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Proteolytic Regulation of Neurite Outgrowth From Neuroblastoma Cells by Thrombin and Protease Nexin-1

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This review summarizes studies on the reciprocal regulation of neuroblastoma neurite outgrowth by thrombin and protease nexin-1 (PN-1). PN-1 recently was shown to possess the same deduced amino acid sequence as the glial-derived neurite-promoting factor. The neurite outgrowth activity of PN-1 depends on its ability to inhibit thrombin. Thrombin not only blocks the neurite outgrowth activity of PN-1, but it also brings about neurite retraction in the presence of PN-1. Thrombin also produces neurite retraction in the absence of PN-1 and other regulatory factors. This suggests that its activity is due to a direct action on cells. The neurite retraction by thrombin depends on its proteolytic activity. It does not occur with the other serine proteases that have been tested, indicating that it is a specific effect and is not due to a general proteolytic effect that could detach neurites from the culture dish. Serum brings about neurite retraction in certain neuroblastoma cells and primary neuronal cultures; most of this activity is due to residual thrombin in the serum. Together, these results suggest that PN-1 and thrombin (or a thrombin-like protease) play a role in regulation of neurite outgrowth.

Key words: hirudin, cAMP, prostaglandin E_1, heparin, neurite retraction

The regulation of neurite outgrowth from neuronal cells has been studied extensively as a fundamental problem of cell differentiation. Many of these studies have focused on the identification and characterization of molecules that can stimulate neurite outgrowth with the realization that this is a necessary step in understanding this complex process. The availability of purified neurotrophic factors and antibodies against these factors can provide the tools to explore whether alterations in these factors occur in certain neurological diseases. Efforts to characterize factors that stimulate neurite outgrowth have also been prompted by the realization that they might have therapeutic applications.

There has been less effort directed to the identification of molecules that inhibit the action of neurite-promoting factors or that directly bring about neurite retraction.

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These factors could be equally important in the overall regulation of neuronal differentiation, especially during early postnatal organization of the central nervous system when axonal elimination is known to occur [1]. Indeed, the complex process of neuronal differentiation is undoubtedly regulated by a combination of positive and negative signals. Factors that bring about neurite retraction also could be important in the pathogenesis of certain neurological diseases.

This review summarizes our studies, which show that thrombin can modulate and reverse neuroblastoma neurite outgrowth and that it can bring about neurite retraction in serum-free medium in the absence of other regulatory factors [2]. These studies were based on findings by Monard and colleagues of a glial-derived neurite-promoting factor whose action is blocked by thrombin and urokinase [3,4]. It was recently shown that this factor is identical with protease nexin-1 (PN-1) [5,6], a 45-kDa protease inhibitor that is synthesized and secreted by a variety of cultured cell types [7,8]. PN-1 rapidly inhibits thrombin, urokinase, and plasmin by forming covalent complexes with the protease catalytic site serine [7,9]. The PN-1 protease complexes bind back to the cells and are rapidly internalized and degraded [10]. This provides a mechanism to regulate and clear regulatory proteases in the extracellular environment [11].

RESULTS

The stimulation of neurite outgrowth by PN-1 and the ability of thrombin to block and reverse this is shown in Figures 1 and 2. Cloned mouse neuroblastoma cells (clone Nb2a) were employed in these studies to ensure that the effects of the purified proteins were directly on these neuron-like cells rather than on contaminating glial cells found in primary neuronal cultures, which could release neurotrophic factors. As in the studies of Monard and colleagues [12], the neuroblastoma cells were cultured in medium containing 10% fetal calf serum, which was changed to medium containing 0.8% fetal calf serum at the time of addition of PN-1. Figure 2A shows that added PN-1 stimulated neurite outgrowth in over 50% of the cells, as judged by the number of cells that possessed neurites equal to or longer than one cell diameter. As shown, this was blocked by thrombin at approximately a stoichiometric concentration. Figure 2B shows the time course of neurite induction of PN-1 and the effect of adding thrombin at the time indicated by the arrow. These results showed that thrombin could not only block neurite outgrowth by PN-1, but it could also bring about retraction of extended neurites [2].

Similar experiments were conducted with hirudin, another inhibitor of thrombin. Monard and colleagues earlier showed that hirudin stimulated neuroblastoma neurite outgrowth [3]. Starting with this observation, we showed that added thrombin blocked the neurite outgrowth activity of hirudin (Fig. 3a). More importantly, thrombin reversed neurite outgrowth and brought about neurite retraction just as it did with PN-1 (Fig. 3B) [2].

The ability of thrombin to reverse neurite retraction with the two quite different thrombin inhibitors, PN-1 and hirudin, indicated that it might play some fundamental role in the regulation of neurite outgrowth. This was evaluated by conducting experiments that did not involve added protease inhibitors (Figs. 1, 4). The approach used took advantage of previous findings that neuroblastoma cells and certain primary neuronal cultures extend neurites when placed in serum-free medium [13–17]. Figures 1c and 4 show the induction of neurite outgrowth in mouse neuroblastoma cells after

switching them to serum-free medium. As can be seen, the time course and extent of neurite outgrowth were very similar to that produced by addition of PN-1 or hirudin. Moreover, addition of thrombin brought about retraction of the extended neurites. When thrombin was added at the time of switching the cells to serum-free medium, neurite outgrowth was blocked; however, neurite outgrowth was induced when this medium was changed to serum-free medium not containing thrombin (Figs. 1f, 4). These results showed that thrombin could block and reverse neurite outgrowth in the absence of added protease inhibitors and suggested a direct regulation by thrombin [2].

The concentrations of thrombin required to block neurite outgrowth and to produce neurite retraction after switching neuroblastoma cells to serum-free medium are shown in Figure 5. As shown, similar concentrations of thrombin were required for both blockage and reversal of neurite outgrowth induced by serum-free medium. A half-maximal effect was observed at 2 ng/ml or 50 pM thrombin; a maximal effect required about 100 ng/ml of thrombin. Similar studies conducted with thrombin inactivated by diisopropylfluorophosphate (DIP-thrombin) revealed that it was 500 to 1,000 times less potent in preventing neurite outgrowth than active thrombin (Fig. 5). This preparation contained about 0.1% residual thrombin activity, indicating that its much reduced ability to block neurite outgrowth was due to remaining active thrombin. Thus, the proteolytic activity of thrombin was necessary for its effects on neurite outgrowth [2].

To test the specificity of these effects of thrombin, we evaluated the ability of other serine proteases to block neurite outgrowth produced by switching the neuroblastoma cells to serum-free medium. Table 1 shows that three other serine proteases were ineffective in blocking neurite outgrowth even at 10 μg/ml. Trypsin could not be used at high concentrations since it caused cell detachment at concentrations greater than 0.3 μg/ml; however, at 0.1 μg/ml it did not block neurite extension (Table I). Thus, the ability of thrombin to block differentiation appeared to result from a specific effect rather than general proteolysis, which might simply detach neurites from the culture dish [2].

The specificity of neurite retraction by thrombin was further evaluated by testing its ability to retract neurites that had been extended by agents that effectively raise intracellular cyclic AMP concentrations [18,19]. In contrast to neurites extended by PN-1, hirudin, or serum-free medium, thrombin did not significantly retract neuroblastoma neurites that had been extended by prostaglandin E_1 or dibutyrl cyclic AMP (Fig. 6). In addition, thrombin did not block or reverse neurite outgrowth induced by nerve growth factor in the PC12 rat pheochromocytoma cell line (D.G. and D.D.C., unpublished data). These results further support the specificity of neurite retraction by thrombin.

DISCUSSION

The studies summarized here show that thrombin can bring about retraction of neuroblastoma neurites after they have been extended by treatment with PN-1 or hirudin or with serum-free medium [2]. This requires the proteolytic activity of thrombin, although it is not a general proteolytic effect, since plasmin, urokinase, or trypsin do not bring about neurite retraction under similar conditions. These results, along with the finding that thrombin does not retract neurites extended by treatment

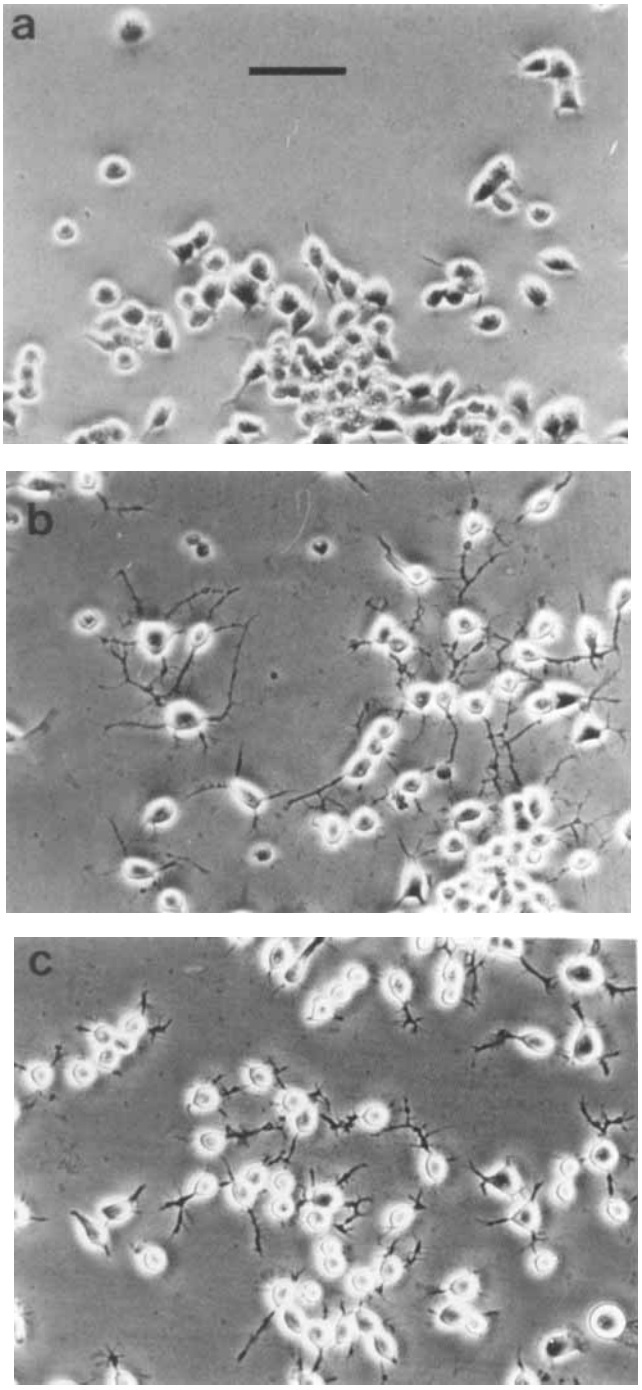

Fig. 1. Photomicrographs of unfixed neuroblastoma cells. Mouse neuroblastoma cells (clone Nb2a) were grown for 2 days in Dulbecco's Modified Eagle's (DME) medium containing 10% Fetal Calf Serum (FCS) and then exposed to the following conditions: **a:** 4 h in DME medium containing 0.8% FCS; **b:** 4 h in DME medium containing 0.8% FCS plus 2 μg/ml PN-1; **c:** 4 h in serum-free DME medium; **d:** 4 h in serum-free DME medium followed by 4 h in the same medium containing 0.1 μg/ml thrombin; **e:** 4 h in serum-free DME medium containing 0.1 μg/ml thrombin; **f:** 4 h in serum-free DME medium with 0.1 μg/ml thrombin followed by 4 h in the same medium without thrombin. Bar = 20 μm.

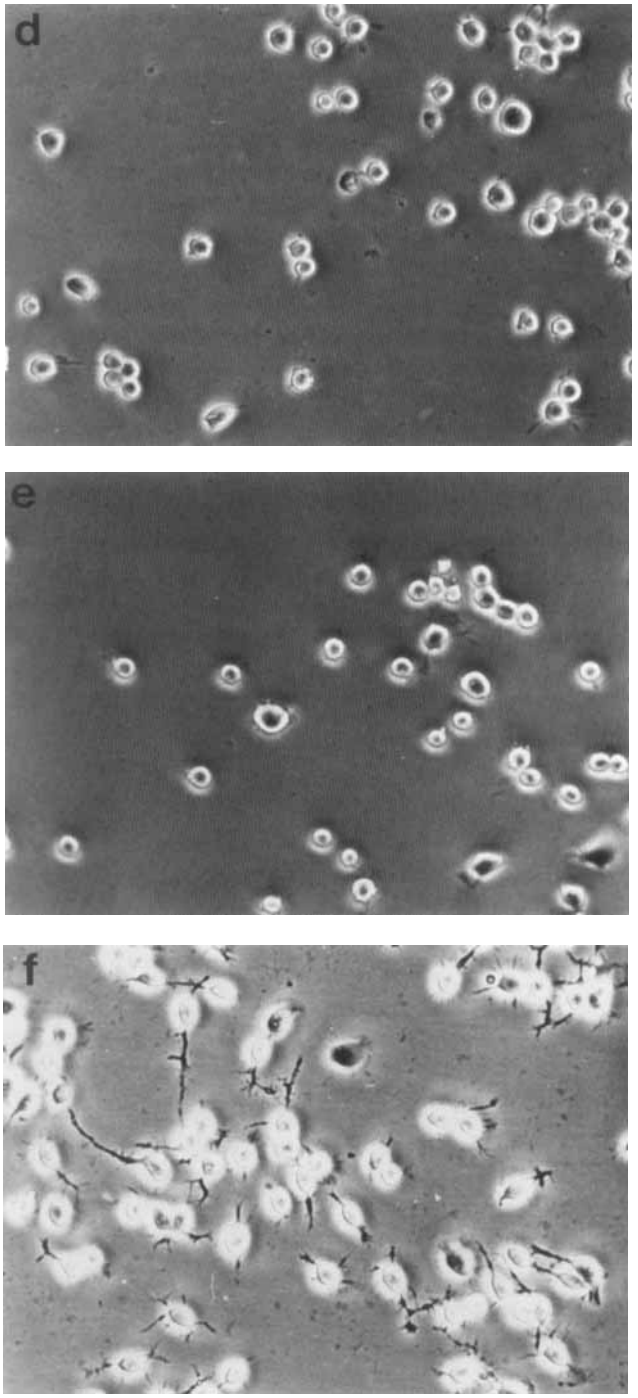

Figure 1d–f.

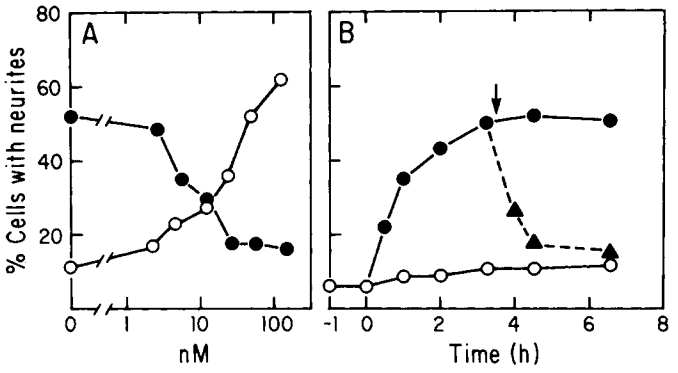

Fig. 2. Induction of neurite outgrowth by PN-1 and its inhibition and reversal by thrombin. **A:** The medium on neuroblastoma cells was changed from DME medium containing 10% FCS to DME medium containing 0.8% FCS. Then, the indicated concentrations of PN-1 (○), or 46 nM PN-1 (2 μg/ml) along with the indicated concentrations of thrombin (●) were added. After 4 h the cells were fixed and scored for neurite outgrowth. **B:** The medium on neuroblastoma cells was changed to DME medium containing 0.8% FCS at time zero, and the percentage of cells with neurites was scored in cultures without additions (○). In parallel cultures, 2 μg/ml PN-1 was added at time zero (●); at 3.5 h (arrow) thrombin (2 μg/ml) was added to some of these cultures (▲). (Reprinted with permission from [2].)

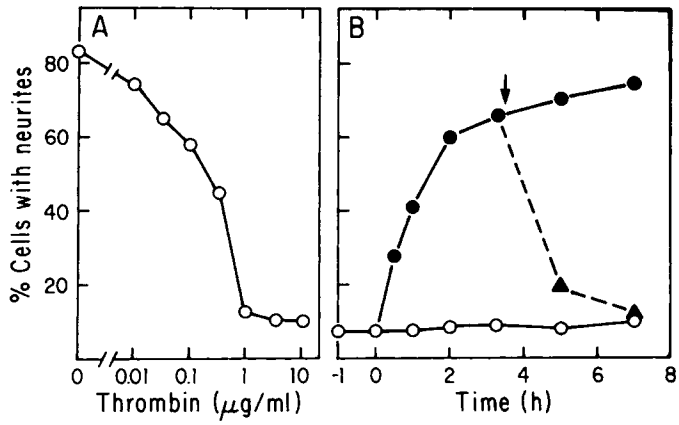

Fig. 3. Induction of neurite outgrowth by hirudin and its inhibition and reversal by thrombin. **A:** The medium on neuroblastoma cells was changed from DME medium containing 10% FCS to DME medium containing 0.8% FCS. Then, hirudin (1 unit/ml) and the indicated concentrations of thrombin were added. After 4 h the cells were fixed and scored for neurite outgrowth. **B:** The medium on neuroblastoma cells was changed to DME medium containing 0.8% FCS at time zero, and the percentage of cells with neurites was scored (○). In parallel cultures, 1 unit/ml hirudin was added at time zero (●); at 3.5 h (arrow) thrombin (1 μg/ml) was added to some of these cultures (▲). (Reprinted with permission from [2].)

with dibutyryl cyclic AMP or prostaglandin E_1, indicate that the thrombin effect is specific and may represent an important regulatory signal in the control of neurite differentiation. The fact that thrombin brings about neurite retraction in the absence of *added* neurotrophic or growth factors indicates a direct action on the neuroblastoma cells, although recent studies show that thrombin inactivates acidic fibroblast growth factor [20], a known neurotrophic agent [21,22]. In future studies, it will be important to determine if the neuroblastoma cells secrete acidic fibroblast growth factor in

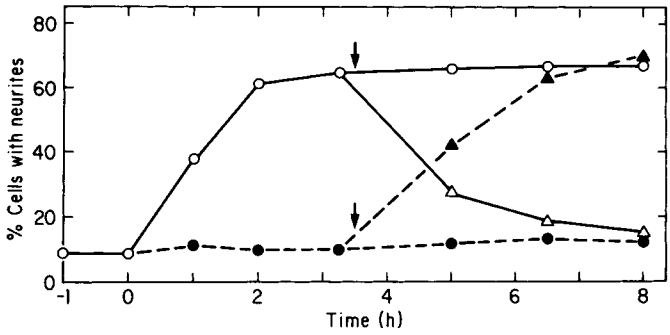

Fig. 4. Time course for induction of neurite outgrowth by serum-free medium and retraction of neurites by thrombin. At time zero neuroblastoma cultures were switched from DME medium containing 10% FCS to serum-free DME medium (○); at 3.5 h (arrow) thrombin (0.1 μg/ml) was added to some of these cultures (△). In a parallel set of cultures, thrombin (0.1 μg/ml) was added when the cells were switched to serum-free medium at time zero (●); at 3.5 h (arrow) some of these cultures were switched to serum-free medium not containing thrombin (▲). (Reprinted with permission from [2].)

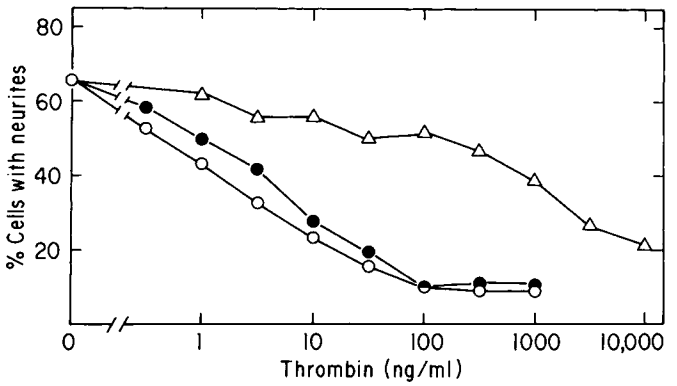

Fig. 5. Ability of thrombin to block neurite outgrowth and produce neurite retraction in serum-free cultures of neuroblastoma cells. Cells were changed from DME medium containing 10% FCS to serum-free DME medium containing the indicated concentrations of thrombin (○) or DIP-thrombin (△) for 5 h prior to fixing cells and scoring for neurite outgrowth. In parallel cultures, cells were first incubated in serum-free DME medium for 2 h to induce neurite outgrowth; the indicated concentrations of thrombin were then added for an additional 3 h prior to fixing the cells and scoring for neurite outgrowth (●). (Reprinted with permission from [2].)

serum-free medium and to determine if this could bring about neurite outgrowth in these cells. It is noteworthy that mouse neuroblastoma cells (clone N1E-115) possess specific binding sites for thrombin [23]. Although the role of these sites has not been established, their existence suggests that neuronal cells might interact directly with thrombin.

It has been shown in many studies that serum markedly inhibits neurite outgrowth and that serum-free culture conditions induce neurite outgrowth in certain neuroblastoma cells and primary neuronal cultures [13–17]. Our recent studies showed that most of this inhibition of neuroblastoma neurite outgrowth by serum was due to residual thrombin that presumably was not inactivated by antithrombin III [2]. First, neurite outgrowth that was induced by removal of serum was prevented by thrombin

TABLE I. Modulation by Proteases of Neurite Outgrowth in Serum-Free Medium*

Addition (per ml)	Percent differentiated cells
Control	57 ± 4
Thrombin (0.1 μg)	12 ± 2
Thrombin (1 μg)	12 ± 3
DIP-thrombin (1 μg)	41 ± 2
Urokinase (10 μg)	49 ± 5
Plasmin (10 μg)	58 ± 4
Trypsin (0.1 μg)	60 ± 3

*The medium on neuroblastoma cultures were changed to serum-free DME medium together with the tested proteins. After 16 h, the cells were fixed and scored for neurite outgrowth as described. The values shown are means of duplicate determinations ± 1 SEM. (Reprinted with permission from [2].)

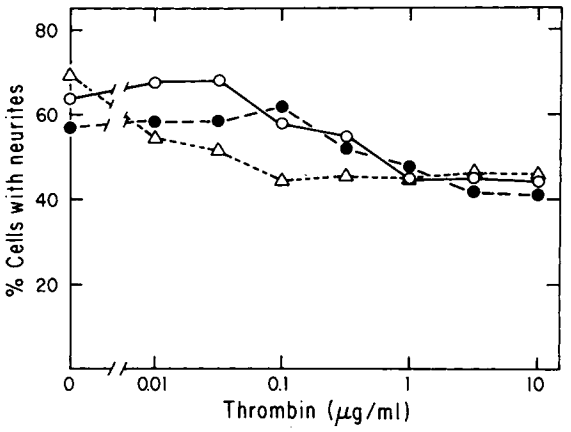

Fig. 6. Inability of thrombin to block neurite outgrowth induced by dibutyryl cAMP or prostaglandin E_1. Neuroblastoma cells were changed from DME medium containing 10% FCS to DME medium containing 0.8% FCS and the indicated concentrations of thrombin together with either 1.0 mM dibutyryl cAMP (○) or 50 μM prostaglandin E_1 (●). In parallel cultures, the medium was changed from DME medium containing 10% FCS to serum-free medium containing the indicated concentrations of thrombin and 1.0 mM dibutyrl cAMP (△). After 5 h, the cells were fixed and scored for neurite outgrowth.

addition. Second, added thrombin produced neurite retraction in neuroblastoma cells whose neurites had been extended by incubation in serum-free medium. Third, assays for thrombin in serum-containing medium that inhibited neurite outgrowth indicated that most of the inhibition could be accounted for by thrombin that was produced during the incubation of this medium at 37°C. Finally, heparin addition to this medium not only reduced thrombin levels in serum-containing medium but also stimulated neurite outgrowth [2]. This effect was presumably due to the well-known ability of heparin to accelerate the inactivation of thrombin by plasma antithrombin III.

Studies with PN-1 (and the identical glial-derived neurite-promoting factor) strongly indicate that their neurotrophic activity is dependent on their ability to inhibit thrombin. First, the two quite different thrombin inhibitors, PN-1 and hirudin,

indistinguishably stimulate neurite outgrowth from mouse neuroblastoma cells [2,3]. Moreover, thrombin similarly blocked the neurotrophic activity of both PN-1 and hirudin and reversed their effects by bringing about neurite retraction in their continued presence [2]. It is noteworthy that urokinase also blocks the neurite outgrowth activity of PN-1 [2,3]; this is undoubtedly a result of the ability of this protease to form complexes with PN-1 and prevent it from inhibiting thrombin [2]. Consistent with this conclusion is the finding that after switching neuroblastoma cells to serum-free medium, urokinase, unlike thrombin, did not block neurite outgrowth or produce neurite retraction [2]. Finally, it is noteworthy that the extracellular matrix accelerates the inactivation of thrombin by PN-1 [24] and blocks its ability to inactivate urokinase or plasmin [25]. The ability of the extracellular matrix to accelerate the inactivation of thrombin by PN-1 is mostly due to heparan sulfate [26].

Evidence is accumulating that suggests that PN-1 and thrombin might also regulate neurite outgrowth in the brain. The first evidence came from experiments that showed that rat brain contains mRNA for the glial-derived growth factor/PN-1 [5]. Recently, it has been shown that human brain contains PN-1, as judged by measurements of its activity and by Western blot analysis (S.L. Wagner and D.D. Cunningham, unpublished data). Also, preliminary studies indicate that human brain contains mRNA for prothrombin (S.L. Wagner, A.L. Lau, P.J. Isackson, and D.D. Cunningham, unpublished data). Finally, binding of thrombin has been detected in primary rat brain cultures [27] and in homogenes of human brain and spinal cord [28]. In future studies it will be important to conduct detailed studies on the presence of PN-1 and thrombin (or a thrombin-like protease) in various parts of the brain during development and in certain neurological diseases to evaluate further the role of these molecules in neural development, function, and regeneration.

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