Thrombin Perturbs Neurite Outgrowth and Induces Apoptotic Cell Death in Enriched Chick Spinal Motoneuron Cultures through Caspase Activation

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Increasing evidence indicates several roles for thrombin-like serine proteases and their cognate inhibitors (serpins) in normal development and/or pathology of the nervous system. In addition to its prominent role in thrombosis and/or hemostasis, thrombin inhibits neurite outgrowth in neuroblastoma and primary neuronal cells *in vitro*, prevents stellation of glial cells, and induces cell death in glial and neuronal cell cultures. Thrombin is known to act via a cell surface protease-activated receptor (PAR-1), and recent evidence suggests that rodent neurons express PAR-1. Previously, we have shown that the thrombin inhibitor, protease nexin-1, significantly prevents neuronal cell death both *in vitro* and *in vivo*. Here we have examined the effects of human α-thrombin and the presence and/or activation of PAR-1 on the survival and differentiation of highly enriched cultures of embryonic chick spinal motoneurons. We show that thrombin significantly decreased the mean neurite length, prevented neurite branching, and induced motoneuron death by an apoptosis-like mechanism in a dose-dependent manner. These effects were prevented by cotreatment with hirudin, a specific thrombin inhibitor. Treatment of the cultures with a synthetic thrombin receptor-activating peptide (SFLLRNP) mimicked the deleterious effects of thrombin on motoneurons. Furthermore, cotreatment of the cultures with inhibitors of caspase activities completely prevented the death of motoneurons induced by either thrombin or SFLLRNP. These findings indicate that (1) embryonic avian spinal motoneurons express functional PAR-1 and (2) activation of this receptor induces neuronal cell degeneration and death via stimulation of caspases. Together with previous reports, our results suggest that thrombin, its receptor(s), and endogenous thrombin inhibitors may be important regulators of neuronal cell fate during development, after injury, and in pathology of the nervous system.

Key words: thrombin; serine proteases; PAR-1; apoptosis; caspases; spinal motoneuron cultures

Thrombin is a prominent member of the serine protease superfamily, of which most members exert their proteolytic activity by cleaving after the amino acid arginine (for review, see Mann, 1994). Thrombin-like serine proteases have been studied extensively in relation to coagulation and thrombosis. However, recent studies have localized these proteases to different cell populations within the nervous system, and the effects of these proteases on particular cell types are beginning to be characterized (see Ho et al., 1994; Smirnova et al., 1994; Festoff et al., 1996; Turgeon and Houenou, 1997). Thrombin has been shown to alter cell morphology and differentiation in astrocyte and neuroblastoma cultures (Gurwitz and Cunningham, 1988, 1990; Zurn et al., 1988; Grabham et al., 1992; Suidan et al., 1992) and to induce proliferation and differentiation in cultured glial cells (Perraud et al., 1987; Loret et al., 1989; Cavanaugh et al., 1990). Therefore, the role of thrombin in the nervous system seems distinct from its function in the coagulation system. However, the molecular mechanisms by which thrombin affects neuron development have only recently begun to be examined (Donovan et al., 1997; Stefanis et al., 1997).

Thrombin is known to exert its effects via a G-protein–coupled, cell surface protease-activated receptor (PAR-1), whose activation involves a "tethered-ligand" mechanism (Vu et al., 1991). Thrombin cleaves between Arg^{42}-Ser^{43} of the extracellular N-terminal domain of the receptor, generating a new N terminal that undergoes a conformational change and binds to another portion of the receptor leading to receptor activation and signal transduction (Vu et al., 1991; for review, see Turgeon and Houenou, 1997). The sequence SFLLRNP in the amino domain of the cleaved human PAR-1 is, in fact, the activator of the receptor and is known as the thrombin receptor-activating peptide (Vu et al., 1991). Synthetic SFLLRNP has been shown to activate PAR-1 and to mimic the effects of thrombin on PAR-1–expressing cells. Thus, SFLLRNP may be a useful tool in receptor function analysis.

Thrombin activity may be regulated by a group of serine protease inhibitors, known as serpins, that include protease nexin-1, heparin cofactor II, antithrombin III, which are all present in mammalian species (Baker et al., 1980; Low et al., 1981; Cunningham et al., 1987; Abraham et al., 1988; Struss et al., 1992). In addition, the nonserpin hirudin from the medicinal leech (*Hirudo medicinalis*) is the only known thrombin-specific inhibitor (Stone and Hofsteenge, 1986; Markwardt, 1989).

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Both neuronal and glial cells have been shown to express prothrombin and thrombin receptor transcripts, although in different patterns, within the CNS (Dihanich et al., 1991; Niclou et al., 1994). Moreover, expression of serpins and/or serine proteases is generally upregulated after injury or in diseased states (Meier et al., 1989; Rao et al., 1993; Vaughan and Cunningham, 1993; Festoff et al., 1994), suggesting that serine proteases play a significant function after disruption of brain tissue homeostasis (Brun and Englund, 1981; Coleman and Flood, 1987; Swash and Swartz, 1992; Houenou et al., 1995). Thus, after injury to the brain or spinal cord, nerve cells may be exposed to abnormally high levels of thrombin that are either locally produced or derived from the surrounding vasculature. Recent studies also show that a motoneuronal cell line and embryonic mouse spinal cord neurons express PAR-1 and are sensitive to thrombin (Smirnova et al., 1998a). However, whether embryonic motoneurons are sensitive to thrombin in a manner consistent with a role in programmed cell death is still unknown.

We show that human α-thrombin inhibited neurite outgrowth and induced apoptosis-like cell death in highly enriched avian motoneuron cultures. These thrombin-induced effects seemed to be mediated via PAR-1 activation, because a synthetic agonist of PAR-1 elicited the same deleterious effects on motoneuron cultures. Furthermore, the effects of thrombin or activation of PAR-1 on motoneurons were completely prevented by cotreatment of the cultures with caspase inhibitors. Our findings suggest that PAR-1 activation in avian spinal motoneurons leads to stimulation of caspases, intracellular molecules known to be mediators of apoptosis in several other cell types.

MATERIALS AND METHODS

Motoneuron cultures. Motoneurons were isolated from stage 28 (embryonic day 5–5.5) embryos, as determined by the staging criteria of Hamburger and Hamilton (1951), and were cultured using modified methods from Dohrman et al. (1986) and Arakawa et al. (1990) recently described by Milligan et al. (1995). Briefly, the ventral portion of the lumbar spinal cords of chick embryos were removed using tungsten needles and were kept in ice-chilled sterile-filtered PBS until dissections were completed. The ventral lumbar spinal cords containing primarily motoneurons were then treated with 0.05% trypsin (in PBS without Ca^{2+} and Mg^{2+}) for 15 min to dissociate the cells. The partially dissociated cells were added to Lebowitz-15 (L-15) defined serum-free media (Life Technologies, Gaithersburg, MD) and further dissociated by running the mixture through a 1 ml pipette followed by centrifugation (400 $\times$ *g* for 15 min; Beckman GS-6R centrifuge) over a layer of 6.8% metrizamide (Sigma, St. Louis, MO). Motoneurons remained in the top half of the metrizamide, forming a visible white band that was collected and added to 5 ml of L-15 media. A 4% BSA cushion was then gently added beneath the cells and centrifuged at 200 $\times$ *g* for 10 min (Breckman centrifuge). The supernatant was discarded, and the pellet was resuspended in 0.5–1.0 ml of L-15 media and filtered through a 50 μm nylon filter. A portion of this preparation was loaded onto a hemocytometer for an initial cell count. From this initial count, the cells were diluted appropriately and plated at a density of 2000 motoneurons per well in 35 mm Petri dishes, each with four wells that were 10 mm in diameter (Greiner dishes). The dishes were precoated with laminin (Sigma) and poly-D-ornithine (Sigma). Cells were incubated in a CO_2 water-jacketed incubator at 37°C and 5.2% CO_2.

Immunostaining with SC-1 and Islet-1 antibodies to assay purity. After 24 hr of incubation, the culture media were removed, and the cells were stained with mouse anti-chick SC-1 monoclonal antibodies, which recognize a membrane glycoprotein expressed on motoneurons during development (hybridoma supernatant diluted 1:5 in PBS; SC-1 hybridomas were provided by Drs. C. Henderson and H. Tanaka). The cells were incubated with SC-1 antibodies for 2 hr at 37°C, washed three times with PBS, and fixed with 10% formaldehyde for 10 min. Alternatively, cells were first fixed with 4% paraformaldehyde for 10 min and then incubated for 2 hr at 37°C with mouse monoclonal antibodies to the *Islet-1* gene product, an early marker for developing motoneurons (hybridoma supernatant diluted 1:250 in PBS; provided by Dr. T. Jessell). After incubation with either primary antibody, the cells were washed with PBS and then incubated with goat anti-mouse IgG for 1 hr at 37°C. After washing with PBS, the cells were incubated in an avidin–biotin complex solution (Vectastain kit; Sigma) for 1 hr. The cells were stained with 3,3′-diaminobenzidine (DAB), washed three times with water, and coverslipped.

Anti-β-tubulin immunostaining of neurites. Culture media were gently removed, and the motoneurons were washed once with 1–2 ml of PBS before the cells were fixed with 4% paraformaldehyde for 15 min at 4°C. The cells were then washed three times with 0.2% Triton X-100/PBS to remove any excess paraformaldehyde. Mouse anti-chick-β-tubulin antibody solution (Sigma) diluted 1:200 in 1% horse serum and 0.2% Triton X-100/PBS was added to the cells, which were then incubated at room temperature for 1–2 hr. After the incubation period, the cells were washed three times with 0.2% Triton X-100/PBS and incubated for 2 hr at room temperature with goat anti-mouse IgG (secondary antibodies) in Triton X-100/PBS. After three additional washes with Triton X-100/PBS, the cells were incubated for 2 hr in avidin–biotin complex. Cells were stained with DAB for 2–5 min or until a brown product was visualized. Finally the cells were washed three times and coverslipped.

Experimental treatment of motoneuron cultures. The cells were grown in Greiner dishes (Bellco) in L-15 media at a density of 2000 cells per well. Two hours after the initial plating time, the cultures were treated with different proteins, including purified human α-thrombin (3400 National Institutes of Health units/mg from Sigma), hirudin (1700 anti-thrombin units/mg from Sigma), the thrombin receptor-activating peptide, SFLLRNP (BACHEM). To begin examining the mechanisms involved in the action of thrombin, we also (co)treated some cultures with two different caspase inhibitors, YVAD-CHO and DEVD-CHO, as described previously (Milligan et al., 1995; Smirnova et al., 1998a; L. Li, D. Prevette, R. W. Oppenheim, and C. E. Milligan, personal communication). The concentrations of these agents are specified in the figures and/or figure legends. Initially, motoneurons were identified using SC-1 and/or Islet-1 immunostaining as specific markers. Cell numbers were obtained by counting the number of viable cells seen across two diameters of each Greiner dish well using a 20$\times$ objective of a phase contrast microscope (Olympus BX2). The criteria used to examine motoneuron survival included the presence of two or more neurites per neuron, with the length of one or more of those neurites being at least twice the diameter of the cell soma, and the absence of vacuoles and/or degenerating neurites (Milligan et al., 1994)

To examine neurite outgrowth, we plated the cells at a lower density of 1000 cells per well to prevent or minimize interferences attributable to cell-to-cell contacts from neighboring cells. After treatment with different agents, the cells were fixed with 4% paraformaldehyde and stained with anti-β-tubulin antibodies as described above. Neurites were traced and their length was determined using a computerized imaging system (Macintosh IIfx 8RAM/160HD microcomputer). Each neuron image was captured and digitized by Perceptics and analyzed using National Institutes of Health Image 1.32 software. Only those neurons with a neurite at least twice the diameter of the soma were included in this analysis that involved 100 neurons per treatment group. In addition to analyzing neurite length, we have determined the number of side branches (primary branches) that occurred on the longest neurite per motoneuron.

Terminal deoxynucleotidyl transferase-mediated biotin–dUTP nick-end labeling histochemistry. This *in situ* technique labels the 3′-DNA ends that are exposed by endonucleases during apoptosis (Gavrieli et al., 1992; Wijsman et al., 1993). It is possible to visualize directly the apoptotic cells (that exhibit DNA fragmentation) *in vitro* using the *in situ* detection kit (catalog #1684795; Boehringer Mannheim, Indianapolis, IN) and the protocol described previously (Gavrieli et al., 1992). Briefly, the cells were fixed with 4% paraformaldehyde for 30 min and incubated for 2 min in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate). The terminal deoxynucleotidyl transferase-mediated biotin–dUTP nick-end labeling (TUNEL) reaction mixture was added to the cells and incubated in a humidified chamber for 1 hr at 37°C. Negative controls were incubated solely with labeling solution containing a fluorescent marker. Positive controls were initially incubated with DNase I for 10 min to induce DNA breaks, followed by addition of the TUNEL reaction mixture. Samples were visualized with a microscope equipped with fluorescence filters.

Transmission electron microscopy. To examine the morphology of motoneurons further, we fixed cultures with 2.5% glutaraldehyde in phosphate, pH 7.3, at room temperature. After 1 hr of fixation, the cells were

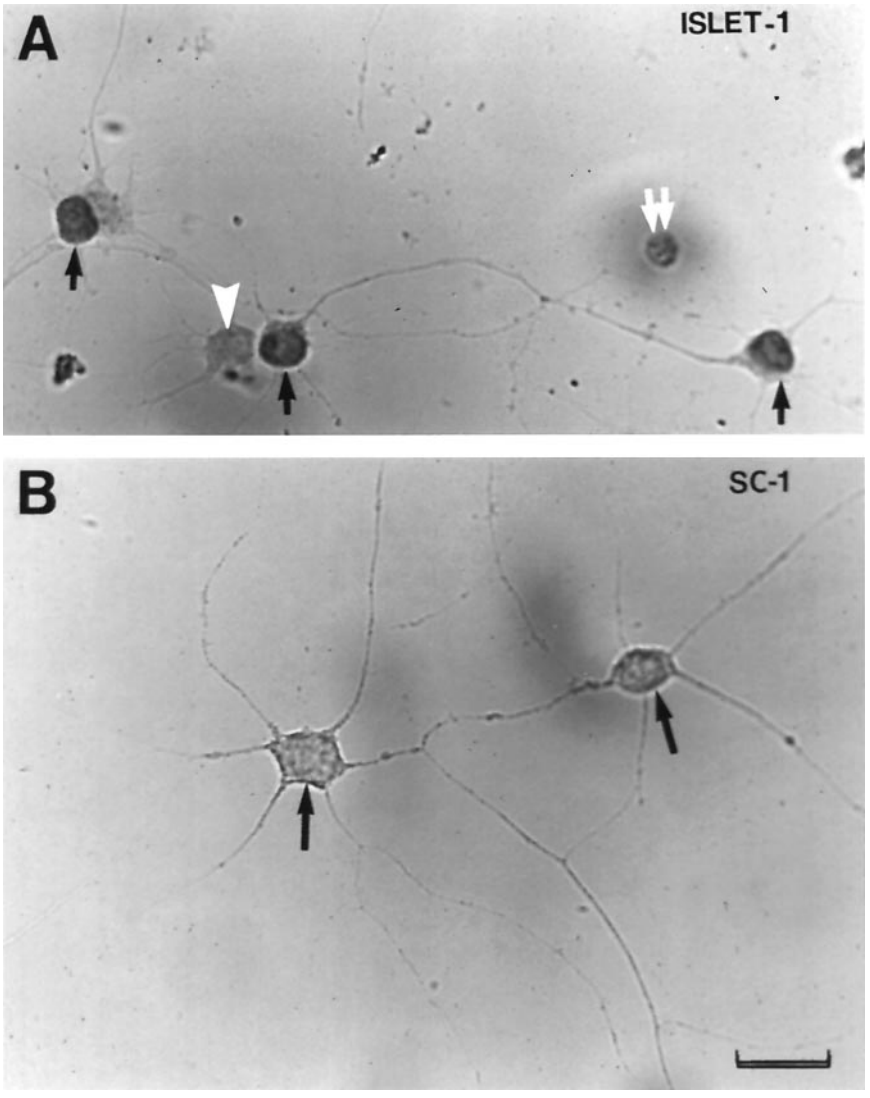

Figure 1. Examples of cultured chick spinal motoneurons stained with Islet-1 (*A*) or SC-1 (*B*) antibodies 24 hr after plating. Motoneuron counts typically showed 83 and 75% of cells positive for Islet-1 and SC-1, respectively, in enriched cultures. *Black arrows* indicate Islet-1– (*A*) or SC-1– (*B*) positive motoneurons. The *white arrowhead* in (*A*) points to an Islet-1–negative cell, whereas the *double white arrows* show a dying neuron. Scale bar, 25 μm.

rinsed in phosphate buffer, pH 7.3, and post-fixed for 1 hr in 2% osmium buffer, followed by three rinses of 10 min each in phosphate buffer. The motoneurons were then dehydrated in a graded series of ethanol for 10 min each. After ethanol dehydration, the cells were dehydrated twice in propylene oxide (PPO) for 10 min. The specimens were infiltrated for 2 hr with a 1:1 mixture of PPO and Spurr resin, followed by overnight infiltration in a 1:2 mixture of PPO and Spurr resin. Finally, the cells were infiltrated in pure Spurr resin for 6 hr and embedded in Spurr resin at 70°C overnight. Ultrathin sections were cut and processed for transmission electron microscopy (TEM) observation.

Data analysis. Data from the different experiments, including surviving or dying motoneuron numbers, neurite length, and neurite branching, were statistically analyzed using the one-way ANOVA followed by the Tukey–Kramer multiple comparison *post hoc* test. For each condition, experiments were performed at least three times, each in triplicate. Results were expressed as mean ± SEM relative to untreated controls or to initially plated cells.

RESULTS

Purity of motoneuron cultures

To establish the purity of our cultures, we stained 1-d-old chick spinal motoneuron cultures with either SC-1 or Islet-1 antibodies. Both antibodies have been shown previously to be specific for early developing motoneurons (Tanaka et al., 1989, 1991; Ericson et al., 1992). Our results show that the Islet-1 antibody staining was specifically confined to the nucleus of the motoneurons (Fig. 1*A*), whereas the SC-1 antibody staining was localized to the cell membrane (Fig. 1*B*). The average cell counts established for the cultures showed 83 and 75% labeling (purity) with Islet-1 and SC-1, respectively. These findings are in agreement with previous reports using the same methods described above (e.g., Milligan et al., 1994).

Thrombin decreased motoneuron survival *in vitro*

Previous studies have shown that exposure of rat primary hippocampal (Smith-Swintowsky et al., 1995), mixed murine primary spinal cord (Festoff et al., 1996), mouse motoneuron cell line, or primary (moto)neuron (Smirnova et al., 1998a) cultures to picomolar concentrations of thrombin resulted in a significant dose-dependent decrease in cell survival. However, thrombin concentrations in the picomolar range were without significant effects on our embryonic chick spinal motoneuron cultures (data not shown), suggesting a lower expression of thrombin receptors and/or sensitivity of embryonic avian spinal motoneurons to human α-thrombin compared with rodent hippocampal or motoneurons. Treatment of embryonic chick motoneuron cultures with 1–1000 nM human α-thrombin resulted in a dose-dependent decrease in motoneuron survival when examined 48 hr after plating (Fig. 2*A*). Neuronal survival after treatment with throm-

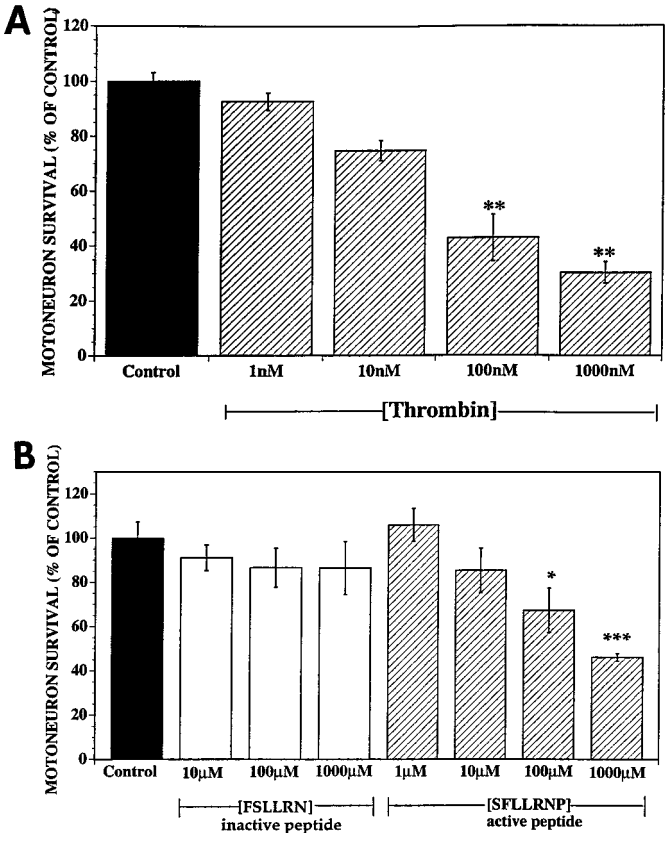

Figure 2. Motoneuron survival (mean ± SEM) in 48 hr cultures after treatment with different concentrations of either human α-thrombin (*A*), inactive FSLLRN, or active SFLLRNP (*B*). Agents were added 2 hr after the initial plating, and cultures were examined for survival 48 hr after the initial plating time. Untreated control motoneurons were grown in L-15 media, and positive controls were treated with soluble chick skeletal muscle extracts (CMX) at 14 μg/ml (data not shown). $^{*}p < 0.05$; $^{**}p < 0.01$ versus control; $^{***}p < 0.01$ versus 100 μM; $n = 3$ separate experiments performed in triplicate.

bin concentrations ≥100 nM was significantly decreased ($p < 0.01$) in comparison with that in the control cultures that were grown solely in L-15 culture media (Fig. 2*A*). Further addition of thrombin after the initial thrombin treatment did not result in increased motoneuron death (data not shown). To determine whether the effects of thrombin on motoneurons were mediated via PAR-1, we added the synthetic thrombin receptor fragment SFLLRNP that has been shown previously to activate PAR-1 in platelets (Seiler et al., 1992) and astrocytes (Beecher et al., 1994) to motoneuron cultures. As a negative control, parallel cultures were treated with the inactive peptide FSLLRN that includes a permutation of the first two amino acids, S and F, of the active receptor agonist (Vu et al., 1991). SFLLRNP produced a dose-dependent decrease in motoneuron survival (Fig. 2*B*) similar to that observed with thrombin (Fig. 2*A*), whereas inactive FSLLRN had no effect on cell survival (Fig. 2*B*). However, as reported in previous studies (Beecher et al., 1994; Vaughan et al., 1995), much higher (100–1000–fold) concentrations of SFLLRNP were required to obtain results similar to those observed with thrombin treatment. Concentrations of SFLLRNP that significantly reduced motoneuron survival were in the micromolar range (Fig. 2*B*), whereas thrombin affected cells in the nanomolar range (Fig. 2*A*). This difference may be caused by the fact that a thrombin molecule can cleave and activate several PAR-1 molecules, whereas one molecule of SFLLRNP can bind to and activate only one PAR-1 (Ishii et al., 1993). Alternatively, it is possible that the receptor peptide is less efficacious than is the protease because of steric hindrance (Smith-Swintowsky et al., 1997), because the exogenous peptide is likely to interact with the intact (noncleaved) N terminal of the receptor. Nevertheless, the finding that SFLLRNP affects cell survival (Fig. 2*B*) suggests that avian spinal cord motoneurons express functional PAR-1.

Thrombin altered the pattern of neurite outgrowth

To characterize further the deleterious effects of thrombin on motoneurons, we examined aspects of neurite outgrowth as an indicator of morphological differentiation and/or alteration that could be correlated with cell survival and/or degeneration after 48 hr in culture. The cultures were examined for neurite length and the number of primary branches that occurred on the longest neurite of each motoneuron. The results show that compared with control cultures (Fig. 3*A*), thrombin altered the morphological variations of the neurites (Fig. 3*B*). Thrombin-treated motoneurons had either one or two very long neurites or several short ones (Fig. 3*B*). Quantitatively, thrombin significantly decreased ($p < 0.001$) the mean neurite length (Fig. 4*A*) and the number of primary branches that occurred on the longest neurite of the motoneurons (Fig. 4*B*) compared with that of controls. These effects of thrombin on neurites were dose-dependent, with effective concentrations of the protease ranging from 1 to 1000 nM (Fig. 4*A*,*B*). However, because our initial cultures were 75–85% enriched in motoneurons, it is possible that the few surviving cells after treatment with thrombin were inter or commissural neurons. This is unlikely because 70% of the remaining cells stained positive for Islet-1 after thrombin treatment (data not shown). However, we cannot exclude the possibility that thrombin down-regulates expression of the Islet-1 antigen in some of the surviving motoneurons.

Hirudin attenuated the effects of thrombin on cultured motoneurons

Examination of motoneuron cultures 48 hr after incubation with different concentrations of hirudin (1–1000 nM) shows that, alone, this specific thrombin inhibitor did not affect neurite outgrowth (Fig. 3*C*; quantitative data not shown) nor did it affect the survival of motoneurons (Fig. 3*E*). Cotreatment with hirudin, however, attenuated the deleterious effects of thrombin (Fig. 3*D*,*E*). Previous or simultaneous treatment of the cultures with 100 nM hirudin resulted in a complete prevention of the motoneuron death induced by 100 nM thrombin (Fig. 3*E*). Treating the cultures with hirudin at 24 hr after incubation with thrombin at $t = 0$ only partially prevented thrombin-induced motoneuron cell death (Fig. 3*E*).

Thrombin induced apoptotic motoneuron cell death

Using the TUNEL cytochemistry, we show that thrombin induced chick motoneuron death by an apoptosis-like mechanism (Fig. 5). Cell cultures collected as early as 6 hr after thrombin treatment stained positive for DNA fragmentation *in situ* (Fig. 5*C*), whereas control cultures were all TUNEL-negative at this time point (Fig. 5*A*). Furthermore, the TUNEL-positive nuclei appeared to shrink over time, so that by 18 hr after thrombin addition, labeled cell nuclei (Fig. 5*D*) were much smaller than those examined at 6 hr (Fig. 5*C*). These two observations (DNA fragmentation and cellular shrinkage) were shown to be hallmark features of apoptotic cell death (Oppenheim, 1991; Wyllie, 1981). Counting positively labeled versus nonlabeled cells at 12 hr re-

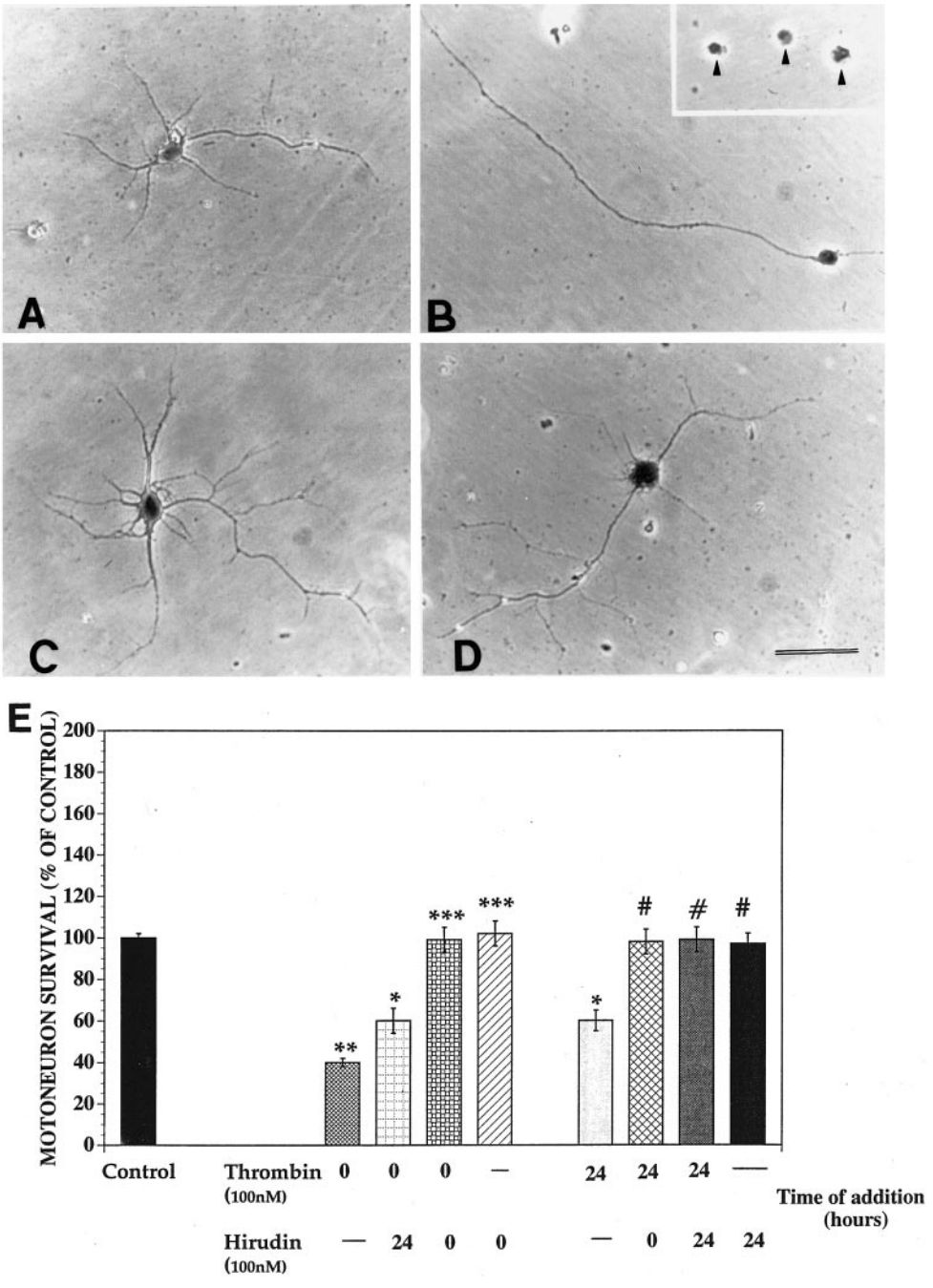

Figure 3. *A–D*, Photomicrographs of 2-d-old motoneurons cultured in L-15 media alone (control) (*A*) or in L-15 media supplemented with 100 nM thrombin (*B*), 100 nM hirudin (*C*), or 100 nM hirudin and 100 nM thrombin (*D*). Note that the thrombin-treated motoneuron exhibits unbranched neurites (*B*), whereas the cells treated with either hirudin alone (*C*) or hirudin and thrombin (*D*) appear similar to the control (*A*). The *inset* in *B* shows degenerating motoneurons (*arrowheads*) after treatment with thrombin. Scale bar, 60 μm. *E*, Surviving motoneuron numbers (mean ± SEM) in 48 hr cultures after treatment with either 100 nM thrombin, 100 nM hirudin, or combinations of these agents at different times. Thrombin and/or hirudin were added to cell cultures at either $t = 0$ or $t = 24$ hr, and all the cultures were examined 48 hr after the initial plating. Cultures treated with thrombin alone at $t = 0$ ($^{**}p < 0.01$) or $t = 24$ hr ($^{*}p < 0.05$) were significantly different from controls. Cotreatment with hirudin completely prevented thrombin-induced death of motoneurons, whereas addition of hirudin at $t = 24$ hr only partially saved the cells treated with thrombin at $t = 0$. The *short horizontal lines* represent no addition of either thrombin or hirudin. $^{***}p < 0.001$ and $\#p < 0.01$ versus thrombin treatment at $t = 0$ and $t = 24$ hr, respectively.

vealed that ~30% of the cells were TUNEL-positive in control cultures (Fig. 5*E*), consistent with previous findings (Milligan et al., 1994). However, after treatment with 100 nM thrombin, the proportion of TUNEL-positive motoneurons significantly increased, compared with control cultures, to 50% (Fig. 5*E*).

Furthermore, TEM was used to examine the ultrastructural changes that occurred during thrombin-induced cell death (Fig. 6*A–C*). Twelve hours after incubation with thrombin (100 nM), motoneuron cultures were collected and processed for TEM analysis. In contrast to healthy control motoneurons, which show no signs of intracellular organelle degeneration (Fig. 6*A*), motoneurons treated with thrombin showed the typical ultrastructural features of apoptosis (Fig. 6*B*,*C*). These included chromatin condensation in the nucleus (Fig. 6*B*) and breakdown and incorporation of the cytoplasmic inclusions into apoptotic bodies (Fig. 6*B*,*C*) (see also Chu-Wang and Oppenheim, 1978). In addition, there were some nonmembrane-bound particles that may be the result of membrane weakening and tearing over time and may be present because of the lack of phagocytic activity *in vitro* (*double arrows* in Fig. 6*B*,*C*).

Caspase inhibitors prevented the effects of thrombin on cultured motoneurons

The mechanisms underlying thrombin-induced neuronal cell death are not yet completely understood. In a recent study, thrombin-induced apoptosis in hippocampal neurons was linked to PAR-1 activation of the 21 kDa *ras* oncogene GTPase RhoA (Donovan et al., 1997). The inhibition of caspase activation in

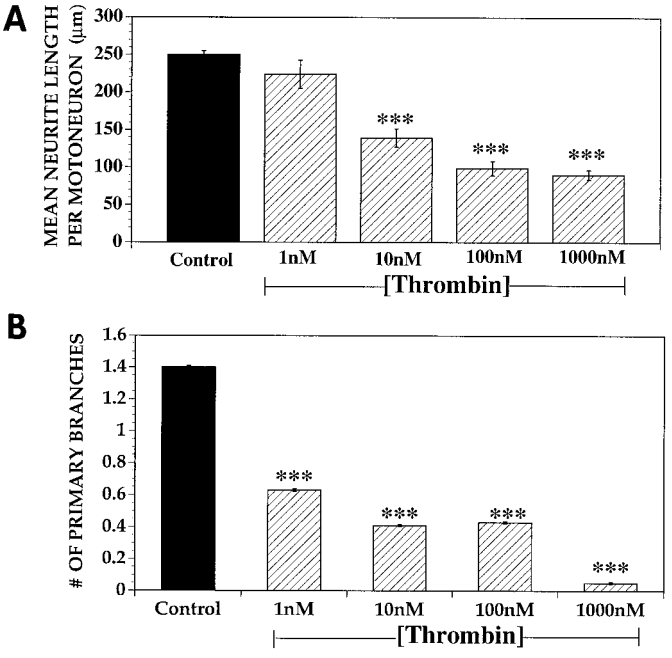

Figure 4. *A*, Mean neurite length (± SEM) per motoneuron in 48 hr cultures after treatment with different concentrations of thrombin (1–1000 nM). Thrombin was added 2 hr after the initial plating, and the cultures were stained with anti-β-tubulin antibodies 48 hr after the initial plating, as described in Materials and Methods. Control cultures were kept in L-15 media. *B*, The number of primary branches (mean ± SEM) that occurred on the longest neurite in the chick motoneuron cultures at 48 hr after treatment with thrombin. $^{***}p \leq 0.001$ for thrombin treatment versus control in *A* and *B*; $n = 100$ motoneurons examined per group.

pheochromocytoma 12 (PC12) cells by serine protease inhibitors suggests a role for caspases in the action of serine proteases (Stefanis et al., 1997). However, a recent study has shown that thrombin is a death signal in both a murine motoneuron cell line and in embryonic primary (moto)neurons, via activation of caspase-3 (CPP32) (Smirnova et al., 1998a).

To determine whether caspases were active players in the chick motoneuron cell death cascade induced by thrombin, we treated the motoneuron cultures with either the caspase-1 inhibitor YVAD-CHO (1 μM) or the caspase-3 inhibitor DEVD-CHO (10 μM). The results show that either of these agents increased cell survival by 90% in comparison with control cultures (Fig. 7), in agreement with previous findings (Milligan et al., 1995; Smirnova et al., 1998a; Li, Prevette, Oppenheim, and Milligan, personal communication). Motoneuron survival was not additive after treatment with both inhibitors (data not shown). However, cotreatment of the cultures with either of these inhibitors completely prevented the death of motoneurons induced by thrombin (Fig. 7). Furthermore, motoneuron cell death induced by the PAR-1 agonist SFLLRNP was also prevented by cotreatment with the caspase inhibitor DEVD-CHO (data not shown). Together, these results suggest the involvement of the caspase pathway in the deleterious effects induced by PAR-1 activation on avian spinal motoneurons in culture.

DISCUSSION

In the present study, we examined whether embryonic chick spinal motoneurons express functional PAR-1 and whether thrombin and a synthetic PAR-1 agonist affect motoneuron survival and morphological differentiation in highly enriched cultures. We found that thrombin significantly decreased motoneuron survival (Fig. 2*A*) and inhibited neurite outgrowth (Fig. 4) in a dose-dependent manner. The neurodegenerative effects of thrombin seemed to involve mechanisms associated with apoptosis (Figs. 5, 6) and to implicate a specific cell surface receptor, because a synthetic thrombin receptor-activating peptide elicited the same deleterious changes as the protease (Fig. 2*B*). In addition, treatment with two different caspase inhibitors, YVAD-CHO and DEVD-CHO, prevented thrombin-induced motoneuron cell death (Fig. 7), implicating caspase activation in the mechanisms underlying the action of PAR-1 activation on motoneurons .

Although levels of CNS prothrombin or thrombin are still unknown, plasma levels of (pro)thrombin are reported to be between 1 and 5 μM (Walz et al., 1985), i.e., significantly higher than the concentrations of thrombin that affected motoneuron survival in the present study. It has been reported previously that concentrations of thrombin above 1 nM cause a dose-dependent neurite retraction in neuroblastoma cells (Gurwitz and Cunningham, 1988). However, picomolar concentrations of thrombin are mitogenic for astrocytes (Low et al., 1982) and toxic for hippocampal neurons (Smith-Swintowsky et al., 1995). We found thrombin to be toxic for motoneurons in a dose-dependent manner between 1 and 1000 nM, as indicated by decreases in motoneuron survival and neurite outgrowth. Similar concentrations of thrombin have been shown to be toxic to murine (moto)neurons and a motoneuronal cell line in culture (Smirnova et al., 1998a).

Previous findings suggest that PAR-1 mediates the activities of thrombin on different cell types. Furthermore, PAR-1 agonists can inhibit neurite outgrowth activity in neuroblastoma cells in culture similar to treatment with thrombin (Jalink and Moolenar, 1992; Suidan et al., 1992). The present results showing that the synthetic peptide SFLLRNP decreases cell survival in a manner comparable with that observed with thrombin treatment further support the idea that embryonic chick motoneurons express functionally active PAR-1. Interestingly, we have recently determined that PAR-1 shares 70% homology with Fas-associated death domain, which has been shown to mediate Fas-induced cell death via caspase activation (for review, see Barinaga, 1996).

Under the conditions of our cultures, we were able to measure decreases in neurite outgrowth after addition of thrombin. It seems that thrombin causes selective alterations, but not a complete inhibition, of the pattern of neurite outgrowth, because it decreases the average length of all neurites and the number of secondary branches without affecting the growth of the longest neurite. Although the mechanisms by which thrombin exerts this action on neurite outgrowth are still unclear, recent reports suggest the involvement of a protein kinase C-dependent pathway (see Shea, 1995; Shea et al., 1995). Previous studies have shown that thrombin degrades laminin and fibronectin (for review, see Monard, 1988), which are important extracellular matrix components for neurite development. Therefore, thrombin treatment may degrade the laminin coating of the culture dishes, thus affecting neurite outgrowth. This explanation seems unlikely because pretreatment of the culture dishes with thrombin followed by extensive washing with L-15 media before plating the motoneurons did not affect the survival of the cells, nor did it alter neurite outgrowth (data not shown). It is, however, likely that thrombin, through PAR-1, activates RhoA, which has been implicated in neurite retraction (Jalink et al., 1994).

Smith-Swintowsky et al. (1995) have shown that administration of thrombin affected calcium homeostasis. It is well established

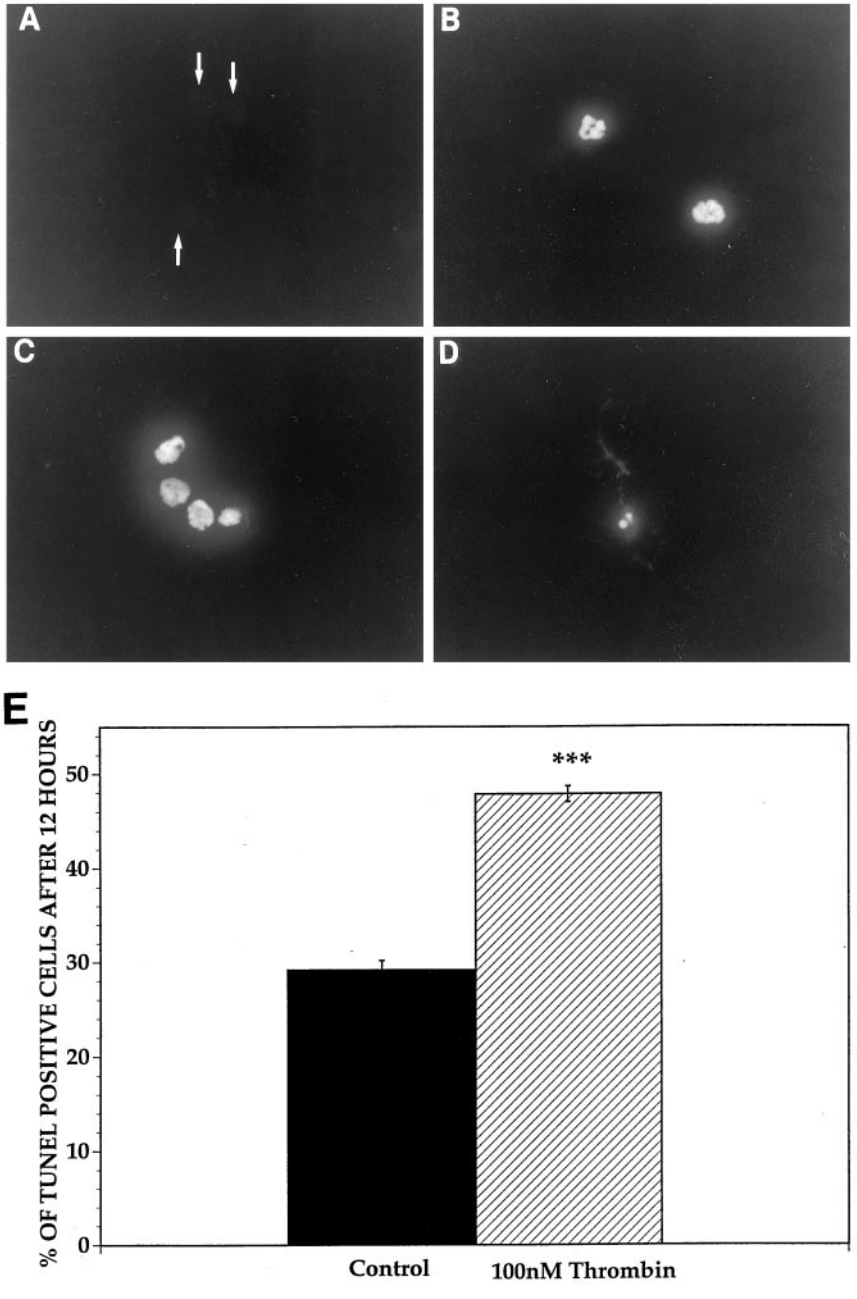

Figure 5. *A–D*, Photomicrographs of cultured chick spinal motoneurons taken at 60× magnification after TUNEL cytochemistry. *A*, A negative control culture in which the motoneurons (*arrows*) were grown solely in L-15 media and were TUNEL-labeled after 6 hr. *B*, Positive control cells grown in complete medium and treated with 1 mg/ml DNase I for 10 min before TUNEL labeling to produce DNA fragmentation. *C*, TUNEL-positive nuclei of motoneurons 6 hr after incubation with 100 nM thrombin. *D*, A TUNEL-positive motoneuron nucleus 18 hr after incubation with 100 nM thrombin. *E*, Percentage of TUNEL-positive motoneurons at 12 hr after culture in L-15 media alone (control) or with 100 nM thrombin. Approximately 30% of the control cells were TUNEL-positive, whereas, after thrombin treatment, the number of labeled cells increased to 50%. Data are mean ± SEM from three different experiments; $^{***}p < 0.001$ for thrombin treatment versus control.

that sustained increases in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) can lead to changes in cytoarchitecture, cell damage, and cell death (Siman et al., 1989; Yanagihara et al., 1990; Mattson et al., 1992). Using Ca^{2+}-imaging analysis, Smith-Swintowsky et al. (1995) have shown that thrombin causes an increase of $[Ca^{2+}]_i$ and induces neurodegeneration in rat hippocampal neuron cultures. Similar findings have also been reported in the murine motoneuron cell line NCSSC19 and in primary mouse (moto)neuron cultures (Smirnova et al., 1998b). Together with the reports that local increases in $[Ca^{2+}]_i$ lead to neurite retraction, whereas agents that lower $[Ca^{2+}]_i$ lead to neurite outgrowth and cell survival (Mattson et al., 1988, 1989; Mattson, 1993), these observations suggest that increases in $[Ca^{2+}]_i$ may contribute to thrombin-mediated neurite retraction.

Inhibitors of caspases prevent the death of motoneurons after trophic factor deprivation *in vitro* and during the period of programmed cell death *in vivo* (Milligan et al., 1995; Li, Prevette, Oppenheim, and Milligan, personal communication). A rapid induction of caspase-3 (CPP32) activity, but not caspase-1 or interleukin 1β-converting enzyme, occurs after trophic factor withdrawal in PC12 cells (Stefanis et al., 1996). In addition, inhibitors of serine proteases that act upstream of the caspases, such as 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and *N*(α)-*p*-tosyl-L-lysine chloromethyl ketone, inhibited the CPP32 and nedd-2-cleaving activities that are induced after withdrawal of trophic support in PC12 cells (Stefanis et al., 1997). A recent report shows caspase-3 activation in murine clonal and primary motoneurons after thrombin treatment (Smirnova et al., 1998a), and the present findings show that cotreatment with YVAD-CHO (a caspase-1 inhibitor) or DEVD-CHO (a caspase-3 inhibitor) inhibits the effects of thrombin. These findings support the involvement of members of the caspase family of

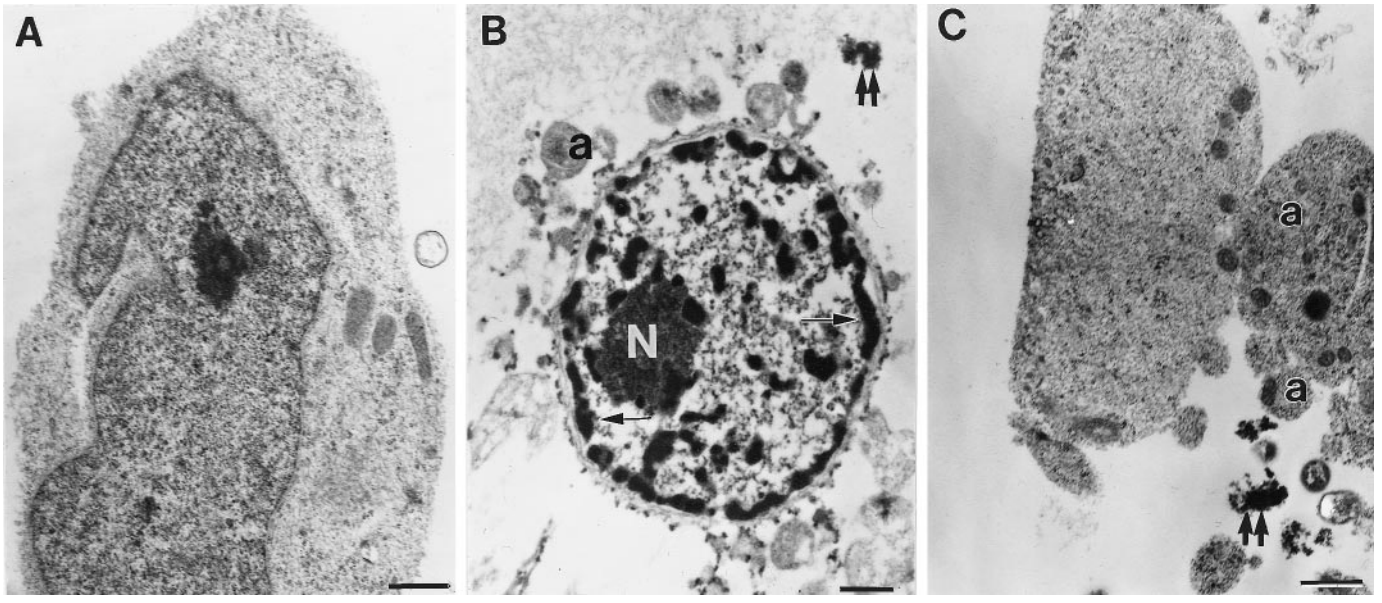

Figure 6. Electron micrographs depicting a healthy control motoneuron from a culture treated with 14 μg/ml CMX (*A*) and cultured motoneurons at 12 hr after treatment with 100 nM thrombin (*B*, *C*). In *B*, the plasma membrane, cytosol, and organelles have all been incorporated into apoptotic vesicles (*a*), and all that remain are the nucleus, which has become small and rounded with an eccentrically placed nucleolus (*N*), and chromatin condensation (*single arrows*) around the nuclear membrane. *C* shows a larger apoptotic vesicle in the process of forming smaller apoptotic vesicles. The apparently poor membrane preservation of the cells has been suggested to be inherent to glutaraldehyde-fixed free (cultured) cells, in contrast to tissue samples (i.e., Robinson et al., 1987). The presence of nonmembrane-bound particles (*double arrows* in *B*, *C*) released in the culture media may be attributable to the lack of phagocytic activity. Scale bars: *A*, *C*, 185 nm; *B*, 14 nm.

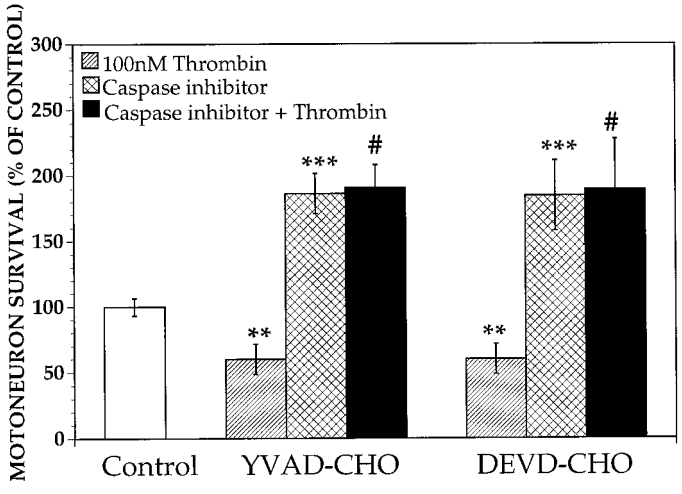

Figure 7. Motoneuron numbers (mean ± SEM) in 48 hr control cultures (*open bar*) and in cultures treated with either 100 nM thrombin (*hatched bars*), 1 μM YVAD-CHO, or 10 μM DEVD-CHO (*cross-hatched bars*) or cotreated with thrombin and either caspase inhibitor (*black bars*). Agents were added 2 hr after plating, and cells were counted 48 hr after the initial plating. Control cultures were grown in L-15 media. Treatment with 100 nM thrombin significantly decreased cell survival (**$p < 0.01$ vs control), whereas treatment with either YVAD-CHO or DEVD-CHO alone increased motoneuron survival by 180% (***$p < 0.001$ vs control). However, the decrease in motoneuron survival after 100 nM thrombin treatment was completely prevented by cotreatment with either YVAD-CHO or DEVD-CHO (#$p < 0.001$ vs 100 nM thrombin treatment); $n = 3$ experiments performed in triplicate.

proteases in the induction of motoneuron death by thrombin. These results also link thrombin as an extracellular signal to activation of intracellular caspase pathways via the cell surface G-protein–coupled receptor PAR-1.

However, whether PAR-1 and its physiological ligand thrombin normally play a role in the nervous system function or pathology *in vivo* is still not clear. Both thrombin and its receptor have been shown to be expressed in different regions of the CNS (Dihanich et al., 1991; Weinstein et al., 1995). The potent thrombin inhibitor, protease nexin-1 (PN-1), is also present at significant levels in the CNS (Mansuy et al., 1993; Reinhard et al., 1994) and skeletal muscle tissue (e.g., Festoff et al., 1994). We have shown previously that exogenous PN-1 prevents the death of spinal motoneurons during the period of programmed cell death in the developing chick embryo and after axotomy in the neonatal mouse (Houenou et al., 1995). Collectively, these observations suggest that serpins, such as PN-1, modulate the activity of thrombin-like proteases *in vivo* to control neuronal cell function, including neurite extension, synaptic plasticity, and cell viability (Suidan et al., 1992; Mansuy et al., 1993; Houenou et al., 1995; Smith-Swintowsky et al., 1995; Tsirka et al., 1995; Vaughan et al., 1995). Consequently, changes in the serpin–protease equilibrium and/or disregulation of PAR-like receptor expression may lead to the pathology of the nervous system. In support of this idea is the finding that PAR-1 expression is significantly increased in the spinal cord of the mouse mutant *wobbler*, a suggested model of motoneuron disease, compared with control (Salcedo et al., 1998). Further support of this notion is the association of an increase in thrombin-like proteases, a reduction in synaptogenesis, and a decrease in neuronal cell viability in the brain of patients with Alzheimer's disease (Wagner et al., 1989). In addition, mice with the disrupted PN-1 gene develop abnormal epileptic activity and hippocampal long-term potentiation (Lüthi et al., 1997), further indicating a role for serine proteases, serpins, and PAR-like receptors in CNS function.

In conclusion, we have shown that (1) the serine protease thrombin induces degeneration and death of developing avian spinal motoneurons in highly enriched cultures; (2) like mouse

motoneurons (Smirnova et al., 1998a), avian spinal motoneurons express functional thrombin receptors (PAR-1), whose activation leads to a reduction in cell viability; and (3) the deleterious effects of thrombin or PAR-1 activation on avian motoneurons seems to be mediated via pathways requiring caspase activities, as also shown in mouse clonal and primary (moto)neurons *in vitro* (Smirnova et al., 1998a). These findings suggest a new role for PAR-1 as a mediator of apoptosis in the nervous system. Taken at their apparent value, our findings, together with previous reports, suggest that a balance between thrombin-like proteases, their receptors, and their naturally occurring inhibitors may play a significant role in neuronal cell survival, differentiation, and plasticity during development, after injury, and/or in pathology of the CNS and PNS (Smirnova et al., 1994; Festoff et al., 1996; Turgeon and Houenou, 1997).

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