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Protease Nexin-1 and Thrombin Modulate Neuronal Ca^{2+} Homeostasis and Sensitivity to Glucose Deprivation-Induced Injury

Virginia L. Smith-Swintosky,[1] Stephen Zimmer,[2] John W. Fenton II,[3] and Mark P. Mattson[1]

[1]Sanders-Brown Research Center on Aging and Department of Anatomy and Neurobiology, University of Kentucky, Lexington, Kentucky 40536-0230, [2]Lucille Parker Markey Cancer Research Center and Department of Microbiology and Immunology, University of Kentucky, Lexington, Kentucky 40536, and [3]Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201-0509

Protease nexin-I (PN-1) is a 44 kDa serine proteinase inhibitor that rapidly inhibits thrombin by forming SDS stable complexes with serine at the catalytic site of the protease. Levels of both PN-1 and thrombin are increased in the brain in response to insults such as ischemia, suggesting roles in neural injury and repair processes. We now report that PN-1–protected cultured rat hippocampal neurons against glucose deprivation- induced damage (GDID), and the protection was abolished by equimolar thrombin. PN-1 reduced resting intracellular free calcium levels ($[Ca^{2+}]_i$) and attenuated the elevation of $[Ca^{2+}]_i$ normally associated with GDID. Thrombin reduced neuronal survival and caused a significant increase in $[Ca^{2+}]_i$. Submaximally toxic levels of thrombin exacerbated GDID. Calcium responses to thrombin were attenuated in neurons contacting PN-1 immunoreactive astrocytes. These findings suggest that PN-1 and thrombin play important roles in modulating neuronal calcium responses, and vulnerability, to metabolic/excitotoxic insults.

[Key words: astrocytes, calcium, excitotoxicity, Fura-2, glutamate, hippocampus, immunocytochemistry, neuronal death, protease nexin-1, thrombin]

Protease nexin-1 (PN-1) is a 44 kDa serine proteinase inhibitor belonging to the nexin family and the serpin superfamily of protease inhibitors. It rapidly inhibits certain proteinases, particularly thrombin, by forming SDS stable complexes with the catalytic site serine of the proteinase (Baker et al., 1980; Low et al., 1981). This complex formation mediates cellular binding, and internalization and degradation of the protease (Low et al., 1981). A PN-1 receptor has yet to be isolated and characterized, and all known biological actions of PN-1 appear to be mediated via its binding to thrombin.

PN-1 is synthesized and secreted by a variety of cultured cells including glial cells and neurons (Rosenblatt et al., 1987; Reinhard et al., 1988, 1994; Milligan et al., 1991). PN-1 is identical to glia-derived nexin (Guenther et al., 1985; Gloor et al., 1986; McGrogan et al., 1988), which has been shown to stimulate neurite outgrowth in neuroblastoma cells (Monard et al., 1983; Diaz-Nido et al., 1991), superior cervical ganglion neurons (Zurn et al., 1988), and rat hippocampal neurons (Farmer et al., 1990). PN-1 inhibits granule cell migration (Lindner et al., 1986), prevents degradation of the extracellular matrix (ECM) in smooth muscle (Rao et al., 1989), and promotes cell survival in superior cervical ganglion neurons (Zurn et al., 1988). In addition, PN-1 modulates thrombin-stimulated cell division (Low et al., 1982). High levels of PN-1 and PN-1 mRNA are present in brain (Reinhard et al., 1988; Wagner et al., 1989a; Simpson et al., 1994).

PN-1 has two distinct functional sites—a reactive center that interacts with a proteinase and a heparin binding site. The reactive center has been shown to be necessary for promotion of outgrowth, while bound heparin increases the rate of association of the proteinase–proteinase inhibitor complex by 40-fold (Nick et al., 1990). It is well established that PN-1 exerts its various actions through the inhibition of thrombin. When PN-1 is bound to the cell surface or ECM it acts as a specific thrombin inhibitor; however, in interstitial fluid it also effectively blocks urokinase and plasmin (Wagner et al., 1989b; Choi et al., 1990).

Thrombin, in addition to playing a central role in the formation of blood clots, is a serine proteinase with diverse bioregulatory activity. It has been shown to cause neurite retraction (Gurwitz and Cunningham, 1988, 1990; Zurn et al., 1988; Grabham et al., 1991; Suidan et al., 1992), stimulate the cleavage and secretion of β-amyloid precursor protein *in vitro* (Igarashi et al., 1992; Davis-Salinas et al., 1994), induce the release of arachidonic acid from spinal cord cultures (Means and Anderson, 1986), cause a significant rise in levels of $[Ca^{2+}]_i$ in endothelial cells (Goligorsky et al., 1989; Tiruppathi et al., 1992) and T-lymphoblastoma cells (Tordai et al., 1993), rapidly stimulate Ca^{2+}-dependent cGMP formation in neuroblastoma cells (Snider and Richelson, 1983), induce secretion of NGF from cultured astrocytes (Neveu et al., 1993), stimulate proliferation and morphological changes in glial cells (Perraud et al., 1987; Loret et al., 1989; Cavanaugh et al., 1990), participate in wound repair (Carney et al., 1992; Cromack et al., 1992; Stiernberg et al., 1993), and increase the metastatic potential of tumor cells (Wojtukiewicz et al., 1993). In addition, there is evidence for thrombin receptors in the brain and spinal cord (McKinney et al., 1983; Means and Anderson, 1986; Rasmussen et al., 1991; Vu et al., 1991; Suidan et al., 1992).

The presence of thrombin receptors in the brain is intriguing since thrombin is not normally found in brain parenchyma. Nev-

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Correspondence should be addressed to Dr. V. L. Smith-Swintosky, 211 Sanders-Brown Building, University of Kentucky, Lexington, KY 40536-0230.

ertheless, prothrombin RNA is found in the CNS (Dihanich et al., 1991). Thrombin is known to alter neuronal and glial processes and disrupt their function (Gurwitz and Cunningham, 1988; Zurn et al., 1988; Cavanaugh et al., 1990; Grabham et al., 1991; Suidan et al., 1992; Beecher et al., 1994). Thus, the presence of PN-1 and its mRNA in the brain, especially that localized in and around blood vessels, suggests that PN-1 may play a protective role against extravasated prothrombin that would be converted to active thrombin, as might occur when the blood–brain barrier is perturbed (e.g., stroke or severe head trauma; Choi et al., 1990). In fact, both PN-1 and thrombin have been implicated in several injury/disease states (Meier et al., 1989; Rao et al., 1990, 1993; Vaughan and Cunningham, 1993; Festoff et al., 1994; Scotti et al., 1994), including Alzheimer's disease (Wagner et al., 1989a; Akiyama et al., 1992; Davies et al., 1993), severe head trauma (Suzuki et al., 1994), and cerebral ischemia (Hoffman et al., 1992; Nitsch et al., 1993). Recent evidence indicated that PN-1 is reexpressed in the adult rat brain after transient forebrain ischemia and persists for at least 1 year after the ischemic event (Hoffman et al., 1992; Nitsch et al., 1993). However, the role of PN-1 in the brain's response to ischemic injury is unknown.

Mammalian central neurons depend on a constant supply of glucose in order to function normally and survive. A reduction in brain glucose transport and utilization is known to occur with aging and, to a greater extent, in ischemia and Alzheimer's disease (Hoyer, 1988; Hoyer et al., 1988; Kalaria and Harik, 1989). Reduced glucose availability results in ATP depletion, failure of Ca^{2+} extrusion/buffering systems, membrane depolarization, excessive glutamate release, and NMDA glutamate receptor activation (Siesjo et al., 1989; Cheng and Mattson, 1992; Martin et al., 1994). Excitotoxicity is believed to contribute to neuronal injury and death in a variety of acute and chronic neurodegenerative disorders (Coyle, 1979; Choi, 1988; Mattson et al., 1993a,b). All of these alterations would contribute to neurotoxic increases in intracellular calcium ($[Ca^{2+}]_i$). Excessive and sustained elevations in $[Ca^{2+}]_i$ can lead to the activation of calcium-dependent proteases, lipases, kinases, and free radicals, which destabilize the cytoskeleton and membranes leading to cell damage and eventual death (Choi, 1988; Mattson and Kater, 1988; Siesjo et al., 1989; Siman et al., 1989; Yanagihara et al., 1990; Johnson et al., 1991; Mattson et al., 1992). Relatively little is known about the role of thrombin and PN-1 in neuronal responses to injury, and their possible effects on neuronal Ca^{2+} homeostasis. In the present study, we tested the hypothesis that PN-1 and thrombin can influence neuronal Ca^{2+} homeostasis and the outcome of metabolic/excitotoxic insults.

Materials and Methods

Hippocampal cultures. Dissociated cell cultures of fetal rat hippocampus (embryonic day 18) were established and maintained as described previously (Mattson and Kater, 1988b; Mattson et al., 1994). Cells were seeded into 35 mm polyethyleneimine-coated plastic culture dishes (Costar) containing 2 ml of Eagle's Minimum Essential Medium supplemented with 26 mM $NaHCO_3$, 40 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum (Sigma), and 0.001% gentamicin sulfate. After a 3 to 5 hr incubation period to allow for cell attachment, cultures were rinsed once with fresh medium and then a final volume of 0.8 ml medium was added. Cultures were maintained in a humidified atmosphere (6% CO_2, 94% room air) at 37°C. All experiments were performed in 7–10-d-old cultures.

Glucose deprivation and experimental treatments. The culture medium was removed and dishes were washed three times with glucose-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, and 5 mM HEPES). Glucose-containing (10 mM) Locke's solution was used in parallel control cultures. Human recombinant PN-1 (a generous gift from Dr. Randy Scott, Incyte Pharmaceuticals) was prepared as 100- to 1000-fold concentrated stocks in water . Human α-thrombin (Sigma and purified by JWF) was prepared as 100- to 1000-fold stocks in saline, and aliquots were stored at −80°C. Prior to experimental treatment the culture medium was removed and replaced with serum-free defined medium (Eagle's MEM supplemented with 5 μg/ml bovine insulin, 100 μg/ml human transferrin, 100 μg/ml BSA (fraction V), 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 42 ng/ml thyroxine, 33 ng/ml tri-iodo-L-thyronine, 1 mM pyruvate, and 20 mM KCl; Sigma) in order to remove any thrombin and PN-1 present in serum.

Assessment of neuronal survival. Methods for assessing neuronal viability have been detailed in previous studies (Mattson et al., 1989, 1994). Briefly, cultures were visualized and photographed with a phase-contrast Nikon Diaphot inverted microscope. Neurons were considered viable if they had neurites that were uniform in diameter and smooth in appearance, and somata that were smooth and round to oval in shape. Degenerating nonviable neurons possessed fragmented and beaded neurites and a rough, swollen, vacuolated soma with an irregular shape. Subsequent to these morphological changes, the degenerated neurons detached from the culture substrate. Viable neurons in premarked microscope fields (each field was approximately 1 mm^2) were counted prior to and up to 24 hr following glucose deprivation. Counts were made without knowledge of the treatment history of the cultures. Statistical comparisons were done using paired and unpaired Student's *t* tests (two-tailed), one-way analysis of variance (ANOVA), and Bonferroni *t* test for multiple pair-wise comparisons.

Immunocytochemistry. Cell cultures were fixed for 30 min in cold 4% paraformaldehyde in 10 mM PBS. Some of the fixed cells were permeabilized by exposure to 0.2% solution of Triton X-100 in PBS for 5 min; other cells were not permeabilized in order to look at the presence of PN-1 on the cell surface. Cultures were then incubated for 30 min in blocking serum (normal goat serum; Vector Labs) followed by a 3 hr incubation in PBS containing the primary antibody. An affinity-purified polyclonal antibody to PN-1, raised in rabbits (a generous gift from Dr. Randy Scott, Incyte Pharmaceuticals) was used at a 1:100 dilution. Cells were further processed using a rabbit IgG ABC kit (Vector Labs) with diaminobenzidine tetrahydrochloride as a substrate. Stained cultures were wet mounted in glycerol, then examined and photographed using an inverted Nikon Diaphot microscope with phase-contrast and bright-field optics.

Measurement of $[Ca^{2+}]_i$. Fluorescence ratio imaging of the Ca^{2+} indicator dye fura-2 was used to quantify $[Ca^{2+}]_i$ as detailed in our previous studies (Mattson et al., 1989, 1991, 1994). For these studies, cells were grown in glass-bottom dishes (Mat-Tek, Inc.) coated with 0.05% polyethyleneimine. Cells were loaded at 37°C for 35 to 40 min with 4 to 6 μM fura-2 AM (Molecular Probes). The loaded cells were then rinsed with Locke's solution (with or without glucose) and incubated an additional 60 min to equilibrate prior to $[Ca^{2+}]_i$ imaging. Experimental treatments were added back to cultures during this equilibration period. Two ratiometric imaging systems were used to quantify neuronal $[Ca^{2+}]_i$. One was a Nikon inverted microscope with a fluoro 40× N.A. 1.3 objective lens and an intensified CCD camera (Quantex) coupled to a Quantex imaging system; QFM software to acquire and process the images. The other was a Zeiss Attofluor system with an Axiovert microscope and 40× N.A. 1.3 objective and intensified CCD camera. The $[Ca^{2+}]_i$ was determined from the ratio of the fluorescence emission using two different excitation wavelengths (340 and 380 nM) as described by Grynkiewicz et al. (1985). Measurements represented the average $[Ca^{2+}]_i$ in the neuronal cell body. For experiments that examined acute $[Ca^{2+}]_i$ responses, images were acquired of the same neurons before and after treatments. For experiments that examined long-term changes in $[Ca^{2+}]_i$ (e.g., glucose deprivation studies), images of populations of neurons were acquired at a single time point. Statistical comparisons were made using Student's *t* test (two-tailed), one-way analysis of variance (ANOVA), and Bonferroni *t* test for multiple pair-wise comparisons.

Results

Protease nexin-1 attenuates glucose deprivation-induced neuronal injury

As described previously (Cheng and Mattson, 1991, 1992), when rat hippocampal cell cultures were deprived of glucose, neuronal damage and death occurred 16 to 24 hr following the onset of glucose deprivation (Figs. 1, 2). Pretreatment of cultures

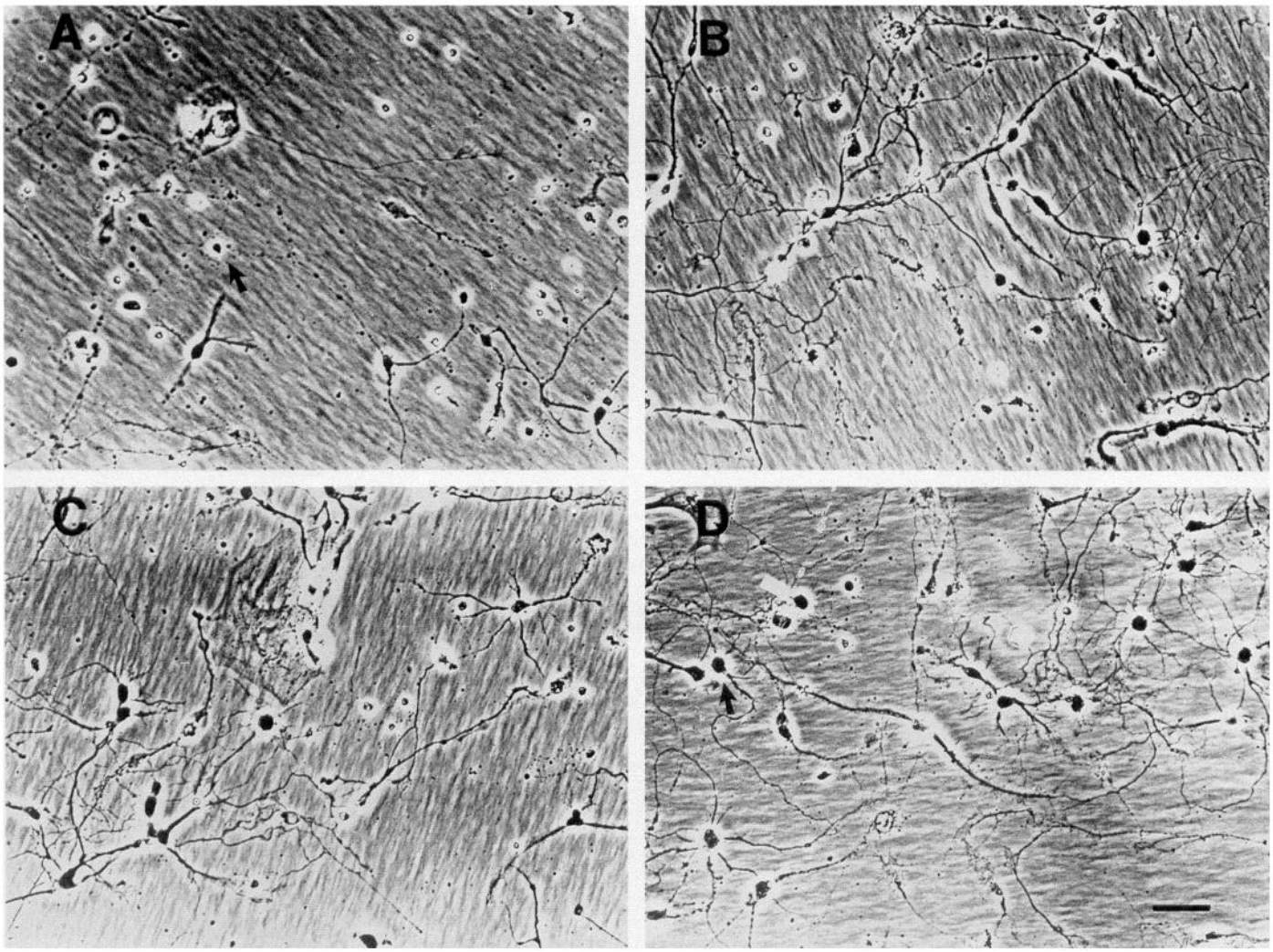

Figure 1. PN-1 protects hippocampal neurons from glucose deprivation-induced damage. Phase-contrast micrographs of cultured hippocampal cells. *A,* Control glucose-deprived culture after 24 hr in glucose-free medium. Note cell loss and damage to somata and neurites of many of the remaining neurons. *Arrow* indicates a degenerating neuron. *B,* Twenty-four hours in medium containing 10 mM glucose. *C,* Twenty-four hours in glucose-free medium lacking calcium. *D,* A culture pretreated for 24 hr with 25 nM PN-1 and then deprived of glucose for 24 hr. Note that neurons appear undamaged. *Arrow* indicates a healthy neuron. Scale bar 50 μM.

with 25 nM PN-1 for 24 hr prior to the onset of glucose deprivation resulted in a highly significant increase in neuronal survival (Fig. 2*A*). Neuronal survival was increased approximately 2.5-fold over survival values in control cultures deprived of glucose. The neuroprotective effect of PN-1 was concentration dependent, with the most effective level at 25 nM concentration (Fig. 2*A*). Higher concentrations of PN-1 (50 to 100 nM) were not protective. However, PN-1 alone (50 to 500 nM) was not neurotoxic when added to cultures maintained in medium containing glucose (Fig. 2*A*).

In order to further characterize the protective effects of PN-1, we administered PN-1 at different times relative to the onset of glucose deprivation. We found that PN-1 significantly protected hippocampal neurons from glucose deprivation-induced damage (GDID) when added to cultures 24 hr before the insult (Fig. 2*B*). Administration of PN-1 at the time of onset of glucose deprivation, or 4 hr following glucose withdrawal, did not increase cell survival significantly (Fig. 2*B*).

PN-1 attenuates the elevation of $[Ca^{2+}]_i$ that mediates glucose deprivation-induced damage

Previous findings demonstrated that the mechanism of GDID in cultured rat hippocampal neurons involves a large elevation of $[Ca^{2+}]_i$ that occurs 12 to 16 hr following the onset of glucose deprivation (Cheng and Mattson, 1991; Cheng et al., 1993). Therefore, we measured $[Ca^{2+}]_i$ in control and PN-1–treated glucose-deprived neurons (Fig. 2*C*). When measured 14 to 16 hr following the onset of glucose deprivation, $[Ca^{2+}]_i$ was elevated approximately 2.5-fold compared to parallel control cultures containing 10 mM glucose. The $[Ca^{2+}]_i$ 14 to 16 hr following the onset of glucose deprivation in neurons pretreated for 24 hr with 25 nM PN-1 was significantly less than in parallel control, glucose-deprived cultures ($p < 0.005$; Fig. 2*C*).

Thrombin is neurotoxic, exacerbates glucose deprivation-induced damage, and destablizes neuronal calcium homeostasis

Exposure of cultures to thrombin (10 pM to 1 μM) resulted in a significant concentration-dependent decrease in neuronal sur-

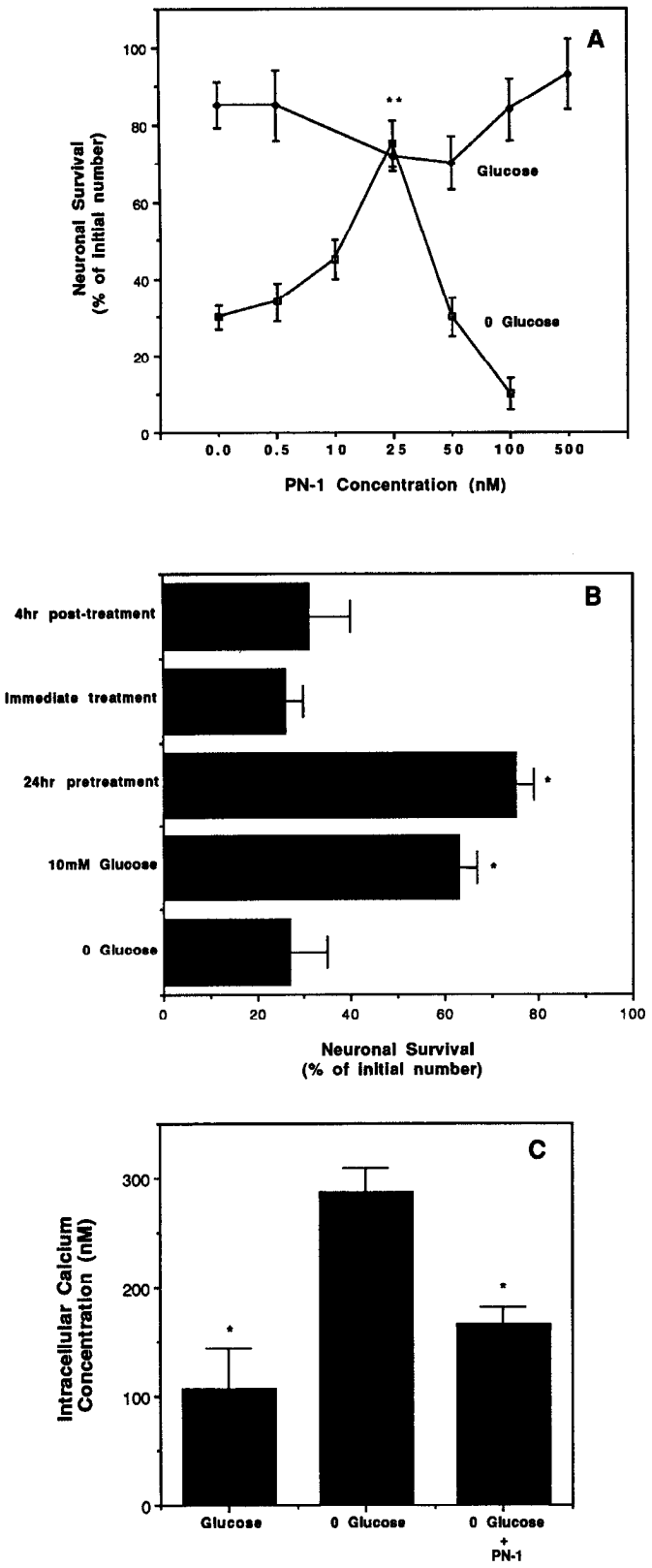

Figure 2. PN-1 protects neurons against glucose deprivation-induced damage and stabilizes $[Ca^{2+}]_i$. *A,* Cultures that had been pretreated for 24 hr with the indicated concentrations of PN-1 were exposed to glucose-free medium or medium containing 10 mM glucose, and neuronal survival was assessed 24 hr later. Values represent the mean and SEM of determinations made in 8–24 fields from two to six separate experiments. *$p < 0.01$; compared with values for cultures exposed to 0 glucose alone. *B,* Cultures were exposed to 25 nM PN-1 24 hr before, at the time of, or 4 hr following the onset of glucose deprivation. Neuronal survival was assessed 24 hr following the onset of glucose deprivation. Values represent the mean and SEM of determinations made in 8–16 fields from two to four separate experiments. *$p < 0.01$ compared with 0 glucose alone. *C,* Cultures were exposed to 25 nM PN-1 24 hr prior to exposure to glucose-free medium. Intracellular calcium levels were measured after 14–16 hr of incubation in the indicated conditions. Values represent the mean and SEM of determinations made in 22–172 neurons. *$p < 0.005$ compared with value for 0 glucose condition.

vival ($p < 0.01$; Fig. 3*A*). The most effective concentrations were in the range of 100 nM to 1 μM. Pretreatment of cultures with a submaximally toxic level of thrombin (25 nM) prior to glucose deprivation resulted in significant exacerbation of GDID ($p < 0.05$; Fig. 3*B*).

Treatment of hippocampal cultures with thrombin for 6 hr led to a significant increase in rest $[Ca^{2+}]_i$ in neurons ($p < 0.0001$; Table 1). The elevation of $[Ca^{2+}]_i$ in response to thrombin involved Ca^{2+} influx through the plasma membrane since thrombin did not elevate $[Ca^{2+}]_i$ when neurons were incubated in Ca^{2+}-free medium (Table 1). Calcium influx, induced by thrombin, was mechanistically involved in neuronal injury since thrombin was not neurotoxic when cells were incubated in Ca^{2+}-free medium ($p < 0.05$; Table 1).

Interactive effects of PN-1 and thrombin on glucose deprivation-induced injury and calcium homeostasis

Co-incubation of PN-1 with equimolar thrombin resulted in a complete blockade of PN-1's protection against GDID ($p < 0.01$; Fig 4*A*). However, increasing concentrations of PN-1 were able to overcome thrombin neurotoxicity ($p < 0.01$; Fig. 4*B*). Likewise, PN-1 and thrombin exerted opposite effects on neuronal $[Ca^{2+}]_i$. Resting $[Ca^{2+}]_i$ was significantly reduced by 42% in hippocampal neurons exposed to 25 nM PN-1 within 10 min (Fig. 5*A*). This effect was reversible with equimolar thrombin administration causing $[Ca^{2+}]_i$ to return to, and rise above rest levels within 10 min (Fig. 5*A*). As described above, hippocampal neurons deprived of glucose experience a significant increase in $[Ca^{2+}]_i$, which is attenuated by pretreatment with PN-1. This $[Ca^{2+}]_i$ stabilizing effect of PN-1 was blocked by co-incubation with thrombin ($p < 0.05$; Fig. 5*B*).

Since previous studies showed that neurons contacting astrocytes are relatively resistant to Ca^{2+}-mediated toxicity (Mattson and Rychlik, 1990) and because PN-1 is produced by astrocytes (Rosenblatt et al., 1987; Reinhard et al., 1988; Milligan et al., 1991; Reinhard et al., 1994), we examined Ca^{2+} responses to thrombin in neurons alone compared to neurons contacting astrocytes. To begin, we found rest levels of $[Ca^{2+}]_i$ to be 1.5-fold higher in neurons not contacting astrocytes as compared with neurons contacting astrocytes. Subsequent thrombin administration caused a highly significant increase of $[Ca^{2+}]_i$ to approximately 215 nM in neurons not contacting astrocytes compared with control cultures. In addition, thrombin induced a significant elevation of $[Ca^{2+}]_i$ to approximately 130 nM in neurons contacting astrocytes compared with control cultures, although the peak of $[Ca^{2+}]_i$ remained significantly lower than in neurons not contacting astrocytes (Fig. 6*A*). In order to establish that the hippocampal astrocytes expressed PN-1, cells were immunostained with a polyclonal antibody to PN-1. Astrocytes were highly immunoreactive with the PN-1 antibody, and nonpermeabilized astrocytes exhibited PN-1 immunoreactivity on their cell surface (Fig. 6*B*). The staining was specific because it was

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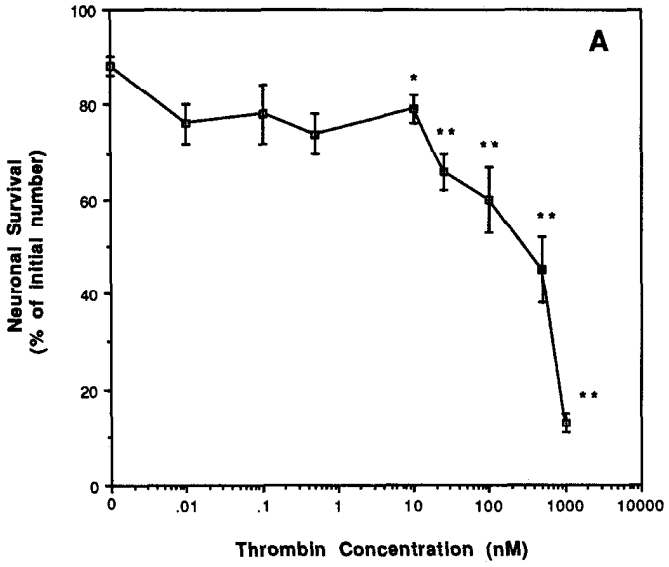


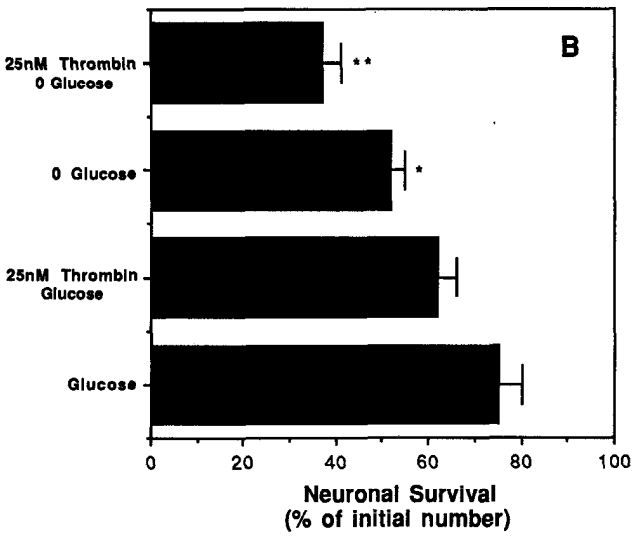


Figure 3. Thrombin is neurotoxic and exacerbates glucose deprivation-induced damage. *A,* Cultures were exposed to thrombin 10 pM-1 μM, and neuronal survival was assessed 48 hr later. Values represent the mean and SEM of determinations in eight fields per treatment group from two separate experiments. $*p < 0.05$; $**p < 0.01$ compared with cultures not exposed to thrombin. *B,* Cultures were pretreated with 25 nM thrombin for 24 hr and then either deprived of glucose for 24 hr or incubated in medium containing 10 mM glucose for 24 hr. Neuronal survival was assessed 24 hr after glucose deprivation. Values represent the mean and SEM (4–24 fields per treatment group from three separate experiments). $*p < 0.05$; $**p < 0.01$ compared with glucose cultures and $**p < 0.01$ compared with 0 glucose cultures.

eliminated when the primary antiserum was preabsorbed with excess PN-1 (data not shown). Neurons were also immunoreactive with the PN-1 antibody.

Discussion

Energy deprivation and excitotoxicity are believed to contribute to neuronal injury in both acute and chronic neurodegenerative

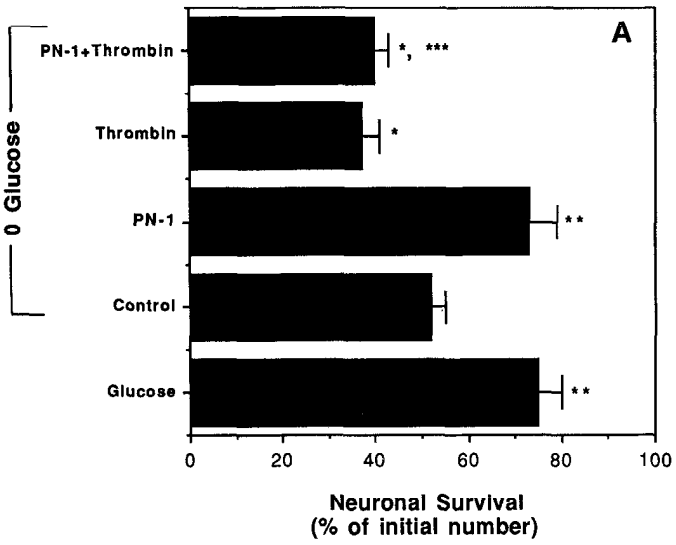
Table 1. $[Ca^{2+}]_i$ measurements and neuron survival for thrombin-exposed and control hippocampal cell cultures

Treatment	Intracellular calcium concentration (nM)	Neuronal survival (% of initial number)
Control without Ca^{2+}	74 ± 4	85 ± 6
100 nM thrombin without Ca^{2+}	70 ± 2	84 ± 8
Control with 1.8 mM Ca^{2+}	71 ± 5	76 ± 6
100 nM thrombin with 1.8 mM Ca^{2+}	212 ± 32**	62 ± 3*

Cultures were exposed to thrombin for 6 hr for $[Ca^{2+}]_i$ measurements and for 24 hr for neuron survival measurements. Values represent mean and SEM for $[Ca^{2+}]_i$ and percentage neuron survival from two to three separate experiments.
$*p < 0.05$.
$**p < 0.0001$; compared with thrombin without Ca^{2+} and control with 1.8 mM Ca^{2+}.

disorders. For example, in both cerebral ischemia and traumatic brain injury, cellular ATP levels are reduced (Martin et al., 1994), and glucose availability appears to be reduced in Alzheimer's disease (Hoyer, 1988). In such brain injuries, levels of PN-1 and thrombin are increased to varying amounts (Hoffman, et al., 1992; Nitsch et al., 1993; Suzuki et al., 1994), and therefore it is important to understand how they influence the injury process. Suzuki et al. (1994) reported that traumatic brain injury results in increased levels of thrombin in the brain up to fivefold. Ischemic brain injury induced a prolonged increase in PN-1 immunoreactivity, which persisted for at least a year (Hoffman et al., 1992; Nitsch et al., 1993), perhaps in response to extravasated blood containing thrombin (Choi et al., 1990). Nishino et al. (1993) demonstrated that intracerebral injections of thrombin resulted in infiltration of inflammatory cells, proliferation of mesenchymal cells, induction of angiogenesis, increased vascular permeability, and increased vimentin-positive astrocytes. The source of increased thrombin in brain injury may be vascular, but also may include endogenous thrombin produced *in situ* in response to brain injury (Beecher et al., 1994). When thrombin induces reactive gliosis, cytokines are released. Cytokines and other injury-related factors have been shown to stimulate PN-1 secretion in cultured brain cells (Vaughan and Cunningham, 1993), suggesting that brain injury-induced cytokine cascades may counteract the potentially damaging presence of thrombin. In the present study, we directly examined the effects of PN-1 and α-thrombin on neuronal Ca^{2+} homeostasis and injury in a hippocampal paradigm of energy deprivation/excitotoxic injury. We found that PN-1 protected hippocampal neurons against GDID, and that thrombin exacerbated the neuronal injury induced by this metabolic insult. Thrombin alone was neurotoxic at high concentrations (> 25 nM).

The concentration of PN-1 that was effective in protecting hippocampal neurons against GDID (25 nM) is similar to concentrations that increased neurite outgrowth in neuroblastoma cells (Monard et al., 1983; Cunningham and Gurwitz, 1989; Gurwitz and Cunningham, 1990) and embryonic hippocampal neurons (Farmer et al., 1990). Levels of PN-1 in CSF were reported to be in the range of approximately 2 to 6 nM (Festoff et al., 1992). Our data suggest that such basal levels of PN-1 may be sufficient to reduce neuronal injury *in vivo,* especially in conjunction with injury-induced increases in PN-1 production, al-



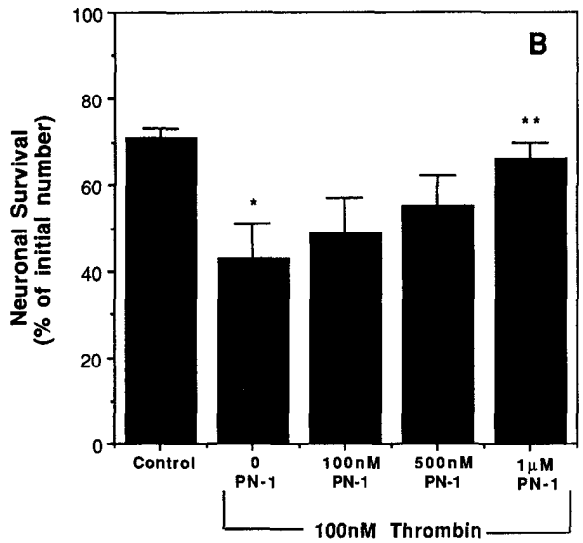
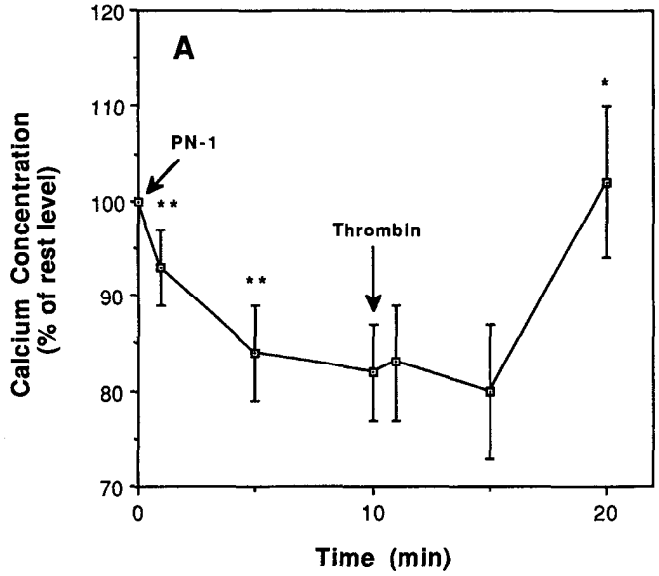


Figure 4. Effects of thrombin on PN-1–induced protection against GDID and PN-1's ability to reverse thrombin neurotoxicity. *A,* Cultures were pretreated for 24 hr with the indicated treatments and then deprived of glucose for 24 hr. PN-1, 25 nM; thrombin, 25 nM. Neuronal survival was assessed 24 hr following the onset of glucose deprivation. Values represent mean and SEM of determinations made in 8–20 fields from two to five separate cultures per treatment group. $*p < 0.05$; $**p < 0.01$ compared with 0 glucose control and $***p < 0.01$ compared with PN-1–treated cultures in 0 glucose medium. *B,* Cultures were treated with 100 nM thrombin and increasing concentrations of PN-1 for 24 hr. Values represent mean and SEM of determinations made in 12–20 fields from three to five separate cultures per treatment group. $*p < 0.01$ compared with control cultures. $**p < 0.01$ compared with thrombin alone.

though this remains to be established. It is unclear why there was a small window of PN-1 concentrations effective in protecting neurons against GDID, such that high concentrations were ineffective. This phenomenon was apparently not the result of neurotoxicity of the high concentrations of PN-1, since these concentrations had no effect on cell survival in cultures maintained in media containing 10 mM glucose. Previous studies have demonstrated similar results with regard to neurite outgrowth in hippocampal cultures such that concentrations between 1 to 6



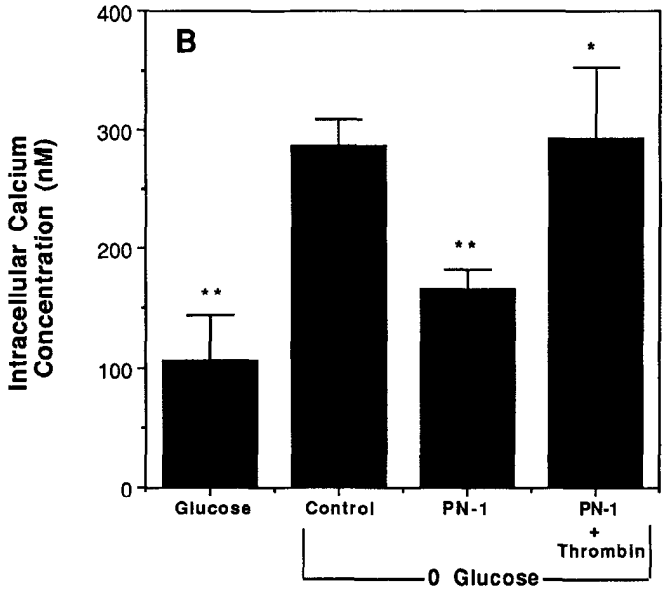
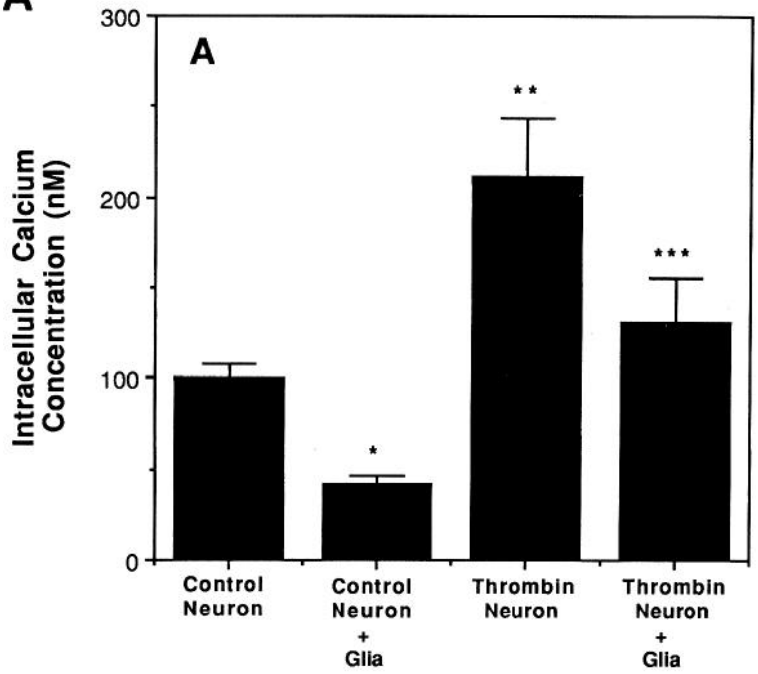
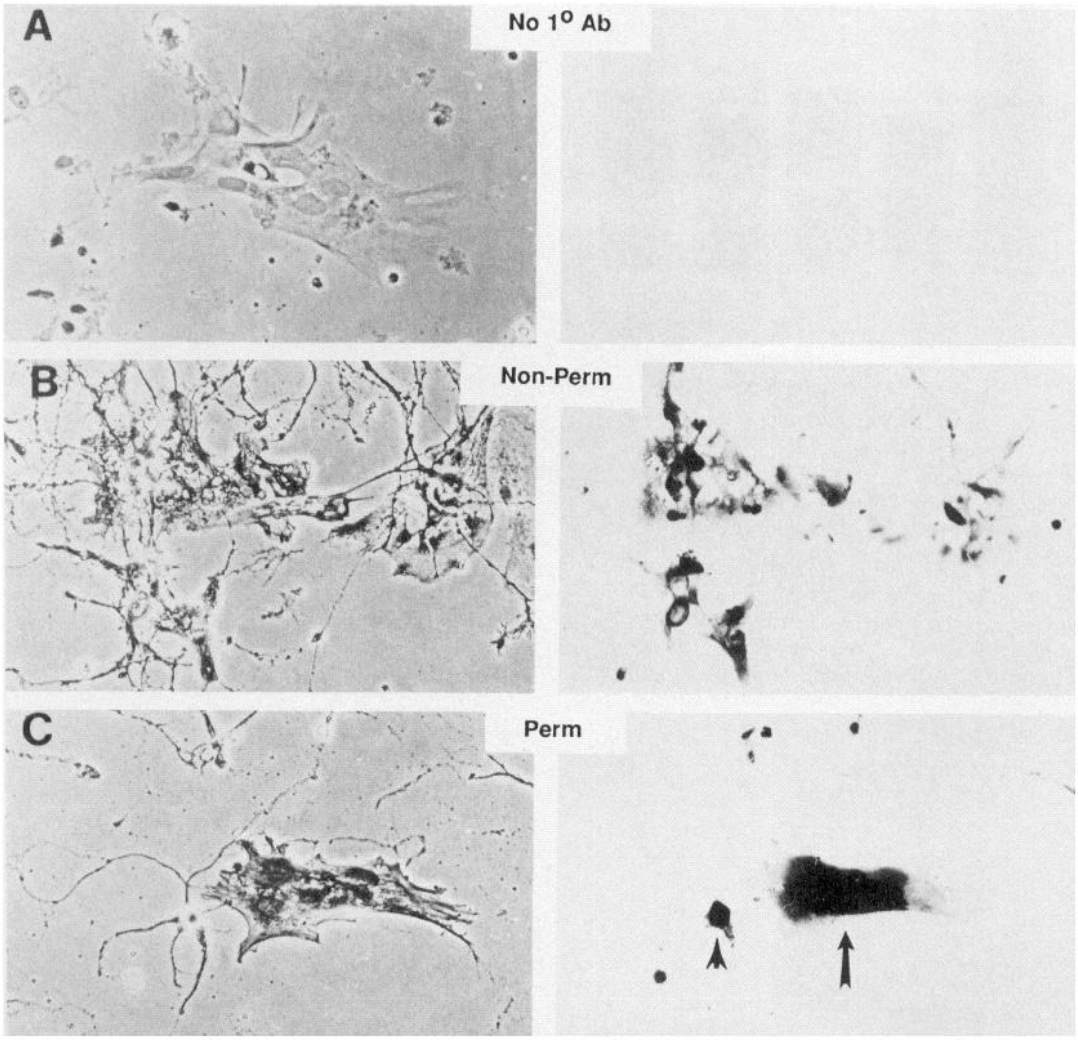


Figure 5. Thrombin reverses the $[Ca^{2+}]_i$ lowering actions of PN-1 in cultured hippocampal neurons. *A,* Immediately following acquisition of images of rest, $[Ca^{2+}]_i$ cultures were exposed to 25 nM PN-1, and additional $[Ca^{2+}]_i$ images were acquired 1, 5, and 10 min later. Thrombin (25 nM) was then added to the cultures, and $[Ca^{2+}]_i$ images were acquired 1, 5, and 10 min later. Values represent the mean and SEM of 20 neurons. Values at 5 and 10 min following exposure to PN-1 were significantly lower than pretreatment values ($p < 0.005$). Thrombin significantly reversed the $[Ca^{2+}]_i$ lowering effect of PN-1 at the 10 min time point following thrombin administration ($p < 0.01$). *B,* Thrombin blocks the $[Ca^{2+}]_i$ stabilizing action of PN-1 in glucose-deprived neurons. Cultures were pretreated with 25 nM PN-1 alone or in combination with 25 nM thrombin, and were then deprived of glucose for 14–16 hr, at which time $[Ca^{2+}]_i$ was quantified. Values represent the mean and SEM of determinations made in 22–172 neurons. $*p < 0.05$ compared with PN-1–treated cultures; $**p < 0.01$ compared with 0 glucose control cultures.

A


A
300
200
100
0
Intracellular Calcium Concentration (nM)
*
**
***
Control Neuron
Control Neuron + Glia
Thrombin Neuron
Thrombin Neuron + Glia

B


A
No 1° Ab
B
Non-Perm
C
Perm

nM PN-1 promoted outgrowth but higher concentrations (11 nM) were ineffective (Farmer et al., 1990). These data support the idea that a delicate balance exists between proteases and their inhibitors with respect to their influences on neurite outgrowth and cell survival.

It is well established that sustained increases in $[Ca^{2+}]_i$ can lead to destabilization of the neuronal cytoarchitecture, leading to cell damage and eventual death (Siman et al., 1989; Yanagihara et al., 1990; Johnson et al., 1991; Mattson et al., 1992). We previously reported that GDID involves activation of glutamate receptors and Ca^{2+} influx (Cheng and Mattson, 1991). In the present study, stabilization of $[Ca^{2+}]_i$ by PN-1 appeared to be mechanistically involved in protection against GDID. The increase in $[Ca^{2+}]_i$ that normally occurred within 14 to 16 hr of the onset of glucose deprivation was suppressed in hippocampal neurons pretreated with PN-1. Moreover, preliminary data indicate that the direct neurotoxicity of 100 μM glutamate is significantly attenuated in cultures pretreated with PN-1 (unpublished data). Therefore, PN-1 appears to be important in maintaining $[Ca^{2+}]_i$ homeostasis in the face of metabolic and excitotoxic insults.

Like other neuroprotective agents, PN-1 protected neurons against GDID when administered within a specific window of time relative to the insult. We found that PN-1 was effective in protecting hippocampal neurons against GDID only when added prior to the onset of glucose deprivation. This characteristic of neuroprotection by PN-1 is similar to that of other neurotrophic factors employed in this system including bFGF (Cheng and Mattson, 1991), IGF-1 and IGF-2 (Cheng and Mattson, 1992a), TNFs (Cheng et al., 1994), and secreted forms of β-amyloid precursor protein (Mattson et al., 1993b). We previously showed that new protein synthesis was required for bFGF to protect cultured hippocampal neurons against glutamate toxicity (Mattson et al., 1989). Although the specific proteins induced by neurotrophic factors that mediate neuroprotection have not been established, recent studies have identified several candidates. For example, bFGF (Collazo et al., 1992) and TNFs (Cheng et al., 1994) increased expression of calbindin, a Ca^{2+}-binding protein believed to play roles in calcium buffering and protection against excitotoxicity (Scharfman and Schwartzkroin, 1989; Mattson et al., 1991). In addition, bFGF reduced the expression of a 71 kDa glutamate binding protein involved in NMDA receptor function in cultured hippocampal neurons (Mattson et al., 1993c). We have found that 100 μg/ml cycloheximide can block PN-1 protection against GDID; however, preliminary results suggest that PN-1 does not induce calbindin expression in cultured hippocampal neurons (V. L. Smith-Swintosky, unpublished data), and the present data are consistent with a mechanism of protection involving thrombin inhibition (see below). Recent evidence indicates that thrombin signalling induces the activation and induction of NF-κB (Nakajima et al., 1994) and AP-1 mediated gene transcription (Trejo et al., 1992) in other cell types, which suggests that thrombin's bioactivity may affect protein synthesis.

Since PN-1 is a potent inhibitor of the serine proteinase, thrombin, we undertook additional studies to determine what effects thrombin might have on cultured hippocampal neurons and the relationship between PN-1 and thrombin in these cultures. The concentrations of thrombin that affected neuronal survival and calcium homeostasis in the present study are within a range that is present in blood and bioactive on neural cells *in vitro*. Although levels of prothrombin have not been quantified in the brain, plasma levels of prothrombin are reportedly within 1–5 μM (Walz et al., 1985)). Gurwitz and Cunningham (1990) reported that thrombin concentrations above 1 nM caused dose-dependent neurite retraction in neuroblastoma cells. Somewhat lower concentrations of thrombin (pM) are mitogenic for astrocytes (Low et al., 1982). We found thrombin to be neurotoxic in a concentration-dependent manner (25 nM to 1 μM). Although, similar concentrations of thrombin were considerably less toxic to embryonic hippocampal neurons at earlier stages of development in culture (1 to 3 d in culture; unpublished data), suggesting that thrombin may differentially affect immature and mature neurons. Moreover, we found pretreatment of cultures with a submaximally toxic level of thrombin (25 nM) followed by glucose deprivation led to a significant exacerbation of GDID, up to 1.5-fold higher than glucose deprivation alone. In addition, we have evidence that pretreatment with 25 nM thrombin can significantly increase glutamate toxicity (unpublished data). These results may have important implications for neurodegenerative disease states such as ischemia and severe head trauma. In cerebral ischemia and head trauma the blood–brain barrier is often compromised, and blood penetrates into the brain parenchyma, allowing thrombin to come in contact with neurons. It is also possible that prothrombin is transported extravascularly and activated by neural cells. Lack of oxygen and glucose to neurons resulting in energy failure and subsequent release of glutamate are classic components of ischemic damage. Our data suggest that thrombin may worsen these events.

Our Ca^{2+} imaging studies showed that PN-1 induced a decrease in rest $[Ca^{2+}]_i$, while thrombin caused an increase of $[Ca^{2+}]_i$ and reversed the $[Ca^{2+}]_i$ lowering action of PN-1. The ability of thrombin to increase $[Ca^{2+}]_i$ and cause neurodegeneration was dependent on Ca^{2+} influx since neither effect of thrombin was observed when cells were incubated in Ca^{2+}-deficient medium. These results suggest that endogenous thrombin may contribute to rest $[Ca^{2+}]_i$ levels through basal activation of thrombin receptors, which may increase neuronal vulnerability to injury. Thrombin is thought to activate its receptor by cleaving the receptor, freeing an amino-terminal peptide, which then binds and activates the thrombin receptor (Vu et al., 1991). In many cell types activation of thrombin receptors results in elevation of $[Ca^{2+}]_i$. For example, Goligorsky et al. (1989) showed that thrombin induced a sustained increase in $[Ca^{2+}]_i$ in cultured endothelial cells, and Tiruppathi et al. (1992) reported that a synthetic 14-amino acid (TRAP) peptide corresponding to the "tethered ligand" portion of the thrombin receptor also induces

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Figure 6. The $[Ca^{2+}]_i$ response to thrombin is suppressed in neurons contacting PN-1 immunoreactive astrocytes. *A*, Rest $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ 6 hr after exposure to 100 nM thrombin were measured in neurons not contacting or contacting astrocytes. Values represent the mean and SEM (n = 25–47 neurons/condition). *$p < 0.001$ compared to value for control neurons not contacting astrocytes. **$p < 0.005$ compared to corresponding control value. ***$p < 0.05$ compared to value for thrombin-treated neurons not contacting astrocytes. *B*, Phase contrast and bright-field micrographs of 7-d-old hippocampal cultures immunostained with a polyclonal anti-rabbit PN-1 antibody. *Top*, Cultures incubated without primary antibody. There is no evidence of nonspecific staining. *Middle*, Cultures not permeabilized prior to incubation with primary antibody. There is notable staining associated with the cell surface of both astrocytes and neurons. *Bottom*, Cultures permeabilized with 0.2% Triton X-100 prior to incubation with primary antibody. Both astrocytes and neurons show dark staining within the cell body. *Arrowhead* indicates a neuron. *Arrow* indicates an astrocyte.

$[Ca^{2+}]_i$ elevation in endothelial cells. In addition, thrombin has been shown to cause a rapid transient increase in $[Ca^{2+}]_i$ in T-lymphoblastoma cells (Tordai et al., 1993). It is reasonable to consider that thrombin-induced increases in $[Ca^{2+}]_i$ may contribute to thrombin-induced neurite retraction, especially in light of our previous findings that local increases in $[Ca^{2+}]_i$ lead to neurite retraction, whereas agents that lower $[Ca^{2+}]_i$ promote neurite outgrowth and cell survival (Mattson et al., 1988, 1989; Mattson, 1993). In this regard, our finding that PN-1 reduced $[Ca^{2+}]_i$ is consistent with its previously reported neurite outgrowth-promoting action (Guenther et al., 1985; Gurwitz and Cunningham, 1990).

Thrombin-induced elevation of $[Ca^{2+}]_i$ was markedly attenuated in neurons contacting astrocytes compared to neurons not contacting astrocytes. Astrocytes are known to produce PN-1 (Gloor et al., 1986), and we found that PN-1 immunoreactivity was associated with the surface of astrocytes in the hippocampal cultures of the present study. Although not directly demonstrated, these observations suggest that astrocyte-derived PN-1 was involved in attenuation of thrombin-induced Ca^{2+} responses. Previous studies have shown that astrocytes can protect neurons against excitatory amino acid toxicity, as well as the toxicity of Ca^{2+} ionophores (Mattson and Rychlik, 1990). Recent evidence suggests that neurons also secrete PN-1 (Reinhard et al., 1994), and it is therefore not clear whether the PN-1 immunoreactivity that we found associated with neurons is glial or neuronal derived. Since known biological actions of PN-1 are mediated by binding thrombin, and because thrombin exacerbated GDID, it is reasonable to consider that endogenous thrombin increases neuronal vulnerability to metabolic/excitotoxic insults and that glia-derived PN-1 protects neurons against such insults. This is supported by our data indicating that thrombin toxicity could be overcome by increasing the concentration of exogenous PN-1 in the culture media. We therefore propose that PN-1 secreted locally by glial cells can bind thrombin, prevent activation of thrombin receptors in neurons, and thereby stabilize calcium homeostasis. Our data indicate that the PN-1–thrombin system modulates metabolic/excitotoxic insults and concomitant disruptions in Ca^{2+}-homeostasis, which are associated with neurodegeneration.

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