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Protease-activated receptor-2 mediates proliferative responses in skeletal myoblasts

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SUMMARY

Protease-activated receptor-2 (PAR-2) is a G protein-coupled receptor that is cleaved by proteases within the N terminus, exposing a new tethered ligand that binds and activates the receptor. Activators of PAR-2 include trypsin and mast cell tryptase. Skeletal myoblasts are known to express PAR-1, a thrombin receptor. The current study was undertaken to determine whether myoblasts express PAR-2. Primary neonatal rat and mouse skeletal myoblast cultures were shown to express PAR-2 in polymerase chain reaction and immunocytochemical studies. Expression of PAR-2 was also demonstrated by immunohistochemistry in developing mouse skeletal muscle in vivo. Trypsin or a synthetic peptide corresponding to the rat PAR-2 tethered ligand caused a dose-dependent elevation in intracellular calcium in cultured rat myoblasts, with an EC_{50} of 13 nM or 56 μM, respectively. Studies aimed at identifying the function of PAR-2 in myoblasts demonstrated no effect of the receptor-activating peptide on survival or fusion in serum-deprived myoblasts. The PAR-2-activating peptide did, however, stimulate proliferation of serum-deprived myoblasts. These results demonstrate that skeletal muscle cells express PAR-2, activation of which leads to stimulation of myoblast proliferation.

Key words: Protease-activated receptor, Myoblast, Trypsin, Thrombin

INTRODUCTION

The protease-activated receptors (PARs) belong to the family of seven transmembrane domain G protein-coupled receptors. The PARs are activated by specific proteolytic cleavage of their extracellular amino terminus, which exposes a tethered ligand domain. PARs -1, -3 and -4 are activated by thrombin, and enzymatic activators of PAR-2 include trypsin and mast cell tryptase (Corvera et al., 1997; Nystedt et al, 1994). For PARs -1, -2 and -4, peptides corresponding to the newly created N terminus are able to activate the relevant receptor in the absence of receptor cleavage.

Expression of PAR-2 has been described in many tissues, including kidney, bone, neutrophils, and the epithelial and smooth muscle components of gastrointestinal tract, respiratory tract, blood vessels and skin (Abraham et al., 2000; Al-Ani et al., 1995; Bohm et al., 1996; Corvera et al., 1997; D'Andrea et al., 1998; Howells et al., 1997; Nystedt et al., 1994; Saifeddine et al., 1996). Activation of PAR-2 leads to a variety of functional responses in different tissues. In the digestive system, activation of PAR-2 causes generation of eicosanoids by enterocytes and activation of ion channels in pancreatic duct epithelial cells (Kong et al., 1997; Nguyen et al., 1999). Smooth muscle contraction in isolated gastric tissue also occurs as a result of PAR-2 activation (Saifeddine et al., 1996). In the vascular system, PAR-2 mediates proliferation of endothelial and smooth muscle cells (Bono et al., 1997; Mirza et al., 1996). Activation of PAR-2 leads to endothelium-dependent vasodilation of isolated vascular preparations, and a lowering of blood pressure in rats (Hwa et al., 1996; Saifeddine et al., 1996).

Expression of PAR-1 in skeletal muscle cells both in vivo and in vitro has been described previously (Abraham et al., 1998; Suidan et al., 1996). Thrombin and rat PAR-1 tethered ligand peptide ('thrombin receptor activating peptide'; TRAP-1) are able to stimulate proliferation of primary myoblast cultures (Suidan et al., 1996) and to inhibit differentiation of myoblastic cell lines (Guttridge et al., 1997). We have recently demonstrated that thrombin inhibits apoptosis and differentiation in primary myoblast cultures, effects that could not be mimicked by TRAP-1 (Chinni et al., 1999). It is known, however, that TRAP-1 activates PAR-2 as well as PAR-1 (Blackhart et al., 1996; Santulli et al., 1995). Thus, a possible interpretation of our results in primary myoblast cultures was that activation of PAR-2 in TRAP-1-treated cells cancelled out the effect of PAR-1 activation. It seemed appropriate, therefore, to investigate whether myoblasts express PAR-2.

In the present study we have used reverse transcriptase polymerase chain reaction (RT-PCR), immunocytochemistry and immunohistochemistry to examine the expression of PAR-

2 in cultured myoblasts and in skeletal muscle in vivo. The results show for the first time that functional PAR-2 is expressed by skeletal muscle cells. We have also investigated functional responses of myoblasts to PAR-2 activation. During skeletal muscle development, mononuclear myoblasts proliferate and then fuse to form multinucleate muscle fibres. Myoblasts persist in adult muscle in the form of satellite cells, which provide a source of muscle nuclei for repair and regeneration of damaged muscle fibres. Myoblast survival, fusion and proliferation are, therefore, all important processes in muscle development and pathology. Our studies on the regulation of these processes by PAR-2 are documented here.

MATERIALS AND METHODS

Materials

Human α-thrombin was prepared as described (Stone and Hofsteenge, 1986). Rat TRAP-1 was a 17-amino-acid peptide (SFFLRNPSENTFELVPL) synthesised by Dr P. Thompson (Department of Biochemistry and Molecular Biology, Monash University). Rat PAR-2-activating peptide (RAP) was a 6-amino-acid peptide (SLIGRL) synthesised by AusPep (Melbourne, Australia). Both peptides were purified by high pressure liquid chromatography (HPLC) and their composition verified by amino acid analysis. Polymerase chain reaction (PCR) 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. Rabbit anti-PAR-2 antibody was a generous gift from the late Prof S. R. Stone, and its characterization has been described (Abraham et al., 2000).

Cell culture

Primary muscle cell cultures were established from the upper hind limb muscles of neonatal rats or mice as described (Chinni et al., 1999; Suidan et al., 1996). Briefly, Dulbecco's modified Eagle's medium (DMEM) with relevant additives was used throughout the process of cell isolation as well as for culture. Muscles were excised and minced with fine scissors, trypsinised (0.25% trypsin) for 40 minutes and triturated in the presence of 10% horse serum. After filtration through a nylon strainer (70 μm), the suspension was centrifuged and cells resuspended in DMEM containing 10% horse serum. Cells were then preplated on plastic for 1.5 hours to remove the more adherent non-myogenic cells. Non-adherent cells were washed and resuspended in DMEM containing 10% horse serum. For immunocytochemistry, apoptosis and fusion assays, cells (10^5/well) were plated directly in 8-chamber glass slides pretreated with poly-L-lysine then coated with laminin. For RNA isolation and $[Ca^{2+}]_i$ assays, cells were plated directly in 25 cm^2 flasks, and for proliferation assays, cells were plated directly in 75 cm^2 flasks. All experiments were conducted on the primary isolates except for $[Ca^{2+}]_i$ and proliferation assays, which were conducted on first passage cells.

In some experiments, fibroblasts removed by preplating were maintained in DMEM in 10% foetal bovine serum, for further experiments. Such cell populations were found to be negative for expression of the myoblast marker desmin in immunocytochemical studies, whereas at least 90% of the myoblast-enriched cell population from which they were separated were desmin-positive.

RNA preparation and polymerase chain reaction analysis

Total cellular RNA was isolated from confluent rat or mouse myoblast cultures in 25 cm^2 flasks using TRI REAGENT (Sigma) according to the manufacturer's instructions. First-strand cDNA was synthesised from 7 μg RNA with Moloney Murine Leukemia Virus reverse transcriptase using oligo(dT) primer (Ready-To-Go You-Prime First-Strand Beads, Pharmacia Biotech). Using the entire first-strand reaction, PCR amplification was performed according to the manufacturer's instructions with the following primer pairs: PAR-1 (intron-spanning): sense 5′ ATG GGG CCC CGG CGC TTG CTG 3′, antisense 5′ CCC TAA GCT AGT AGC TTT TTG TAT ATG 3′ (predicted fragment size 1290 bp); PAR-2: sense 5′ CAC CAC CTG TCA CGA TGT GTC 3′, antisense 5′ CCC GGG CTC AGT AGG AGG TTT TAA CAC (predicted fragment size 472 bp); GAPDH: sense 5′ ACC ACC ATG GAG AAG GCT GG 3′, antisense 5′ CTC AGT GTA GCC CAG GAT GC 3′. The samples were placed in a thermal cycler for 32 cycles of the following profile: denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and polymerization at 72°C for 1 minute. The PCR products were electrophoresed in 1.8% (w/v) agarose gels and labelled with ethidium bromide for photography.

Immunohistochemistry

Embryos were harvested from C57BL/6J mice on embryonic day 17 (E17). Dissected limbs from E17 embryos and adult C57BL/6J mice were fixed briefly in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. After fixation, the tissues were cryoprotected by immersion in 25% sucrose and frozen in OCT compound (Bayer) for storage at −70°C. Cryostat sections (10 μm) were collected onto 3-aminopropyltriethoxysilane-coated slides and stored at −70°C prior to immunostaining to detect the presence of PAR-2.

Tissue sections were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and washed in PBS. Endogenous peroxidase activity was inhibited by a 30 minute incubation in methanol containing 0.3% v/v hydrogen peroxide. After being washed in PBS, sections were blocked with 1% BSA in PBS for 30 minutes. Anti-PAR-2 (2.5 μg/ml in PBS/0.1% BSA) was applied to the sections overnight at 4°C. Control sections were incubated in the absence of primary antibody or with anti-PAR-2 preincubated with antigenic peptide (2.5 mM) for 30 minutes at 4°C prior to tissue staining; both methods resulted in similar levels of background staining. Primary antibody was followed by the avidin-biotin-peroxidase complex (ABC) procedure (Immunopure® ABC Peroxidase Staining Kit, Pierce) and peroxidase activity detected by diaminobenzidine (Sigmafast® Peroxidase Substrate Tablet Set, Sigma) according to the manufacturers' instructions. Sections were counterstained with haematoxylin, dehydrated and mounted under coverslips in nonaqueous mountant.

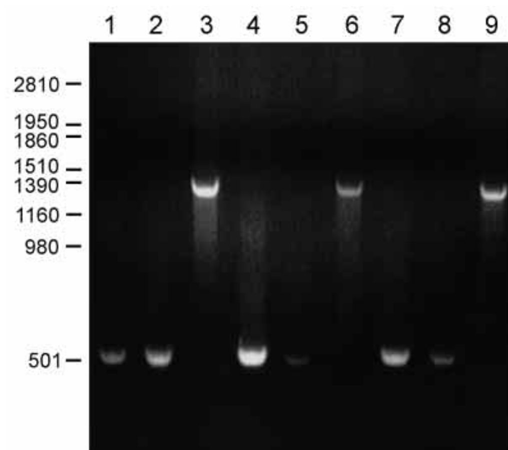

Fig. 1. RT-PCR analysis of expression of protease-activated receptors by primary rat myoblast-enriched cultures (lanes 1-3) and fibroblasts (lanes 4-6), and primary mouse myoblast-enriched cultures (lanes 7-9). Lanes 1, 4 and 7: GAPDH; lanes 2, 5 and 8: PAR-2; lanes 3, 6 and 9: PAR-1. The positions of marker RNAs are shown.

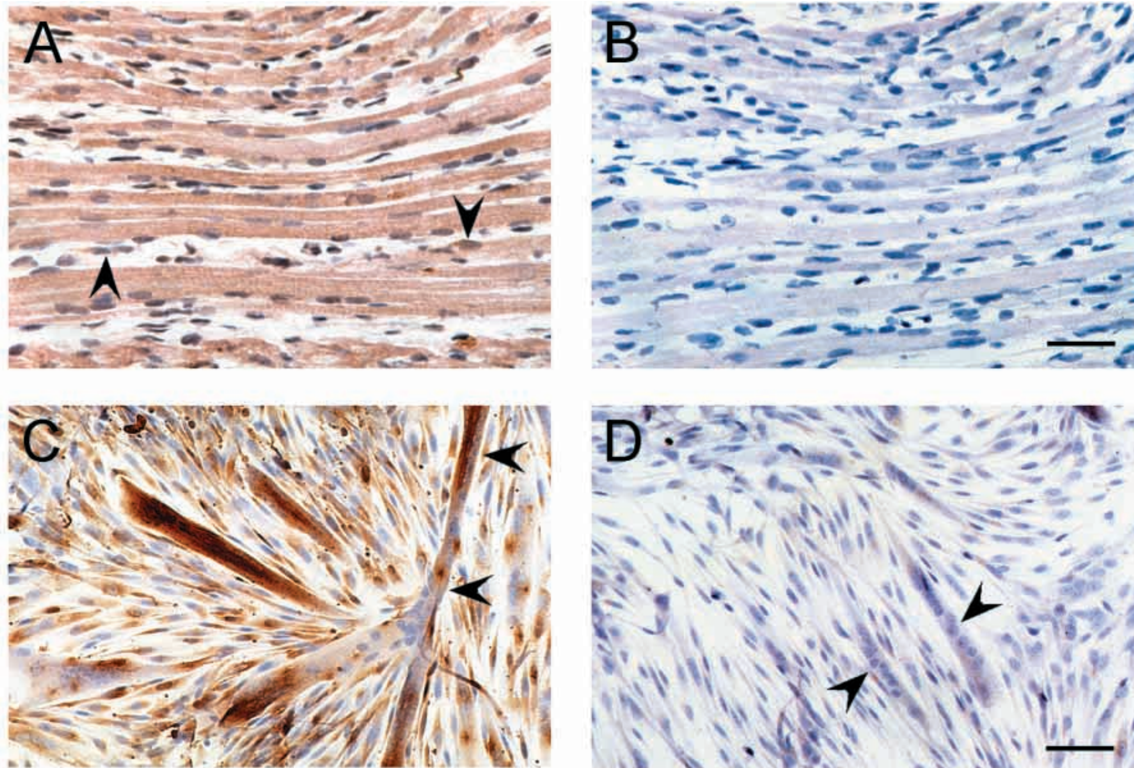

Fig. 2. Immunochemical detection of PAR-2 in muscle cells in vivo and in vitro, using the avidin-biotin-peroxidase complex system (Haematoxylin counterstain). (A,B) Cryosections of E17 mouse hindlimb muscle stained with anti-PAR-2 (A) or no primary antibody (B). Examples of PAR-2-positive mononuclear cells are indicated by arrowheads. Bar, 50 μm. (C,D) Cultured rat myoblasts, some of which have undergone fusion to form myotubes (arrowheads), stained with anti-PAR-2 (C) or no primary antibody (D). Bar, 100 μm.

Immunocytochemistry

Cells in 8-chamber slides were grown until subconfluent in the presence of 10% horse serum, then the medium was replaced with Ham's F-10 containing 4% serum to induce fusion of myoblasts to form myotubes. After 24 hours, cells were washed gently in Hanks' balanced salt solution (HBSS) at room temperature, fixed in paraformaldehyde (4%) in HBSS for 10 minutes, then washed in PBS. Cultures were blocked for 1 hour in PBS containing foetal calf serum (FCS; 10%) then incubated in anti-PAR-2 antibody (2.5 μg/ml) overnight at 4°C. Primary antibody was detected using the ABC procedure, and slides prepared for microscopic examination as for tissue sections.

Intracellular Ca^{2+} measurement

For $[Ca^{2+}]_i$ assays, cells plated in 25 cm^2 flasks were trypsinised then plated in 75 cm^2 flasks, grown to confluence then detached with non-enzymatic dissociation medium (Sigma). $[Ca^{2+}]_i$ assays were conducted essentially as described by Jenkins et al. (1993) except that the buffer used for loading the cells with Fura-2 and subsequent steps was 10 mM Hepes, containing 138 mM NaCl, 5 mM KCl, 1 mM MgCl, 1.5 mM $CaCl_2$, 5 mM D-glucose and BSA 0.1% (w/v), pH 7.4. Briefly, cells were washed twice with HBSS then loaded with the fluorescent indicator Fura-2/AM (1 μM; Molecular Probes). Cells were washed and incubated for 30 minutes to allow hydrolysis of intracellular Fura-2/AM, then centrifuged and resuspended in fresh buffer for fluorescence measurements at 37°C in stirred cuvettes. Trypsin, RAP, thrombin and EGTA (at concentrations indicated in the figures) were tested for their ability to influence $[Ca^{2+}]_i$. The response to bradykinin (1 μM) was tested at the end of each assay to ensure that intracellular calcium stores had not been depleted.

Apoptosis and fusion assays

Apoptosis and fusion assays were carried out as described (Chinni et al., 1999). Briefly, cells were grown until 80-90% confluent in 8-chamber slides, then deprived of serum for 24 hours in the presence or absence of RAP (300 μM), thrombin (100 nM) or RAP (300 μM) and thrombin (100 nM). Cells were then fixed and stained with anti-desmin and the nuclear dye 4′,6 diamidino-2-phenylindole (DAPI). Morphologically apoptotic nuclei in desmin-positive cells were counted and expressed as a percentage of total desmin-positive cells (myoblasts). For fusion assays, myoblast nuclei in myotubes were counted and expressed as a percentage of total myoblast nuclei. In each case at least 100 nuclei per well were counted, and results from triplicate wells were expressed as means ± s.e.m. Results were analysed using Student's *t*-test. Experiments were carried out twice to confirm the validity of the data.

Table 1. Effect of activation of PAR-2 on myoblast apoptosis and fusion

Treatment	Apoptotic cells (%)[a]	Myoblast nuclei in myotubes (%)[a]
Control	44±2.1	22±2.4
RAP (300 μM)	38±0.7[b]	17±0.7[b]
Thrombin (100 nM)	8±1.0[c]	10±1.8[c]
RAP (300 μM) + Thrombin (100 nM)	11±0.6[d]	15±3.8[d]

[a]Results are expressed as mean ± s.e.m. (*n*=3).
[b]Not significantly different from control value.
[c]Significantly different from control value (P<0.01).
[d]Not significantly different from value for thrombin alone.

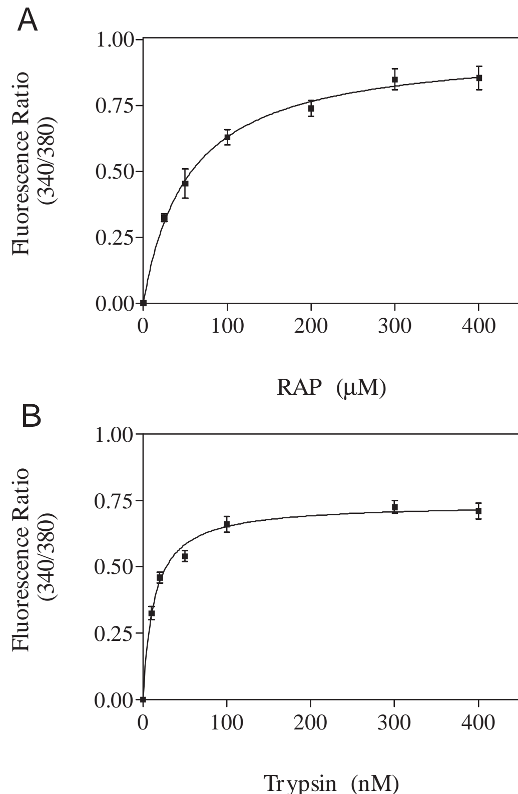

Fig. 3. $[Ca^{2+}]_i$ mobilization in rat myoblast-enriched cells in response to activation of PAR-2. Dose-responses to RAP (A) and trypsin (B) in Fura-2-loaded cells.

Proliferation assays

For cell number assays, cells were grown to confluence in 75 cm^2 culture flasks then trypsinised and plated into 24-well plates (7.5×10^4 cells/well). After 24 hours (T_0), cells in four wells were trypsinised, washed, stained with Trypan Blue, and live cells counted in a haemocytometer. The remaining cells were deprived of serum and RAP (300 μM) or thrombin (100 nM) was added to groups of four wells, while four wells were left with serum-free medium alone. After a further 24 hours, cells were trypsinised, washed, stained with Trypan Blue and counted.

For bromodeoxyuridine (BrdU) incorporation assays, cells were grown to confluence in 75 cm^2 culture flasks then trypsinised and plated into 96-well plates (10^4 cells/well). After 16 hours, cells were deprived of serum and RAP (300 μM) or thrombin (100 nM) was added to groups of eight wells, while eight wells were left with serum-free medium alone. BrdU incorporation was assayed using a commercial colorimetric ELISA kit (Boehringer-Mannheim) according to the manufacturer's instructions; BrdU was added to the wells 24 hours after addition of test substances, then cells were incubated for 4 hours before fixation and processing for BrdU detection. The detection system made use of peroxidase-labelled anti-BrdU and tetramethylbenzidine as substrate. Results were quantitated in a spectrophotometric plate-reader at 450 nm.

Results of both assays were expressed as mean ± s.e.m. Results were analysed using Student's *t*-test. Both cell number and BrdU incorporation experiments were carried out twice to confirm the validity of the data. In addition, parallel cultures were prepared in 8-chamber slides; cells were fixed 24 hours after addition of test substances then stained for the presence of desmin. Desmin-negative cells were counted and expressed as a proportion of total cell number in RAP-treated and control cells (mean ± s.e.m.; n=4).

RESULTS

Expression of PAR-2 by skeletal myoblasts

The expression of PAR-2 by primary mouse and rat skeletal myoblast cultures was investigated by RT-PCR. Bands of the appropriate size for the PAR-2 primers, as well as for the PAR-1 primers used as a positive control, were detected in RNA from myoblast-enriched cultures (Fig. 1, lanes 2,3,8,9). Since up to 10% of the cells in these cultures are usually fibroblasts, RNA was extracted from fibroblasts harvested from the preplating step of the rat myoblast isolation and investigated for PAR-2 expression. PAR-2 transcript was detected in fibroblast RNA. In a single RT-PCR run investigating equal amounts of RNA from fibroblasts and myoblast-enriched cultures, the intensity of the PAR-2 band relative to that of the GAPDH band was very much weaker for fibroblasts (Fig. 1, lanes 4,5) than for myoblasts (Fig. 1, lanes 1,2), indicating that the contaminating fibroblasts could not be entirely responsible for the PAR-2 transcript detected in myoblast-enriched cultures.

To confirm that muscle cells express PAR-2, both muscle tissue and myoblast-enriched cultures were investigated by immunochemical methods. Strong specific PAR-2 staining of muscle fibres and mononuclear cells was observed in sections of embryonic mouse muscle (Fig. 2A,B), as well as in adult muscle (not shown). In cultures incubated under conditions designed to induce fusion, both mononuclear myoblasts and multinucleate myotubes were stained specifically by the anti-PAR-2 antibody (Fig. 2C,D).

Effect of activators of PAR-2 on $[Ca^{2+}]_i$ in cultured myoblasts

To determine whether the PAR-2 present in muscle cells is coupled to intracellular effectors, cultured myoblasts were loaded with Fura-2 to detect intracellular Ca^{2+} transients, and stimulated with two activators of PAR-2, trypsin and the rat PAR-2 agonist peptide, RAP; RAP is an effective tool for this purpose since it specifically activates PAR-2 (Blackhart et al., 1996). Both RAP and trypsin stimulated transient, dose-dependent increases in $[Ca^{2+}]_i$; the EC_{50} for RAP was 56 μM and that for trypsin was 13 nM (Figs 3A,B, 4A). Following RAP or trypsin treatment, $[Ca^{2+}]_i$ showed a sharp peak followed by a plateau, as compared with the rapid return to baseline seen in myoblasts with activation of PAR-1 by thrombin (Fig. 4A,B). When extracellular calcium was depleted by the chelating agent EGTA, $[Ca^{2+}]_i$ in RAP-treated cells immediately returned to the original baseline level, indicating that the prolonged elevation results from entry of extracellular calcium (Fig. 4A).

Similar experiments were also carried out on fibroblastic cells derived from the preplating of myoblast preparations. RAP or trypsin caused an increase in cytosolic $[Ca^{2+}]$ in Fura-2 loaded fibroblasts (Fig. 4C,D), although the amplitude of the response to RAP was considerably smaller than that of myoblasts. Thrombin also caused an increase in $[Ca^{2+}]_i$ when added to the fibroblastic cells.

Lack of effect of activation of PAR-2 on fusion and apoptosis in myoblasts

Since the current study was initiated to determine whether PAR-2 expression by primary myoblast cultures could account

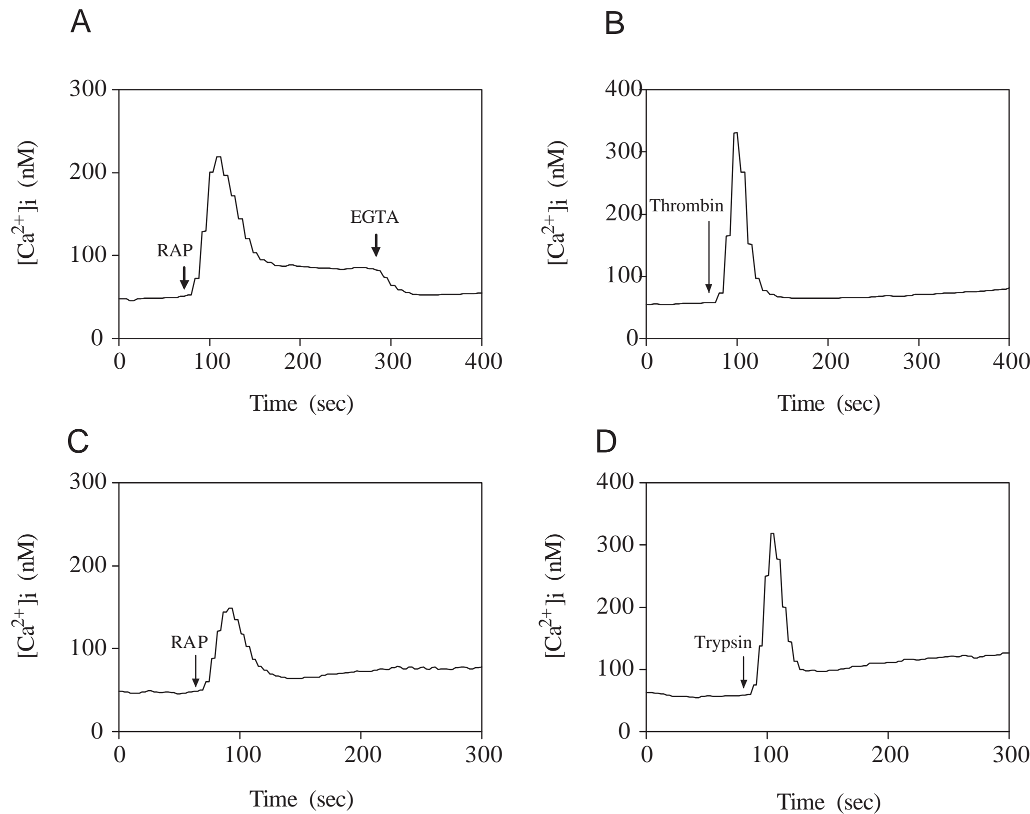

Fig. 4. $[Ca^{2+}]_i$ mobilization in rat myoblast-enriched cells or muscle-derived fibroblasts in response to activation of PARs. Traces for Fura-2-loaded myoblasts (A,B) and fibroblasts (C,D) treated with RAP (300 µM), EGTA (5 mM), thrombin (100 nM) or trypsin (100 nM).

for differences in responses of myoblasts to thrombin (which activates PAR-1) and TRAP-1 (which activates PAR-1 and PAR-2) in apoptosis and fusion assays (Chinni et al., 1999), the activity of RAP in these assays was investigated. Thrombin was used as a positive control, and RAP and thrombin were also used in combination, to determine whether activation of PAR-2 concurrently with PAR-1 resulted in the abrogation of effects of PAR-1 activation. As shown in Table 1, RAP did not influence either control or thrombin-inhibited levels of apoptosis. The value for myoblast fusion in the presence of RAP was lower than the control value, but the difference was not significant. The fusion value for RAP in combination with thrombin was higher than for thrombin alone, but the difference was not significant (Table 1). The results presented here were obtained from experiments conducted on primary isolates so as to be directly comparable with those conducted previously with thrombin and TRAP-1. Similar experiments were conducted on first passage myoblasts, and similar results were obtained (data not shown).

Effect of PAR-2 activation on myoblast proliferation

The ability of RAP to influence myoblast proliferation was investigated. Due to the rapidity with which primary cultured myoblasts undergo apoptosis, these experiments were necessarily conducted during the first 24 hours following serum withdrawal. First passage myoblasts were grown in serum-containing medium until subconfluent. At this time (T_0), cells in some wells were trypsinised and counted, while cells in the remaining wells were deprived of serum and treated with or without RAP (300 µM) or thrombin (100 nM). Cell number increased slightly over the subsequent 24 hours in control cells, but significantly more ($P<10^{-4}$) in cells treated with RAP (Fig. 5A). The greatest increase in cell number above control values at 24 hours was seen in cells treated with thrombin. Cells subjected to a treatment regimen similar to that used for cell number assays were also tested for BrdU incorporation. RAP caused a 46% increase in BrdU incorporation above that seen in cells treated with control medium (Fig. 5B); this difference was highly significant ($P<10^{-5}$). Thrombin caused a greater than twofold increase in BrdU incorporation (Fig. 5B). In parallel cultures stained at the end of the RAP treatment period for the presence of the myoblast marker, desmin, 7.2±0.4% of RAP-treated cells were non-myogenic, compared with 7.2±0.5% of control cells. These observations indicate that RAP's effect on cell proliferation in the mixed population was not exerted differentially on non-myogenic cells.

DISCUSSION

The results presented here demonstrate that PAR-2 is expressed by skeletal muscle cells both in vivo and in vitro. Cultured myoblasts are able to respond to activation of PAR-2 through both calcium mobilization and increased proliferation.

The main purpose in initiating this study was to determine whether myoblastic expression of PAR-2 could account for differences in responses of these cells to thrombin and the PAR-1 tethered ligand peptide. We have previously

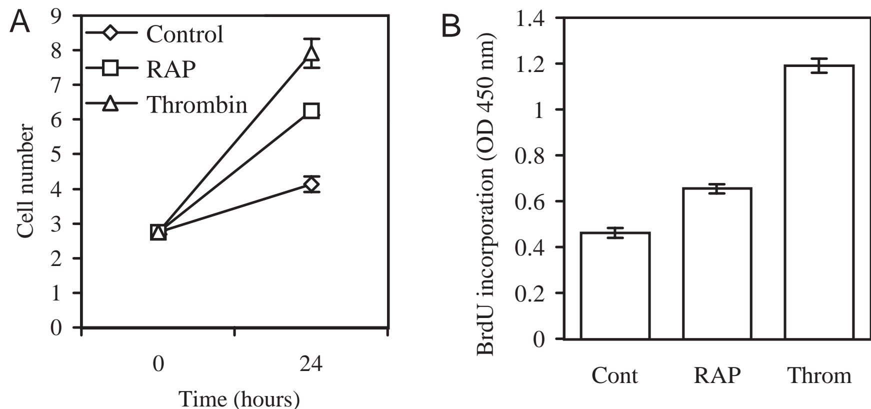

Fig. 5. (A) Effect of PAR activation on cell number in serum-deprived myoblast-enriched cultures. Cells were treated with RAP (300 μM), thrombin (100 nM) or no additive (Control) at the time of serum deprivation (T_0). Cell counts were conducted at T_0 and 24 hours later. Results are expressed as cell number $\times 10^{-4}$/well (mean ± s.e.m.; *n*=4). (B) BrdU incorporation in serum-deprived myoblast-enriched cultures. Cells were treated with RAP (300 μM), thrombin (100 nM; Throm) or no additive (Cont). Results are expressed as optical density (OD) at 450 nm (mean ± s.e.m.; *n*=8).

demonstrated that thrombin is a potent inhibitor of apoptosis and fusion of serum-deprived skeletal myoblasts (Chinni et al., 1999). It is known that myoblasts express one of the known thrombin receptors, PAR-1 (Suidan et al., 1996), but they do not express either of the other two, PAR-3 or PAR-4 (Chinni et al., 1999). Surprisingly, the PAR-1 tethered ligand peptide, TRAP-1, was unable to mimic thrombin's effect on apoptosis or fusion. There were two possible explanations for this observation, the first being that thrombin's effects on myoblast apoptosis and fusion are mediated by a novel thrombin receptor. Alternatively, since TRAP-1 activates PAR-2 as well as PAR-1, it seemed possible that the explanation for the lack of response was that concomitant activation of PAR-2 led to masking of the PAR-1 response. Although the results presented here demonstrate that, indeed, PAR-2 is expressed by myoblasts, we have ruled out the possibility that coactivation of PAR-1 and PAR-2 could explain the lack of effect of TRAP-1, since RAP had no significant effect on basal or thrombin-inhibited apoptosis (or fusion) of serum-deprived myoblasts. It seems, therefore, that thrombin's effects on apoptosis and fusion are mediated by a novel receptor, rather than by PAR-1.

In our studies on $[Ca^{2+}]_i$ mobilization in myoblasts, activators of PAR-2 induced $[Ca^{2+}]_i$ responses with a different profile to those induced by thrombin. Following treatment with RAP or trypsin, the initial peak was followed by a plateau due to Ca^{2+} entry, whereas thrombin-induced responses showed no sign of Ca^{2+} entry. Similar differences between responses to activators of PAR-1 and PAR-2 have been observed in osteoblasts (Abraham et al., 2000).

PAR-2 activation has previously been shown to stimulate proliferation of vascular endothelial and smooth muscle cells (Bono et al., 1997; Mirza et al., 1996), observations that led us to investigate the possibility that PAR-2 activation has the same effect in skeletal muscle cells. Serum-deprived myoblasts showed a significant increase in both cell number and BrdU incorporation in the presence of RAP, leading to the conclusion that RAP stimulates proliferation. It is interesting to note that neither the increase in cell number nor the increase in BrdU incorporation in RAP-treated cells was as great as that seen in thrombin-treated cells. Thrombin causes a profound inhibition of apoptosis as well as stimulating proliferation under the culture conditions used for these experiments (Chinni et al., 1999; Suidan et al., 1996), whereas RAP did not significantly inhibit apoptosis. It is likely, therefore, that part of the difference in cell number between the RAP and thrombin treatment groups was a result of differences in the number of apoptotic cells.

We have demonstrated expression of PAR-2 in rat muscle during late embryonic development, a time at which there is a rapid expansion in muscle mass. Thus, PAR-2-mediated proliferation is likely to be of some importance during muscle development. A role for PAR-2 in muscle development would also depend, however, on the presence of an activator of PAR-2. The only known mammalian activators are trypsin and mast cell tryptase, and it is unlikely that either of these is available in developing skeletal muscle. It is possible, however, that an unidentified muscle protease capable of activating PAR-2 exists. PAR-2 was also shown to be expressed in adult muscle, where it may play a role in responses to pathological insults involving mast cell infiltration and degranulation. Resident mast cells are found in low numbers in normal muscle, but in a number of pathological conditions including various forms of muscular dystrophy there is a substantial increase in mast cell numbers (Helliwell et al., 1990; Nahirney et al., 1997). There is some evidence that the contents of mast cell granules cause damage in dystrophin-deficient muscle (Gorospe et al., 1994). On the other hand, mast cells are more numerous in muscles of young *mdx* mice that are able to regenerate than in muscle of older animals in which there is little regeneration, observations that have led to the suggestion that mast cells contribute to muscle regeneration (Lefaucheur et al., 1996). Our observation of PAR-2-mediated proliferation in muscle cells points to a mechanism by which tryptase, one of the molecular constituents of mast cell granules, may promote muscle regeneration.

Expression of PAR-2 in skeletal muscle has not previously been described. In fact, Nystedt et al. (1994) were unable to detect PAR-2 expression by northern analysis in RNA extracted from mouse skeletal muscle; it is possible that the immunohistochemical and RT-PCR techniques used in the current study are more appropriate for detection of a membrane protein such as PAR-2, which is likely to be expressed at extremely low levels relative to other muscle proteins. Santulli et al. (Santulli et al., 1995) failed to detect expression of PAR-2 by dermal fibroblasts, whereas Akers et al. (Akers et al., 2000) observed expression of PAR-2 by lung-derived and dermal fibroblasts. We observed functional expression of PAR-2 on fibroblasts of neonatal skeletal muscle origin, although RT-PCR and calcium studies indicated that PAR-2 was less

abundant in fibroblasts than in myoblasts. These observations suggest that fibroblasts from different sources may vary in their expression of PAR-2.

In conclusion, PAR-2 is expressed by skeletal muscle cells in vivo and in vitro, and mediates proliferative responses in these cells. PAR-2-mediated proliferation is likely to be of some importance in pathological conditions of muscle involving mast cell infiltration and degranulation, and may also be important during muscle development and growth.

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