral nervous system (PNS) Schwann cells from dorsal root ganglia (Krystosek and Seeds, 1984; Baron-Van Evercooren *et al.*, 1987), superior cervical ganglia (Alvarez-Buylla and Valinsky, 1985), and sciatic nerve (Kalderon, 1984), as well as central nervous system (CNS) astrocytes from developing cerebrum (Toshniwal *et al.*, 1987), show associated PA activities.

Plasminogen activator activity in neural tissue appears to correlate with the regenerative capacity of nerves, or the ability of a specific nerve to regenerate. A large induction of PA activity is observed following sciatic nerve crush (degeneration of axons followed by regeneration) or transection (degeneration only) (Bignami *et al.*, 1982; Hantaï *et al.*, 1988). In contrast, an increase in PA activity is not found following transection of rat optic nerve (Bignami *et al.*, 1982), a vertebrate CNS nerve that cannot regenerate. To test if increased PA activity is a general phenomenon of nerve growth and regeneration, we examined a vertebrate CNS nerve that has retained the capacity for functional regeneration, the goldfish optic nerve, for the presence and induction of PA.

The adult goldfish visual pathway is characterized by a capacity for continuous growth (Johns and Easter, 1977; Meyer, 1978) and the ability to regenerate optic axons after nerve crush (Attardi and Sperry, 1963; Sperry, 1963). These characteristics, and its suitability for experimental manipulation, have rendered this pathway a useful adult model system for the study of nerve growth and regeneration in the absence of other developmental processes found with embryonic systems (for reviews see: Grafstein, 1986; Schechter *et al.*, 1989). After optic nerve crush, axonal fibers distal to the site of injury begin to degenerate. New fibers extend from the crush zone and reach the optic tectum 7-10 days after injury, for 15-20 cm fish (Murray, 1976). Measured increases of specific mRNAs such as tubulin (Heacock and Agranoff, 1976) and intermediate filaments (Tesser *et al.*, 1986) accompany the extension of new axons from the retinal ganglion cells. The rise in mRNA levels is followed by increases in synthesis and axonal transport of these proteins (McQuarrie and Grafstein, 1982).

We report here evidence of the existence of the plasmin-PA system in goldfish and PA production in response to injury of the goldfish optic nerve. In nerves, the activity is detected transiently in crude homogenates during degeneration and regeneration, but appears to be present in a latent form at low levels even in uninjured nerves.

Results

Purification of piscine plasminogen

Goldfish and trout plasminogens were purified from sera by lysine-sepharose chromatography. Using non-reducing SDS-PAGE, both proteins showed an apparent molecular weight of ~80 kd, that underwent a shift to M_r = 95 kd

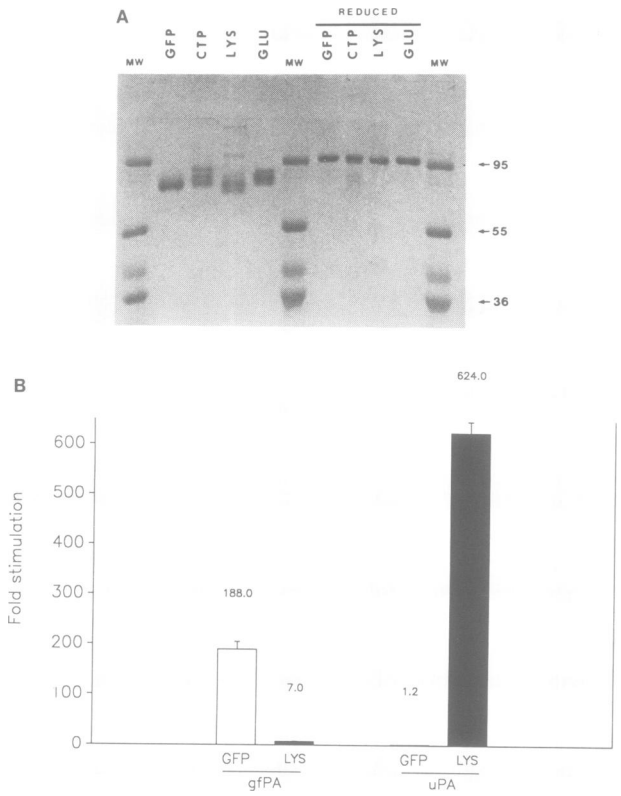

Fig. 1. Analysis of goldfish plasminogen. **(A)** Coomassie blue stained gel of SDS−PAGE analysis of goldfish plasminogen (GFP); trout plasminogen (CTP); human Lys-plasminogen (LYS); and human Glu-plasminogen (GLU). Samples are shown in both nonreduced and reduced-alkylated forms. Each lane contains 4 μg total protein. **(B)** Activation of GFP or LYS by either human urokinase-type plasminogen activitor (uPA) or goldfish aortal homogenate containing PA activity (gfPA). In the chromogenic assay (Materials and methods), 1 μg of either GFP or LYS was incubated in the presence of either 0.15 IU of uPA or 6.3 μg of aortal homogenate. Initial rates of hydrolysis were determined. Data are presented as fold activation or the increase in initial rates of hydrolysis relative to that of plasminogen alone (GFP IR = 0.6×10^{-7} ΔA/min^2, LYS IR = 2.4×10^{-7} ΔA/min^2). Bars represent standard error, n = 3.

upon reduction. This shift in molecular weight is similar in type and magnitude to that seen with the human proenzymes (Figure 1a). Several bands can be seen in all lanes under nonreduced conditions which collapse into single or doublet bands upon reduction. The doublet bands are present in trout, and both of the human plasminogens. For the human plasminogens these bands represent differences in glycosylation (Wallen, 1980). The goldfish plasminogen has no intrinsic activity but is converted to a two-chain, active protease (heavy chain, M_r = 50 kd; light chain M_r = 35 kd) by PA derived from goldfish aorta (unpublished data). The use of the piscine instead of human plasminogen increased the sensitivity of the assay for goldfish PA more than 25-fold (Figure 1b). Curiously, human activators did not significantly activate any of the piscine plasminogens (Figure 1b, unpublished observations); therefore, goldfish aorta, which contained a large amount of PA activity, was used as our standard PA.

Plasminogen activator activity increases after nerve injury

No proteolytic activity could be detected in crude homogenates of intact adult optic nerve by the chromogenic

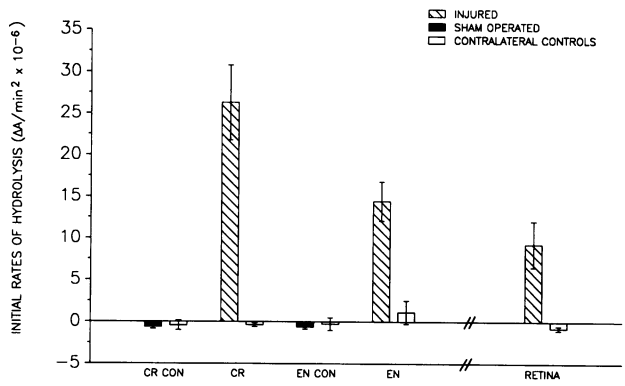

Fig. 2. PA activity in injured and uninjured goldfish optic nerve and retina. Initial rates of hydrolysis derived from the chromogenic assay using 5 μg of total protein for each nerve (11 days post-op). Sham operated controls for crushed fish consisted of Tricane anesthetization followed by dissociation of the eye's connective tissue and removal of any fatty deposits. Controls for the enucleated fish were the same as for crushed fish with the additional transection of the eye's supporting musculature. Care was taken not to damage the nerves. Retina (15 μg) of corresponding crushed optic nerves were also assayed for PA activity. However, quantitative comparisons of activity between optic nerve and retina are complicated by the presence of vitreous humor in the retinal homogenates. INJURED, right eye, injured nerves; SHAM OPERATED, right eye, sham operated nerves; CONTRALATERAL CONTROLS, left eye, contralateral uninjured control nerves; CR CON, crushed sham operated control; CR, crushed; EN CON, enucleated sham operated control; EN, enucleated; RETINA, retina isolated from corresponding crushed nerve. Bars represent standard error; for optic nerves, n = 4, retina, n = 3.

assay. After enucleation (removal of nerve cell bodies—degeneration only) or optic nerve crush (injury—degeneration with subsequent regeneration), however, a large amount of proteolytic activity was detected (Figure 2). The activity was entirely plasminogen-dependent (unpublished data). To determine if PA production was due to specific changes occurring within the optic nerve or to inflammation caused by trauma, sham operated controls were examined (see the legend to Figure 2). Activity was only seen with samples in which the nerve had been injured (crushed or enucleated). No activity could be detected in the sham operated or in the contra-lateral uninjured control nerves (Figure 2). Plasminogen activator activity was also seen in the retina of corresponding crushed optic nerves.

The time course of the appearance of PA activity after nerve injury was determined (Figure 3). Extensive precautions were taken to allow quantitative comparison between the time points (see Materials and methods). Plasminogen activator activity was found as early as one day after injury (crush or enucleation). The amount of activity reached its highest mean values at 8 days for both groups. The activity in the enucleated samples decreased significantly faster than that seen in the crushed optic nerve. By 40 days, the activity returned to negligible levels. No activity was detected in the contra-lateral uninjured control nerves.

In order to characterize the activity present in optic nerve, casein zymography after SDS−PAGE was used. The activity from freshly isolated, crushed optic nerve homogenates migrated as a doublet at 60−65 kd whereas the activity from the aorta migrated as a single band of similar molecular weight (Figure 4). The optic nerve PA is comparable to a doublet seen for platyfish PA which had a slightly lower molecular weight (M_r = 50 to 55 kd) (Takahashi *et al.*,

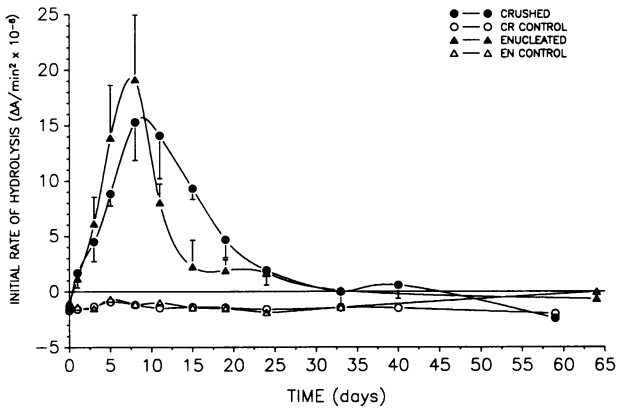

Fig. 3. Time course of gfPA expression. Initial rates of hydrolysis derived from the chromogenic assay using 5 μg of total protein for each nerve (see Materials and methods for experimental details and internal controls). CRUSHED, crushed (right) optic nerve, n=4; CR CONTROL, contralateral uninjured (left) control optic nerve for crushed, n=2; ENUCLEATED, enucleated (right) optic nerve, n=4; EN CONTROL, contralateral uninjured (left) control optic nerve for enucleations, n=2. Bars represent standard error.

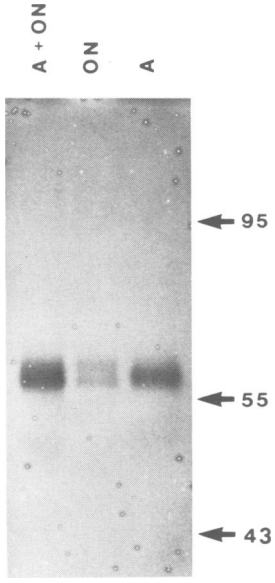

Fig. 4. Casein zymograph of gfPA. Casein zymograph analysis of gfPA activity found in fresh aortal and injured optic nerve homogenates. ON, 5 μg total protein from 11 day post-crush optic nerve; A, 5 μg total protein from goldfish aorta; A + ON, 5 μg each sample combined demonstrating similarity in their molecular weight. The activity was strictly plasminogen dependent.

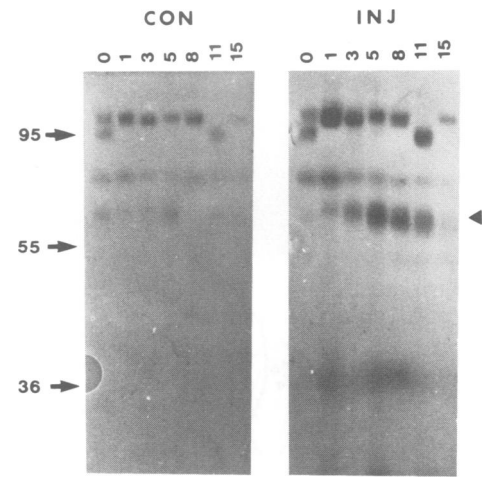

Fig. 5. Casein zymograph of time course optic nerve samples. Protein samples from each point in the time course experiment (Figure 3) were immediately denatured after isolation and stored at −70°C until all samples were collected. Casein analysis was conducted on the same casein−agarose bed for both injured and uninjured contralateral controls in each group. Each lane represents 5 μg of ON proteins from one individual goldfish at its respective time point. The lane from the injured samples half of the gel and its corresponding lane in the controls half of the gel originate from the same goldfish. Zymograph shown here represents samples from enucleated fish. The triangle points to the putative native gfPA. CON, contralateral uninjured (left) optic nerve; INJ, enucleated (right) optic nerve. All bands were plasminogen dependent.

1987). Although the putative native goldfish PA (gfPA) has a molecular weight similar to mammalian tissue-type PA (tPA) (M_r = 68−72 kd), its activity is not enhanced in the chromogenic assay by human fibrinogen fragments as is tPA (Hoylaerts *et al.*, 1982). By immunoinhibition, gfPA cross-reacts to a greater extent with human urokinase-type PA (uPA) antisera than with human tPA antisera (unpublished data). To examine PA production throughout the time of axonal degeneration and regrowth, equivalent amounts of protein from the different time points were denatured immediately after isolation and stored at −70°C until all samples were collected. Denatured samples were then analyzed by casein zymography. For both enucleations (Figure 5) and crushed samples (unpublished data), the intensity of the PA band at 65 kd paralleled the results from the chromogenic assay. Surprisingly, several new plasminogen-dependent bands were detected. These bands were only observed with samples that had been stored denatured and frozen; freshly isolated or samples frozen prior to denaturation only displayed activity of the species at 65 kd (Figure 4 versus Figure 5). Three high molecular weight bands at M_r = 75, 95 and 120 kd could be detected in both injured and the contra-lateral uninjured nerves (see Discussion). The low molecular weight band at 36 kd is a degradation product, since PA activity from 10 μg of aortal homogenate is entirely degraded to the 36 kd species within 10 min when incubated with 1 μg purified human lysine−plasmin (unpublished data).

Tissue culture analysis

Retinal explants (Landreth and Agranoff, 1976, 1979) were analyzed by fibrin overlay zymography. Lytic zones generally appeared after 4 h and expanded rapidly (Figure 6). All explants analyzed (>150) produced lytic zones only in the presence of plasminogen. Lytic zones were observed surrounding the explant squares and around individual cells that had either migrated or settled away from the explants (Figure 6a,b). Lytic zones were never associated with any of the retinal ganglion-derived neuritic bundles (Landreth and Agranoff, 1976, 1979) growing out from the explants. Primary cell cultures from retina and optic nerve were also analyzed by fibrin overlay zymography. Certain cells in both cultures were seen to produce lytic zones (Figure 6c; unpublished data). Due to the lack of unambiguous markers

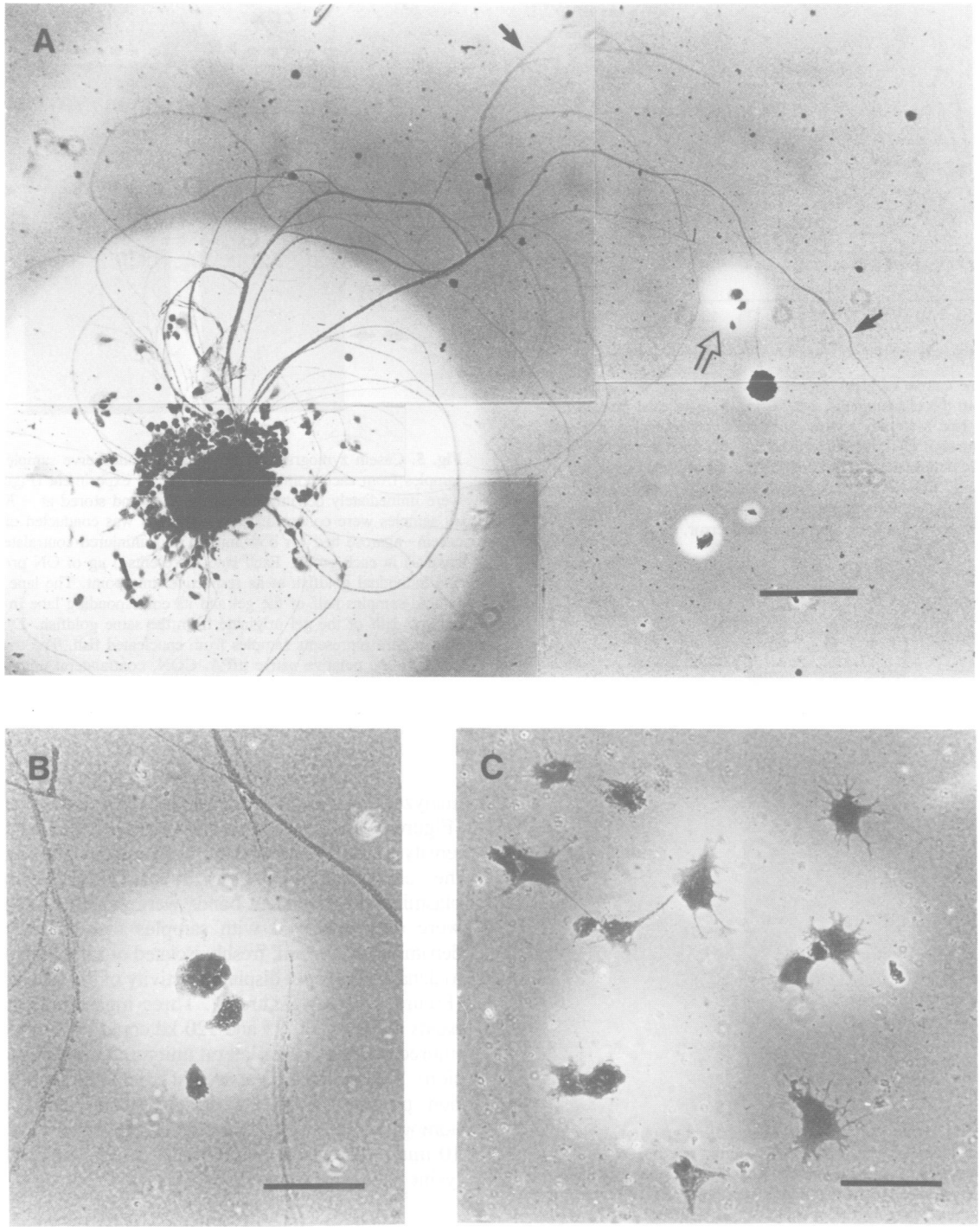

Fig. 6. Fibrin overlay of retinal explant and primary cell cultures. (**A**) Coomassie blue stained fibrin overlay zymograph of 7 day cultured retinal explant. Lytic zones can be visualized as clear light areas against the granular dark background. Plasminogen dependent lytic zones can be seen surrounding both the explant tissue block and presumed glial cells that have migrated from the explant (open arrow). No lytic zones were seen around the neuritic bundles (arrows) extending from the explant. (**B**) higher magnification of presumed glial cells seen in A (open arrow). (**C**) Cells expressing PA activity in 1 day old primary retinal cell culture. Note that not all cells of similar morphology produce lytic zones. Bar: A, 500 μm; B and C, 150 μm.

for goldfish retinal cell types (Jones and Schechter, 1987) an exact identification of cells with PA associated activity was not possible. The collective data suggest that at least the cells of glial origin express PA activity.

Discussion

We report here the induction in response to nerve damage of a plasminogen activator in the goldfish visual pathway.

Murray (1976) has shown that degradation of the axonal processes occurs concurrently with new axonal growth, and that new axonal fibers begin to reach the tectum ~7–10 days after crush. Therefore, maximum axonal outgrowth occurs during the peak of PA activity observed following optic nerve crush (Figure 3). The PA activity observed in the enucleations represents only the contribution of the degenerative process since in this case, there is no regeneration.

Casein analysis of the degenerative and regenerative process reveals a correlation between the intensity of the band at 60–65 kd and the amount of PA activity observed in the chromogenic assays. The lower molecular weight band (M_r = 36 kd) is an enzymatically active degradation fragment (see Results). The three higher molecular weight bands (M_r = 75, 95 and 120 kd) were also present in the contra-lateral uninjured samples and were plasminogen-dependent. This PA activity was not detected in the chromogenic assay. One likely explanation for these observations is the coexistence of a proteolytic inhibitor(s). This inhibitor might mask the PA activity present in the crude homogenates of control nerves by inhibiting the PA and possibly plasmin; PA–inhibitor complexes are often stable during SDS–PAGE and migrate more slowly, but regain activity in the casein underlay (Wohlwend *et al.*, 1987). Upon optic nerve damage, a large increase in gfPA (as visualized in Figure 5 by the increased intensity of the high molecular weight bands after injury) could overwhelm the amount of inhibitor present allowing manifestation of PA activity. In fact, the participation of proteolytic inhibitors in nerve growth is becoming apparent in light of their neurite promoting activity (Guenther *et al.*, 1985; Gloor *et al.*, 1986; Patterson, 1988; Zurn *et al.*, 1988; Hawkins and Seeds, 1989).

We have attempted to demonstrate directly the presence of PA inhibitors by incubation of optic nerve extracts with radiolabeled human uPA followed by SDS–PAGE to identify PA–inhibitor complexes (Wohlwend *et al.*, 1987). A convincing demonstration of an inhibitor could not be obtained. However, the piscine inhibitor may react poorly with the human enzyme, since for plasminogen activation the mammalian and piscine components show marked specificity (Figure 1b). Therefore, for these experiments to be successful, it may be necessary to use a purified piscine form of PA which is not yet available.

In fibrin overlay assays of retinal explants, no lytic zones were observed around the neuritic bundles or the tips of the neuritic growth cones. Retinal and optic nerve primary cell cultures showed lytic zones around certain cell types, but not all cells of similar morphology expressed PA activity. It has been observed tht less than 10% of Schwann cells in culture produce lytic zones (Alvarez-Buylla and Valinsky, 1985). The data presented here suggest that the PA produced is of glial origin. First, the presence and induction of PA in enucleations (Figures 2, 3 and 5) supports the claim that PA is, at least in part, produced by glial cells. Secondly, optic nerve primary cultures, containing no neuronally derived cells, produced lytic zones (unpublished data). Finally, in fibrin overlays of retinal explants, lytic zones were associated with the tissue block but not with the neuritic bundles. However, since a precise identification of the different cell types was not possible, we cannot exclude the possibility that some of the PA is neuronally derived. The PA observed is not likely to be solely derived from tissue macrophages invading the lesion, or fibroblasts, since in the mammalian CNS damage to the optic nerve does not result in increased expression of PA yet the above factors are present.

In contrast to our results, no increase in PA activity was observed in the rat optic nerve after injury (Bignami *et al.*, 1982); thus the induction of PA correlates with the goldfish visual pathway's capacity for nerve growth. The underlying mechanism behind this induction is not known but could be attributed to glial proliferation which is diminished in nonregenerating CNS nerves (Bignami *et al.*, 1982). Plasminogen activator produced by proliferating glia could be used for glial migration and myelination. Alternatively, PA produced by glial cells could be captured and used by neuronal cells. Mouse cerebellar granule neurons have recently been shown to bind tPA (Verrall and Seeds, 1989). The putative inhibitor could serve to modulate proteolysis while stimulating neuritic growth (see above). Further analysis of the system is necessary to establish conclusively the presence of an inhibitor and to determine if an alternate proteolytic mechanism is present as in cultures of rat sympathetic and sensory neurons (Pittman, 1985).

The demonstration of PA activity in the goldfish visual pathway extends our understanding of the similarities between higher and lower vertebrates in the mechanism for nerve growth and regeneration. It also reinforces the usefulness of this system as a biochemical model system for the study of proteases and possibly their inhibitors in nerve growth.

Materials and methods

Purification of piscine plasminogen

Whole blood was collected by heart puncture of ice anesthetized goldfish. The blood was allowed to clot on ice for one hour and then centrifuged. The serum (100 μl/fish) was decanted and stored at −70°C until purification. Frozen sera were pooled and applied to a lysine–sepharose column equilibated with PBS (Deutsch and Mertz, 1970). The lysine–sepharose was prepared according to the CNBr method of March *et al.* (1974) using Sepharose 4B (Pharmacia). After binding, the column was sequentially washed with 2 column volumes each of PBS, 0.3 M $KHPO_4$, pH 8.0, PBS and plasminogen was then eluted with PBS containing 0.2 M of the lysine analog ϵ-amino caproic acid (ϵACA) (Sigma). Protein fractions were pooled, dialyzed against PBS overnight at 4°C, and treated with diisopropyl fluorophosphate (DFP) (Sigma) to destroy any contaminating plasmin activity. The pooled sample was adjusted to 30 mM DFP by three additions of 5 M DFP with 30 min intervals at 37°C, and then dialyzed overnight against 0.1 M Tris–HCl pH 8.1 containing 0.01% Tween 80. The samples were analyzed by SDS–PAGE and in the chromogenic assay using goldfish aortal homogenates (see below) to test for purity and activity. The addition of 0.01% Tween 80 to all buffers improved the protein yield. Since the yield of sera from goldfish is low, trout serum was purchased from Cocalico Biologicals, Reamstown, PA, and the plasminogen purified as above. Protein determinations were performed with the Bradford Biorad method using BSA as a standard.

Surgical procedures

Common goldfish (*Carassius auratus*) were obtained commerically from Mt. Parnell Fisheries, Mercersburg, PA. All fish were 8–11 cm long and were kept in 40 gallon tanks with constant filtering and frequent water changes. All surgical procedures were conducted under a dissecting microscope. The fish were anesthetized in a 0.5 mg/ml solution of Tricane (3-amino-benzoic acid ethyl ester) (Sigma). The connective tissue surrounding the eye was disrupted. The eye was rolled forward and fatty tissue from

behind the eye was removed by aspiration to expose the optic nerve. For experiments requiring optic nerve crush (Jones *et al.*, 1989; Tesser *et al.*, 1986), the nerve was pinched immediately behind the eye with a curved pair of forceps for five seconds. A clear zone could be seen extending across the optic nerve confirming axotomy. Care was taken not to transect the nerve. For enucleation, the nerve was first severed immediately behind the eye followed by transection of all supporting musculature and the eye was removed leaving the optic nerve attached to the tectum. A small piece of gauze was placed in the orbit after enucleation to prevent excessive bleeding. Fish were allowed to recover in shallow aerated water before being returned to their tanks. Survival was typically 100%.

Sample preparations

Optic nerves were isolated from ice anesthetized fish, rinsed in ice cold PBS, and individually homogenized in 20 μl of 0.1 M Tris−HCl pH 8.1 containing 0.5% Triton X-100 in a micro tissue grinder homogenizer (Kontes). The homogenizer was then rinsed with 20 μl of the same buffer and the wash pooled with the homogenate. Samples were centrifuged in a microcentrifuge for 20 min at 4°C and the supernatants collected. In batch preparations of optic nerve, specimens were homogenized together in a larger volume of buffer. The left optic nerve (contra-lateral nerve) served as an internal control for all manipulations to the right optic nerve.

For the time course experiment, several precautions were taken to allow comparison between the time points. First, all surgical procedures were performed on the same day, with the exception of the zero day time point. For the zero day time point, the fish were either crushed or enucleated, allowed to recover to 0.5 hours and then processed. Second, all buffers were made in bulk in advance, sterilized, and aliquoted. Third, the chromogenic substrate solution, trout plasminogen, and a high specific activity aortal homogenate, were aliquoted and stored at −70°C. The aortal homogenate served as an internal control for the assays since the samples were assayed on different days. Four μg of aortal homogenate were assayed in triplicate in individual wells. The aortal activity measurements were averaged and used as a reference point to adjust all readings.

Retinas were isolated as described by Quitschke *et al.* (1985). For crude homogenates, pieces of retina were rinsed extensively with cold PBS to remove the majority of the vitreous. The pieces were then homogenized, quantitated for protein, and handled as described for the optic nerve. Fish aorta were isolated, rinsed extensively with cold PBS, homogenized and handled as above.

Plasminogen activator assays

The chromogenic assay used to detect activity in crude homogenates was conducted as described by Andrade-Gordon and Strickland (1986), with the following changes. Each reaction contained 150 μl 0.1 M Tris, pH 8.1, 0.01% Tween 80; 20 μl of 3 mM substrate H_D-Val-Leu-Lys-pNA (S-2251, Kabi, Sweden) in the Tris−Tween buffer; 10 μl of 0.25 μg/μl plasminogen; and 5 μg of each sample in 20 μl 0.1 M Tris−HCl, pH 8.1, 0.5% Triton X-100. The assays were conducted at room temperature in microtiter plates and the change of absorbance at 405 nm was monitored over the course of 3 h on a Bio-Tek microplate reader.

Visualization of PA was conducted using the casein underlay technique (Granelli-Piperno and Reich, 1978; Vassalli *et al.*, 1984). Homogenates were fractionated by SDS−PAGE, proteins were renatured by washing the gel in Triton X-100 followed by water. The gel was placed over a casein agarose film that contained 14 μg/ml of piscine plasminogen. Plasminogen activator was observed after incubation at room temperature as a dark lytic zone against a white background. Assays were conducted in the presence or absence of plasminogen to determine the amount of plasminogen dependent activity.

The fibrin overlay technique was used to visualize PA production by retinal explants and primary cell cultures using a slight modification of the procedure as described (Strickland *et al.*, 1976). Using fibrin rather than casein as the substrate gave slightly better resolution in the assay, but both substrates gave similar results. Low melting point agarose (1%) was used with 50 μg/ml piscine plasminogen and 3 mg/ml fibrinogen. The final concentration of media was 0.25×. Coverslips were rinsed in L-15 media minus serum, to remove exogenous plasminogen for the minus plasminogen controls. 100 μl of the fibrin−agarose mix at 40°C was poured over the coverslips containing either seven day old explants or primary cell cultures. The overlays were kept at room temperature in a humidified chamber. Lytic zones began to develop within 4 h. Coverslips were then fixed in 10% acetic acid, 50% methanol and stained with 2.5% Coomassie blue.

Tissue culture

Retinal explants were isolated from goldfish seven days post-crush as previously described (Jones *et al.*, 1989; Landreth and Agranoff, 1976, 1979). The 500 μm squares were plated on polylysine (Sigma) coated coverslips secured to 6-well Falcon tissue culture dishes by melting spots of the dish surrounding the coverslip with a flamed needle. Glass coverslips were autoclaved and incubated for 2−3 h in 5 mg/ml poly-L-lysine (M_r = 38.5 kd) in sterile filtered 0.1 M sodium borate buffer, pH 8.4. The coverslips were then washed with several changes of sterile water for 24 h and stored at room temperature in sterile water until use.

For primary cell culture the following procedure was used (Jochen Kleinschmidt, personal communication). Retinal pieces or optic nerve segments were treated with a 15 U/ml solution of Papain (Sigma) in sterile CA^{2+}, Mg^{2+} free Ringer's solution (140 nM NaCl, 3 mM KCl, 5 mM glucose, 10 mM HEPES, pH 7.4). The papain solution was activated by preincubation with 0.8 mg/ml cysteine for 20 min at 32°C. The samples were incubated with the papain for 30 min at 32°C gently agitating the tubes every few minutes. The papain was then activated by washing the samples in Ringer's solution containing 0.5 mg/ml BSA at room temperature for 15 min. The samples were placed in 0.5−1 ml of L-15 media and triturated gently using a 1 ml Pipetman. During the trituration, aliquots were examined under the microscope to optimize single cell yield and minimize excessive trituration. Cells were then plated as with the explants. Explants and cells were maintained in Gibco L-15 media supplemented with 20 mM HEPES pH 7.2, 0.1 mg/ml gentamicin sulfate (Sigma), and 5% fetal bovine serum in a humidified chamber at room temperature.

Acknowledgements

The authors are grateful to Drs Paul Jones, Anne Estreicher and Jochen Kleinschmidt for their generous assistance in this project. We thank Toni Daraio for her help in preparing the manuscript. This work was supported by grants from the National Institutes of Health (EY05212) to N.S. and (HD17875; HD25922) to S.S.

References

Alvarez-Buylla,A. and Valinsky,J.E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3519−3523.
Andrade-Gordon,P. and Strickland,S. (1986) *Biochemistry*, **25**, 4033−4040.
Attardi,D.G. and Sperry,R.W. (1963) *Exp. Neurol.*, **7**, 46.
Baron-Van Evercooren,A., Leprince,P., Rogister,B., Lefebvre,P.P., Delree,P., Selak,I. and Moonen,G. (1987) *Dev. Brain Res.*, **36**, 101−108.
Bignami,A., Cella,G. and Chi,N.H. (1982) *Acta Neuropathol.*, **58**, 224−228.
Danø,K., Andreasen,P.A., Grondahl-Hansen,J., Kristensen,P., Nielsen,L.S. and Skriver,L. (1985) *Adv. Cancer Res.*, **44**, 139−266.
Deutsch,D.G. and Mertz,E.T. (1970) *Science*, **170**, 1095−1096.
Gloor,S., Odink,K., Guenther,J., Nick,H. and Monard,D. (1986) *Cell*, **47**, 687−693.
Grafstein,B. (1986) in Adler,R. and Farber,D. (eds), *The Retina. A Model for Cell Biology Studies. Part II.* Academic Press, Inc., New York, pp. 275−335.
Granelli-Piperno,A. and Reich,E. (1978) *J. Exp. Med.*, **148**, 223−234.
Guenther,J., Nick,H. and Monard,D. (1985) *EMBO J.*, **4**, 1963−1966.
Hantaï,D., Rao,J.S. and Festoff,B.W. (1988) *Rev. Neurol. (Paris)*, **144**, 680−687.
Hawkins,R.L. and Seeds, N.W. (1989) *Dev. Brain Res.*, **45**, 203−209.
Heacock,A.M. and Agranoff,B.W. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 828−832.
Hoylaerts,M., Rijken,D.C., Lijnen,J.R. and Collen,D. (1982) *J. Biol. Chem.*, **257**, 2912−2919.
Johns,P.R. and Easter,S.S. (1977) *J. Comp. Neurol.*, **176**, 331−341.
Jones,P.S. and Schechter,N. (1987) *J. Comp. Neurol.*, **266**, 112−121.
Jones,P.S., Tesser,P., Borchert,J. and Schechter,N. (1989) *J. Neurosci.*, **9**, 454−465.
Kalderon,N. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7216−7220.
Krystosek,A. and Seeds,N.W. (1981a) *Science*, **213**, 1532−1534.
Krystosek,A. and Seeds,N.W. (1981b) *Proc. Natl. Acad. Sci. USA*, **78**, 7810−7814.
Krystosek,A. and Seeds,N.W. (1984) *J. Cell Biol.*, **98**, 773−776.
Landreth,G.E. and Agranoff,B.W. (1976) *Brain Res.*, **118**, 299−303.
Landreth,G.E. and Agranoff,B.W. (1979) *Brain Res.*, **161**, 39−53.
March,S.C., Parikh,I. and Cuatrecasas,P. (1974) *Anal. Biochem.*, **60**, 149−152.
McQuarrie,I.G. and Grafstein,B. (1982) *Brain Res.*, **235**, 213−223.
Meyer,R.L. (1978) *Exp. Neurol.*, **59**, 99−111.
Murray,M. (1976) *J. Comp. Neur.*, **168**, 175−196.
Ossowski,L., Quigley,J.P., Kellerman,G.M. and Reich,E. (1973) *J. Exp. Med.*, **138**, 1056−1064.
Patterson,P.H. (1988) *Neuron*, **1**, 263−267.

Pittman,R.N. (1985) *Dev. Biol.*, **110**, 91–101.
Quitschke,W., Jones,P.S. and Schechter,N. (1985) *J. Neurochem.*, **44**, 1465–1476.
Reich,E. (1978) In Ruddon,R.W. (ed.), *Biological Markers of Neoplasia. Basic and Applied Aspects.* Elsevier-North Holland, Inc. NY, pp. 491–498.
Sappino,A.-P., Huarte,J., Belin,D. and Vassalli,J.-D. (1989) *J. Cell Biol.*, **109**, 2471–2479.
Schechter,N., Jones,P.S., Quitschke,W. and Tesser,P. (1989) In Woodhead,A.D. (ed.), *Nonmammalian Animal Models for Biomedical Research.* CRC Press, Inc., Boca Raton, FL pp. 225–244.
Soreq,H. and Miskin,R. (1983) *Dev. Brain Res.*, **11**, 149–158.
Soreq,H., Miskin,R., Zutra,A. and Littauer,U.Z. (1983) *Dev. Brain Res.*, **7**, 257–269.
Sperry,R. (1983) *Proc. Natl. Acad. Sci. USA*, **50**, 703.
Strickland,S., Reich,E. and Sherman,M.I. (1976) *Cell*, **9**, 231–240.
Takahashi,K., Wakamatsu,Y., Ozato,K. and Wakayama,Y. (1987) *Cell Struct. Funct.*, **12**, 11–22.
Tesser,P., Jones,P.S. and Schechter,N. (1986) *J. Neurochem.*, **47**, 1235–1243.
Toshniwal,P.K., Tiku,M.L., Tiku,K. and Skosey,J.L. (1987) *J. Neurol. Sci.*, **80**, 307–321.
Unkeless,J.C., Gordon,S. and Reich,E. (1974) *J. Exp. Med.*, **139**, 834–850.
Valinsky,J.E. and Le Douarin,N.M. (1985) *EMBO J.*, **4**, 1403–1406.
Vassalli,J.-D., Dager,J.-M., Wohlwend,A. and Belin,D. (1984) *J. Exp. Med.*, **159**, 1653–1668.
Verrall,S. and Seeds,N.W. (1989) *J. Cell Biol.*, **109**, 265–271.
Wallen,P. (1980) in Kline,D.L. and Reddy,K.N.N. (eds), *Fibrinolysis.* CRC Press, Inc., Boca Raton, FL, pp. 1–24.
Wohlwend,A., Belin,D. and Vassalli,J.-D. (1987) *J. Immunol.*, **139**, 1278–1284.
Zurn,A.D., Nick,H. and Monard,D. (1988) *Dev. Neurosci.*, **10**, 17–24.

Received on February 22, 1990; revised on April 30, 1990