Heparan Sulfate Mimetics Modulate Calpain Activity During Rat Soleus Muscle Regeneration

M. ZIMOWSKA,1 D. SZCZEPANKOWSKA,1 W. STREMINSKA,1 D. PAPY,2 M.C. TOURNAIRE,2 J. GAUTRON,2 D. BARRITAULT,2 J. MORACZEWSKI,1 AND I. MARTELLY2*

1Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Poland
2Laboratoire de Recherche sur la Croissance Cellulaire, la Régeneration et la Réparation Tissulaires (CRRET), UPR ESA CNRS 7053, Faculté de Sciences et Technologie, Université Paris XII, Avenue du Général de Gaulle, Créteil, 94010 Cedex, France

Skeletal muscle regenerates after injury. Tissue remodelling, which takes place during muscle regeneration, is a complex process involving proteolytic enzymes. It is inferred that micro and milli calpains are involved in the protein turnover and structural adaptation associated with muscle myolysis and reconstruction. Using a whole-crush injured skeletal muscle, we previously have shown that in vivo muscle treatment with synthetic heparan sulfate mimetics, called RGTAs (for ReGeneraTing Agents), greatly accelerates and improves muscle regeneration after crushing. This effect was particularly striking in the case of the slow muscle Soleus that otherwise would be atrophied. Therefore, we used this regeneration model to study milli and micro calpain expressions in the regenerating Soleus muscle and to address the question of a possible effect of RGTAs treatment on calpain levels. Micro and milli calpain contents increased by about five times to culminate at days 7 and 14 after crushing respectively, thus during the phases of fibre reconstruction and reinnervation. After 64 days of regeneration, muscles still displayed higher levels of both calpains than an intact uninjured muscle. Milli calpain detected by immunocytochemistry was shown in the cytoplasm whereas micro calpain was in both nuclei and cytoplasm in small myofibres but appeared almost exclusively in nuclei of more mature fibres. Interestingly, the treatment of muscles with RGTA highly reduced the increase of both milli and micro calpain contents in Soleus regenerating muscles. These results suggest that the improvement of muscle regeneration induced by RGTA may be partly mediated by minimising the consequences of calpain activity. J. Cell. Physiol. 9999: 1–10, 2001.

INTRODUCTION

Skeletal muscle is able to regenerate after a trauma. The regeneration process includes muscle fibre degeneration followed by fibre reconstruction, which occurs through the activation of satellite cells (Schmalbruch, 1976; Schultz et al., 1985).

Tissue remodelling, which takes place during muscle regeneration is a complex process involving proteolytic enzymes (Maltz and Oron, 1990; Salminen, 1984). Among proteinases that are believed to be of major importance in the regeneration context, is the calcium-activated neutral cysteine proteinase, referred to as calpain (Murachi, 1984; Melloni and Pontremoli, 1989; Johnson, 1990). The calpain family comprises ubiquitous calpains, namely the micro calpain that requires 3–50 μM Ca²⁺ and the milli calpain requiring 200–1000 μM Ca²⁺ (Melloni and Pontremoli, 1989; Suzuki and Ohno, 1990; Suzuki and Sorimachi, 1998). Muscles also express the calpain 3 (p94 calpain), a skeletal muscle-calpain.

Contract grant sponsor: Association Française contre les Myopathies (AFM); Contract grant sponsor: Naturalia and Biologia; Contract grant sponsor: CNRS; Contract grant sponsor: Ministère de l’Enseignement supérieur, de la recherche et de la technologie; Contract grant sponsor: Action Intégrée; Contract grant number: 96439; Contract grant sponsor: French Ministère des Affaires Étrangères; Contract grant number: 251087A.

*Correspondence to: Prof. I. Martelly, Laboratoire CRRET, Faculté de Sciences et Technologie, Université Paris XII, Avenue du Général de Gaulle, Créteil, 94010 Cedex, France. E-mail: martelly@univ-paris12.fr

Received 10 July 2000; Accepted 13 February 2001
Published online in Wiley InterScience, XX Month 2001.
specific isotype (Jones et al., 1999; Kinbara et al., 1998; Sorimachi et al., 1993). Mutation in calpain 3 is involved in limb girdle muscular dystrophy (Richard et al., 1995) and indirectly increases myonuclear apoptosis through alteration of IκBα/NF-κB pathway (Baghdjian et al., 1999). Despite the increasing number of data that associate ubiquitous calpain activity and muscle pathologies (Rabbani et al., 1984; Nagy and Samaha, 1986; Turner et al., 1993; Spencer et al., 1995; Kumamoto et al., 1995; Voisin et al., 1996; Spencer and Tidball, 1996; Kumamoto et al., 1997; Badalamente and Stranger, 2000), the physiological functions of the ubiquitous calpains remain to be clarified (Carafoli and Molinari, 1998). The broad histological distribution of calpains suggests that they play an important role in all animal cells by initiating limited proteolysis (Suzuki et al., 1992; Suzuki et al., 1995; Suzuki and Sorimachi, 1998). They were found to be involved in degradation of muscle fibres (Huang and Forsberg, 1998; Saito et al., 1994). An increase in calpains was seen mostly in atrophic fibres from patients with Duchenne’s or Becker’s muscular dystrophy (Kumamoto et al., 1995) and in mdx dystrophic mice (Kumamoto et al., 1995; Spencer et al., 1995; Spencer and Tidball, 1996). These results suggest that in dystrophic muscles, where successive muscle fibre degradation and reconstruction occur, the increase in calpain activity principally correlates with fibre degradation. The level and activity of calpains are also regulated during in vitro myoblast differentiation (Schollmeyer, 1986; Brustis et al., 1994; Cottin et al., 1994; Barroy et al., 1997; Stockholm et al., 1999), thus suggesting a role for calpains in fibre reconstruction.

We developed a model of skeletal muscle regeneration where muscles are denervated and crushed from tendon to tendon (Bassaglia and Gautron, 1995). In this model of regeneration, an exhaustive myolysis occurs within the first 3 days, which is followed by muscle fibre reconstruction that concerns the entire muscle. This permits quantitative biochemical investigation at the whole muscle level. In this model, the extensor digitorum longus (EDL) fast muscle regenerates properly and shows a quasi-normal structure starting day 16 after wounding. In contrast, the regeneration, which follows muscle fibre degeneration and reconstruction, is heterogeneous, and after 16 days post wounding, regeneration and innervation do not progress further. Finally, muscle degenerates and becomes fibrotic.

We have found that a new class of compounds composed of heparan sulfate mimetics is able to stimulate the repair of several tissues (Fredj-Reygrobellet et al., 1994; Blanquaert et al., 1995; Meddah et al., 1996a; Desgranges et al., 1997). In particular, these compounds improve muscle regeneration as evaluated by the number and the size of newly formed myotubes, the morphology of the neuromuscular junction, and the revascularisation characteristics (Gautron et al., 1995; Aamiri et al., 1995a,b; Desgranges et al., 1995). Muscle regeneration improvement is especially remarkable in the Soleus muscle. Treatment of regenerating Soleus muscle with these heparan sulfate mimetics stabilizes reinnervation and reduces fibrosis (Aamiri et al., 1995b).

These molecules, called RGTA (for ReGenerating Agent) are dextran derivatives which behave in vitro as mimics of heparan sulfate with regard to their ability to interact with and protect heparin binding growth factors (HBGF) against proteolytic degradation (Tardieu et al., 1992; Meddah et al., 1996c). To our knowledge, RGTA is the only example of a treatment for which denervated and crushed Soleus muscle recovers function and does not turn into a fibrotic tissue. In addition, these compounds have been shown to inhibit plasmin (Meddah et al., 1995; Ledoux et al., 2000) and elastase from leukocyte (Meddah et al., 1996c), thus reducing the consequences of inflammation in muscular tissue. Altogether, it seems that RGTA molecules improve regeneration because they increase the bioavailability of growth factors and can modify certain proteinase activities during muscle degeneration and regeneration.

The crush-induced regeneration model that we established gives the opportunity to investigate changes of calpain contents during the regeneration process and allows addressing the question of a possible effect of pharmacological agents such as heparan sulfate mimetics (RGTA) on these proteinase contents. In a previous study, we have shown that RGTA treatment highly stimulates differentiation of satellite cells grown in primary cultures. Interestingly, this RGTA treatment also reduced micro and milli calpain mRNA by about 50% as compared to untreated satellite cells (Stockholm et al., 1999). It was therefore of interest to establish the variations of milli and micro calpain contents in the course of Soleus muscle regeneration and to investigate whether RGTA treatment modifies calpain activity in these muscle.

We showed that an increase in micro and milli calpain concentrations occurs in untreated regenerating muscles, in particular during muscle fibre reconstruction. This increase was suppressed in crushed muscles treated with the heparan sulfate mimetic RGTA. We concluded that acceleration and improvement of muscle regeneration could be at least partially due to a reduced activity of certain proteinases such as calpains.

**MATERIALS AND METHODS**

**Materials**

The RGTA presently used, namely RG1192, is a dextran derivative obtained by controlled and sequential substitutions on the glucose residues of a batch of dextran T40 (average Mr 37,000; Pharmacia, Sweden) according to Mauzac and Jozeofonvicz (1984). In brief, dextran T40 was carboxymethylated on OH residues, then a partial amidation of the carboxylated residues with benzylamine was performed followed by an O-sulfonation. The chemical characterisation of the final product was established by acidic titration for CH₂COONa and SO₃Na contents and by elementary analysis for N and S contents of CH₂CONHCH₂C₆H₅ and SO₃Na respectively. Infrared spectroscopy was used to confirm the presence of carboxyl, amide, and sulfate groups. ¹H-nuclear magnetic resonance (¹H-NMR) allowed a precise characterisation of the substitute in C2 of glucose units. The structure of the final product is shown in Figure 1. The average molecular weight of the RG1192 was 140,000 as determined by high-performance size exclusion chromatography in 0.1 M NaNO₃, using KB 804 and KB 805 aqueous gel filtration columns.
CALPAIN IN REGENERATING RAT SOLEUS MUSCLE

Fig. 1. Schematic structure of the dextran derivative RG1192. Polymers were elaborated from T40 dextran by chemical substitution as described in Material and Methods. The different percentages indicated in the figure were calculated from the degree of substitution (d.s.) relative to the position of each group in a glucose unit, assuming that the maximum of d.s. is 3 since the carbons at C2, C3, and C4 positions are susceptible to react. For an easier representation, the substituted glucosidic units were arranged in an arbitrary combination. Their respective proportions within each polymer were calculated according to the nature of the group linked to the C2 position determined by \(^1\)H-NMR. “R” represents the proportion (percentage) of each substituted group in the positions C3 + C4 taken globally.

(Shodex, Japan) applied in series. This polymer did not present any significant anticoagulant activity (less than 5 IU/mg as compared to 173 IU/mg for heparin).

The phenyl-sepharose CL-4B was from Pharmacia and the ion exchange chromatography (DEAE-cellulose 52) was from Whatman. The polyclonal antibodies raised against micro and milli calpains and purified micro calpain were from Calbiochem (San Diego, California). The chemiluminescence detection kit was from Roche (Meylan, France). All the other products were from Aldrich (St. Quentin, Fallavier, France).

**Regeneration experiment**

The regeneration of Soleus muscles was induced in 2 months old, male Wistar rats according to (Bassaglia and Gautron, 1995). All procedures complied with the “Principles of Laboratory Animal Care” and “Guide for the Care and Use of Laboratory Animals” (NIH Publication No80-23, revised 1985). In brief, rats were anaesthetised by ether and the involved muscle was exposed. Muscle was denervated by sectioning the motor nerve at the muscle entrance, then it was crushed with forceps from tendon to tendon. RGTA (RG1192, 100 µg diluted in 100 µl 150 mM NaCl) was then injected into the injured muscle. Other animals having injured muscles received the same volume of 150 mM NaCl and were referred to as controls. After 3, 7, 14, and 64 days of regeneration, the animals were euthanised by ether and the regenerating and contralateral muscles of each animal were harvested and weighed. Muscles were then either directly used for calpain assays or snap frozen in isopentane cooled by liquid nitrogen for further histological studies. At each time, at least three rats were used and each experiment was repeated three times. Intact Soleus muscles were taken from uninjured animals in order to determine the levels of micro and milli calpains in these muscles. The concentration of calpains in crushed muscles or their contralateral muscles were expressed as a percentage of the concentrations of calpains found in these intact muscles.

**Preparation of cellular extracts and calpain purification**

Muscles were homogenised in a buffer containing 20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 250 mM sucrose, 1% Triton X-100, 0.01% leupeptin, 0.5 mM PMSF, and 10 mM (mercaptoethanol) at pH 7.5. Crude extracts were centrifuged at 10,000 g for 10 min and the obtained supernatants were clarified by filtration through glass wool. All operations were performed on ice. Calpains were purified according to Moraczewski et al. (1996) using hydrophobic and ion-exchange chromatography. The clarified supernatant was loaded onto a phenyl-sepharose CL-4B column previously equilibrated with 20 mM Tris-HCl buffer pH 7.4, containing 2 mM EDTA, 2 mM EGTA, and 1 M NaCl. The column was washed with decreasing concentrations of NaCl. The calpain fraction was eluted by buffer without NaCl, with 1% ethylene glycol (Karlsson et al., 1985). This step enabled the separation of calpastatin, the calpain inhibitor, from calpains. Then, the fraction containing calpains was loaded onto a DE52 column previously equilibrated with 20 mM Tris-HCl buffer pH 7.4, containing 2 mM EDTA, 2 mM EGTA. Milli calpain was eluted at concentration of 0.5 M NaCl where the micro calpain-containing fraction was eluted at concentration 0.2 M NaCl (Birkhold and Sams, 1994). The entire procedure was performed at 4 °C in a cold room.

**Assay of calpain activity**

The calpain activity assay was performed using a substrate specific to calpains (N-succinyl-Leu-Tyr-7-amide-4-methylcoumarin), (Sasaki et al., 1984). A buffer containing 50 mM calpain substrate and 10 mM CaCl\(_2\), or 10 mM EGTA was incubated with the calpain fraction for 30 min at 25 °C. The reaction was stopped with 9 mM EDTA. Fluorescence was measured in a Shimadzu spectrofluorimeter at 495 nm (excitation) and 520 nm (emission). The activity obtained was expressed as arbitrary units representing the difference between samples to which CaCl\(_2\) had been added and the basal activity with EGTA. Experiments included several dilutions of purified micro calpain as a standard. The activity of calpains was finally expressed as nmol of calpain equivalent. Results are given as a percentage of enzyme concentration in Soleus regenerating or contralateral muscles compared to intact muscles. In an intact muscle of uninjured animal, micro and milli calpains reached 16.96±2.8 nmol/mg protein and 14.96±0.94 nmol/mg protein respectively.

**Zymography**

Zymography was made according to Raser et al. (1995). In brief, 200 µg of protein/well were loaded onto 10% acrylamide gels containing 0.2% α-casein. Protein measurement was made according to Bradford (1976).
After electrophoresis, the gels were incubated with buffer containing 20 mM Tris-HCl, 4 mM CaCl₂ and 10 mM DTT for 48 h. Gels were then stained with Coomassie blue and scanned using the Gel.Doc. Computer program.

**Immunolocalisation and immunoblotting of calpains**

Muscles frozen in isopentane cooled by liquid nitrogen were cut into transversal crosssection (10 μm thick) at −20°C using a cryostat. Sections were fixed with paraformaldehyde. Activity of endogenous peroxidase was blocked with 0.3% H₂O₂, 40% methanol in PBS and endogenous biotin was blocked with avidin. Non-specific sites were blocked with PBS containing 0.2% Tween-20, 3% BSA, and 10% goat serum in PBS. Sections were incubated with primary antibody raised against milli or micro calpain (Calbiochem) at final dilution of 1:100 in 0.2% Tween-20 and 3% BSA in PBS. After two washes with PBS, the sections were incubated with biotin-conjugated anti rabbit IgG antibody at final dilution of 1:100 for 1.5 h and then with avidin conjugated with peroxidase for 30 min. Finally, sections were incubated with diaminobenzidine for 10 min and washed with several changes of deionised H₂O.

The specificity of antibodies raised against calpains was checked by Western blot technique after SDS-polyacrylamide (10%) electrophoresis according to standard procedure. Primary cultures of satellite cells isolated from leg muscles of 2 months old rat were performed. Some cultures were treated with RGTA at 25 μg/ml the day of plating as previously described in Stockholm et al. (1999). Samples containing 20 μg of protein extract prepared at day 6 of culture were electrophoresed and transferred to PVDF membranes (Immobilon-P, Millipore). Micro-calpain (calpain I) monoclonal antibody was used at 1/800 and milli calpain (calpain II) polyclonal antibody was used at 1/400. Peroxidase conjugated secondary antibody was diluted at 1/1,000. Detection of bands was carried out by chemiluminescence.

**RESULTS**

**Variations of milli and micro calpain concentrations in untreated Soleus regenerating muscle**

The milli calpain concentration in Soleus regenerating muscle injected with NaCl only (controls) was, at day 3, at a level about twice the level found in intact muscles of non uninjured animals (P < 0.01) (Fig. 2A). Milli calpain concentration further increased to reach its highest level at day 7 of regeneration. It was then about five times that of intact muscles of uninjured animals (P < 0.001). Milli calpain concentration then decreased slowly to reach at day 64 after crush a level which was about three times the level found in an intact muscle (P < 0.001) (Fig. 2A).

The level of micro calpain concentration in controls at day 3 was about 150% of the level found in intact untreated muscle (P < 0.05) (Fig. 2B). The activity of micro calpain then increased from day 3 after crushing, to reach its highest level at day 14 when it was five times higher than found in the intact muscle (P < 0.001). It subsequently decreased at day 64 after the operation. At this time however, it remained at about twice the level found in intact muscle of uninjured animals (P < 0.001) (Fig. 2B).

In contralateral muscles, the concentrations of both milli (Fig. 2A) and micro (Fig. 2B) calpains were increased at day 3 compared to intact muscle level, and were even higher than in crushed muscles. Then, the calpains concentrations decreased between days 7 and 14 in these unwound contralateral muscles to levels statistically equivalent to those found in intact muscles of uninjured animals.

**Effect of RGTA on milli and micro calpain concentrations in Soleus regenerating muscle**

The presence of RGTA did not have an effect on the initial rise in milli calpain amount at day 3 in the crushed muscles (Fig. 3A). In the following days, treatments with RGTA abolished the increase of milli calpain concentration that was observed in controls of non-injured animal (shown as a line at 100%). White bars: regenerating muscles; black bars: contralateral muscles. Statistical analysis (Student test): at days 3 and 7 calpain content in contralateral muscles differed from intact muscle of non-injured animal with P < 0.001 and P < 0.05 at day 3 and P < 0.001 at the other days.
Untreated muscles injected with NaCl. At day 64, the enzyme concentration in RGTA treated muscles was reduced to the level found in intact muscle of uninjured animals but never fell below the normal range (Fig. 3A). RGTA has no significant effect on calpain activity in contralateral muscles compared to the control situation (Fig. 3C).

Treatments with RGTA also abolished during the whole period of observation the increase in micro calpain concentration that occurred in control (NaCl injected regenerating muscles from day 3 to 64 after crush (Fig. 3B). In contralateral muscles, RGTA injection did not significantly alter calpain level compared to the corresponding contralateral muscles of control animals (Fig. 3D).

Zymography studies

Representative zymographies of regenerating muscles are shown in Figure 4. At day 3 the activities of calpains were similar in control (NaCl treated) and
RGTA treated muscles. The differences between control and RGTA treated muscles at days 14 and 64 were not very striking in the case of milli calpain. At day 14 or 64, the activities of micro calpain found in extracts from RGTA-treated muscles were reduced compared to the activity found in control muscles. These observations correlated with the results obtained with enzymatic activity assays.

**Immunolocalisation of calpains during muscle regeneration**

We have examined the localisation of milli and micro calpain in intact Soleus muscle (Fig. 5A and D). We also examined calpain localisation during regeneration of Soleus muscles injected with RGTA and in muscles injected with NaCl at days 3, 7, and 14 of regeneration (Fig. 5B shows muscles at days 7 and 14 after crush). In the absence of the primary antibodies raised against calpains, no labelling was observed (Fig. 5G and H). As shown in Figure 6, the calpain antibodies recognised the large subunits of the enzyme in protein extracts from satellite cells grown in primary cultures. Similar results were also obtained with skeletal muscle extracts (Moraczewski et al., 1996). Accessorily, it can be noticed the RGTA treatment slightly reduced calpain content in these satellite cell extracts, especially micro calpain.

In intact muscle fibres, milli calpain was shown in the cytoplasm of fibres and in cells surrounding the fibres (Fig. 5A). Micro calpain appeared almost exclusively in nuclei and in cellular structures surrounding the fibres (Fig. 5D). At day 3 after injury, we observed pronounced myolysis and the fibres were completely disorganized. In these muscles, inflammatory cells...
were seen, which were identified mainly as polymorphonuclear lymphocytes and to a less extent as macrophages. Activated satellite cells, detected as desmin positive cells, and debris of muscle fibres were observed in these muscles. In all the observed cross sections, both milli calpain immunostainings were visible in inflammatory cells and mononucleated cells, possibly satellite cells (desmin positive cells) (not shown).

At day 7 after crushing in control muscles injected with NaCl, regression of inflammatory edema had occurred but a high number of blood cells and macrophages were still visible. At that time, as previously observed (Gautron et al., 1995), Soleus muscles injected with RGTA showed a more advanced regeneration in comparison to control muscles treated with NaCl (Fig. 5B and E). A greater number of newly formed myotubes of larger size, in which nuclei were localized centrally, were observed. Milli calpain was found in the cytoplasm of young fibres (Fig. 5B, white arrows). In some newly formed fibres, milli calpain was also shown around the nucleus (Fig. 5B, black arrow). A sustained label was also visible at the periphery of the fibres (Fig. 5B). In these muscles, micro calpain was observed in the cytoplasm of small myotubes and mononucleated cells, possibly satellite cells (Fig. 5E, white arrow). In larger fibres, micro calpain was mainly found in the nuclei often localized in the middle of the fibre, or in nuclei that begins to shift to a lateral position in the fibre (Fig. 5E, black arrow). It was also shown at the periphery of muscle fibres (Fig. 5E). At day 14 of regeneration, the inflammation cells had disappeared. In muscles injected with NaCl, new muscle fibres with irregular size were observed (not shown). Treatments with RGTA improved the regeneration in comparison to control muscles treated with NaCl, in particular the diameter of fibres were larger and of more homogenous size. Milli calpain was observed in cytoplasm of myotubes (Fig. 5C), whereas micro calpain concentrated almost exclusively in the nuclei of mature fibres as in intact muscle fibres (Fig. 5F).

DISCUSSION

Different experimental methods for inducing degeneration and regeneration have been established (Chambers and McDermott, 1996). These methods include exhaustive exercise (Belcastro et al., 1998; Smith et al., 1999), muscle ischemia (Carlson and Gutmann, 1975; Desgranges et al., 1999), and single injection of toxin (Whalen et al., 1990; Marsh et al., 1998). Many of these treatments do not induce homogenous muscle fibre destruction and the regenerative response in not uniform. In the experimental model presently used, the muscle undergoes complete degeneration within the first 3 days and then the reconstruction of muscle fibres takes place. Thus, it was appropriate to investigate changes in calpain contents during muscle regeneration and to address the pertinent question proposed by others (Tidball and Spencer, 2000) of a possible pharmacological modulation of calpain through RGTA treatment.

We first established the variations of calpains during the regeneration of Soleus muscle. In order to assess a possible systemic reaction induced in the contralateral muscle by the crushing, we used intact muscles from non-injured rats as reference for calpain concentration. Indeed, contralateral muscles showed a transient increase in calpain amounts compared to intact muscles of non-injured animals, within the first days following injury. Several reasons could account for this transitory increase. This possibly reflects a sustained solicitation of the Soleus contralateral muscle because of the unilateral crush. It might also reflect a systemic reaction of the organism to the injury, in particular to the nerve cutting, possibly through a transmedullar process or through blood circulation. It has been shown that unilateral sciatic nerve injury induced up-regulation of genes in both denervated and contralateral muscles (Tang et al., 2000). A lesion in the sciatic nerve increased the expression of II-1β and TGF-β1 in the opposite root ganglia and had a transmedullar effect on contralateral nerve (Ryoke et al., 2000). Inflammatory cells were not seen in contralateral muscles in our experiments. It cannot be excluded however that inflammatory signals such as cytokine or mediators of neurogenic inflammation might reach these contralateral muscles either through the spinal cord (Decaris et al., 1999) or through blood circulation, and alter calpain expression in these muscles.

At day 3 in crushed NaCl-treated Soleus muscles (controls) both micro and milli calpain amounts were higher than in intact muscles of non-injured animals. This increase in calpain expression, which was more important in the case of milli calpain, was probably...
associated to inflammatory cells numerous in these muscles. It could also be associated to fibre degeneration that occurs up to days 3–4 in our model. Several studies have shown indeed that calpain activation was associated with proteolysis and fibre degradation. An increase in calpains was seen mostly in atrophic fibres of muscles from patients with Duchenne’s or Becker’s muscular dystrophy (Kumamoto et al., 1995).

We have further examined calpain concentrations in regenerating Soleus muscles up to day 64 after crushing. Milli and micro calpains reached their maximum of concentration respectively at day 7, during the phase of muscle fibre reconstruction, and at day 14, when an attempt of re-innervation occurs. This suggests that these calpains might have different regulation determinism and/or function in the regenerating process.

Interestingly, following a single injection of RGTA performed into Soleus muscle just after crushing, the increases in concentrations of both calpains were highly reduced when muscle fibres reformed. Sixty-four days after crush and RGTA injection, calpain activity had nearly resumed the normal level of an intact uninjured muscle. The fact that RGTA reduced calpain content in vivo is consistent with our previous observation. Indeed, it was shown that RGTA treatment, which stimulates satellite cell differentiation, reduces the mRNA levels of micro and milli calpains in differentiating primary cultures of rat satellite cells (Stockholm et al., 1999).

In addition, milli and micro calpains also showed different localisation during regeneration. In intact muscles, whereas micro calpain was almost exclusively in nuclei, milli calpain was intracellular mostly at the peripheral areas of cytoplasm. In regenerating muscles, milli calpain was generally found in the cytoplasm of fibres, and was sometimes found around the nuclei of young fibres. This localisation of milli calpain was consistent with observations made by others (Kumamoto et al., 1992; Spencer and Tidball, 1996). In contrast, micro calpain, which was temporarily present in the cytoplasm of young neo-formed myotubes, was visible mainly in the nuclei of more mature muscle fibres (Fig. 5). Such differential localisations of milli and micro calpains have not yet been described in other tissue or in regenerating muscle and suggest differential function of these calpains in the muscular tissue.

The role of calpain in myogenesis is still controversial and most information comes from in vitro studies. It had been proposed that milli calpain is required for membrane fusion of foetal myoblasts (Brustis et al., 1994). Modifications of some cytoskeleton proteins would facilitate fusion of the cells (Dourdin et al., 1999). On the other hand, studies on L8 cell line myoblasts showed that milli and micro calpain do not change significantly in fusing cultures. The ratio of calpain to calpastatin, the intracellular calpain inhibitor, is possibly the most important parameter to consider in regulating myoblast fusion (Barony et al., 1997). Since RGTA treatment moderates the increase in calpain content and improves the overall regeneration process, a direct role of these proteases on myoblast fusion and muscle fibre reconstruction is unlikely to be prevalent. We have observed that the re-innervation of Soleus muscle fibres is abortive in our model in the absence of RGTA treatment (Aamiri et al., 1995b). This suggests that a reduced calpain concentration would favor the stabilisation of innervation. According to this reasoning, it was reported that inhibition of calpain could have therapeutic potential (Wang and Yuen, 1994). Leupeptin application improved re-innervation in rats (Harding et al., 1999). Interestingly, leupeptin injection was also shown to improve muscle repair of mdx dystrophic mice (Stratcher, 1999; Badalamente and Stracher, 2000). RGTA has been shown to inhibit calpain activity in an in vitro biochemical assay (unpublished results). Thus RGTA, which alleviates calpain content, could operate also through a direct inhibitory effect on calpain enzyme in vivo.

The presence of RGTA in regenerating muscle might have pleiotropic effects, including heparin binding growth factors protection and bioavailability, and preservation of matrix structure. It also might reduce inflammation effects since RGTA inhibited the activity of plasmin and of leukocyte elastase (Meddahi et al., 1995; Meddahi et al., 1996c; Ledoux et al., 2000). The present study indicates that RGTA also might potentially reduce protein degradation since it limits the amounts of calpains in the muscles. This might be an important factor in the success of regeneration. Taken together, these RGTA effects would re-establish a subtle equilibrium between different molecules that control the regeneration process and would preserve a cellular environment favorable to satellite cell activation and differentiation.

The present work suggests that RGTA mediates this effect by acting on calpain overall activity. The ability of RGTA to facilitate regeneration in Soleus muscle suggests a new therapeutic opportunity for the treatment of various neuromuscular disorders.

Acknowledgments

The collaboration between the French and Polish teams was supported by an Action Integre´e. The French Ministére des Affaire Etrange`res was awarded to M.Z.

Literature Cited


