

# Association of calpains 1 and 2 with protein kinase C activities

Michel Savart, Mohammed Belamri, Véronique Pallet and André Ducastaing

*Laboratoire de Biochimie et Technologie des Aliments, Département Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cédex, France*

Received 9 March 1987

Calpains 1 and 2 co-eluted with protein kinase C activities after hydrophobic (phenyl-Sepharose) and anion-exchange (Mono Q) chromatographies of a  $100\,000 \times g$  supernatant which was defined as cytosol. After centrifugation of the cytosol at  $200\,000 \times g$  for 16 h, the major part of calpain 1 and of its associated protein kinase C activity was recovered in the pellet, when the major part of calpain 2, also associated to a protein kinase C activity, was present in the resulting supernatant. Polyacrylamide gel electrophoresis of the fractions eluted from the Mono Q column, which contained calpains 1 or 2 and their associated protein kinase C activities, revealed two main bands with a molecular mass of 80 and 28 kDa.

Calpain 1; Calpain 2; Protein kinase C

## 1. INTRODUCTION

Protein kinase C can be activated by  $\text{Ca}^{2+}$ -limited proteolysis with calpains 1 and 2, and calpain 1 from brain copurifies with protein kinase C [1]. Recent hypotheses on the mechanism of activation of protein kinase C by  $\text{Ca}^{2+}$ -dependent proteases are based on the differential partitioning of these enzymes between the cytosol and the plasma membranes [2]. However, the presence of proteins in the cytosol does not necessarily signify that they are normally soluble proteins of the cytoplasm as has been observed with the desensitized  $\beta$ -adrenergic receptors [3,4].

In this study we show that in skeletal muscle calpains 1 and 2 are associated with protein kinase C activities. Furthermore, cytosolic calpain 1 and its associated protein kinase C are not soluble enzymes, but are bound to light fractions pelleted at  $200\,000 \times g$ .

Correspondence address: M. Savart, Laboratoire de Biochimie et Technologie des Aliments, Département Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cédex, France

## 2. MATERIALS AND METHODS

### 2.1. Materials

Casein was obtained from Merck (art.2244). [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci/mmol) was purchased from Amersham International. L- $\alpha$ -Phosphatidyl-L-serine, 1,2-dioleoyl-rac-glycerol and histone type IIS from calf thymus were obtained from Sigma. Phenyl-Sepharose CL-4B and the HPLC anion-exchange column (MonoQ, HR 5/5) were supplied by Pharmacia.

### 2.2. Preparation of cytosol from rabbit skeletal muscle

All operations were conducted at  $4^\circ\text{C}$ . Adult male New-Zealand rabbits, killed by cervical dislocation, were used as the starting material. Muscles were rapidly dissected and homogenized with 5 vols of 30 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 2 mM EGTA and 1 mM EDTA in a Waring Blendor, for 30 s at low speed followed by 30 s at high speed. This homogenate was centrifuged at  $12\,000 \times g$  for 15 min. The supernatant was filtered through cheese-cloth to remove free floating fat and centrifuged at  $100\,000$

$\times g$  for 60 min. The resulting supernatant is defined as the cytosol. Protein was determined by the method of Bradford [5] using the Bio-Rad reagent, and BSA as standard.

### 2.3. Preparation of the fractions from the 200000 $\times g$ centrifugation

After centrifuging the cytosol at 200000  $\times g$  for times varying from 2 h to 16 h, the resultant pellets were suspended in 1 mM EGTA/1 mM EDTA/20 mM Tris-HCl (pH 7.5), with a Polytron PCU-2 at setting 4 for 15 s. These homogenates were used for the determination of calpain and protein kinase C activities. The supernatant and the homogenate of pellets obtained after 16 h of centrifugation were kept for subsequent purification.

### 2.4. Calpain assays

Calpains were assayed as described with some modifications [6], by measuring the formation of trichloroacetic-soluble material with casein as substrate. The reaction mixture (0.8 ml) contained 2 mg casein and 2 mM free  $\text{Ca}^{2+}$  or 2 mM EGTA in 2 mM 2-mercaptoethanol buffered with 100 mM Tris-HCl at pH 7.5. The enzymatic reaction was started by the addition of the different fractions to study. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 0.8 ml of cold 10% trichloroacetic acid (w/v), immediately followed by immersion in an ice bath. Tubes containing EGTA were used as blanks. After centrifugation at 4000  $\times g$  for 15 min the absorbance at 280 nm of the supernatant of the tubes incubated in the presence of  $\text{Ca}^{2+}$  was read against the blank. One unit of calpain activity was defined as the amount of enzyme which catalyzed an increase of 0.001 absorbance unit at 280 nm after one min incubation.

### 2.5. Protein kinase C assay

Protein kinase C activity was measured by the method of Kitano et al. [7] with a slight modification. The reaction mixture (0.25 ml) contained 20 mM Tris-HCl at pH 7.5, 5 mM magnesium acetate, 200  $\mu\text{g}/\text{ml}$  histone type IIIS, 10  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP ( $10^5$  cpm/nmol), 10  $\mu\text{g}$  phosphatidylserine, 0.2  $\mu\text{g}$  diolein, 0.5 mM  $\text{CaCl}_2$  and the enzyme preparation to be assayed. Basal activity was measured in the presence of 0.5 mM EGTA in-

stead of  $\text{CaCl}_2$ , phosphatidylserine and diolein. After 3 min at 30°C, the reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid. The acid-precipitable material was collected on Whatman GF/C glass microfibre filters. The filters were then washed with 4  $\times$  2 ml of 10% trichloroacetic acid and treated for 30 min with 0.4 ml of 10 mM KOH at room temperature. The radioactivity was measured using Ready-solv EP scintillation fluid (Beckman) in a Beckman LS 1 800 scintillation counter.

### 2.6. SDS-polyacrylamide gel electrophoresis

Samples and reference proteins were denatured at 100°C for 2 min in 30 mM Tris-HCl buffer (pH 7.4) containing 0.05% SDS and 3%  $\beta$ -mercaptoethanol. Electrophoresis was carried out with 12% (w/v) acrylamide slab gels (135  $\times$  120  $\times$  1.5 mm) by the method of Laemmli [8]. Gels were run at 35 mA for 4 h.

## 3. RESULTS

### 3.1. Phenyl-Sepharose CL-4B chromatography

Solid NaCl was added to the cytosol, the 200000  $\times g$  supernatant or the 200000  $\times g$  pellet to obtain a final concentration of 0.5 M. The samples were loaded on a column (1.6  $\times$  12 cm) of phenyl-Sepharose CL-4B previously equilibrated with 20 mM Tris-HCl buffer at pH 7.5 containing 1 mM EDTA, 1 mM EGTA and 0.5 M NaCl (buffer A). The column was washed with buffer A and the proteins bound were eluted in a single peak, containing both calpain and protein kinase C activities, with 20 mM Tris-HCl buffer at pH 7.5 containing 1 mM EDTA, 1 mM EGTA and 1% (v/v) ethylene glycol (buffer B). No calpain or protein kinase C activities were detected in the proteins passed straight through the column. In agreement with Karlsson et al. [9] we observed that the endogenous calpain inhibitor calpastatin, whose presence in rabbit muscle has been demonstrated in our laboratory [10,11], was not bound to phenyl-Sepharose.

### 3.2. Anion-exchange analysis of the cytosol

The peak activity of the phenyl-Sepharose column from the cytosol (65 ml, 10.27 mg protein) was loaded on a mono Q column. Two peaks of calpain and protein kinase C activities co-eluted

(fig.1A). The first peak was eluted at 0.20–0.25 M, and the second between 0.40 and 0.45 M. After dialysis overnight against 20 mM Tris-HCl at pH 7.5 of the fractions containing these  $\text{Ca}^{2+}$  proteases, their sensitivity to calcium was studied. The fraction eluted at about 0.20 M NaCl was maximally activated by  $1 \times 10^{-4}$  M  $\text{Ca}^{2+}$  and corresponded to calpain 1 [12]. The fraction eluted around 0.4 M plateaued at  $1 \times 10^{-3}$  M  $\text{Ca}^{2+}$  and corresponded to calpain 2 [6]. The kinases associated to calpains 1 and 2 corresponded to protein kinase C according to the assay defined by Kitano et al. [7] and detailed in section 2.

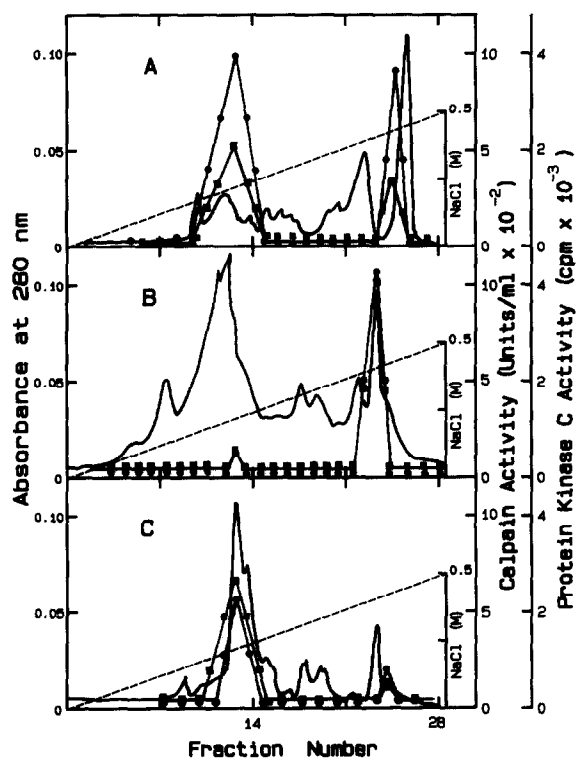


Fig.1. Mono Q separation of calpains and protein kinase C activities from skeletal muscle. The Mono Q column was equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EGTA and 1 mM EDTA, and eluted with a 30 ml of 0–0.5 M NaCl gradient in the same buffer. The gradient was run over 30 min and 1 ml fractions were collected. (●) Protein kinase C activity; (■) calpain activity; (—) absorbance at 280 nm. (A) Cytosol; (B) 200000  $\times$  g pellet; (C) 200000  $\times$  g supernatant.

### 3.3. Anion-exchange analysis of the 200000 $\times$ g supernatant

The supernatant obtained following a 200000  $\times$  g centrifugation for 16 h was submitted to a phenyl-Sepharose chromatography and the active fractions obtained (60 ml, 10 mg protein) were loaded on a Mono Q column. As shown in fig.1B, a first minor peak of calpain 1 associated to protein kinase C activity was eluted, followed by a second major peak of calpain 2, always associated with protein kinase C activity. Thus, a possible sedimentation at 200000  $\times$  g of calpain 1 and of its associated protein kinase C was considered.

### 3.4. Screening for calpain 1 and protein kinase C activities in the 200000 $\times$ g pellet

The pellet obtained after 16 h of centrifugation was re-suspended as reported in section 2 and submitted to phenyl-Sepharose chromatography. The peak activity (65 ml, 2.4 mg protein) was applied on a Mono Q column. Fig.1C shows that the major part of calpain 1 co-eluted with a protein kinase C activity at 0.20–0.25 M NaCl as in fig.1A. Thus, it appeared that the major part of calpain 1 and of its associated protein kinase C activity sedimented in particulate fractions when centrifuged at 200000  $\times$  g for 16 h. If the concentration of EGTA and EDTA present in buffers was decreased to 0.1 mM, the percentage of calpain 2 and of its associated protein kinase C increased in the 200000  $\times$  g pellets but decreased in the 200000  $\times$  g supernatant. Analogous variations in the concentrations of chelators were without effect on the distribution of the calpain 1-protein kinase C complex between supernatants and pellets obtained at 200000  $\times$  g. A study was undertaken to verify if the sedimentation of calpain 1-protein kinase C was observable for shorter times of centrifugation. From 2 h of centrifugation at 200000  $\times$  g the presence of calpain 1 and protein kinase C was observed in the pellets, with high specific activities, but the total activity recovered in the pellets was greater after 16 h. Therefore, this latter time was chosen for all experiments.

### 3.5. Purification of calpains 1 or 2 and of protein kinase C activities from the 200000 $\times$ g fractions

As already mentioned, after hydrophobic chromatography of the 200000  $\times$  g supernatant,

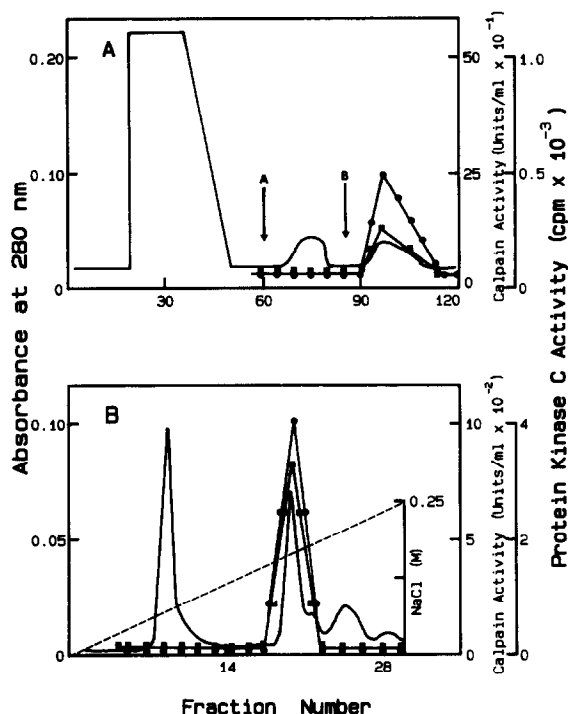


Fig.2. Purification of calpain 1 and protein kinase C from the  $200000 \times g$  pellet. (A) Phenyl-Sepharose column. The chromatography was conducted as indicated in section 3, but elution of enzyme activities with buffer B (arrow B) was preceded by a step with 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA and 0.15 M NaCl (arrow A). (B) Mono Q column. Equilibration and running of the 0–0.25 M NaCl gradient were performed as in fig.1. (●) Protein kinase C activity; (■) calpain activity; (—) absorbance at 280 nm.

fractions containing calpain 2 and protein kinase C activities were obtained around 0.4 M NaCl after gradient elution on a Mono Q column (fig.1B). When these fractions were analysed by gel electrophoresis, two main bands appeared of molecular mass 80 and 28 kDa (fig.3A). These bands correspond to the high and light subunits of calpain 2 in rabbit skeletal muscle, as described by Penny et al. [13]. Calpain 1 and protein kinase C activities obtained from the  $200000 \times g$  pellet, which eluted around 0.2 M (fig.1C), gave electrophoretic patterns with many bands. So, a new type of purification was performed by modifying both the experimental conditions for phenyl-Sepharose chromatography (fig.2A) and the gradient elution for the Mono Q column (fig.2B). Us-

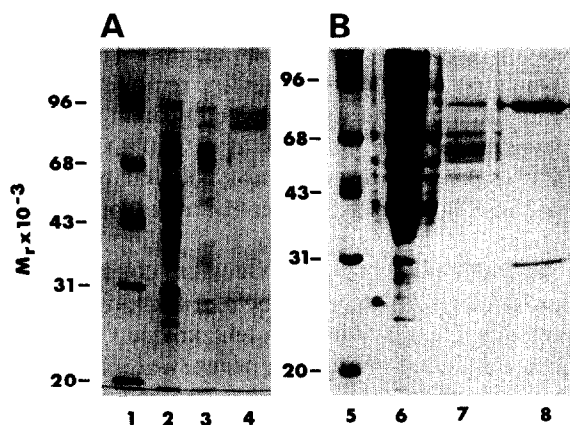


Fig.3. SDS-polyacrylamide gel electrophoresis of calpain 1, calpain 2 and protein kinase C from each stage of preparation. Lanes 1 and 5 are standard marker proteins. (A)  $200000 \times g$  supernatant. Lanes: 2 from  $200000 \times g$  supernatant; 3 from phenyl-Sepharose; 4 from Mono Q (fig.1B). (B)  $200000 \times g$  pellet. Lanes: 6 from  $200000 \times g$  pellet; 7 from phenyl-Sepharose (fig.2A); 8 from Mono Q (fig.2B). About  $6 \mu\text{g}$  of proteins were applied to each lane, except lane 2 ( $20 \mu\text{g}$ ) and lane 6 ( $30 \mu\text{g}$ ).

ing a 0–0.25 M NaCl gradient, the associated calpain 1 and protein kinase C activities eluted at 0.16 M (fig.2B). Gel electrophoresis of the active fractions shows 80 and 28 kDa bands (fig.3B) corresponding to the subunits of calpain 1 in rabbit skeletal muscle [13] and two light bands at 85 and 76 kDa.

#### 4. DISCUSSION

Protein kinase C was found in both cytosol and plasma membranes, and models based on its intracellular translocation have been proposed for its activation [14–17]. The presence of calpain 1 has been reported in plasma membranes from erythrocytes [18]. Our results demonstrate that the major part of cytosolic calpain 1 and of its associated protein kinase C is bound to particles obtained after a  $200000 \times g$  centrifugation. So, we suggest that these two enzymes are linked to light plasma vesicles obtained during cell homogenization.

As indicated in section 3, the calpain 2 and protein kinase C activities present in cytosol decreased when the concentration of chelators present in buf-

fers increased. It has also been reported that both protein kinase C [2] and calpain [19] were dissociated from membranes by removing  $\text{Ca}^{2+}$ . So, if calpain 2 and its associated protein kinase C appear as soluble enzymes after extraction with  $\text{Ca}^{2+}$  chelators, this does not necessarily signify that these enzymes are present in the cytoplasm, *in vivo*.

Another point to be discussed concerns the biological significance of the association between these enzyme activities. Protein kinase C was initially defined as an inactive proenzyme which may be activated by limited proteolysis with  $\text{Ca}^{2+}$ -dependent proteases [20]. In brain, calpain 1 copurifies with protein kinase C and activates it [1]. In skeletal muscle, we presently report that both calpain 1 and 2 are associated to protein kinase C whose relationships with the three forms of protein kinase C recently discovered [21] are under investigation in our laboratory. For us, these associations between calpains and protein kinase C until the final step of purification are not fortuitous, and we hypothesize that proteolytic regulation of protein kinase C could correspond to physiological reality.

#### ACKNOWLEDGEMENTS

We thank Bruno Varichon for the iconography and Cynthia Dubourg for her skilful secretarial assistance. This work was supported in part by a grant from the National Institute for Agricultural Research (INRA).

#### REFERENCES

- [1] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) *J. Biol. Chem.* 258, 1156–1164.
- [2] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. and Horecker, B.L. (1985) *Proc. Natl. Acad. Sci. USA* 82, 6435–6439.
- [3] Stadel, J.M., Strulovici, B., Nambi, P., Lavin, T.N., Briggs, M.M., Caron, M.G. and Lefkowitz, R.J. (1983) *J. Biol. Chem.* 258, 3032–3038.
- [4] Strulovici, B., Stadel, J.M. and Lefkowitz, R.J. (1983) *J. Biol. Chem.* 258, 6410–6414.
- [5] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [6] Azanza, J.L., Raymond, J., Robin, J.M., Cottin, P. and Ducastaing, A. (1979) *Biochem. J.* 183, 339–347.
- [7] Kitano, T., Go, M., Kikkawa, V. and Nishizuka, Y. (1986) *Methods Enzymol.* 124, 349–352.
- [8] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [9] Karlsson, J.O., Gustavsson, S., Hall, C. and Nilsson, E. (1985) *Biochem. J.* 231, 201–204.
- [10] Cottin, P., Vidalenc, P.L. and Ducastaing, A. (1981) *FEBS Lett.* 136, 221–224.
- [11] Cottin, P., Vidalenc, P.L., Merdaci, N. and Ducastaing, A. (1983) *Biochim. Biophys. Acta* 743, 299–302.
- [12] Murakami, T., Hatanaka, M. and Murachi, T. (1981) *J. Biochem. (Tokyo)* 90, 1809–1816.
- [13] Penny, I.F., Taylor, M.A.J., Harris, A.G. and Etherington, D.J. (1985) *Biochim. Biophys. Acta* 829, 244–252.
- [14] Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) *J. Biol. Chem.* 257, 13193–13196.
- [15] Hirato, K., Hirota, T., Aguilera, G. and Catt, K.J. (1985) *J. Biol. Chem.* 260, 3243–3246.
- [16] Wolf, M., Le Vine, H. iii, May, W.S. jr, Cuatrecasas, P. and Sahyoun, N. (1985) *Nature* 317, 546–549.
- [17] May, W.S. jr, Sahyoun, N., Wolf, M. and Cuatrecasas, P. (1985) *Nature* 317, 549–551.
- [18] Hatanaka, M., Yoshimura, N., Murakami, T., Kannagi, R. and Murachi, T. (1984) *Biochemistry* 23, 3272–3276.
- [19] Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Horecker, B.L. (1985) *Biochem. Biophys. Res. Commun.* 128, 331–338.
- [20] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) *J. Biol. Chem.* 252, 7610–7616.
- [21] Huang, K.P., Nakabayashi, H. and Huang, F.L. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8535–8539.