Biochem. J. (1990) 265, 575–579 (Printed in Great Britain)

Investigation of the structural basis of the interaction of calpain II with phospholipid and with carbohydrate

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Two forms of pig kidney calpain II were isolated, both of which appeared to contain an intact 80 kDa large subunit, but which showed specific proteolytic degradation at the *N*-terminal end of the 30 kDa small subunit. The structure of each of these molecules was investigated by amino acid sequence analysis. The forms corresponded to molecules with small subunits starting at residue 38 (degraded calpain A) and at residue 62 (degraded calpain B) of the complete sequence. These molecules were tested for their ability to interact with phosphatidylinositol and with carbohydrate (agarose gel-filtration media). Calpain and degraded calpain A, but not degraded calpain B, would interact with phosphatidylinositol. Thus the sequence $(G)_{17}$TAMRILG (residues 38–61) is essential for the interaction. Neither calpain nor the degraded forms of the enzyme showed specific interaction with carbohydrate.

INTRODUCTION

Calpains are cytoplasmic Ca^{2+}-dependent cysteine proteinases (for a review see Suzuki *et al.*, 1987). The enzymes have two subunits. The large subunit (80 kDa) has a papain-like active-site domain and a *C*-terminal calmodulin-like Ca^{2+}-binding domain. The small subunit (30 kDa) has an *N*-terminal glycine-rich domain and a *C*-terminal calmodulin-like Ca^{2+}-binding domain. Two forms of the enzyme are well characterized: calpain I, which is activated *in vitro* by micromolar concentrations of Ca^{2+}, and calpain II, which requires millimolar Ca^{2+} for activity. Calpains I and II have identical small subunits but distinct large subunits. When Ca^{2+} binds to calpain the enzyme is activated and also begins to autodigest. Initially autolysis results in conversion of the enzyme into a form with subunit molecular masses of 75 kDa plus 18 kDa and increased Ca^{2+}-sensitivity (Coolican *et al.*, 1986; DeMartino *et al.*, 1986; Imajoh *et al.*, 1986*b*). Subsequently additional cleavages occur and enzyme activity is eventually lost (Crawford *et al.*, 1987). Interaction with phospholipid, particularly phosphatidylinositol, has been shown to lower the Ca^{2+} concentration required to initiate autolysis (Coolican & Hathaway, 1984; Pontremoli *et al.*, 1985*b*). These observations have led to the idea that interaction of calpain with membranes and subsequent autolysis are crucial steps in the activation of the enzyme *in vivo* (Pontremoli *et al.* 1985*a*,*c*,*d*; Kuboki *et al.*, 1987; Suzuki *et al.*, 1987). Calpain is thought to interact with phospholipid through the *N*-terminal region of the small subunit (Imajoh *et al.*, 1986*a*). This region of the molecule has also been proposed to bind carbohydrate (Zimmerman & Schlaepfler, 1988).

During the purification of calpain II, enzyme molecules lacking various sections of the small subunit can be isolated (Parkes *et al.*, 1985). The present paper analyses the ability of phosphatidylinositol to decrease the Ca^{2+}-requirement for autolysis of these enzyme forms, and also their ability to bind to carbohydrate. The aim was to try to identify more precisely the phospholipid-binding sites and carbohydrate-binding sites within the *N*-terminal region of the small subunit of calpain.

EXPERIMENTAL

Materials

Phosphatidylinositol (wheat germ) was purchased from Lipid Products, South Nutfield, Redhill, Surrey, U.K. The material was dissolved in methanol containing 0.05% butylated hydroxytoluene. The concentration of the solution was determined by phosphate analysis as described by Ames & Dubin (1960). Before use, a sample of the solution was dried under N_2, 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 5 mM-EDTA, 0.01% monothioglycerol and 0.01% NaN_3 was added and the solution was sonicated in a water bath for 1 h at room temperature.

Bio-Gel P-10 was from Bio-Rad Laboratories, Watford, Herts., U.K. Sepharose 4B was from Pharmacia, Milton Keynes, Bucks., U.K.

Methods

Purification of calpain and calpain fragments. Calpain II was purified from pig kidney by using the procedure described for the purification of calpain from chicken gizzard smooth muscle (Parkes *et al.*, 1985), with the following modifications. Pig kidney (approx. 1.4 kg of fresh tissue) was minced and homogenized at 4 °C in a final 2.5 litres of 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 25 mM-EDTA, 0.01% monothioglycerol, 0.1 mM-phenylmethanesulphonyl fluoride, 0.01% NaN_3 and 0.25 M-sucrose. The homogenate was spun at 9000 *g* for 30 min, and the supernatant was filtered through glass-wool. $(NH_4)_2SO_4$ was added to the supernatant to give a 60%-saturated solution, and this was stirred for 1 h at 4 °C. The pellet obtained after centrifugation at 9000 *g* for 30 min was redissolved in

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50 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-EDTA, 0.01% monothioglycerol, 0.1 mM-phenylmethanesulphonyl fluoride and 0.01% NaN_3. The sample was dialysed against this buffer, centrifuged at 120000 *g* for 45 min, filtered through glass-wool and sequentially chromatographed on DEAE-Sepharose, Reactive Red–agarose and Pharmacia Mono-Q (h.p.l.c.) as described previously (Parkes *et al.*, 1985). Except for chromatography on Mono-Q all steps were carried out at 4 °C. Calpain activity was eluted from the Mono-Q column as one major and three minor peaks. The major peak corresponded to the intact calpain molecule. The minor peaks were calpain molecules with 80 kDa large subunits but with small subunits of 29 kDa, 26 kDa and 18 kDa. The 80 kDa + 18 kDa species appears identical on SDS/polyacrylamide-gel electrophoresis with the initial autolytic product of calpain. The other two minor species are not observed as intermediates during autolysis and may arise as the result of the action of other proteinases during purification.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was with gels of 12.5% (w/v) polyacrylamide and the buffer system described by Laemmli (1970). The samples were prepared by adding an equal volume of 125 mM-Tris/HCl buffer, pH 6.8, containing 20% (w/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 5% (v/v) saturated Bromophenol Blue, and incubating in a boiling-water bath for 5 min. The gels were stained with Coomassie Brilliant Blue and scanned with an LKB 2202 Ultroscan laser densitometer.

Amino acid sequence analysis. Samples for amino acid sequence analysis were initially run on SDS/polyacrylamide-gel electrophoresis. Samples were then electroblotted on to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore, Watford, Herts., U.K.) with a Bio-Rad Trans-Blot cell at 500 mA for 1 h. The Immobilon-P membrane was stained, after blotting, with 0.1% Ponceau-S dye in aq. 1% (v/v) acetic acid for 60 s. After washing three times with 1% acetic acid and twice with distilled water, the bands of interest were excised and destained with 10 mM-NaOH, washing with ten 1 ml portions of distilled water (Matsudaira, 1987; Applied Biosystems User Bulletin no. 36, March 1988). The destained bands were cut into 4 mm × 2 mm pieces and subjected to sequencing on an Applied Biosystems 470A/120A protein sequencer with on-line phenylthiohydantoin analysis using the 03CPTH program (version 3.0 ABI standard sequencing software; Applied Biosystems, Warrington, Lancs., U.K.). Data analysis and integration were performed with a Waters 840 Data Station (Millipore).

Amino acid analysis. Samples for amino acid analysis were run on SDS/polyacrylamide-gel electrophoresis and blotted on to Immobilon-P and bands were excised as described above. The samples were hydrolysed in gaseous constant-boiling HCl at 150 °C for 75 min. The amino acid composition was determined after pre-column formation of derivatives with phenyl isothiocyanate with the Waters Pico-Tag system.

RESULTS

Calpain, two degraded forms of the enzyme and autolysed calpain were purified as described in the Experimental section. SDS/polyacrylamide-gel electrophoresis of these enzyme forms is shown in Fig. 1. The 80 kDa + 29 kDa subunit form is referred to below as degraded calpain A and the 80 kDa + 26 kDa subunit form as degraded calpain B. The small subunits of calpain, degraded calpain A, degraded calpain B and autolysed calpain were subjected to *N*-terminal amino acid sequence analysis as described in the Experimental section. The results of sequence analysis of the small subunits are shown in Fig. 2. The molecules have successively longer sections missing from the *N*-terminus. The calpain small subunit gave no *N*-terminal sequence, consistent with the observation that it is blocked (Sakihama *et al.*, 1985). Degraded calpain A had a small subunit beginning at residue 38 of the native polypeptide, and degraded calpain B small subunit started at residue 62. The autolysed calpain small subunit started at residue 90. This is the same cleavage site as that observed for rabbit calpain (Imajoh *et al.*, 1986*b*). *C*-Terminal amino acid sequence analysis was attempted with samples of the various forms of the small subunit blotted on to Immobilon-P membranes. No conclusive results were obtained. Amino acid analyses of the samples were, however, consistent with no loss of polypeptide from the *C*-termini.

The large subunit of degraded calpain B was subjected to *N*-terminal amino acid sequence analysis. It showed no *N*-terminal sequence despite loading approx. 500 pmol on to the gel and successful blotting demonstrated by Ponceau S staining. It was therefore concluded that the large subunit of degraded calpain B has a blocked *N*-terminus. Intact calpain also has a blocked *N*-terminus

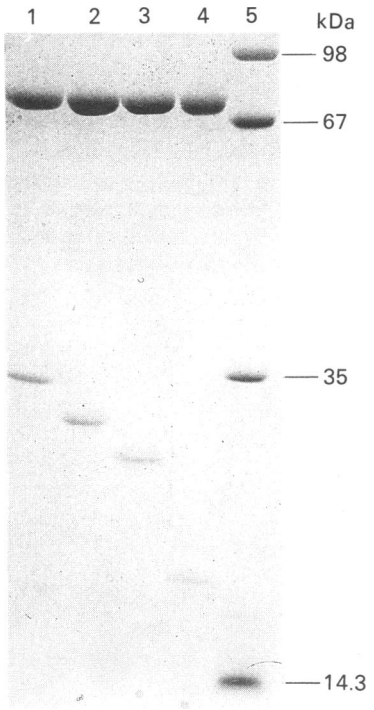

Fig. 1. SDS/polyacrylamide-gel electrophoresis of calpain and degraded calpain forms

Lane 1, calpain; lane 2, degraded calpain A; lane 3, degraded calpain B; lane 4, autolysed calpain; lane 5, molecular-mass markers.

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    1                 10                  20
(a) M F L V N S F L K G G G G G G G G G G L G G G L
(b)
(c)
          B B B B
                    T T T T T T T T T T T T T T T T

          30                  40                  50
(a) G N V L G G L I S G A G G G G G G G G G G G G G
(b)                       * G G G G G G G G G G G(G)G
(c)
    B B B B B B B B B B B B
                      T T T T T T T T T T T T T T T

                    60                  70
(a) G G G G T A M R I L G G V I S A I S E A A A Q Y N
(b) G G G G T A M R I L G G
                        + G V I S A I S E A A
(c)                               H H H H H H H H
          B B B B B B B B B B B B B
    T T T T T                                   T T

          80                  90                  100
(a) P E P P P P R T H Y S N I E A N E S E E V R Q F R
(b)                         # A N E S E E V R Q F
(c)

    T T T T T T T T T T T T T
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Fig. 2. Amino acid sequence analysis of degraded calpain forms

(a) Amino acid sequence of pig calpain small subunit, from Sakihama *et al.* (1985). (b) *N*-Terminal amino acid sequences of the small subunits of degraded calpain A (*) and B (+), and of autolysed calpain (#). The (G) is a residue that was not identified in the sequence analysis. (c) Secondary-structure prediction found with the use of the University of Leeds suite of programs. Key: H, α-helix; B, β-sheet; T, turn.

(Ohno *et al.*, 1984). These observations indicate that the structure of the large subunit is the same in the various calpain forms investigated. Calpain and the two degraded forms of calpain showed the same Ca^{2+}-sensitivity, with half-maximal activity at approx. 0.6 mM-Ca^{2+}. As these molecules have the same large subunit, this observation is consistent with the view that the increased Ca^{2+}-sensitivity that occurs during autolysis is due to cleavage of the large subunit (Imajoh *et al.*, 1986*b*).

The ability of phosphatidylinositol to decrease the Ca^{2+} concentration required for autolysis of the calpain forms was analysed as follows. Calpain or degraded calpain (70 pmol) was incubated at room temperature in 35 μl of 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 0.01% monothioglycerol, 0.01% NaN_3 and various concentrations of Ca^{2+}, with or without 0.47 mM-phosphatidylinositol. After 1 min autolysis was stopped by the addition of 20 μl of gel sample buffer made 10 mM in EDTA and the samples were analysed by SDS/polyacrylamide-gel electrophoresis. The degree of autolysis was measured by densitometric scanning of each gel track and quantifying the conversion of the small subunit into the 18 kDa autolytic product. The results are shown in Fig. 3. They demonstrate that phosphatidylinositol lowers the Ca^{2+} concentration required for autolysis of calpain and degraded calpain A, but not for degraded calpain B. Thus phosphatidylinositol can interact with calpain and degraded calpain A but not with degraded calpain B. The effects of phosphatidylinositol on calpain and degraded calpain A were not identical. Less autolysis occurred at the lowest Ca^{2+} concentration for degraded calpain A in the presence of phosphatidylinositol than for calpain itself. This may be because phosphatidylinositol does not bind in precisely the same way to degraded calpain A as it does to calpain, or that once bound the phosphatidylinositol does not produce the same structural changes and effects on Ca^{2+} binding in the two molecules, and these differences are reflected in the degree of autolysis.

The carbohydrate-binding properties of calpain were investigated by using a procedure similar to that described by Zimmerman & Schlaepfer (1988). These authors demonstrated that calpain bound to agarose-based gel-filtration media in 10 mM-Hepes/NaOH buffer, pH 7.4, containing 10 mM-NaCl, 1 mM-leupeptin, 1 mM-dithioerythritol and 5 mM-Ca^{2+}, and that the enzyme was eluted

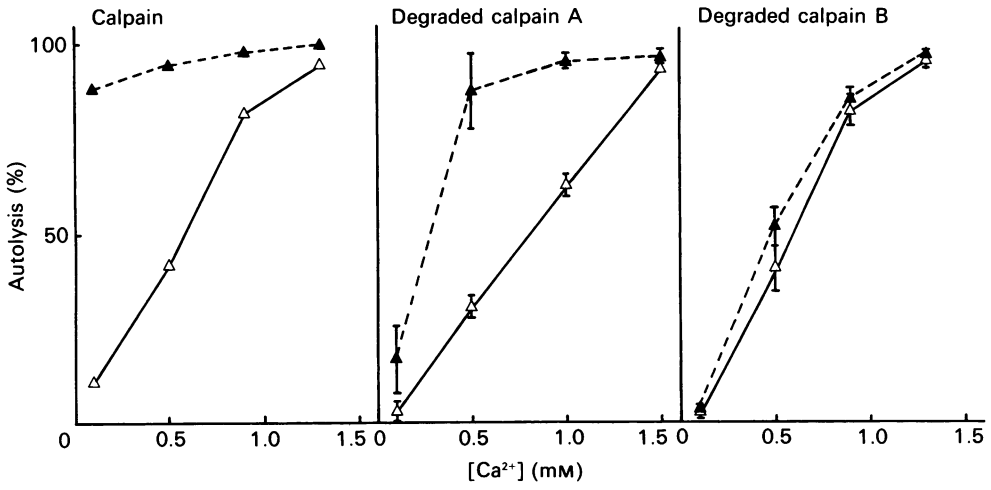

Fig. 3. Interaction of calpain forms with phosphatidylinositol

Calpain forms were incubated with or without phosphatidylinositol at various Ca^{2+} concentrations for 1 min at room temperature and the samples were run on SDS/polyacrylamide-gel electrophoresis. The percentage conversion of the small subunit into the 18 kDa form (autolysed calpain) was found by scanning the gels. For details see the Results section. ——, Without phosphatidylinositol; – – – –, with phosphatidylinositol. The error bars represent S.E.M. (n = 4).

Table 1. Interaction of calpain with gel-filtration media

Note: Sepharose is agarose-based and Bio-Gel is acrylamide-based. For experimental details see the Results section.

	Protein bound in the presence of Ca^{2+}?	Protein eluted with EDTA?
Sepharose 4B, 10 mM buffer		
Calpain	Yes	Yes
Degraded calpain A	Yes	Yes
Degraded calpain B	Yes	Yes
Autolysed calpain	Protein was insoluble in the buffer used, adding EDTA restored solubility	
Sepharose 4B, 50 mM buffer		
Calpain	No	–
Autolysed calpain	No	–
Bio-Gel P-10, 10 mM buffer		
Calpain	Yes	Yes
Bio-Gel P-10, 50 mM buffer		
Calpain	No	–

by washing with buffer containing EGTA. A 0.5 ml column of Sepharose 4B was therefore prepared and equilibrated in 10 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-NaCl, 0.25 mM-leupeptin, 0.01% monothioglycerol, 0.01% NaN_3 and 5 mM-Ca^{2+}. Calpain (50 μg in column buffer) was loaded on to the column, and the column was washed with 2 ml of buffer and then with 2 ml of buffer made 10 mM in EDTA. Fractions (0.5 ml) were collected and analysed for protein content by recording the absorbance at 280 nm and running samples on SDS/polyacrylamide-gel electrophoresis. The experiment was repeated with degraded calpains A and B, and with autolysed calpain prepared by incubating calpain with Ca^{2+} in the absence of leupeptin for 5 min at room temperature before adding leupeptin and running the column. The results of these experiments are given in Table 1. The autolysed calpain was clearly insoluble in the 10 mM buffer. Therefore it seemed possible that the other forms of calpain were only marginally soluble in the presence of Ca^{2+}, causing them to stick to the column, and that solubility was restored by EDTA, resulting in elution. The experiments were therefore repeated with a buffer of slightly higher ionic strength, 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 0.25 mM-leupeptin, 0.01% monothioglycerol, 0.01% NaN_3 and 5 mM-Ca^{2+}. Under these conditions the enzyme did not bind to the column. These results suggested that the binding of calpain to Sepharose 4B may be not a specific interaction with carbohydrate but a solubility phenomenon. To investigate whether carbohydrate was important, the experiments were repeated with a Bio-Gel P-10 (acrylamide) column. As shown in Table 1, calpain bound to this column in 10 mM buffer and was eluted by EDTA; however, the enzyme did not bind in 50 mM buffer. These results are consistent with the suggestion that calpain is not a carbohydrate-binding protein, but is merely of marginal solubility at low ionic strength in the presence of Ca^{2+}. Zimmerman & Schlaepfer (1988) may not have been aware of the solubility problem for the following reasons. They applied Ca^{2+}-free calpain to columns equilibrated in Ca^{2+} buffers and so precipitation could only occur within the columns, they did not do experiments with autolysed calpain analogous to those described above, and, also, detection of insolubility is presumably critically dependent on protein concentration.

DISCUSSION

The experiments to investigate the interaction of phosphatidylinositol with calpain show that the $(G)_{17}$TAMRILG region of the small subunit must be present for phosphatidylinositol to decrease the Ca^{2+} concentration required for autolysis. This confirms and extends the results of Imajoh *et al.* (1986*a*), who suggested that the *N*-terminus of the small subunit was required. It is, however, clear that the hydrophobic residues at the very *N*-terminus of the subunit (residues 1–8) are not crucial for the interaction, nor is the presence of both polyglycine sequences. The large subunit appears not to be involved in the interaction since the structure of this subunit is the same in the three calpain forms examined. It should be noted that the experiments did not measure binding directly, only the consequence of the interaction. Attempts to demonstrate Ca^{2+}-independent binding of phosphatidyl[^{3}H]inositol to the calpain forms by using the procedure described by Garret *et al.* (1988) were unsuccessful. Thus $(G)_{17}$TAMRILG may constitute the phosphatidylinositol-binding site of calpain. There are, however, other possibilities. For example, removal of this region of the protein may cause a conformational change in the remainder preventing phosphatidylinositol binding, or may still allow phosphatidylinositol to bind but prevent communication of the effect to the Ca^{2+}-binding region of the molecule.

The amino acid sequence of the *N*-terminal domain of the calpain small subunit is unusual. There are two strings of polyglycine and a proline-rich region. This suggests that the structure might consist of very flexible regions interspersed with regions of more ordered secondary structure. Secondary-structure prediction, with the use of the University of Leeds suite of programs, is shown in Fig. 2 and confirms this suggestion. The expected mode of binding of protein to a phospholipid bilayer would involve either simple electrostatic interaction with the phospholipid head group or both hydrophobic and electrostatic interactions if the protein penetrated some way into the bilayer. For the calpactin/endonexin family of Ca^{2+}- and phospholipid-binding proteins it has been postulated that interaction with phospholipid is mainly electrostatic, with Ca^{2+} acting as a bridge between protein and anionic phospholipid (Geisow *et al.*, 1987; Klee, 1988). In the case of protein kinase C, an enzyme similarly dependent on Ca^{2+} and interaction with membrane for activity, it is thought that both electrostatic and hydrophobic interactions are important (Snoek *et al.*, 1988). For calpain it is clear that some interaction between the protein and the phospholipid head group is occurring because the effect on Ca^{2+}-sensitivity is seen particularly with phosphatidylinositol. Whether other phospholipids,

anionic or otherwise, can substitute for phosphatidylinositol is controversial (Coolican & Hathaway, 1984; Pontremoli *et al.*, 1985*b*). If hydrophobic interactions also occur one might expect to see amphiphilic α-helices or β-strands in the *N*-terminal region of the small subunit (Jain & Zakim, 1987; Kaiser & Kezdy, 1987; von Heijne, 1988). These are not apparent in the structure prediction in Fig. 2. In particular, the region $(G)_{17}$TAMRILG, which has been identified in the experiments described here as a possible phospholipid-binding site, is predicted as a flexible region followed by a β-strand, but the latter has no obvious hydrophobic sidedness. Thus no clear picture of how calpain might bind phospholipid emerges. The experiments show that it is not necessary to have both polyglycine sequences, or the *N*-terminal hydrophobic regions MFLVNSFL or LGNVLGGLISG, for calpain to interact with phospholipid. However, the $(G)_{17}$TAMRILG sequence is essential.

The experiments designed to investigate the carbohydrate-binding properties of calpain suggest that the specific interaction proposed is artifactual.

We thank Ms. J. Parsons for performing the electroblotting of gels before amino acid sequence analysis. This work was supported by a Project Grant from the Medical Research Council.

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Received 30 June 1989/11 August 1989; accepted 15 August 1989