Ca²⁺-dependent hydrophobic-interaction chromatography

Isolation of a novel Ca²⁺-binding protein and protein kinase C from bovine brain

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Several bovine brain proteins have been found to interact with a hydrophobic chromatography resin (phenyl-Sepharose CL-4B) in a Ca²⁺-dependent manner. These include calmodulin, the Ca²⁺/phospholipid-dependent protein kinase (protein kinase C) and a novel Ca²⁺-binding protein that has now been purified to electrophoretic homogeneity. This latter protein is acidic (pI 5.1) and, like calmodulin and some other high-affinity Ca²⁺-binding proteins, exhibits a Ca²⁺-dependent mobility shift on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with an apparent *M_r* of 22 000 in the absence of Ca²⁺ and *M_r* 21 000 in the presence of Ca²⁺. This novel calcioprotein is distinct from known Ca²⁺-binding proteins on the basis of *M_r* under denaturing conditions, Cleveland peptide mapping and amino acid composition analysis. It may be a member of the calmodulin superfamily of Ca²⁺-binding proteins. This calcioprotein does not activate two calmodulin-dependent enzymes, namely cyclic nucleotide phosphodiesterase and myosin light-chain kinase, nor does it have any effect on protein kinase C. It may be a Ca²⁺-dependent regulatory protein of an as-yet-undefined enzymic activity. The Ca²⁺/phospholipid-dependent protein kinase is also readily purified by Ca²⁺-dependent hydrophobic-interaction chromatography followed by ion-exchange chromatography, during which it is easily separated from calmodulin. A preparation of protein kinase C that lacks contaminating kinase or phosphatase activities is thereby obtained rapidly and simply. Such a preparation is ideal for the study of phosphorylation reactions catalysed *in vitro* by protein kinase C.

It is well known that calmodulin, the ubiquitous and multifunctional Ca²⁺-binding protein (Cheung, 1980; Klee & Vanaman, 1982; Means *et al.*, 1982; Walsh & Hartshorne, 1983), undergoes a substantial conformational change on binding Ca²⁺, with exposure of a hydrophobic site(s) (LaPorte *et al.*, 1980; Tanaka & Hidaka, 1980). Similar Ca²⁺-dependent exposure of hydrophobic sites has been demonstrated for troponin C (Tanaka & Hidaka, 1981) and other Ca²⁺-binding proteins termed 'calcimedins' (Moore & Dedman, 1982*a,b*). This property has been exploited in the purification of calmodulin by Ca²⁺-dependent hydrophobic-interaction chromatography (Gopalakrishna & Anderson, 1982). We have found that

this technique has broader applicability and we have used this method as a powerful initial column-chromatographic step in the purification from bovine brain of the Ca²⁺/phospholipid-dependent protein kinase (protein kinase C) and a novel Ca²⁺-binding protein.

Protein kinase C is a kinase that requires Ca²⁺ and a phospholipid for activity (for review see Takai *et al.*, 1982). This kinase is receiving a great deal of attention presently for two major reasons. Firstly, it may mediate the effects of a variety of extracellular messengers, including various peptide hormones, growth factors, muscarinic cholinergic agents and α -adrenergic agents, which induce phosphatidylinositol turnover with transient production of diacylglycerol. Unsaturated diacylglycerol has been shown to increase significantly the Ca²⁺-sensitivity of protein kinase C (Takai *et*

Abbreviations used: SDS, sodium dodecyl sulphate; 21 kDa CaBP, Ca²⁺-binding protein of *M_r* 21 000.

al., 1979; Kishimoto *et al.*, 1980) and has been postulated as a second messenger capable of activating protein kinase C without a change in cytosolic $[Ca^{2+}]$. Secondly, phorbol esters, which act as tumour promoters, can substitute for unsaturated diacylglycerol in the activation of protein kinase C; indeed, protein kinase C appears to be the phorbol ester receptor (Castagna *et al.*, 1982; Nidel *et al.*, 1983; Kikkawa *et al.*, 1983). Much attention is now being focused on the identification of substrates of protein kinase C, which include a 40000- M_r cytosolic protein of platelets (Ieyasu *et al.*, 1982), the 20000- M_r light chain of myosin of platelets (Naka *et al.*, 1983) and smooth muscle (Endo *et al.*, 1982), vinculin of smooth muscle (Werth *et al.*, 1983), troponin I and troponin T of cardiac muscle (Katoh *et al.*, 1983), the 40S-ribosomal-subunit protein S6 (LePeuch *et al.*, 1983) and cardiac phospholamban (Iwasa & Hosey, 1984). We considered that, since protein kinase C consists of a single polypeptide chain (Kikkawa *et al.*, 1982; Wise *et al.*, 1982; Schatzman *et al.*, 1983) and requires Ca^{2+} but no additional protein factor for activity, it must bind Ca^{2+} directly and may thereby expose a hydrophobic site(s) in analogous fashion to calmodulin. It may therefore be amenable to purification by Ca^{2+} -dependent hydrophobic-interaction chromatography. Since the published procedures for protein kinase C purification are long (about 2 weeks) and tedious and result in low yields (Kikkawa *et al.*, 1982; Wise *et al.*, 1982; Schatzman *et al.*, 1983), we sought to develop a rapid and simple purification procedure for this enzyme. During development of this procedure, we detected a novel Ca^{2+} -binding protein, which we have purified and partially characterized.

Materials and methods

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10–40 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.), phenyl-Sepharose CL-4B, DEAE-Sephacel, M_r -marker proteins, histone III-S, L- α -phosphatidyl-L-serine and 1,3-diolein were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), *Staphylococcus aureus* V8 proteinase was from Pierce Chemical Co. (Rockford, IL, U.S.A.) and hydroxyapatite was from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). Calmodulin was purified from bovine brain as described by Gopalakrishna & Anderson (1982). Myosin and myosin light-chain kinase, both from chicken gizzard, were purified as described by Walsh *et al.* (1983). Bovine brain cyclic nucleotide phosphodiesterase, calcineurin and a novel 24000- M_r bovine brain calciprotein were generously given by Dr. R. K. Sharma, Ms.

Catherine Pallen and Dr. David M. Waisman respectively, all of the Department of Medical Biochemistry, University of Calgary. Canine skeletal myofibrils (used as M_r -marker proteins) were prepared as described by Etlinger & Fischman (1972).

Enzyme assays

Myosin light-chain kinase activity was measured as previously described (Walsh *et al.*, 1983). Myosin (0.5 mg/ml) was incubated at 25°C with 25 mM-Tris/HCl buffer, pH 7.5, containing 4 mM-MgCl₂, 60 mM-KCl and either 0.1 mM-CaCl₂ or 1 mM-EGTA in the presence and in the absence of calmodulin (30 µg/ml) or 21 kDa CaBP (40 µg/ml). Reactions were initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approx. 4000 c.p.m./nmol) to a final concentration of 0.75 mM. Reactions (0.45 ml) were quenched after 5 min by addition to an equal volume of 25% (w/v) trichloroacetic acid/2% (w/v) sodium pyrophosphate, and protein-bound ^{32}P was quantified as previously described (Walsh *et al.*, 1983).

Cyclic nucleotide phosphodiesterase activity was assayed as described by Wang *et al.* (1972). Protein kinase C activity was assayed essentially by the method of Kikkawa *et al.* (1982). The enzyme sample (80 µl/ml of reaction mixture) was incubated at 25°C or 30°C (see the text below) with histone III-S (0.2 mg/ml) in 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgCl₂, phosphatidylserine (40 µg/ml), 1,3-diolein (0.8 µg/ml) and either 0.5 mM-CaCl₂ or 1 mM-EGTA. Reaction was initiated by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approx. 50000 c.p.m./nmol) to a final concentration of 10 µM. Reactions (0.25 ml) were quenched by addition to 0.5 ml of 25% (w/v) trichloroacetic acid/2% (w/v) sodium pyrophosphate, and ^{32}P -bound phosphate was quantified as described by Walsh *et al.* (1983). In separate experiments, the quenching procedure was shown to precipitate the histone quantitatively.

Electrophoresis

Electrophoresis was performed in the presence of 0.1% SDS in 7.5–20% or 15–20% polyacrylamide-gradient slab gels by the procedure of Laemmli (1970).

Amino acid composition analysis

Triplicate samples of bovine brain 21 kDa CaBP (0.73 nmol) were hydrolysed *in vacuo* at 110°C in 6M-HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for the periods indicated in the text before amino acid composition analysis in a Beckman model 121M amino acid analyser. Tryptophan was determined by methanesulphonic acid hydrolysis as described by Simpson *et al.* (1976), and cysteine by performic acid oxidation as described by Hirs (1967).

Results

Preparation of protein kinase C

Step 1: Homogenization and centrifugation. All procedures were carried out at 4°C, unless indicated otherwise. Frozen bovine brain (500g) was chopped into small pieces and homogenized in a Waring blender for 2 × 30s in 3 vol. (1500ml) of 0.25M-sucrose in 20mM-Tris/HCl buffer, pH 7.5, containing 2mM-EDTA, 10mM-EGTA and 1mM-dithiothreitol. The homogenate was centrifuged at 30000g for 15min. The supernatant was filtered through glass-wool and centrifuged at 100000g for 60min. The supernatant was again filtered through glass-wool. Solid (NH₄)₂SO₄ was added with constant stirring to a final saturation level of 75% (i.e. 480g/l) to concentrate the protein in the extract. The mixture was stirred for 30min and centrifuged at 30000g for 45min. The pellet was redissolved in a minimum volume (<200ml) of 20mM-Tris/HCl buffer, pH 7.5, containing 1mM-EDTA, 1mM-EGTA and 1mM-dithiothreitol (buffer A) and dialysed overnight against 2 × 10 litres of buffer A. After dialysis, the solution was clarified by centrifugation at 30000g for 30min.

Step 2: Ca²⁺-dependent hydrophobic-interaction chromatography. To the supernatant, solid MgCl₂ and solid CaCl₂ were added with stirring to final concentrations of 2mM each. The sample was loaded on a column (1.5cm × 30cm) of phenyl-Sepharose CL-4B previously equilibrated with 20mM-Tris/HCl buffer, pH 7.5, containing 0.1mM-CaCl₂ and 1mM-dithiothreitol (buffer B). The column was washed with buffer B until A₂₈₀ reached baseline, and then with buffer B containing 1M-NaCl to elute any proteins electrostatically bound to the column. Finally, proteins that were bound hydrophobically in a Ca²⁺-dependent manner were eluted with 20mM-Tris/HCl buffer, pH 7.5, containing 1mM-EGTA and 1mM-dithiothreitol. Selected fractions were examined by SDS/15–20%-polyacrylamide-gradient-slab-gel electrophoresis and assayed for protein kinase C activity as described in the Materials and methods section. The absorption profile for protein eluted from the phenyl-Sepharose CL-4B column is shown in Fig. 1(a). Note that most of the protein in the column load passed straight through the column (compare lanes b–d in Fig. 1b); this gave a very large A₂₈₀ peak, which has been omitted from Fig. 1(a). Fig. 1(a) therefore shows only the profile after application of buffer B containing 1M-NaCl. No protein kinase C activity was detected in the flow-through peak (not shown), indicating that all the enzyme was bound to the column in the presence of Ca²⁺. When EGTA was included in the eluting buffer, a number of proteins were eluted from the column. The peak fraction of protein

kinase C activity (fraction 50) coincided with the bulk of the calmodulin eluted with EGTA (see Fig. 1b, fraction 50). Fig. 1(b) demonstrates that proteins in addition to calmodulin and protein kinase C were also eluted from the hydrophobic column with EGTA-containing buffer. These constituents consisted mainly of two proteins (M_r 25000 and 21000), which must be Ca²⁺-binding proteins since, like calmodulin, they expose a hydrophobic site in the presence of bound Ca²⁺. The higher-M_r protein may be identical with a 24000-M_r Ca²⁺-binding protein previously isolated from bovine brain (Waisman *et al.*, 1983). Two minor proteins eluted after the 21kDa CaBP (fractions 70–90). These are distinct Ca²⁺-binding proteins of M_r greater than 21000. In the present study we have pursued the purification of the protein kinase C and the 21kDa CaBP. Two pools (A and B) were obtained (Fig. 1a): pool A contained protein kinase C (not visible on the gel) and calmodulin; pool B contained the 21kDa CaBP and contaminating Ca²⁺-binding proteins. The next section describes the further purification of protein kinase C; subsequently, the purification of the 21kDa CaBP to homogeneity is presented.

Step 3: ion-exchange chromatography. Pool A from phenyl-Sepharose CL-4B (Fig. 1a) was dialysed overnight against 2 × 10 litres of 20mM-Tris/HCl buffer, pH 7.5, containing 1mM-EGTA and 1mM-dithiothreitol (buffer C) and loaded on a column (1cm × 30cm) of DEAE-Sephacel previously equilibrated with buffer C. The column was washed with buffer C until A₂₈₀ reached baseline, and bound proteins were eluted with a linear salt gradient. Selected fractions were assayed for protein kinase C activity as described in the Materials and methods section (Fig. 2). The major peak of A₂₈₀, which was eluted at 0.28M-NaCl, contained calmodulin, as shown by gel electrophoresis (results not shown). A peak of protein kinase C activity was detected that was eluted at approx. 0.16M-NaCl. This activity required both Ca²⁺ and phospholipid. Gel electrophoresis of protein kinase C-containing fractions followed by silver staining indicated that the kinase activity correlated with a band of M_r 85000.

Whereas the protein kinase C preparation (pooled fractions 69–76 from Fig. 2) was not entirely homogeneous (Fig. 6b; 84% purity as determined by densitometric scanning of SDS/polyacrylamide-gel electrophoretograms), it was not contaminated by other protein kinases and also contained no phosphatase activity, as shown in Fig. 3. This Figure illustrates the time course of phosphorylation of histone III-S by protein kinase C in the presence of 0.5mM-Ca²⁺. After 11 min incubation, excess EGTA was added to inhibit the kinase, and protein-bound phosphate was moni-

Fig. 1. Ca^{2+} -dependent hydrophobic-interaction chromatography of bovine brain soluble proteins
 The 100000g supernatant of bovine brain, after concentration with $(\text{NH}_4)_2\text{SO}_4$, dialysis and addition of excess free Mg^{2+} and Ca^{2+} , was applied to a column (1.5 cm $\times$ 30 cm) of phenyl-Sepharose CL-4B previously equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM- CaCl_2 and 1 mM-dithiothreitol (buffer B). Unbound protein was washed out with buffer B until A_{280} reached baseline; the very large peak of protein in the flow-through is not shown in (a). Next the column was washed with buffer B containing 1 M-NaCl (applied at arrow 1, in a). Finally the column was washed with 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EGTA and 1 mM-dithiothreitol (applied at arrow 2, in a). The chromatogram was developed at 30 ml/h, and 4 ml fractions were collected. (a) shows the elution profile of protein (A_{280} , —) and protein kinase C activity, measured at 25°C as described in the Materials and methods section, in the presence ($\circ$ — $\circ$) and absence (Δ — Δ) of phospholipid (phosphatidylserine), both assayed in the presence of Ca^{2+} . Protein kinase C activity is expressed as pmol of P_i incorporated into histone III-S substrate under standard assay conditions described in the Materials and methods section. Assays conducted in the absence of Ca^{2+} indicated that protein kinase C activity was absolutely dependent on Ca^{2+} (results not shown). Selected fractions were also examined by SDS/15–20%-polyacrylamide-gradient-slab-gel electrophoresis (b). Key to gel lanes: a, M_r markers (bovine serum albumin, M_r 66000; ovalbumin, M_r 45000; glyceraldehyde-3-phosphate dehydrogenase, M_r 36000; carbonic anhydrase, M_r 29000; trypsinogen, M_r 24000; trypsin inhibitor, M_r 20100; α -lactalbumin, M_r 14200); b, column load; c, flow-through (beginning); d, flow-through (end); numbers correspond to fraction numbers in (a). Fractions in (a) were pooled as indicated by the bars: pool A, calmodulin+protein kinase C (fractions 50–58); pool B, 21 kDa CaBP (fractions 59–65). The arrow in (b) indicates the 21 kDa CaBP.

Fig. 2. Ion exchange chromatography of protein kinase C

Pool A from Fig. 1 was dialysed against buffer C and applied to a column (1 cm $\times$ 30 cm) of DEAE-Sephacel previously equilibrated with buffer C. The column was washed with buffer C until A_{280} reached baseline, and bound proteins were eluted with a linear salt gradient (----) generated from 200 ml each of buffer C and buffer C containing 0.4 M NaCl. The flow rate was 12 ml/h, and the fraction size was 4 ml. The elution profile of protein (A_{280} , —) was monitored. Selected fractions were assayed at 25°C for protein kinase C activity under four conditions: +Ca²⁺ + phospholipid (○); -Ca²⁺ + phospholipid (□); +Ca²⁺ - phospholipid (△); -Ca²⁺ - phospholipid (▽). Protein kinase C-containing fractions were pooled as indicated by the bar (fractions 69–76).

Fig. 3. Assay of phosphatase activity in protein kinase C preparation

Protein kinase C (pooled fractions from Fig. 2) was incubated at 30°C under standard kinase assay conditions as described in the Materials and methods section. The reaction volume was 3.5 ml. Samples (0.2 ml) of reaction mixture were withdrawn at times 2, 4, 6, 8 and 10 min for determination of protein-bound phosphate. At time 11 min, EGTA was added to a final concentration of 5 mM to inhibit the kinase, and samples (0.2 ml) were withdrawn at times 12, 14, 16, 18, 20 and 25 min for determination of protein-bound phosphate.

Fig. 4. Ca²⁺- and phospholipid-dependence of protein kinase C activity

Protein kinase C activity was assayed as described in the Materials and methods section under the following conditions: +Ca²⁺ + phospholipid (○); -Ca²⁺ + phospholipid (□); +Ca²⁺ - phospholipid (△); -Ca²⁺ - phospholipid (▽). Samples (0.2 ml) were withdrawn at the indicated times for determination of protein-bound phosphate.

tored up to 25 min incubation. If phosphate contamination were present in the protein kinase C preparation, the amount of protein-bound phosphate would decrease after addition of EGTA; this is clearly not the case.

Fig. 4 illustrates clearly in time-course experiments the Ca²⁺- and phospholipid-dependence of the protein kinase C preparation. This preparation, which can be obtained rapidly (within 5 days) from frozen bovine brains, is ideally suited for studies requiring specific phosphorylation of sub-

strate proteins by protein kinase C. We have found that the preparation is labile to freezing and thawing. Hence the active fraction was stored on ice and experiments in which it was used were performed within approx. 5 days of completion of the preparation.

Preparation of 21 kDa CaBP

Step 1: heat-treatment. Pool B from phenyl-Sepharose CL-4B (Fig. 1a) was enriched in the 21 kDa CaBP. It is well known that calmodulin is heat-stable in the presence, but not in the absence, of bound Ca^{2+} (Teo *et al.*, 1973), a fact that has often been exploited in the purification of calmodulin (for review see Walsh & Hartshorne,

1983). On this basis, we investigated the possibility of using heat-treatment to purify the 21 kDa CaBP (Fig. 5). When a portion of pool B was heated at 80–85°C for 2 min in the presence of 2 mM- Ca^{2+} followed by centrifugation, the 21 kDa CaBP was recovered, along with two low- M_r contaminants in the supernatant (Fig. 5, lane 2); many minor contaminants had been denatured and removed by centrifugation. If the heat-treatment was performed in the absence of Ca^{2+} , however, no such purification was achieved (Fig. 5, lane 3). On the basis of the results of this pilot experiment, further purification of the 21 kDa CaBP from pool B was achieved as follows. To the remainder of pool B, solid CaCl_2 was added with stirring to a final concentration of 2 mM. The solution was immersed in a boiling-water bath and heated to 80°C. The temperature was maintained at $\sim 85^\circ\text{C}$ for 2 min, and then the sample was placed on ice and centrifuged at 48 000 *g* for 30 min.

Step 2: hydroxyapatite-column chromatography. The supernatant was dialysed overnight against 2×10 litres of 1 mM-potassium phosphate buffer,

Fig. 5. Heat-treatment of 21 kDa CaBP in the presence and in the absence of Ca^{2+}

A sample of pool B from Fig. 1, which was enriched in 21 kDa CaBP, was divided into two portions (0.1 ml each). CaCl_2 was added to one to a final concentration of 2 mM (i.e. 1 mM excess over EGTA); the second was maintained at 1 mM-EGTA. Each sample was heated in a boiling-water bath at 80–85°C for 2 min, cooled on ice and centrifuged for 5 min in an Eppendorf 3200 centrifuge. Samples (15 μl) of pool B before (lane 1) and after heat-treatment in the presence (lane 2) and in the absence (lane 3) of Ca^{2+} were examined by SDS/7.5–20%-polyacrylamide-gradient-slab-gel electrophoresis. The 21 kDa CaBP is indicated by the arrow.

Fig. 6. SDS/polyacrylamide-gel electrophoresis of purified 21 kDa CaBP and protein kinase C

(a) The major protein peak from hydroxyapatite column chromatography was examined by SDS/15–20%-polyacrylamide-gradient slab-gel electrophoresis (5 μg of protein applied to gel). (b) The protein kinase C preparation (pooled fractions 69–76 from Fig. 2) was examined by SDS/7.5–20%-polyacrylamide-gradient-slab-gel electrophoresis (5 μg of protein applied to gel).

pH 7, containing 0.2M-KCl (buffer D) and loaded on a column (1 cm × 20 cm) of hydroxyapatite previously equilibrated with buffer D. The column was washed with buffer D until A_{280} reached baseline. Bound proteins were eluted with a linear gradient generated from 150 ml each of buffer D and 0.4M-potassium phosphate buffer, pH 7, containing 0.2M-KCl. Fractions of volume 4 ml were collected at a flow rate of 10 ml/h. The major protein peak, which was eluted as a broad peak in the middle of the gradient, contained the 21 kDa CaBP, which was judged to be homogeneous by electrophoresis under denaturing conditions (Fig. 6a).

Properties of the 21 kDa CaBP

Ca²⁺-dependent electrophoretic mobility shift and M_r determination. Calmodulin and other Ca²⁺-binding proteins are known to migrate differently during SDS/polyacrylamide-gel electrophoresis depending on the presence or the absence of Ca²⁺ (see, e.g., Cartaud *et al.*, 1980). Fig. 7 illustrates that the 21 kDa CaBP exhibits a similar Ca²⁺-dependent mobility shift: as in the case of calmodulin

(lanes 2 and 3) and the β -subunit of calcineurin, another major Ca²⁺-binding protein of brain (lanes 6 and 7), the 21 kDa CaBP migrates more rapidly in this gel-electrophoresis system in the presence (lane 4) than in the absence (lane 5) of Ca²⁺. On the basis of the M_r -marker proteins electrophoresed simultaneously (lanes 1 and 8), the novel brain Ca²⁺-binding protein was determined to have M_r 21 000 in the presence of Ca²⁺ and M_r 22 000 in the absence of Ca²⁺.

Cleaveland peptide mapping. It is apparent from the gel electrophoretograms in Fig. 7 that the 21 kDa CaBP is a distinct Ca²⁺-binding protein from calmodulin and the β -subunit of calcineurin. This conclusion was confirmed by comparative peptide mapping of these three calcioproteins by the procedure of Cleaveland *et al.* (1977). Samples of calmodulin, 21 kDa CaBP and calcineurin were electrophoresed in a 0.1% SDS/15–20%-polyacrylamide-gradient slab gel as in Fig. 7. This step was performed to separate the α - and β -subunits of calcineurin, interest being in the Ca²⁺-binding β -subunit. The gel was then briefly stained and destained to allow detection of the bands correspond-

Fig. 7. Ca²⁺-dependent electrophoretic-mobility shift of Ca²⁺-binding proteins

Samples of calmodulin (5 μ g), 21 kDa CaBP (2 μ g) and calcineurin (10 μ g) were electrophoresed in the presence of 10 mM-Ca²⁺ (lanes 2, 4 and 6 respectively) and 10 mM-EGTA (lanes 3, 5 and 7 respectively) in an SDS/15–20%-polyacrylamide-gradient slab gel. Lanes 1 and 8 contained the following M_r -marker proteins: ovalbumin, M_r 45 000; actin, M_r 42 000; troponin T, M_r 37 000; tropomyosin, M_r 35 000; carbonic anhydrase, M_r 29 000; myosin light chain, M_r 25 000; troponin I, M_r 24 000; trypsin inhibitor, M_r 20 100; troponin C, M_r 20 000; myosin light chain 2, M_r 18 500; myosin light chain 3, M_r 16 500.

Fig. 8. *Cleveland peptide mapping of calmodulin, 21 kDa CaBP and calcineurin β -subunit*

Samples of calmodulin (9.5 μ g), 21 kDa CaBP (9.5 μ g) and calcineurin (47.5 μ g) were subjected to SDS/15–20%-polyacrylamide-gradient-slab-gel electrophoresis. The gel was stained for 5 min in 0.5% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol/10% (v/v) acetic acid and destained in 40% (v/v) methanol/10% (v/v) acetic acid. Protein bands were visible within 30 min, and bands corresponding to calmodulin, 21 kDa CaBP and calcineurin β -subunit were cut out of the gel and incubated with occasional stirring with 62.5 mM-Tris/HCl buffer, pH 6.8, containing 1% (w/v) SDS, 1 mM-EDTA and 10% (v/v) glycerol (equilibration buffer) at room temperature for 60 min. Gel slices were then transferred to the wells of a second SDS/15–20%-polyacrylamide-gradient slab gel (with a 10 cm stacking gel) and overlaid with 20 μ l of equilibration buffer containing 0 (lanes 2–4), 0.05 μ g (lanes 5–7) and 0.25 μ g (lanes 8–10) of *S. aureus* V8 proteinase. Electrophoresis was carried out at 25 mA (1.5 mm gel thickness). The gel was then stained with Coomassie Blue and destained. Key to lanes: 1, M_r -marker proteins; 2, 5 and 8, 21 kDa CaBP + 0, 0.05 and 0.25 μ g respectively of V8 proteinase; 3, 6 and 9, calmodulin + 0, 0.5 and 0.25 μ g respectively of V8 proteinase; 4, 7 and 10, calcineurin β -subunit + 0, 0.05 and 0.25 μ g respectively of V8 proteinase.

ing to calmodulin, 21 kDa CaBP and calcineurin β -subunit, which were cut out of the gel and treated with different amounts of *Staphylococcus aureus* V8 proteinase as described in the legend to Fig. 8. Peptides were separated by SDS/15–20%-polyacrylamide-gradient-slab-gel electrophoresis (Fig. 8). The peptide patterns generated from the three calcuproteins were clearly different. Under conditions that resulted in significant proteolysis of calmodulin and calcineurin β -subunit, the 21 kDa CaBP was quite resistant to digestion. The lower- M_r band observed in lanes 2, 5 and 8 was probably derived from the 21 kDa CaBP by acid hydrolysis during the brief staining and destaining period that followed excision of the 21 kDa CaBP band from the first gel.

Amino acid analysis. Table 1 compares the amino acid composition of the 21 kDa CaBP, purified through the hydroxyapatite chromatography step, with bovine brain calmodulin and rabbit skeletal-muscle troponin C, which is known to exhibit significant sequence homology with calmodulin.

Whereas the 21 kDa CaBP is clearly a distinct calcuprotein, it does exhibit certain similarities to members of the calmodulin family of Ca^{2+} -binding proteins. For example, the 21 kDa CaBP has a high content of acidic amino acids. Calmodulin-related proteins are all acidic proteins, many of the carboxylate side chains being involved in co-ordination of Ca^{2+} . The 21 kDa CaBP has a significantly higher lysine content than calmodulin and troponin C, and this is reflected in a higher isoelectric point: the 21 kDa CaBP has pI 5.1, whereas the pI of calmodulin is 4.0 (Stevens *et al.*, 1976) and that of troponin C 4.1–4.4 (Hartshorne & Dreizen, 1972). The 21 kDa CaBP has significantly higher contents of serine, cysteine and tyrosine, and, in common with the other calcuproteins, lacks tryptophan residues.

Functional studies. The purified 21 kDa CaBP was tested as a possible Ca^{2+} -dependent activator of two calmodulin-dependent enzymes, namely bovine brain cyclic nucleotide phosphodiesterase and chicken gizzard myosin light-chain kinase.

Table 1. Comparison of the amino acid compositions of bovine brain 21 kDa CaBP, bovine brain calmodulin and rabbit skeletal-muscle troponin C

Samples of 21 kDa CaBP (40 μ l each of a solution of concentration 0.4 mg/ml in water) were hydrolysed at 110°C *in vacuo* in 6M-HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for 24, 48 and 72 h. Values represent the means for three determinations. Values for cysteine were obtained by performic acid oxidation (Hirs, 1967). Values for tryptophan were obtained by methanesulphonic acid hydrolysis (Simpson *et al.*, 1976).

	Composition (mol of residue/mol)					
	21 kDa Ca ²⁺ -binding protein				Calmodulin (Watterson <i>et al.</i> , 1980)	Troponin C (Collins <i>et al.</i> , 1977)
Amino acid	24h	48h	72h	Average		
Lysine	16.3	16.3	16.1	16.2	8*	9
Histidine	3.3	3.4	3.4	3.4	1	1
Arginine	8.9	8.9	8.8	8.9	6	7
Aspartic acid	21.3	21.7	19.9	21.0	23	22
Threonine	8.1	7.5	8.1	7.9	12	6
Serine	17.5	16.3	17.0	16.9	4	7
Glutamic acid	28.3	27.2	25.7	27.1	27	31
Proline	6.4	5.1	6.7	6.1	2	1
Glycine	19.1	19.0	18.6	18.9	11	13
Alanine	12.3	11.7	11.4	11.8	11	13
Cysteine	3.3	—	—	3.3	0	1
Valine	7.6	7.7	7.6	7.6	7	7
Methionine	5.1	5.3	5.4	5.3	9	10
Isoleucine	7.7	7.5	7.9	7.7	8	10
Leucine	13.6	13.4	13.2	13.4	9	9
Tyrosine	4.7	4.6	5.0	4.8	2	2
Phenylalanine	10.8	10.6	10.6	10.7	8	10
Tryptophan	0.0	—	—	0.0	0	0

* Contains one residue of ϵ -N-trimethyl-lysine.

Neither enzyme was affected in any way by the 21 kDa CaBP. Furthermore, the novel brain calci-protein had no effect on the activity of bovine brain protein kinase C, prepared as described above.

Discussion

Bovine brain contains several proteins that interact with phenyl-Sepharose in a Ca²⁺-dependent manner. These include calmodulin (the major component), the Ca²⁺- and phospholipid-dependent protein kinase (protein kinase C), and the novel Ca²⁺-binding protein of subunit *M*_r21 000, which we describe here for the first time. Calmodulin and protein kinase C are eluted together when EGTA is applied to the hydrophobic column, whereas the 21 kDa CaBP is eluted slightly later, suggesting that the 21 kDa CaBP may have higher affinity for Ca²⁺ than do calmodulin and protein kinase C. Direct verification of this conclusion will require more detailed studies of the Ca²⁺-binding properties of the 21 kDa CaBP.

Ca²⁺-dependent hydrophobic-interaction chromatography is a very useful initial step in the

purification of protein kinase C, enabling rapid removal of over 90% of total brain soluble proteins. The kinase C prepared in this way is simply separated from calmodulin by ion-exchange chromatography. This method is therefore suitable for the simultaneous isolation of protein kinase C and calmodulin. Protein kinase C from the ion-exchange column, though not electrophoretically homogeneous, is free of any contaminating kinase or phosphatase activities, and its activity, as expected, is dependent on Ca²⁺ and phospholipid. The preparation of kinase C is complete within 5 days and yields a product suitable for studies *in vitro* involving specific phosphorylation of protein substrates by kinase C. The method of preparation of protein kinase C described in the present paper has been successfully applied to smooth muscle (chicken gizzard) as well as bovine brain, suggesting that it may be useful in the preparation of the enzyme from diverse sources.

After the Ca²⁺-dependent hydrophobic-interaction chromatography, the 21 kDa CaBP was purified to electrophoretic homogeneity by heat-treatment in the presence of Ca²⁺ and hydroxyapatite column chromatography. This calci-protein is distinct from calmodulin and the β -subunit of

calcieneurin as shown by polyacrylamide-gel electrophoresis, isoelectric point (5.1), Cleveland peptide mapping and amino acid composition analysis. The 21 kDa CaBP is also distinct from other known Ca^{2+} -binding proteins of the brain (S100, the protein analogous to the vitamin D-induced CaBP, the regulatory light chain of myosin and parvalbumin) on the basis of molecular mass and amino acid composition. Like other known Ca^{2+} -binding proteins, including calmodulin and calcineurin β -subunit, the 21 kDa CaBP undergoes a Ca^{2+} -dependent mobility shift on SDS/polyacrylamide-gel electrophoresis. Comparison of the amino acid composition of the 21 kDa CaBP with members of the calmodulin family of Ca^{2+} -binding proteins indicates many similarities, including a high content of acidic side chains, suggesting that this novel brain calprotein may belong to the calmodulin family. Whether or not this is so will require more detailed structural and Ca^{2+} -binding studies of the 21 kDa CaBP. Waisman *et al.* (1983) have described the isolation of a novel bovine brain Ca^{2+} -binding protein that exhibited M_r 24000 on SDS/polyacrylamide-gel electrophoresis. However, our 21 kDa CaBP is a distinct calprotein, since it has a different amino acid composition, and purified samples of both proteins do not co-electrophorese in denaturing gels (results not shown). An 18 kDa CaBP recently isolated by Manalan & Klee (1984) has a distinct amino acid composition from that of the 21 kDa CaBP described here.

From a functional point of view, the 21 kDa CaBP has been examined for its ability to activate cyclic nucleotide phosphodiesterase and myosin light-chain kinase, both calmodulin-dependent enzymes. It activates neither enzyme. In addition, the 21 kDa CaBP has no effect on the activity of the Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C). The possibility arises that this novel brain calprotein may be involved in the Ca^{2+} -mediated regulation of an as-yet-unidentified enzymic activity. We have not detected the 21 kDa CaBP in chicken gizzard, suggesting that it may be restricted in its tissue distribution to neural tissue.

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