Three members of the family of protease-activated receptors (PARs), PARs-1, -3 and -4, have been identified as thrombin receptors. PAR-1 is expressed by primary myoblast cultures, and expression is repressed once myoblasts fuse to form myotubes. The current study was undertaken to investigate the hypothesis that thrombin inhibits myoblast fusion. Primary rodent myoblast cultures were deprived of serum to promote myoblast fusion and then cultured in the presence or absence of thrombin. Thrombin inhibited myoblast fusion, but another notable effect was observed; 50% of control cells were apoptotic within 24 h of serum deprivation, whereas less than 15% of thrombin-treated cells showed signs of apoptosis. Proteolysis was required for the effect of thrombin, but no other serine protease tested mimicked the action of thrombin. Neither a PAR-1- nor a PAR-4-activating peptide inhibited apoptosis or fusion, and myoblast cultures were negative for PAR-3 expression. Myoblasts exposed to thrombin for 1 h and then changed to medium without thrombin accumulated apoptosis inhibitory activity in their medium over the subsequent 20 h. Thus the protective action of thrombin appears to be effected through cleavage of an unidentified thrombin receptor, leading to secretion of a downstream apoptosis inhibitory factor. These results demonstrate that thrombin functions as a survival factor for myoblasts and is likely to play an important role in muscle development and repair.

Thrombin is a trypsin-like serine proteinase that has a central role in hemostasis and thrombosis. The role of thrombin in these processes is not only dependent on its cleavage of fibrinogen but also on its ability to regulate the activity of cells such as leukocytes, platelets, and endothelial cells (1–4). Many of the cellular actions of thrombin are known to be mediated by a seven-transmembrane domain, G-protein-coupled receptor PAR-1 that is activated by a thrombin cleavage event in its extracellular domain. Proteolysis generates a new N terminus that acts as a tethered ligand, binding to another site in the receptor and activating a signal transduction cascade (5, 6).

Results with mice genetically incapable of expressing PAR-1, however, have shown that not all of the effects of thrombin are mediated by this receptor, leading to the recent discovery of two additional thrombin receptors, PAR-3 and PAR-4, which are closely related to PAR-1 (7–9). Synthetic peptides corresponding to the tethered ligand sequence of PAR-1 and PAR-4 (but not PAR-3) have been shown to activate their respective receptors in the absence of proteolysis (5, 8, 9).

During the process of muscle development, myoblasts proliferate and then undergo differentiation, fusing to form multinucleated myotubes. A knowledge of the factors that determine whether myoblasts will undergo proliferation or differentiation is essential for an understanding of postnatal growth and repair in skeletal muscle. A number of growth factors have been shown to influence these processes in cultured myoblasts. For example, fibroblast growth factor and epidermal growth factor stimulate myoblast proliferation and inhibit differentiation (10, 11).

Several lines of evidence have recently indicated that thrombin is an important regulator of muscle development. Thrombin has been proposed to play a role in synapse elimination occurring at the neuromuscular junction during development, because the specific thrombin inhibitor hirudin blocks synapse elimination both in vitro and in vivo (12, 13). Prothrombin is expressed in developing muscle (14), and in muscle cultures transcript levels and thrombin activity in the medium are increased by cholinergic stimulation (15). PAR-1 is expressed by cultured myoblasts, but expression is repressed once myoblasts fuse to form myotubes (16). In addition, recent immunohistochemical studies in developing muscle indicate that loss of PAR-1 expression by myoblasts soon after fusion also occurs in vivo (17). Thrombin was found to cause an increase in the number of cultured myoblasts, possibly through an increase in proliferation (16). The aim of the current study was to investigate the ability of thrombin to influence myoblast differentiation. Expression patterns of PAR-1 in cultured myoblasts led to the hypothesis that thrombin inhibits myoblast differentiation. Initial experiments were designed to test this hypothesis, but during the course of these studies it was observed that thrombin exerted a potent inhibitory effect on myoblast apoptosis. The results presented here document the effects of thrombin on fusion and apoptosis in primary myoblast cultures.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin was prepared as described (18). PPACK-thrombin was prepared by treating thrombin with the inhibitor PPACK-thrombin was prepared by treating thrombin with the inhibitor 7-Phe-Pro-Arg-CH2Cl according to Stone et al. (19) and displayed less than 0.1% of the initial activity of the protease. Factor Xa was a generous gift from Dr. B. Le Bonnic (INSERM, Paris). Urokinase, plasmin, chymotrypsin, and tissue plasminogen activator were from Sigma. Rat PAR-1-activating peptide (thrombin receptor agonist peptide; TRAP-1) was a 17-amino acid peptide (SFFLRNPSENTFELVPL) synthesized by Dr. P. Thompson (Department of Biochemistry and Molecular Biology, Monash University). Mouse PAR-4-activating peptide (TRAP-4) was a 6-mer (GYPGKF) synthesized by Dr. H. Keah.
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(Department of Biochemistry and Molecular Biology, Monash University). Both peptides were purified by high pressure liquid chromatography, and their compositions were verified by amino acid analysis. Polymerase chain reaction primers were obtained from Pacific Oligos (Lismore, Australia). Cell culture media were from Life Technologies, Inc., and other reagents were from Sigma unless otherwise stated.

Cell Culture—Primary muscle cell cultures were established from the hind limb muscles of 2-day-old newborn rats or mice. Cells were isolated as described, except that the preplating step was carried out for 1.5 h, and the final Percoll gradient step was omitted (20). After preplating, nonadherent cells were collected, centrifuged, and resuspended in 10% (v/v) horse serum in Ham's F-10 medium.

For fusion and apoptosis assays, cells were plated at a concentration of 2 × 10^5 cells/ml onto Nunc Lab Tek II 8-chamber glass slides pre-treated with poly-L-lysine then coated with laminin as described (16). Cell suspension (0.5 ml) was added to each well, and the slides were placed in a humidified atmosphere with 5% CO_2 at 37 °C. Nonadherent cells and other debris were removed when the medium was changed the following day. Experiments on myoblasts were performed when cells were approximately 80–90% confluent, which was usually 2–3 days after plating. At this stage, fusion to form myotubes was minimal. The medium in the wells was removed, and the cells were washed three times with Ham's F-10 medium containing bovine serum albumin (1 mg/ml). The cells were left for 5 min in the last wash before treatment with thrombin or other test substances in Ham's F-10/bovine serum albumin solution. The cells were returned to the incubator for various times (24 h unless otherwise mentioned) before immunocytochemical analysis. In the experiment shown in Fig. 4B, thrombin-containing medium was removed at various times, and then the cells were washed and incubated in fresh serum-free medium for the remainder of the 24 h. The myogenic nature of the cells was verified by staining with antibodies to myo D1 or desmin (21, 22). Our cultures were shown to be approximately 80% positive for either marker. Rat cells were used for all experiments presented except where particular reagents required the use of mouse cells, as specified in the text. The same response to thrombin in apoptosis and fusion assays was observed in mouse cells as in rat cells.

For production of conditioned medium, cells were plated in 6-well plates and grown until 80–90% confluent before serum deprivation and treatment with thrombin-containing (100 nM) or control medium for plates and grown until 80–90% confluent before serum deprivation and in rat cells.

Experiments on myoblasts were performed when cells were cultured for 24 h in the presence of thrombin (100 nM; A and B) or without additives (C and D). A and C, cultures stained with DAPI; B and D, the corresponding fields stained for the presence of desmin. The large arrows indicate myotubes, and the small arrows indicate some of the nuclei detectable as undergoing apoptosis. Bar, 50 μm.

error. Results were analyzed using Student's t test.

RNA Preparation and Polymerase Chain Reaction Analysis—Total cellular RNA was isolated from rat myoblast cultures or mouse spleen tissue using TRI REAGENT (Sigma) according to the manufacturer’s instructions. First strand cDNA was synthesized from 7 μg of RNA with Moloney murine leukemia virus reverse transcriptase using oligo(dT) primer (Ready-To-Go You-Prime First-Strand Beads, Amersham Pharmacia Biotech). Using the entire first-strand reaction, polymerase chain reaction amplification was performed according to the manufacturer’s instructions with the following primer pairs: PAR-1 (intron spanning): sense 5'-ATG GGG CCC CGG CGG TTG CTG-3'; antisense 5'-CCCTA GCT AGT AGC TTG TTG TAT ATG-3'; PAR-3: sense 5'-ACA ACA TCC TGT AGC CCG GTC T-3'; antisense 5'-TAA CAG AAT ATG ATC ACA A-3'; GAPDH: sense 5'-ACC ACC ATG GAG AGG GCT GG-3'; antisense 5'-CTG GTA GCC GAC GAT GC-3'. The samples were placed in a thermal cycler for 32 cycles of the following profile: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and polymerization at 72 °C for 1 min. The polymerase chain reaction products were electrophoresed in 1.8% (w/v) agarose gels and labeled with ethidium bromide for photography. Before incubation with mouse myoblast RNA, PAR-3 primers were validated by the finding that they produced a band of the appropriate size with mouse spleen RNA, which is known to express PAR-3 (7).

Intracellular Ca^{2+} Measurement—For [Ca^{2+}], assays, cells were plated in flasks (75 cm^2) and grown to confluence then detached with nonenzymatic dissociation medium (Sigma). [Ca^{2+}], was measured at 37 °C using the fluorescent indicator Fura-2 (Molecular Probes) essentially as described by Jenkins et al. (23) except that the buffer used for loading the cells with Fura-2 and subsequent assays was 10 mM Hepes buffer, pH 7.4, containing 138 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.5 mM CaCl_2, 5 mM glucose, and 0.1% bovine serum albumin (w/v).

RESULTS

Thrombin Inhibits Skeletal Muscle Cell Differentiation—To investigate whether thrombin modulates myoblast fusion, rat cells were grown until almost confluent in serum-containing medium. Cells were then deprived of serum and cultured in the presence or absence of thrombin. Cultures were evaluated for myotube formation 12, 24, and 32 h after serum deprivation. In control cultures after 24 h, 35% of myoblast nuclei were present in multinucleated myotubes (Figs. 1, C and D, and 2A). In contrast, fusion was inhibited in thrombin-treated myoblasts, with less than 10% of myoblast nuclei present in myotubes at 24 h (Figs. 1, A and B, and 2A). The myotubes present in

**Fig. 1. Effect of thrombin on myoblast fusion and survival.** Subconfluent myoblast-enriched cultures were deprived of serum then cultured for 24 h in the presence of thrombin (100 nM; A and B) or without additives (C and D). A and C, cultures stained with DAPI; B and D, the corresponding fields stained for the presence of desmin. The large arrows indicate myotubes, and the small arrows indicate some of the nuclei detectable as undergoing apoptosis. Bar, 50 μm.
thrombin-treated cultures were considerably smaller and had fewer nuclei than those in control cultures. In cells incubated for over 24 h, thrombin was added again to counteract any loss in activity. Examination at 32 h showed that a considerable number of cells in both thrombin-treated and untreated populations had died. However, untreated wells still contained a much greater proportion of nuclei in myotubes than did the thrombin-treated wells. To determine the concentration dependence of the thrombin effect, primary rat muscle cultures were exposed to various doses of thrombin for 24 h. The maximal efficacy was achieved at a concentration of 100 nM (Fig. 2B), which is the concentration required for optimal receptor-mediated responses to thrombin in a number of cell systems (9, 23–25).

The involvement of PAR-1 activation in thrombin-mediated effects was examined by determining whether similar effects occurred in response to TRAP-1. Cells were treated with TRAP-1, which was added every 4 h over a 24-h period. Neither 50 μM nor 100 μM TRAP-1 was able to mimic the effect of thrombin on myoblast fusion (Fig. 2C). Proteolytically inactive PPACK-thrombin had no effect on myotube formation, showing that the enzymatic activity of thrombin is required (Fig. 2C).

**Thrombin Prevents Myoblast Death**—Cells cultured under conditions that induced myoblast fusion (described above) were found to undergo cell death, which was already detectable 12 h after serum deprivation. In contrast, thrombin treatment caused a significant inhibition of cell death (Figs. 1 and 3). The dying cells showed the typical morphological features of apoptotic cells, with clumping and aggregation of chromatin (Fig. 3, A and B). This phenomenon was paralleled by the occurrence of DNA strand breaks, detectable by the TUNEL reaction (Fig. 3, B and D). In many culture systems, induction of apoptosis is dependent on de novo protein synthesis (26). To assess whether protein synthesis was also required for cell death in our system to occur, cells were treated with cycloheximide (5 μM) for 30 min at the time of serum deprivation, and then the medium was replaced with fresh serum-free medium, and the cell viability was determined 24 h later. Cell death in cultures treated with cycloheximide (19.5 ± 0.5%) was significantly lower (p < 10⁻³) than in control cultures (41.7 ± 1.1%).

In time course studies, cells were treated with thrombin (100 nM) continuously from the time of serum deprivation but were fixed and analyzed for apoptosis at various times. The thrombin effect was already detectable at 12 h. At least 50% of cells in control cultures were apoptotic at 24 h, whereas in thrombin-treated cultures less than 15% of cells were apoptotic at 24 h. Similarly, thrombin still exerted a significant protective effect. A further time course experiment was carried out to determine whether the continued presence of thrombin was required. Cells were exposed to thrombin for 0, 1, 6, 12, or 24 h following serum deprivation, at which time medium was replaced with fresh serum-free medium. Cells were fixed and examined at 24 h. The results demonstrated that a 1-h treatment with thrombin at the time of serum deprivation was sufficient to provide almost complete protection from apoptosis (Fig. 4B). Thrombin

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**Fig. 2.** Myoblast fusion is inhibited in the presence of thrombin. A, subconfluent myoblast-enriched cultures were incubated in serum-free medium in the presence or absence of thrombin (100 nM) for the times indicated. Cultures were fixed, stained for the presence of desmin, and then counterstained with DAPI. Nuclei in myotubes were counted as a proportion of total nuclei in desmin-positive cells; results are presented as the means ± S.E. B, subconfluent myoblast-enriched cultures were incubated for 24 h in serum-free medium containing thrombin at the concentrations indicated. Cultures were processed and nuclei counted as above. C, subconfluent myoblast-enriched cultures were incubated for 24 h in serum-free medium without additives or in the presence of thrombin, TRAP-1 or PPACK-thrombin. Cultures were processed and nuclei counted as above.

**Fig. 3.** Effect of thrombin on myoblast survival. Subconfluent myoblast-enriched cultures were incubated for 24 h in serum-free medium containing thrombin (100 nM; A and B) or without additives (C and D). A and C, cultures stained with DAPI. B and D, the corresponding fields stained with the TUNEL reagents. Arrows indicate individual apoptotic myoblasts detectable both by DAPI and TUNEL staining. Bar, 50 μm.
Inhibited apoptosis over a range of concentrations, with optimal protection occurring at 100 nM (Fig. 4C).

In the presence of catalytically inactive PPACK-thrombin, the level of apoptosis was 88 ± 1.5% of that seen in control cultures, indicating that proteolytic activity is required for the protective effect of thrombin. Experiments were carried out to investigate whether the inhibition of apoptosis was specific for thrombin. None of the other serine proteases tested was able to inhibit apoptosis. In the presence of urokinase or factor Xa (100 nM), apoptosis as a percentage of control values was 100 ± 3.3 or 96 ± 1.0%, respectively. Plasmin, tissue plasminogen activator, or chymotrypsin at 100 nM caused death and detachment of all cells from the substratum.

Mechanism of the Effects of Thrombin on Myoblasts—Further experiments were carried out to determine whether the effect of thrombin was mediated by one of the known thrombin receptors, PAR-1, -3, or -4. Inhibition of apoptosis by thrombin appears not to be mediated via PAR-1 activation, because TRAP-1 did not mimic the effect of thrombin at concentrations able to elicit [Ca\(^{2+}\)] responses in such cells. In the presence of TRAP-1 at 50 or 100 \(\mu\)M, apoptosis as a percentage of control values was 93 ± 9.8 or 87 ± 5.3%, respectively. TRAP-1 was added every 4 h over a period of 24 h to compensate for a possible loss of the peptide activity. In one experiment in which the aminopeptidase inhibitor, amastatin, was added to the culture medium to rule out the possibility that TRAP-1 was being degraded, the peptide was still unable to inhibit apoptosis. Reverse transcriptase-polymerase chain reaction was carried out to investigate whether myoblasts express PAR-3. Because the sequence for PAR-3 is unknown, PAR-3 primers based on the mouse sequence were designed and tested in RNA extracted from mouse cells. This approach is valid because thrombin was found to exert the same protective effect in mouse myoblasts as in rat cells. As a positive control, PAR-3 primers detected PAR-3 transcript in mouse spleen. The same primers failed to detect PAR-3 in mouse myoblasts. Experiments investigating the role of PAR-4 made use of mouse TRAP-4 and mouse myoblasts because the rat PAR-4 sequence has previously been shown to activate PAR-4 in mouse cells (8).

Because the above experiments demonstrated that effects of thrombin on myoblast apoptosis and fusion are not mediated by any of the known thrombin receptors, further experiments were carried out to determine whether the substrate of thrombin is a cell surface protein or a protein secreted into the medium of myoblast cultures. When thrombin was added to
medium collected from myoblast cultures 12 or 24 h after serum deprivation and then inactivated by the addition of PPACK, the resulting medium had no apoptosis inhibitory activity (Table I). Medium was also collected from cells exposed to thrombin for 24 h after serum deprivation. Such medium was treated with PPACK until no thrombin activity was detectable. This medium contained inhibitory activity in apoptosis and fusion assays almost as potent as that of thrombin (Table I). In a final experiment, cells were treated with thrombin for 1 h following serum deprivation and then the medium was replaced with fresh serum-free medium without thrombin. The latter medium was harvested and used in apoptosis and fusion assays and found to have accumulated inhibitory activity with time (Fig. 5).

**DISCUSSION**

Expression patterns of PAR-1 in primary cultures of myoblasts undergoing differentiation (16), as well as in developing muscle (17), led us to predict that thrombin inhibits myoblast fusion. At the time that our experiments were being undertaken, a description of the inhibition of fusion by thrombin in the C2C12 myoblast cell line was published (25). These results, in combination with our own showing that thrombin causes a PAR-1-independent inhibition of fusion, suggest that thrombin regulates myoblast differentiation through multiple pathways.

Our observation that thrombin inhibits myoblast apoptosis is novel and of considerable interest. Vaughan et al. (28) have shown that thrombin protects rat primary astrocytes and hippocampal neurons from cell death induced by hypoglycaemia and oxidative stress but did not describe the cell death inhibited by thrombin as apoptosis. In the current study the myoblast death inhibited by thrombin has been characterized as an apoptotic process not only on the basis of nuclear morphology and oxidative stress but also because of its requirement for protein synthesis demonstrated by the experiments with cycloheximide. The thrombin-induced protection from cell death observed by Vaughan et al. (28) appears to be mediated by PAR-1, because it can be reproduced using TRAP-1. The protective effect of thrombin in the current study uses a different pathway, because it is not mimicked by TRAP-1.

The failure of primary myoblast cultures to express PAR-3 and their failure to respond to TRAP-4 in terms of apoptosis, fusion, or [Ca\textsuperscript{2+}], indicate that neither PAR-3 nor PAR-4 mediates the observed responses to thrombin. If none of the known thrombin receptors mediates inhibition of fusion and apoptosis in myoblasts, how does thrombin exert these effects? Both effects appear to be dependent on the proteolytic activity of thrombin, because PPACK thrombin was inactive in both assays. The effect is specific for thrombin, because no other serine protease tested was able to mimic the activity. Fig. 6
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Thrombin plays a role in the recovery from muscle damage involving gene fusion, and our results suggest that thrombin may be important in the absence of vascular disruption. Recent studies investigating the expression of prothrombin in developing muscle confirm that the protein is present in this tissue (14). In addition, thrombin activity is increased in myotube cultures after cholinergic stimulation, in part by increasing prothrombin transcription (15). Our results suggest that thrombin produced by myotubes during muscle development would take part in a negative feedback loop, limiting the number of myoblasts that fuse, and ensuring that some cells capable of proliferation remain to meet future needs.

In normal adult muscle apoptosis appears to be a rare event (29), and our results suggest that thrombin may be important in maintaining this situation. It is also likely that thrombin plays a role in the recovery from muscle damage involving vascular disruption, partly through its effect on myoblast survival. Indeed, a thrombin concentration of 360 nM has been measured in the fluid phase of clotted blood (30), well above the levels required for maximal responses in our culture system. In a number of pathological situations, including exercise-induced muscle damage and muscular dystrophy, the proportion of muscle cells undergoing apoptosis increases (29, 31, 32). It is of considerable interest to note that the incidence of apoptosis is elevated in skeletal muscle subjected to disuse or denervation, as compared with normal muscle (33, 34). Because cholinergic stimulation of muscle fibers results in an increase in thrombin activity (15), it is tempting to speculate that a decrease in thrombin activity in denervated muscle allows the resultant apoptosis to occur.

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