FEBS Letters 359 (1995) 60–64 FEBS 15086

Isolation and identification of a μ-calpain-protein kinase Cα complex in skeletal muscle

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Received 5 December 1994

Abstract **A μ-calpain-PKC complex was isolated from rabbit skeletal muscle by ultracentrifugation and by anion-exchange chromatography. The PKC associated to μ-calpain was stimulated by calcium, phosphatidylserine and diacylglycerol, and corresponds to a conventional PKC (cPKC). This complex presents an apparent molecular mass close to 190 kDa and is composed of one μ-calpain molecule and of one cPKC molecule. Using monoclonal antibodies specific for the different cPKC isoforms, the isoenzyme associated to μ-calpain was identified as cPKCα. Immunofluorescence staining reveals a co-localization of μ-calpain and cPKCα on the muscle fibre plasma membranes.**

Key words: Protein kinase C; Calpain; Skeletal muscle

1. Introduction

Calpains are intracellular Ca^{2+}-dependent proteases whose biological functions are still unknown despite their widespread presence in species ranging from honeybee [1] to man [2]. The major difficulty to determine their action in vivo, is inherent to the fact that their physiological substrates have still not been identified with certainty. Two ubiquitous isoenzymes, μ and m-calpains, with distinct calcium sensitivities in vitro, were originally isolated [3,4]. Recently, tissue-specific calpains have been identified in skeletal muscle [5] and in the stomach [6]. μ and m-calpains hydrolyse protein kinase C (PKC) in vitro, and generate an N-terminal regulatory domain and a C-terminal catalytic domain [7]. PKCs are divided into three groups [8]. One is Ca^{2+}-sensitive and phorbol ester-responsive (conventional PKCα, β1, β2, γ), another is Ca^{2+}-insensitive and phorbol ester-responsive (novel PKC δ, ε η θ) and the last group, called atypical, contains isoenzymes as PKC ξ μ and ι which are Ca^{2+}-independent and are not activated by diacylglycerol or phorbol esters [9–11].

We have previously reported that μ-calpain co-elutes with a PKC activity through hydrophobic and anion exchange chromatographies of cytosolic fractions obtained from skeletal muscle [12,13]. The co-elution of μ-calpain and PKC on these columns could correspond either to the presence of a multienzymatic complex or to the fact that these enzymes have the same hydrophobicity and the same net charge. In order to clarify this point, we analysed the μ-calpain-PKC co-purifying enzymes from skeletal muscle by gel filtration and by non-denaturing gel electrophoresis and observed that these two enzymes were physically associated. Then, we used denaturing electrophoresis to assess the subunit composition of this complex. Using different cofactors, we have also determined to which group belonged the PKC associated with μ-calpain and, by Western blotting, we characterised the PKC isoform corresponding to this group. Finally, by immunohistochemical analysis, we have localised μ-calpain and cPKCα in the muscle fibre.

2. Materials and methods

2.1. Materials

Casein was obtained from Merck, Darmstadt, Germany (art. 2244). Redivue [γ-^{32}P]ATP (5000 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Q-Sepharose Fast Flow and Sephacryl S-300 HR were supplied by Pharmacia Biotech (Uppsala, Sweden). Monoclonal antibodies specific for α β and γ PKC isoenzymes (clones MC-3a, MC-2a and MC-la, respectively) were from Seikagaku Corp. (Tokyo, Japan). Anti-calpain polyclonal antibodies specific for the 80 kDa subunits of μ and m-calpains from rabbit were raised in goats by Dr. Levieux (INRA, Theix, France) and purified in our laboratory as previously described [14]. Immobilon-P membrane was purchased from Millipore (Bedford, MA). Phenylmethanesulfonyl fluoride (PMSF) was from Fluka Chemie (Buchs, Switzerland). 1-Oleoyl-2-acetyl-rac-glycerol used as diacylglycerol, the gel calibration kits and the other chemicals, were from Sigma (St. Louis, MO).

2.2. Preparation of cytosol

All operations were conducted at 4°C. Muscles from adult, male New Zealand rabbits, killed by cervical dislocation, were rapidly dissected and homogenised in a Waring Blender, for 30 s at low-speed followed by 30 s at high speed, with 5 vols. of extraction buffer (30 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 1 mM PMSF, 10 mM benzamidine, 10 mM EGTA, 2 mM EDTA, 0.5 mM DTT and 1 mM NaN_3). The homogenate was centrifuged at 12,000 × *g* for 15 min. The supernatant was filtered through cheese-cloth to remove free-floating fat and centrifuged at 100,000 × *g* for 60 min. The pH of the supernatant obtained after ultracentrifugation was adjusted to 7.4 with NaOH. The resulting preparation is defined as the cytosol.

2.3. Anion-exchange chromatography

Approximately 300 ml of cytosol (3 g protein) were loaded onto a Q-Sepharose fast flow column (2.6 × 11 cm) previously equilibrated with buffer A (30 mM Tris-HCl, pH 7.4, containing 1 mM PMSF, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT and 1 mM NaN_3). The column was then washed with buffer A until the outflow reached the baseline. The adsorbed proteins were eluted with 6 bed volumes of a 0–250 mM NaCl linear gradient in the same buffer. An aliquot of each fraction was assayed for calpain and PKC activities.

2.4. Gel filtration on Sephacryl S-300 HR

The fractions obtained from the Q-Sepharose column and containing the μ-calpain and PKC activities were pooled (approximately 30 mg of protein) and concentrated on an Amicon PM 30 membrane. These

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SSDI 0014-5793(95)00014-3

active fractions were loaded onto a Sephacryl S-300HR column (1.6 × 40 cm) previously equilibrated in buffer A containing also 0.15 M NaCl (buffer B). The proteins were eluted with buffer B at a flow rate of 25 ml/h. Fractions of 3 ml were collected and their enzyme activities assayed. The partition coefficient K_{av} for calibration of the column was calculated as: $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_e = elution volume of the protein, V_o = void volume and V_t = total bed volume.

2.5. Enzyme assays

Calpain activity was assayed as described by Wolfe et al. [15] using fluorescein-casein as substrate and measuring the fluorescence of a 2.5% trichloroacetic acid supernatant. Casein was labeled with fluorescein isothiocyanate according to the method of Twining [16]. Fluorescence was measured with a Shimadzu (Kyoto, Japan) RF-551 S spectrofluorimeter using an excitation wavelength of 495 nm and an emission wavelength of 518 nm. Specific activity was expressed as fluorescence units (FU)/mg protein per min. Protein kinase C activity was measured as previously described using histone type III S as substrate [17].

2.6. Polyacrylamide gel electrophoresis

Non-denaturing electrophoresis was carried out in linear 5–18% polyacrylamide gels. Samples, (50 μg/lane) were electrophoresed in a cold room at 4°C for 24 h, at a constant voltage of 150 V. Molecular weight standards were run on the same gel and included apoferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (67 kDa). SDS-PAGE electrophoresis were carried out on 8% separating gels and samples were run at 25 mA for 2 h 30 min.

2.7. Immunoblotting

The proteins obtained after gel electrophoresis were electrotransferred (2.5 mA/cm^2, 30 min) to an Immobilon-P membrane using a Millipore blot semi-dry electroblotter (Millipore Corp., Bedford, MA). The non-specific sites were blocked for 2 h at 25°C with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.3) containing 5% (w/v) skim milk. After washing 4 times for 5 min in PBS, the membrane was incubated for 4 h at room temperature with the α β or γ antibodies diluted 1:200 with 0.1% (w/v) skim milk in PBS or with the calpain antibodies diluted 1:100. Antibody binding was detected with IgG conjugated to peroxydase (dilution 1:1000) after SDS-PAGE of the proteins obtained by gel filtration. After SDS-PAGE of the proteins obtained by non-denaturing electrophoresis, which were present in low quantity, the transfer membrane was incubated overnight with IgG conjugated to alkaline phosphatase (dilution 1:10 000).

2.8. Immunohistochemical analysis

Sectioned rabbit skeletal muscles were fixed in 4% formaldehyde for 1 h at 4°C. After washing with 20% sucrose in phosphate-buffered saline (PBS), the muscles were frozen in isopentane chilled by liquid nitrogen and sectioned at 8 μm in a cryostat at −20°C. The sections were disposed on glass slides coated with gelatin and incubated for 10 min with bovine serum albumin to block the non-specific sites. The sections were then incubated for 10 min in PBS containing the anti-PKCα or anti-μ-calpain antibodies. Following washing, sections were incubated for 1 h with anti-mouse IgG or anti-goat IgG conjugated to FITC. Control sections lacked the primary antibodies but were treated identically to experimental sections. Preparations were examined with an epiilluminated fluorescence microscope (Leitz, Wetzlar/Germany) and photographed on Ilford HP-5 plus black and white 400 ASA film.

3. Results

As presented in Fig. 1, μ-calpain co-eluted with a PKC activity from a Q-Sepharose column between 160 and 180 mM NaCl using 6 bed volumes of a 0–250 mM gradient. In agreement with Koohmaraie [18] we observed that, by using at least five column volumes of salt gradient, μ-calpain was effectively separated from the calpain inhibitor calpastatin. In this manner, calpastatin did not interfere with the measurement of μ-calpain activity. The m-calpain was eluted between 0.35–0.4 M NaCl as previously described [12], and consequently, was not present in the fractions eluted with the 0–250 mM gradient. Fig. 2 shows that after gel filtration on Sephacryl S-300 HR, the μ-calpain and PKC co-purifying activities obtained from Q-Sepharose are again strictly superposed. This result is highly reproducible on condition that the serine-protease inhibitors were present in the extraction medium and during all chromatographic steps. It appears that μ-calpain and PKC activities co-eluted just after β-amylase from sweet potato (200 kDa) and that, after calibration of the gel filtration column, this multi-

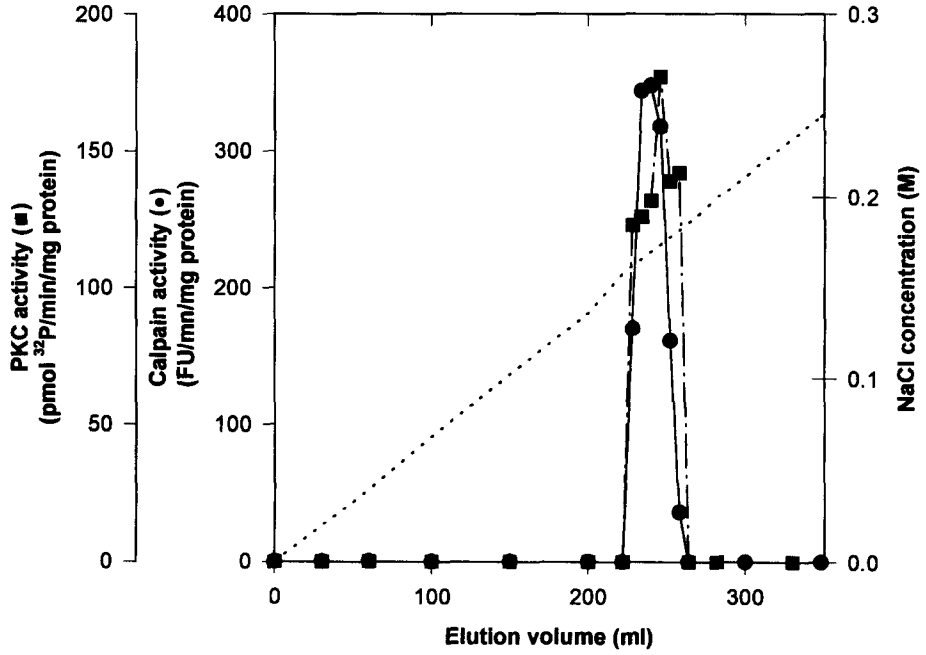

Fig. 1. Chromatography of the cytosolic fraction on a Q-Sepharose column.

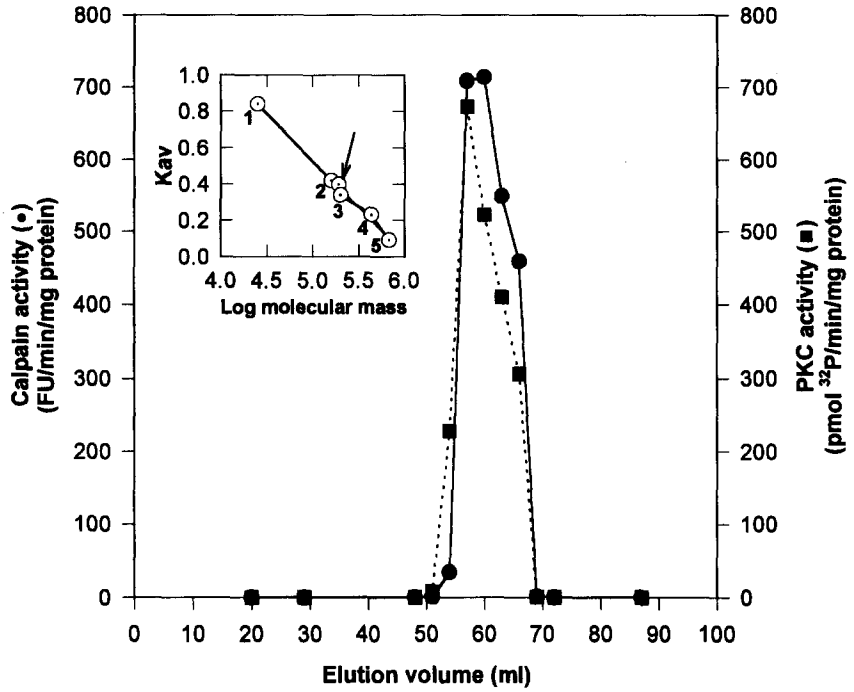

Fig. 2. Chromatography of Q-Sepharose purified extracts on a Sephacryl S-300 HR column. Inset: calibration of the Sephacryl S-300 HR column with: (1) chymotrypsinogen (25 kDa), (2) aldolase (158 kDa), (3) β-amylase from sweet potato (200 kDa), (4) ferritin (443 kDa), (5) thyroglobulin (669 kDa). The arrow indicates the position of the μ-calpain-PKC complex.

enzymatic complex has an apparent molecular mass of approximately 190 kDa (Fig. 2, inset).

As shown in Fig. 3, the kinase activity present in the complex was both Ca^{2+} and phosphatidylserine (PS) dependent. Addition of diacylglycerol to the assay medium, which already contained Ca^{2+} and PS, greatly enhanced kinase activity. In the absence of calcium, no activity was detectable in the presence of PS. These results imply that the PKC associated with μ-calpain belongs to the conventional PKC (cPKC) group.

To determine the type of cPKC present in the complex obtained after gel filtration, Western blot analysis were conducted with monoclonal anti-cPKC antibodies. Western blot yielded an immunoreactive band of around 80 kDa only with the PKCα antibody (Fig. 4). No reaction occurred with the β or γ PKC antibodies. These results are in agreement with a previous Western blot study we have carried out on μ-calpain-PKC co-eluting enzymes obtained after Q-Sepharose [13]. However, the antibodies used for this previous experiment did not permit a discrimination between the α and β isoforms as in the present study.

As presented in Fig. 5A, non-denaturing gel electrophoresis resolves the co-purifying enzymes obtained after Q-Sepharose into major bands between 67 and 190 kDa. Only the 190 kDa band was resolved into major protein bands around 80 kDa (Fig. 5B), one of which being subsequently recognised by the μ-calpain antibody, but not by the m-calpain antibody (Fig. 5C). A 28–30 kDa band corresponding to the molecular mass of the small subunit of calpains appeared from time to time after the second dimension electrophoresis. However, due to the duration of the non-denaturing electrophoresis and to the lability of this protein, this result was not constant. A 78 kDa band was also recognised by the PKCα antibody (5D). In addition, the active fractions were shown not to contain detectable PKC β or γ, using the corresponding monoclonal antibodies (5D). μ-calpain from rabbit is a heterodimer composed of a 80 kDa catalytic subunit [19] and a 28 kDa subunit [20]. PKCs from rabbit have a relative molecular mass around 77 kDa [21]. All of these data imply the association of one molecule of μ-calpain with one molecule of cPKCα.

Immunocytochemistry conducted with anti-μ-calpain and

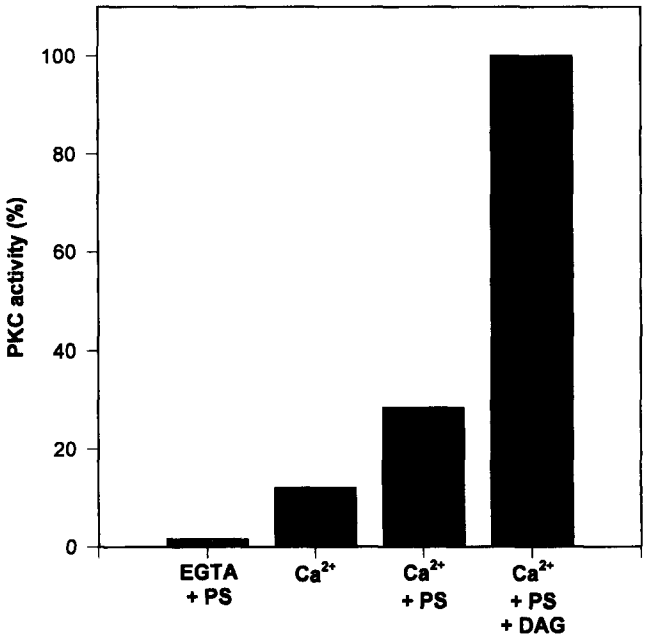

Fig. 3. Effect of calcium, phosphatidylserine (PS) and diacylglycerol (DAG) on the PKC activity associated to μ-calpain. Results were normalised to the maximum activity obtained in the presence of $CaCl_2$ (0.2 mM), PS (20 μg/ml) and DAG (2 μg/ml).

anti-cPKCα antibodies reveals a co-localization of these enzymes in muscle sarcolemma (Fig. 6A,B). Moreover, μ-calpain and cPKCα were always absent from cytoplasm in the various sections of myotubes that we have observed. Concerning the localisation of cPKCα, these results obtained with skeletal muscle from rabbit are in agreement with those of Nakano et al. [22], who report also the presence of the cPKCα isoenzyme on the surface of the plasma membrane in human skeletal muscle.

4. Discussion

In previous papers [12,13] we have shown that μ-calpain co-purified with a PKC activity after chromatography on hydrophobic and anion-exchange columns. In the continuity of these prior studies we report here, for the first time, the existence of a 190 kDa complex constituted by the association of a μ-calpain molecule with a cPKCα molecule, the most universally expressed PKC amongst the various members of the PKC family. The reality of this complex is based, firstly, on results obtained both by gel filtration and by non-denaturing gel electrophoresis. Data from these two methods show that μ-calpain and the co-purifying PKC are associated in a complex with an apparent molecular weight of 190 kDa.

By using SDS gel-electrophoresis and immuno-blotting it appears that this complex is composed of one μ-calpain molecule (110 kDa) and of one PKCα molecule (80 kDa). This association seems to be very specific, because throughout different extractions, biochemical tests with co-factors always revealed a conventional activity and Western blotting always indicated that amongst the cPKCs only the isoenzyme α was selectively associated to μ-calpain. These data support evidence that this complex is not due to an accidental association during homogenisation of the tissue but corresponds to a native form. The existence of this specific complex is also reinforced by the immunohistochemical analysis showing a co-localization of μ-calpain and cPKCα at the plasma membrane level. This co-localization of μ-calpain and PKCα on plasma membrane agrees with our previous study showing that μ-calpain and its associated PKC were co-precipitated by a 200,000 × g centrifugation of the 100,000 × g supernatant [12]. Therefore, in this previous report, we suggested that these two enzymes were linked to light plasma vesicles obtained during cell homogenisation [12].

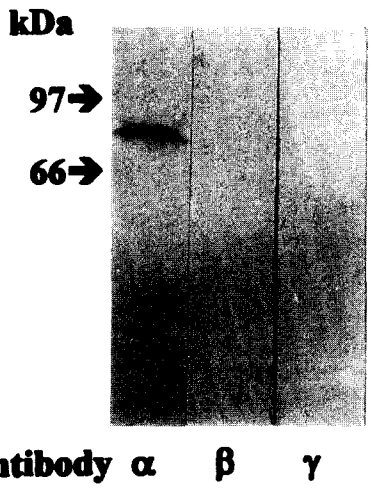

Fig. 4. Immunoblot analysis of cPKC isoenzymes obtained after chromatography on a Sephacryl S-300 HR column and co-eluting with the μ-calpain activity.

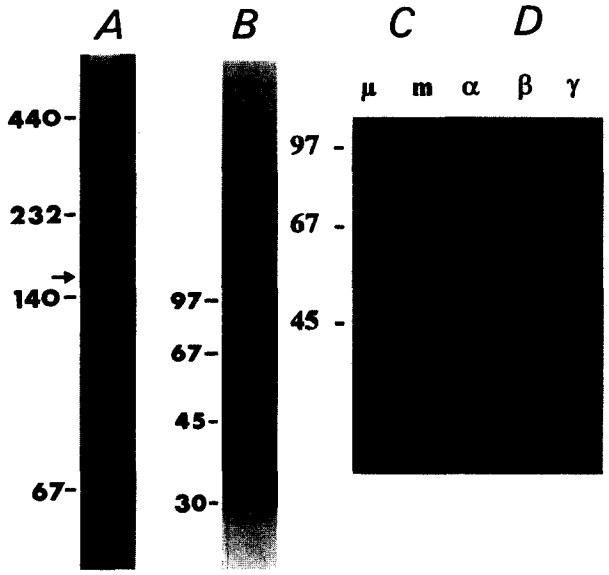

Fig. 5. (A) Non-denaturing polyacrylamide gel electrophoresis of the fractions obtained on Q-Sepharose and containing the μ-calpain and PKC activities. (B) SDS-PAGE of the protein band indicated by an arrow in (A). Immunoblotting obtained after separation by SDS-PAGE; (C) μ and m-calpain. (D) PKCα , β and γ. The sizes of the marker proteins are indicated in kilodaltons.

Concerning the molecular interactions between μ-calpain and PKCα, it appears that binding between these two enzymes does not depend on calcium because isolation of the complex is carried out with high concentrations of EGTA. Further, this complex was isolated without addition of phosphatidylserine and diacylglycerol which are activators of PKCα, indicating that conformational modification of this kinase by these effectors does not seem to be required for binding of PKCα to μ-calpain.

As regards the relationships between μ-calpain and PKCα inside this complex, two major possibilities can be considered. Firstly, cPKCα could be involved in phosphorylation of μ-calpain. In vitro, phosphorylation of a cardiac calpain has been reported [23], but attempts to detect phosphorylation of μ and m-calpains in a leukemia cell line have been unsuccesful [24]. Secondly, cPKCα is cleaved in vitro by μ-calpain in the V3 hinge region to generate two types of catalytic fragments (PKM) of 45 and 46 kDa [25]. Therefore, cPKCα appears as a potential substrate for μ-calpain. Finally, it will be interesting to study whether or not this μ-calpain–PKCα complex is present in other tissues than skeletal muscle. Indeed, recent results have shown an implication of PKM in different cellular processes [26–32], so in this perspective, the existence of a μ-calpain-PKCα complex in various tissues will be of considerable interest.

Acknowledgements: The authors are grateful to Dr. J.J. Brustis for helpful discussions on immunohistochemistry. This study was supported in part by a grant from the National Institute for Agronomic Research (INRA). C.V. is the recipient of a fellowship from the Superior Teaching and Research Ministry (MESR).

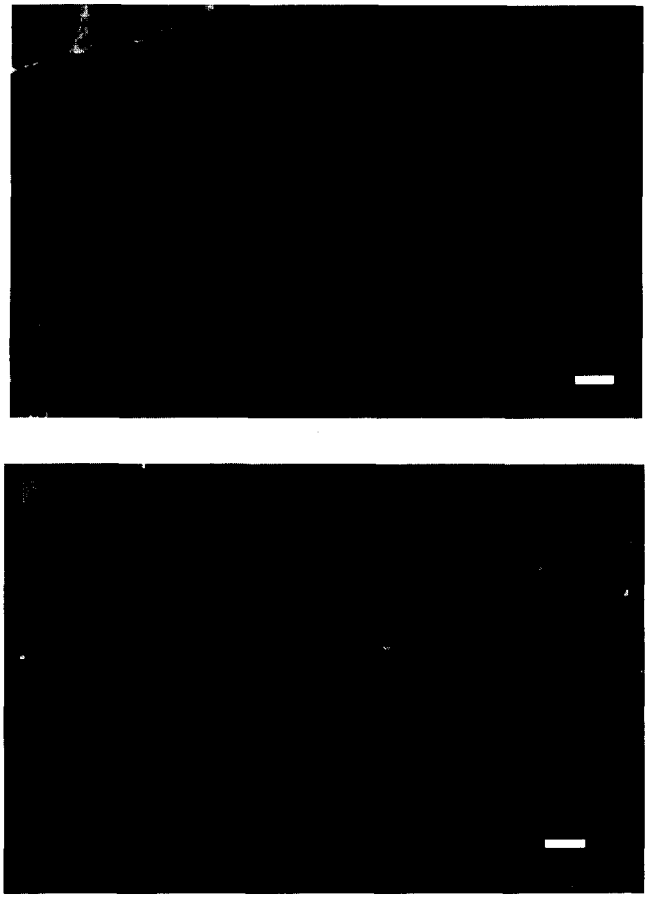

Fig. 6. Immunolocalisation of μ-calpain (A) and PKCα (B) in rabbit skeletal muscle. Scale white bars *(lower right)* are 5 μm.

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