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AVIAN MOTONEURON APOPTOSIS FOLLOWING PROTEASE ACTIVATED RECEPTOR -1 ACTIVATION IS TRIGGERED NEITHER THROUGH INCREASED FREE INTRACELLULAR CALCIUM LEVELS NOR DECREASED NEUROTROPHIC SUPPORT

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ABSTRACT

Activation of protease activated-receptor 1 (PAR-1) has been shown to decrease motoneuron survival. This G protein receptor is activated when thrombin cleaves the original NH_2-terminus generating a new sequence that binds to and activates a transmembrane receptor portion. However, the signaling pathway following motoneuron PAR-1 activation has not been elucidated. Research suggests that a calcium set point, a balance between Ca^{2+}_i and neurotrophic factors, is necessary for motoneuron survival. The first study hypothesized that PAR-1 activated motoneuron death *in vitro* would correspond with increased levels of Ca^{2+}_i. In addition, motoneuron death often results in the concurrent loss of target muscle cells, which are a known source of neurotrophic factors. However, such an event would induce a greater degree of motoneuron cell death than we had previously observed. Therefore, the second study hypothesized that PAR-1 activation *in vivo* would not affect skeletal muscle cell survival. A Tukey's post-hoc test of the Ca^{2+}_i assay did not support the hypothesis that PAR-1 activation would lead to increased Ca^{2+}_i. However, histological studies did support the hypothesis that *in vivo* activation of PAR-1 does not alter the number of surviving skeletal muscle cells. Quantitative western blot analysis of neurotrophic factors did not detect any differences in their levels before or after PAR-1 activation. Taken together, these data suggest that Ca^{2+}_i does not serve as a second messenger in the cell death pathway following activation of avian motoneuron PAR-1, while target skeletal muscle cells and potential levels of muscle derived growth factors are unaltered.

INTRODUCTION

One known trigger of neuronal apoptosis is thrombin, a serine protease whose most-understood role is the conversion of fibrinogen to fibrin during the coagulation cascade (Esmon, 1993). However, mRNA for prothrombin, thrombin's zymogen precursor, has been localized within the developing nervous system, suggesting that thrombin is produced (Dihanich et al., 1991) and is functional (for reviews see Suidan et al., 1992; Turgeon et al., 2000) within the nervous system itself. Thrombin has been shown to cause apoptosis in avian motoneurons, both *in vitro* (Turgeon et al., 1998) and *in vivo* (Turgeon et al., 1999), and to induce neurite retraction (Gurwitz and Cunningham, 1988; Suidan et al., 1992) through activation of a cell surface thrombin receptor.

To date, several thrombin receptors have been identified, and these receptors are all members of a family of membrane proteins known as protease-activated receptors (PARs). Thrombin has been shown to activate both PAR-1 and PAR-3, while another serine protease, trypsin, has been shown to primarily activate PAR-2 (Déry et al., 1998; Ubl et al., 1998). Furthermore, both thrombin and trypsin have been shown to activate PAR-4 (Xu et al., 1998). Each of these receptors has a seven-transmembrane domain and is activated by a tethered ligand. The protease, itself, does not directly activate the receptor; instead, it cleaves the extracellular amino terminus exposing a new amino terminus that undergoes a conformational change allowing it to bind to another region within its own receptor, thus activating it (Vu et al., 1991). All PARs are classified as G protein-linked and the subsequently activated second messenger depends on the specific PAR (Kanthou and Benzakour, 1995). While PARs are present in many cell types throughout the vertebrate body, studies relevant to this project have established that both PAR-1 (Turgeon et al., 1999) and its mRNA (Niclou et al., 1994) are present within the vertebrate nervous system.

The amino acid sequence of PAR-1's tethered ligand is serine-phenylalanine-leucine-leucine-arginine-asparagine-proline (SFLLRNP) (Vu et al., 1991). The addition of a tethered ligand sequence has the ability to activate its cognate receptor without cleaving the original receptor (Vu et al., 1991) so that use of these sequences is beneficial to determine the activation of a specific PAR. For example, the addition of SFLLRNP has been shown to mimic the neurodegenerative effects of thrombin on motoneurons *in vitro* (Turgeon et al., 1998) and *in vivo* (Turgeon et al., 1999). Since PAR-1 is known to be activated only by this specific peptide, these studies provide strong evidence that PAR-1 is indeed the receptor by which thrombin initiates the cell death process in motoneurons. Because PARs are known to activate a variety of G protein subunits, multiple second messenger pathways are possible and within different cell types PAR activation may produce different responses (Déry et al., 1998). Activation of motoneuron PAR-1 by thrombin has been shown to initiate apoptosis, presumably through activation of the caspase cascade (Turgeon et al., 1998). However, the specific intracellular signaling pathway linking PAR-1 to caspase activation in neuronal apoptosis is unknown. While PAR-1 has been shown to activate different second messengers depending on the cell type the most plausible second messenger pathway is the phospholipase C (PLC)/inositol triphosphate (IP_3) cascade through which IP_3 leads to increased levels of intracellular calcium (Ca^{2+}_i) and protein kinase C (Nichols et al., 2001).

Tong et al. (1996) demonstrated that below-normal concentrations of Ca^{2+}_i in cultured sensory neurons led to apoptosis, which was preventable by the addition of nerve growth factor (NGF) that raised the Ca^{2+}_i concentration back to normal. This research led to the formulation of the calcium set-point hypothesis, which proposes that normal levels of Ca^{2+}_i suppress the cell death pathway, while low levels of Ca^{2+}_i necessitate that growth factors be present to override the apoptosis signal (Tong et al., 1996). In support of this hypothesis, Koike and Tanaka (1991) showed that sympathetic neurons also depend on increased Ca^{2+}_i concentrations in the absence of NGF. Furthermore, Smith-Swintosky et al. (1995b) demonstrated that abnormally high Ca^{2+}_i concentrations corresponded with thrombin-induced cell death in hippocampal neurons, which correlated thrombin-induced cell death with changes in Ca^{2+}_i concentrations. The researchers modified the calcium set-point hypothesis by suggesting that abnormally high, as well as abnormally low, Ca^{2+}_i concentrations contributed to neuronal cell death.

Complicating the understanding of the signaling process is the role of the myriad growth factors that influence the vertebrate nervous system. The neurotrophic hypothesis states that neuronal survival depends on access to neurotrophic factors (Houenou and Oppenheim, 1994). Research has also shown that certain neurotrophic factors (NTFs) can at least partially rescue motoneurons from apoptosis after thrombin exposure *in vitro* (Turgeon and Houenou, 1999) and after axotomy *in vivo* (Oppenheim et al., 1995). These growth factors include brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF). Both BDNF and CNTF are synthesized by the skeletal muscle targets and have been shown to inhibit the effects of PAR-1 activation (Turgeon and Houenou, 1999).

Removal of embryonic limb buds eliminates the target skeletal muscle cells for the lumbar spinal cord motoneurons and subsequently eliminates the multiple neurotrophic factors necessary for neuronal survival. Caldero et al. (1998) demonstrated that following limb bud removal in the chick embryo approximately 90% of motoneurons die in comparison to the 40-70% that die during normal programmed cell death events. Interestingly, grafting an extra limb onto a chick embryo reduces motoneuron death below the normal range (Milligan et al., 1994). Thus, the presence of growth factors plays a significant role in controlling motoneuron apoptosis. Therefore, it is possible that the motoneuron cell death observed following activation of PAR-1 *in vivo* is partly due to a loss of target skeletal muscle cells, since PAR-1 is localized to skeletal muscle cells during neuromuscular junction formation (Lanuza et al., 2003).

It is very possible that the neurotrophic rescue pathway and the thrombin death pathway have overlapping second messengers at some point(s) in their respective signal cascades (for review see, Turgeon and Houenou, 1999). The rescue pathway theoretically prevails by canceling the action of a second messenger in the death pathway. Evidence suggests that the rescue pathway may accomplish this by increasing the amount of certain intracellular caspase inhibitors (Perrelet et al., 2002) perhaps initiated in certain situations by increased Ca^{2+}_i concentrations.

The goal of this study was two-fold – first, to determine whether PAR-1 activation altered Ca^{2+}_i concentrations and second, to determine whether PAR-1 activation altered skeletal muscle cell survival. Chick lumbar motoneuron cultures treated with chicken muscle extract (CMX; positive control), NTFs (CNTF or BDNF), thrombin (+/- CMX or either NTF), or the synthetic PAR-1 activating peptide, SFLLRNP (+/- CMX or either NTF) were assessed for changes in Ca^{2+}_i. Chick embryos were also treated with SFLLRNP to access any changes in neurotrophic factor (BDNF, CNTF) concentrations and the number of surviving hindlimb skeletal muscle cells.

MATERIALS AND METHODS

Motoneuron cultures. Fertilized chicken (*Gallus gallus)* eggs were obtained from the Morgan Poultry Center at Clemson University (Clemson, SC) and incubated through embryonic day five (E5) at 37° C (Percival Scientific 36 Series incubator). Motoneurons were isolated and purified through careful dissection and differential centrifugation through metrizamide (Sigma, St. Louis, MO) and BSA (Sigma) gradients (Milligan et al., 1994). The ventral portion of embryonic chick lumbar spinal cords were sub-dissected from E5 embryos, dissociated in a 30 mM suberimidate (Sigma) solution (200 mg dimethyl

suberimidate dihydrochloride, 1M KOH, 85% 0.1M TRIS, and 1% DMSO), and loaded onto a 6.8% metrizamide (w/v in culture medium; Sigma). After centrifugation at 200 x g (Beckman Centrifuge) for 15 min, large cells, i.e. motoneurons, remained in the top half of the gradient, whereas smaller cells, such as interneurons and glial cells, would sediment in a pellet at the bottom of the tube. Motoneurons were collected and grown at a density of 2,000 cells/ml in a defined serum-free medium (Leobovitz's L-15 medium; Gibco, Gaithersburg, MD) supplemented with sodium bicarbonate (25 mg/ml; Sigma), glucose (20mM; Sigma), progesterone ($2x10^{-8}$M; Sigma), sodium selenite ($3x10^{-8}$M; Sigma), conalbumin (0.1 mg/ml; Sigma), putrescine (10^{-4}M; Sigma), and penstrep (Gibco, Carlsbad, CA). The motoneurons were allowed to adhere to the plates for 2 hrs in the incubator before the addition of complete L-15 medium and the appropriate treatments. Culture dishes were pre-treated with the ECM components, 1% laminin (Sigma) and 0.15% poly-D-ornithine (Sigma), to mimic an *in vivo* system.

Thrombin administration to the assigned dishes resulted in final concentrations of 10 nM and 100 nM (Sigma). CMX prepared following the protocol previously described by Oppenheim et al. (1993) was added to achieve a final concentration of 14ng/?l in the appropriate cultures. Finally, BDNF or CNTF (Gibco) was added to appropriate cultures to produce a concentration of 20 ng/ml. In all, 12 cultures were established. After addition of treatments, the cultures were incubated for approximately 24 hours at 37° C and 5% CO2.

Intracellular calcium assay. Intracellular Ca^{2+} was measured using Sigma Kit 587-M. The working solution from the kit was made per instructions, and 1 ml of this solution was added to 2.9 ml polystyrene cuvettes. Just prior to the assay 1,000 living cells were collected from each of the treatment groups and placed in a 15 ml centrifuge tube. In order to lyse the motoneurons and release Ca^{2+}_{i}, 20 ìl of 0.1% Triton x-100 was added to each centrifuge tube for 2 minutes. Then, 10 ì1 of medium from each culture was pipetted into a cuvette containing the working solution. In addition to samples from the 12 cultures, one cuvette contained 10 ìl of dH_2O to be used as a blank, one cuvette contained 10 ì 1 of a 2.50 mM Ca^{2+} standard (Sigma), and one cuvette contained 10 ì 1 of only complete L-15 medium.

A diode-array spectrophotometer (Hewlett-Packard 8453) was used to determine the absorbance of each sample at a wavelength of 575 nm. Absorbance values were then used to calculate actual Ca^{2+} concentrations with this equation: $[Ca^{2+}]$ = (absorbance of sample/ absorbance of Ca^{2+} standard) x $[Ca^{2+}$ standard]. Results were analyzed using a 95% confidence interval, one-way ANOVA and a Tukey's post-hoc test.

Hindlimb skeletal muscle histology. Fertilized *Gallus gallus* eggs were incubated at 37° C. On E3, small windows were cut into the shells and covered with clear tape to prevent contamination. Beginning on E5, 200 ìl of 100 ìM SFLLRNP (a concentration previously shown to induce avian motoneuron apoptosis *in* ovo [Turgeon et al., 1999]; Bachem, Torrance, CA; in 1x PBS) was added daily to embryos in the experimental group. Control embryos received 200 ìl of 1x PBS. On E10, the embryos were sacrificed and the right hindlimbs were carefully removed to ensure that each one was cut at the uppermost portion of the hip joint; toes were left intact. The limbs were placed into Carnoy's fixative (Fisher Scientific, Atlanta, GA) for 24 hours, after which they were placed into 70% ethanol for at least 24 hours. The limbs were paraffin embedded, serially sectioned into 10 ìm slices using a rotary microtome (American Optical820, Cambridge Scientific Products, Cambridge, MA), and stained with hemotoxylin and eosin. Each slide was coverslipped using Permount (Fisher

Scientific) and allowed to dry for 24 hours. Then, the three midpoint slices of each limb were examined and the number of skeletal muscle cells were determined at 400x magnification. Results were analyzed using an independent t-test with a 95% confidence interval.

Protein isolation and western blot analysis. *In* ovo embryos were treated as described above with either SFLLRNP or PBS. Hindlimb muscles from E10 chicks were removed at the hip joint. Proteins were extracted using lysis buffer containing aprotonin (Sigma) and leupeptin (Sigma) to prevent protein degradation. Cytosolic proteins (i.e., serine proteases) were isolated from the membrane-bound proteins (i.e., PARs) using differential centrifugation. Following separation, total protein concentration for the cytosolic proteins were determined using a Bradford Assay (Sigma) and UV-Diode Array Spectrophotometer (Hewlett Packard) to ensure equal protein loading during the separation procedure. Proteins were separated with a 10-20% SDS-PAGE (Cambrex, Walkersville, MD) and transferred to nitrocellulose paper. The transferred proteins were then immunoblotted with specific antibodies to CNTF and BDNF (R&D Systems, Minneapolis, MN). Quantitative analysis of the protein bands were determined using the Kodak Gel Documentation System.

RESULTS

Cytoplasmic intracellular calcium levels are not altered following PAR-1 activation. One-way ANOVA and a Tukey's post-hoc test of motoneuron cultures did not reveal any significant differences in the mean Ca^{2+}_i concentrations between the groups treated with PAR-1 agonists, CNTF or the control ($p = 0.06$; Table 1), whereas CMX and BDNF both significantly increased Ca^{2+}_i ($p=0.02$). Microscopic analysis of simultaneously established motoneuron cultures confirmed that PAR-1 was activated through the observation of increased neuronal cell death and that the CMX, BDNF, and CNTF were functional through the observation of decreased neuronal cell death (data not shown).

Table 1. Mean cytosolic calcium concentrations of motoneurons assayed 24 hrs following PAR-1 activation and/or treatment with neurotrophic factors (n=3).

Treatment	$[Ca^{2+}_i]$ mM $\pm$ SE
Control (no treatment)	1.17430 $\pm$ 0.12434
100nM Thrombin*	1.03081 $\pm$ 0.50978
100µM SFLLRNP*	1.02332 $\pm$ 0.51460
14ng/µl CMX**	2.33550 $\pm$ 0.37340***
20ng/ml CNTF**	1.02232 $\pm$ 0.52446
20ng/ml BDNF**	2.34751 $\pm$ 0.67231***
Thrombin + CMX	2.31344 $\pm$ 0.55132***
Thrombin + CNTF	1.02433 $\pm$ 0.53372
Thrombin + BDNF	2.37754 $\pm$ 0.57993***
SFLLRNP + CMX	2.33388 $\pm$ 0.63429
SFLLRNP + CNTF	1.00657 $\pm$ 0.53382***
SFLLRNP + BDNF	2.44385 $\pm$ 0.55297***

*Final concentration of thrombin or SFLLRNP added to E5 chick motoneuron cultures
**Represents the stock concentration, which was added in 80ml volumes

***$p \leq 0.02$ vs. control, thrombin, and SFLLRNP treated groups

Neither target skeletal muscle cells nor muscle-derived growth factors are altered following PAR-1 induced motoneuron cell death. Following SFLLRNP-induced motoneuron cell death no significant difference in muscle cell number existed between the groups treated with SFLLRNP or PBS (control; p = 0.365; Fig 1).

Furthermore, using SDS-PAGE and western blot analysis there were no detectable differences in CNTF or BDNF production following SFLLRNP treatment (Table 2).

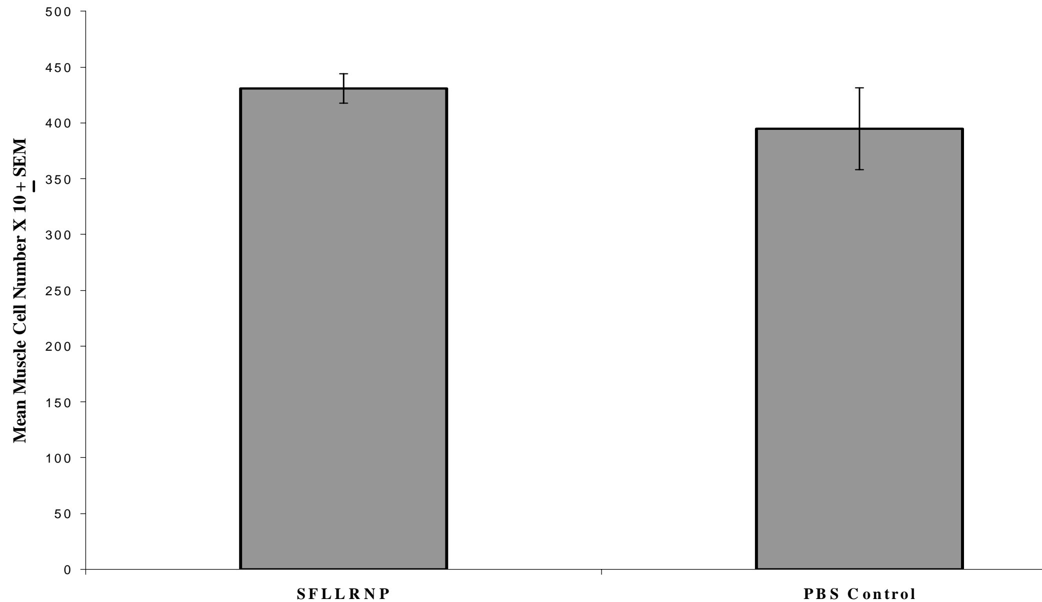

Figure 1. Mean number (± SEM) of iliotibialis muscle cells - as observed in cross-section from E10 chick hindlimbs. Embryos were given a daily dose beginning on E5 of either 200 ìL of 100 ìM SFLLRNP or 200 ìL of 1x PBS. All muscle cells within the most centralized region of three midpoint sections were counted at 400x magnification. A Student's t-test revealed no significant difference between the groups (p= 0.365; n = 6 organisms).

Table 2. Quantification of neurotrophic factors from E10 chick hindlimb skeletal muscle before and after PAR-1 activation with SFLLRNP*

Neurotrophic Factor	Concentration (mg/ml)	
	Before PAR-1 Activation	**After PAR-1 Activation**
CNTF	3.144	3.110
BDNF	4.526	4.563

*Total proteins from the right hindlimb of 20 embryos were removed, separated on a 4-12% tris-glycine gel, and quantified by the Kodak Gel Documentation System that compares the number of pixels in the standard lane bands to those of interest in the experimental lanes. n=3.

DISCUSSION

Cytoplasmic intracellular calcium levels are not altered following PAR-1 activation. The Ca^{2+}_i assay of the motoneuron cultures provided no statistical evidence that Ca^{2+}_i plays a role in the avian PAR-1-induced apoptosis-signaling pathway (Table 1). Since no significant changes in Ca^{2+}_i concentrations were observed, the calcium set-point hypothesis cannot yet be extrapolated to explain PAR-1 induced motoneuron cell death and suggests that IP_3 is not the second messenger employed following avian neuronal PAR-1 activation. Furthermore, previous studies have also failed to link CNTF activity to changes in Ca^{2+}_i (Stoop and Poo, 1996; Peterson and Brudin, 1999), whereas BDNF (Stoop and Poo, 1996) and CMX, which contains BDNF, were expected to increase Ca^{2+}_i levels confirming the efficacy of the assay.

There are several possibilities to explain why Ca^{2+}_i concentrations did not significantly change following PAR-1 activation. As mentioned previously, Tong et al. (1996) and Koike and Tanaka (1991) linked altered Ca^{2+}_i concentrations with sensory and sympathetic neuronal apoptosis most likely from the release of Ca^{2+} from the ER via PLC and IP_3. Furthermore, Smirnova at al. (1998) provided another important link by demonstrating that PAR-1 directly caused both short- and long-term elevation of Ca^{2+}_i in murine motoneurons via G proteins. They hypothesized that the elevated calcium levels were sufficient to cause cell death. However, since all of the research into the neuronal PAR-1 second messenger pathway has been performed in mammalian species and/or non-motoneurons, it is possible that the avian motoneuron PAR-1 acts through a different pathway.

Another possibility is that the Ca^{2+}_i assay accurately determined the concentration of free calcium ions, but that the apoptosis signaling process caused Ca^{2+}_i to be bound or sequestered. Often when Ca^{2+}_i functions as a second messenger, it binds strongly with calmodulin. The complex then functions itself as part of the signal cascade, often activating gene transcription factors via kinases (Lodish et al., 2000). Thus, free calcium ions are removed from the cytoplasm. Rosenthal et al. (1998) determined that apoptosis in keratinocytes depended on Ca^{2+}-calmodulin second messengers. Therefore, further studies are underway to examine the relationship between calmodulin levels and PAR-1 activation in avian motoneurons. Also, recent evidence demonstrates that mitochondria are able to sequester excess Ca^{2+}_i from the cytoplasm (Kruman and Mattson, 1999) suggesting the possibility that Ca^{2+}_i concentrations increased prior to the 24-hr assay but were not detected due to sequestering by the mitochondria.

It is also possible that previously observed increases in Ca^{2+}_i concentrations were not due to release from the ER but instead from ions entering the cell via membrane channels. Smith-Swintosky et al. (1995b) demonstrated that thrombin-induced death in rat hippocampal neurons corresponded with increased Ca^{2+}_i concentrations. However, when the neurons were cultured in a Ca^{2+}-free medium, thrombin did not cause cell death. Ubl et al. (1998) produced evidence that increased Ca^{2+}_i concentrations in astrocytes resulted from both the release of Ca^{2+} from intracellular stores and entry through the cell membrane. These observations indicate that a signal cascade independent of PLC and IP_3 at least partially accounts for Ca^{2+}_i flux in certain cell types. Since our cultures are grown in a relatively low Ca^{2+} concentration medium the decreased availability of extracellular calcium may partially account for the insignificant differences in Ca^{2+}_i concentrations. We are currently trying to determine what levels of calcium can be added to our medium to investigate this possibility without confounding the normal viability of the avian motoneurons.

A last possibility is that Ca^{2+}_i is not part of the PAR-1 –induced apoptosis cascade at all. There are several other potential signal pathways that can be initiated by a PAR linked to G proteins. One of these is the adenylate cyclase (AC)-cyclic AMP (cAMP) pathway where the G protein subunit activates AC to synthesize cAMP. The cAMP serves as a powerful second messenger that activates many cytoplasmic proteins and can alter gene expression. Activation of PAR-1 by SFLLRNP has been shown to decrease cAMP concentrations (Salman et al., 2003). One intriguing possibility is that both cAMP and Ca^{2+}_i might be part of the apoptosis cascade. Nichols et al. (2001) stated that Ca^{2+}-calmodulin complexes can regulate the activity of adenylate cyclase, and thus alter levels of cAMP. Thus, if Ca^{2+}-calmodulin complexes are determined to be altered during PAR-1 activation the next step would be to investigate the activity of adenylate cyclase.

In addition, Snider (1986) stated that neuroblastoma cells treated with thrombin experienced increased cyclic GMP synthesis that corresponded with increased activity of phospholipase A_2 and creation of arachidonic acid. While Ellis et al. (1999) reported that activation of PAR-1 inhibited phosphorylation of MAP kinase in endothelial cells. Thus, PAR-1 is apparently capable of initiating several different cascades in multiple cell types.

Neither target skeletal muscle cells nor muscle-derived growth factors are altered following PAR-1 induced motoneuron cell death. In agreement with Lubischer and Arnold (1995), we surmised that if muscle-derived growth factors had been unable to rescue the motoneurons from the SFLLRNP administration, then skeletal muscle atrophy and death would have occurred in the absence of innervation. Previous studies have shown that prior or co-treatment of motoneurons with muscle-derived growth factors and PAR-1 agonists inhibited PAR-1 induced motoneuron cell death (Turgeon et al., 1999), suggesting that skeletal muscle targets and/or growth factor levels were unchanged despite the previous localization of PAR-1 to skeletal muscle cells (Lanuza et al., 2003). This present study verified this hypothesis. There was no significant difference in hindlimb muscle cell number (Fig. 1) or growth factor concentrations (Table 2) between embryos treated with SFLLRNP and control embryos. Thus, it appears that muscle-derived growth factor availability is unaltered and remains present to perhaps partially override PAR-1 induced cell death. Preliminary studies from this lab indicate an increase in motoneuron cell death following simultaneous NTF inhibition and PAR-1 activation, above the motoneuron cell death observed with PAR-1 activation alone (unpublished results).

The medical implications of understanding how thrombin and/or PAR-1 activation causes motoneurons to die are of utmost significance. Both spinal cord trauma and hemorrhagic stroke rupture the blood-brain barrier causing blood to come into direct contact with nervous tissue, exposing neurons to huge amounts of thrombin. The delicate balance between thrombin and its inhibitors (serpins) in the nervous tissue is then altered. It is likely that much of the damage from these injuries results from neuronal death caused by thrombin; furthermore, thrombin inhibits neuronal regeneration by instigating an inflammatory response at injury sites (Nishino et al., 1993).

Many discoveries have established strong links between apoptosis, thrombin, and Alzheimer 0 #¡– s disease. One of the classic characteristics of Alzheimer 0 #¡– s is the accumulation of extracellular plaques composed primarily from â-amyloid protein (Aâ), which causes neuronal apoptosis both *in vitro* and *in vivo* (Loo et al., 1993; LaFerla et al., 1995). In addition, thrombin and PN-1 (serpin) are also plaque components (Akiyama

et al., 1992; Smith-Swintosky et al., 1995a) and past experiments have demonstrated that thrombin increases neuronal susceptibility to Aâ-induced apoptosis, while PN-1 has a protective effect (Mattson et al., 1993). Since Aâ aggregations also increased Ca^{2+}_i concentrations in neurons (Smith-Swintosky et al., 1995b), perhaps both Aâ and thrombin work in synergy to raise Ca^{2+}_i and trigger apoptosis. These findings are of fundamental importance to understanding the disease, since its cognitive symptoms are caused by a loss of neurons in the cerebral cortex, amygdala, and hippocampus (LaFerla et al., 1995).

PAR-1 agonists may also play a role in the development of amyotophic lateral sclerosis and/or spinal muscle atrophy, since activation of this receptor results in motoneuron cell death (Turgeon et al., 1998, 1999), the population of cells targeted by these diseases. By ascertaining the complete intracellular signal cascade by which activated PAR-1 commits motoneurons to apoptosis, it may then be possible to discover methods of blocking this signal.

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