Ca^{2+} -binding proteins from bovine brain including a potent inhibitor of protein kinase C

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We have previously described the use of Ca^{2+} -dependent hydrophobic-interaction chromatography to isolate the Ca^{2+} -phospholipid-dependent protein kinase (protein kinase C) and a novel heat-stable 21000- M_r Ca^{2+} -binding protein from bovine brain [Walsh, Valentine, Ngai, Carruthers & Hollenberg (1984) *Biochem. J.* **224**, 117–127]. The procedure described for purification of the 21000- M_r calprotein to electrophoretic homogeneity has been modified to permit the large-scale isolation of this Ca^{2+} -binding protein, enabling further structural and functional characterization. The 21000- M_r calprotein was shown by equilibrium dialysis to bind approx. 1 mol of Ca^{2+} /mol, with apparent K_d approx. 1 μM . The modified large-scale purification procedure revealed three additional, previously unidentified, Ca^{2+} -binding proteins of M_r 17000, 18400 and 26000. The 17000- M_r and 18400- M_r Ca^{2+} -binding proteins are heat-stable, whereas the 26000- M_r Ca^{2+} -binding protein is heat-labile. Use of the transblot/ $^{45}\text{CaCl}_2$ overlay technique [Maruyama, Mikawa & Ebashi (1984) *J. Biochem. (Tokyo)* **95**, 511–519] suggests that the 18400- M_r and 21000- M_r Ca^{2+} -binding proteins are high-affinity Ca^{2+} -binding proteins, whereas the 17000- M_r Ca^{2+} -binding protein has a relatively low affinity for Ca^{2+} . Consistent with this observation, the 18400- M_r and 21000- M_r Ca^{2+} -binding proteins exhibit a Ca^{2+} -dependent mobility shift on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, whereas the 17000- M_r Ca^{2+} -binding protein does not. The amino acid compositions of the 17000- M_r , 18400- M_r and 21000- M_r Ca^{2+} -binding proteins show some similarities to each other and to calmodulin and other members of the calmodulin superfamily; however, they are clearly distinct and novel calproteins. In functional terms, none of the 17000- M_r , 18400- M_r or 21000- M_r Ca^{2+} -binding proteins activates either cyclic nucleotide phosphodiesterase or myosin light-chain kinase, both calmodulin-activated enzymes. However, the 17000- M_r Ca^{2+} -binding protein is a potent inhibitor of protein kinase C. It may therefore serve to regulate the activity of this important enzyme at elevated cytosolic Ca^{2+} concentrations.

INTRODUCTION

The calmodulin superfamily of intracellular Ca^{2+} -binding proteins, which are found in a wide variety of tissues and species, serves to transduce the Ca^{2+} second message into specific physiological responses (Cheung, 1980; Kretsinger, 1980; Klee & Vanaman, 1982; Means *et al.*, 1982; Walsh & Hartshorne, 1983). For example, the binding of Ca^{2+} to calmodulin following nervous stimulation of a smooth-muscle cell leads to contraction (Walsh & Hartshorne, 1982). A number of these proteins, including calmodulin (Cheung, 1970; Kakiuchi *et al.*, 1970), calcineurin B (Klee *et al.*, 1979) and S-100a and S-100b (Isobe *et al.*, 1978, 1981), are found in brain. More recently, several novel low- M_r Ca^{2+} -binding proteins have been isolated from bovine brain (Waisman *et al.*, 1983; Manalan & Klee, 1984; Südhof *et al.*, 1984; Walsh *et al.*, 1984), although their roles in Ca^{2+} -mediated regulation have not been elucidated.

Protein kinase C, originally described by Inoue *et al.* (1977), has attracted a great deal of attention in recent years because it may couple extracellular messenger-induced polyphosphoinositide turnover with diverse cellular events. This kinase requires Ca^{2+} and a phospholipid, e.g. phosphatidylserine, for activity (Nishizuka, 1984). Unsaturated diacylglycerol is produced intracellularly by the breakdown of membrane-bound

phosphatidylinositol 4,5-bisphosphate in response to a variety of extracellular messengers, such as various peptide hormones, growth factors, muscarinic cholinergic agents and α -adrenergic agents. The other product of phosphatidylinositol 4,5-bisphosphate hydrolysis is inositol 1,4,5-trisphosphate, which is believed to cause release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum (Berridge, 1984; Berridge & Irvine, 1984). Protein kinase C can be activated *in vitro* at low concentrations of Ca^{2+} by unsaturated diacylglycerol, which increases the Ca^{2+} -sensitivity of the kinase (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). Activated protein kinase C is then capable of phosphorylating specific protein substrates, leading ultimately to the desired physiological response. Several substrates of protein kinase C have been identified to date: 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 and filamin of smooth muscle (Werth *et al.*, 1983; Kawamoto & Hidaka, 1984), troponin I and troponin T of cardiac muscle (Katoh *et al.*, 1983) and skeletal muscle (Mazzei & Kuo, 1984), the 40S-ribosomal-subunit protein S6 (LePeuch *et al.*, 1983), muscle glycogen synthase (Ahmad *et al.*, 1984), the epidermal-growth-factor receptor (Cochet *et al.*, 1984) and cardiac phospholamban (Iwasa & Hosey, 1984).

We have previously described the use of Ca^{2+} -dependent hydrophobic-interaction chromatography in the isolation of protein kinase C and a novel 21000- M_r Ca^{2+} -binding protein (21 kDa CaBP) from bovine brain (Walsh *et al.*, 1984). We now describe some more detailed properties of the 21 kDa CaBP and the identification of three other CaBPs (26 kDa, 18.4 kDa and 17 kDa). Two of these latter CaBPs are heat-stable and have been purified. The observation that calmodulin and S-100 protein are capable of inhibiting protein kinase C (Albert *et al.*, 1984) prompted us to examine the effects of these novel brain calcioproteins on protein kinase C activity. The 17 kDa CaBP proved to be a potent inhibitor of kinase C, suggesting a possible physiological role for this Ca^{2+} -binding protein in the regulation of signal-transduction processes involving protein kinase C.

MATERIALS AND METHODS

Materials

[γ - ^{32}P]ATP (10–40 Ci/mmol) and $^{45}\text{CaCl}_2$ (10–40 mCi/mg of Ca) were purchased from Amersham (Oakville, Ont., Canada). DEAE-Sephacel, phenyl-Sepharose CL-4B, L- α -phosphatidyl-L-serine, histone III-S, 1,3-diolein, cyclic AMP, 5'-nucleotidase and M_r -marker proteins were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydroxyapatite was from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.), and nitrocellulose membranes (Trans-blot transfer medium) and electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). General laboratory reagents used were analytical grade or better and were purchased from Fisher Chemical Co.

Calmodulin was purified from bovine brain by the procedure of Gopalakrishna & Anderson (1982) as modified by Walsh *et al.* (1984). The 21 kDa CaBP was purified from bovine brain as previously described (Walsh *et al.*, 1984). Chicken gizzard myosin and myosin light-chain kinase were purified as described by Persechini & Hartshorne (1981) and Ngai *et al.* (1984) respectively. Rabbit skeletal-muscle actin was isolated by the method of Zot & Potter (1981). Bovine brain protein kinase C was isolated as described by Walsh *et al.* (1984). Bovine brain cyclic nucleotide phosphodiesterase was generously supplied by Dr. R. K. Sharma, Department of Medical Biochemistry, University of Calgary.

Enzyme assays

Myosin light-chain activity was measured 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), as described in detail by Walsh *et al.* (1984), with histone III-S as the substrate. Details of enzymic assay reaction conditions are provided in the text.

Ca^{2+} binding

Ca^{2+} -binding parameters were determined by equilibrium dialysis. The 21 kDa CaBP was dialysed overnight against 10 litres of buffer A [20 mM-Mops (pH 7.1)/0.15 M-KCl/0.1 mM-EGTA/3.0 mM- MgCl_2] containing 0.1 mM-EDTA, followed by dialysis against two changes (10 litres each) of buffer A. The dialysed protein (1.0 mg/ml) was divided into several 1.5 ml portions in

dialysis bags, which were immersed in flasks containing 100 ml of buffer A to which was added $^{45}\text{Ca}^{2+}$ (20 μCi) and various amounts of CaCl_2 . The flasks were agitated gently for 60–65 h at 4 °C. Protein concentrations inside the dialysis bags were determined by using an absorption coefficient, $A_{277}^{1\%}$, of 9.1 (see the Results section) and the amount of ^{45}Ca inside both the flasks and dialysis bags was determined by liquid-scintillation counting. The free $[\text{Ca}^{2+}]$ was calculated assuming a pK_d (Ca^{2+}) for EGTA of 10.9555 (Fabiato, 1981). The accuracy of the equilibrium-dialysis methodology was verified with calmodulin from bovine brain. In this case we obtained 3.6 mol of Ca^{2+} bound/mol of calmodulin with dissociation constants in the micromolar range, i.e. similar to previously published values (Wolff *et al.*, 1977; Potter *et al.*, 1983).

Electrophoresis

Electrophoresis was performed in 7.5–20% or 15–20% polyacrylamide-gradient slab gels (1.5 mm thick), with a 5% acrylamide stacking gel, in the presence of 0.1% (w/v) sodium dodecyl sulphate at 36 mA, with the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol/10% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and diffusion-destained in 10% (v/v) acetic acid. Subunit M_r values were determined by comparison of electrophoretic mobilities in denaturing gels with those of M_r -marker proteins with the use of the method of Lambin (1978).

Amino acid composition analysis

Triplicate samples of bovine brain Ca^{2+} -binding proteins were hydrolysed *in vacuo* at 110 °C in 6 M-HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for 24, 48 and 74 h before amino acid composition analysis with a Beckman model 6300 amino acid analyser. Tryptophan was determined after methanesulphonic acid hydrolysis as described by Simpson *et al.* (1976), and cysteine after performic acid oxidation as described by Hirs (1967). N-Terminal sequence analysis was performed in a Beckman 890C sequencer equipped with a Sequemat autoconverter. The resultant amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. in a Beckman Ultrasphere-ODS reverse-phase C-18 column.

Other methods

Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding assay (Spector, 1978) with dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), by amino acid analysis or by spectrophotometric measurements in the cases of calmodulin ($A_{277}^{1\%} = 1.9$; Klee, 1977), 21 kDa CaBP ($A_{277}^{1\%} = 9.1$; see the Results section), actin ($A_{290}^{1\%} = 6.5$; Rees & Young, 1967) and myosin ($A_{280}^{1\%} = 5.6$; Greene *et al.*, 1983). U.v.-absorption spectra were recorded in a Pye-Unicam PU 8800 spectrophotometer.

Electrophoretic transfer of proteins to nitrocellulose membranes and subsequent incubation with $^{45}\text{CaCl}_2$ were performed as described by Maruyama *et al.* (1984). Dried nitrocellulose membranes were exposed to Kodak X-Omat AR film in a Kodak X-Omat AR cassette equipped with intensifying screens for 3–7 days. Nitrocellulose membranes were stained with Amido Black as described by Maruyama *et al.* (1984).

Fig. 1. ^{45}Ca autoradiography of transblotted brain Ca^{2+} -binding proteins

21 kDa CaBP was purified by Ca^{2+} -dependent hydrophobic-interaction chromatography on phenyl-Sepharose, heat-treatment and hydroxyapatite-column chromatography as previously described (Walsh *et al.*, 1984). Samples obtained at different stages during the purification procedure were subjected to 0.1% sodium dodecyl sulphate/7.5–20% polyacrylamide-gradient slab-gel electrophoresis [(a) shows the Coomassie Blue-stained gel]. Proteins separated in an identical gel were transblotted on to a nitrocellulose membrane and overlaid with $^{45}\text{CaCl}_2$ as described by Maruyama *et al.* (1984). The dried membrane was autoradiographed to detect ^{45}Ca bound to protein (b). Key to lanes: 1, calmodulin (2 μg) + 10 mM- Ca^{2+} ; 2, calmodulin (2 μg) + 10 mM-EGTA; 3, phenyl-Sepharose column load; 4, phenyl-Sepharose column flow-through; 5, pool 2 from phenyl-Sepharose column (enriched in 26 kDa CaBP); 6, pool 2 from phenyl-Sepharose column after heat-treatment as described by Walsh *et al.* (1984); 7, pool 1 from phenyl-Sepharose column (enriched in 21 kDa and 18.4 kDa CaBPs); 8, pool 1 from phenyl-Sepharose column after heat treatment; 9, purified 21 kDa CaBP after hydroxyapatite column chromatography. The 21 kDa CaBP band is indicated (21 kDa). Numbers at the sides indicate molecular masses (in kDa) of protein standards.

RESULTS

Identification of Ca^{2+} -binding proteins by transblotting/ ^{45}Ca autoradiography

The 21 kDa CaBP was purified from bovine brain as previously described (Walsh *et al.*, 1984), i.e. by Ca^{2+} -dependent hydrophobic-interaction chromatography of the 100000 *g* supernatant, heat-treatment and hydroxyapatite-column chromatography. Samples obtained at different stages during the purification were subjected to 0.1% sodium dodecyl sulphate/7.5–20% polyacrylamide-gradient slab-gel electrophoresis (Fig. 1a). Several proteins were specifically eluted from the phenyl-Sepharose CL-4B column by chelation of Ca^{2+} with EGTA, as observed previously (Walsh *et al.*, 1984). These were collected in two pools in order of elution from the hydrophobic column with EGTA and included proteins of M_r 26000, 18400 and 21000 (lanes 5 and 7 in Fig. 1). The 26 kDa CaBP was found to be heat-labile, whereas the 21 kDa and 18.4 kDa proteins were both heat-stable (compare lane 5 with 6 and lane 7 with 8 in Fig. 1a). Identical samples separated in an identical gel were transblotted on to nitrocellulose membranes, and renatured high-affinity Ca^{2+} -binding proteins were detected by incubation with $^{45}\text{CaCl}_2$ and subsequent autoradiography as described in the Materials and methods section (Fig. 1b). This technique clearly identified the 21 kDa and 18.4 kDa proteins as high-affinity Ca^{2+} -binding proteins. The heat-labile 26 kDa protein is a Ca^{2+} -binding protein, since it interacts with phenyl-Sepharose in a Ca^{2+} -dependent manner; however, it is not detected by the transblotting/ ^{45}Ca -autoradiographic technique (lanes 5–8 in Fig. 1b). Therefore the 26 kDa CaBP is either a low-affinity Ca^{2+} -binding protein or it does not renature sufficiently to recover its

Ca^{2+} -binding ability after electrophoresis and transblotting. In view of its thermal instability, the latter possibility is likely. The efficiency of transblotting was verified by Amido Black-staining of the nitrocellulose membrane in Fig. 1(b) (results not shown).

The Ca^{2+} -dependent electrophoretic-mobility shift exhibited by calmodulin is apparent in lanes 1 and 2 (Figs. 1a and 1b). Three Ca^{2+} -binding proteins are visible in the phenyl-Sepharose column load (lane 3 in Fig. 1b) by the transblotting/ ^{45}Ca -autoradiographic technique. The prominent most rapidly migrating component is calmodulin. The next band may be the 21 kDa CaBP. The highest- M_r component is not the 26 kDa CaBP and remains unidentified. A small amount of calmodulin remains unbound to the phenyl-Sepharose column (lane 4 in Fig. 1b). The brain contains insufficient amounts of the 18.4 kDa CaBP to be visible in the phenyl-Sepharose column load (lane 3 in Fig. 1b). It becomes visible later in the preparation after further purification and concentration (lanes 6–9).

Purification of novel Ca^{2+} -binding proteins

Large-scale purification of the 21 kDa CaBP was attempted by combining the heat-treated phenyl-Sepharose column pools from 20 preparations (500 g of brain each) carried out as described by Walsh *et al.* (1984) before the hydroxyapatite-column chromatographic step. The 21 kDa CaBP eluted from this column was, however, contaminated with other proteins, thus necessitating further purification. This was achieved by dialysing the pooled material from the hydroxyapatite column overnight against two changes (10 litres each) of 10 mM- NH_4HCO_3 followed by freeze-drying. The freeze-dried protein was redissolved in approx. 20 ml of 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/0.5 mM-dithiothreitol

Fig. 2. Ion-exchange chromatography of brain Ca^{2+} -binding proteins

The hydroxyapatite-column pool obtained in the large-scale purification of the 21 kDa CaBP was dialysed, freeze-dried and applied to a DEAE-Sephacel column as described in the Results section. The flow rate was 12 ml/h, and the fraction size was 2.4 ml. Bound proteins were eluted with a linear NaCl gradient (----). Protein elution was monitored by A_{280} (—). Selected fractions were examined by 0.1% sodium dodecyl sulphate/7.5–20% polyacrylamide-gradient slab-gel electrophoresis (see gel inset). Key to lanes: 1, 21, M_r -marker proteins (see the legend to Fig. 1); 2–20, fractions 16–34 respectively; 22–36, fractions 35–41, 65, 85, 95, 100, 105, 110, 115 and 120 respectively. Fractions were pooled as indicated by the bars: A, 17 kDa CaBP; B, 21 kDa CaBP; C, 18.4 kDa CaBP.

and loaded on a column (1 cm $\times$ 40 cm) of DEAE-Sephacel previously equilibrated with this buffer. The column was washed with equilibration buffer (a small protein peak was eluted), and bound proteins were eluted with a linear NaCl gradient (Fig. 2). This final purification step yielded highly purified 21 kDa CaBP (pool B) and revealed the presence of additional Ca^{2+} -binding proteins, including Ca^{2+} -binding proteins of M_r 17000 (pool A) and 18400 (pool C). Some structural and functional properties of these novel Ca^{2+} -binding proteins are now described below.

U.v.-absorption spectrum of the 21 kDa CaBP

The u.v.-absorption spectrum of the 21 kDa CaBP is presented in Fig. 3. An absorption maximum at 277 nm is apparent, and a shoulder at 290 nm is indicative of the presence of tryptophan. This latter observation was confirmed by direct tryptophan analysis after methane-

sulphonic acid hydrolysis as described in the Materials and methods section, which revealed the presence of 2 mol of tryptophan residues/mol of 21 kDa CaBP (Table 1). We showed previously that the 21 kDa CaBP lacked tryptophan (Walsh *et al.*, 1984); however, improved methodology (more sensitive amino acid analysis) and the prior removal of NH_3 , which probably masked the tryptophan peak previously, has enabled us to detect and quantify tryptophan accurately. The presence of tryptophan and the relatively higher contents of phenylalanine and tyrosine account for the fact that the absorption coefficient of the 21 kDa CaBP ($A_{277}^{1\%} = 9.1$) is significantly higher than that of calmodulin ($A_{277}^{1\%} = 1.9$) (Klee, 1977), which lacks tryptophan residues. In determining the absorption coefficient of the 21 kDa CaBP, the protein concentration was precisely determined by amino acid analysis. *N*-Terminal sequence analysis revealed that the 21 kDa CaBP is blocked at the *N*-terminus.

Fig. 3. U.v.-absorption spectrum of the 21 kDa CaBP

The 21 kDa CaBP (pool B, Fig. 2) was dialysed overnight against two changes (10 litres each) of 10 mM- NH_4HCO_3 , freeze-dried and redissolved in distilled deionized water (final concentration 0.94 mg/ml as determined by amino acid analysis). The u.v.-absorption spectrum was recorded in a Pye-Unicam PU 8800 spectrophotometer.

Ca^{2+} -binding properties of the 21 kDa CaBP

The Ca^{2+} -binding parameters of the 21 kDa CaBP were determined by equilibrium dialysis as described in the Materials and methods section. In the presence of 3.0 mM- MgCl_2 and 0.15 M-KCl, the calprotein bound 0.92 mol of Ca^{2+} /mol of protein with an apparent K_d of approx. 1 μM .

The 21 kDa Ca^{2+} -binding protein is not cofilin

A 21000- M_r protein named cofilin has been isolated from pig brain (Maekawa *et al.*, 1984; Nishida *et al.*, 1984). This protein interacts with both G-actin and F-actin with 1:1 stoichiometry and causes shortening and slight thickening of actin filaments (Nishida *et al.*, 1984). Although cofilin was not described as a Ca^{2+} -binding protein, we considered the possibility that the 21 kDa Ca^{2+} -binding protein may be identical with cofilin. We therefore examined the ability of the 21 kDa CaBP to interact with F-actin of skeletal muscle in the presence and in the absence of Ca^{2+} by a simple centrifugation technique. F-actin was incubated alone or with the 21 kDa CaBP in the presence and in the absence of Ca^{2+} before ultracentrifugation to sediment the actin filaments and bound protein, leaving unbound protein in the

supernatant. The protein contents of both the pellets and the supernatants were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown). This approach clearly demonstrated that the 21 kDa CaBP does not interact with F-actin either in the presence or in the absence of Ca^{2+} , and therefore it is a distinct protein from cofilin.

Amino acid compositions

Table 1 compares the amino acid compositions of the 17 kDa and 18.4 kDa proteins with those of several other known Ca^{2+} -binding proteins, including the 21 kDa CaBP. The data clearly show that the 17 kDa and 18.4 kDa CaBPs are distinct and novel Ca^{2+} -binding proteins, although they share certain characteristics with the members of the calmodulin superfamily of Ca^{2+} -binding proteins. For example, both proteins possess a high content of acidic amino acids: the 17 kDa CaBP contains approx. 23% aspartic acid/asparagine + glutamic acid/glutamine residues and the 18.4 kDa CaBP approx. 28% aspartic acid/asparagine + glutamic acid/glutamine residues. Of particular importance is the fact that the 17 kDa and 18.4 kDa CaBPs are clearly distinguishable from a novel 18 kDa brain CaBP (CBP-18) described by Manalan & Klee (1984).

Ca^{2+} -dependent electrophoretic-mobility shifts and $^{45}\text{Ca}^{2+}$ binding to calmodulin and 21 kDa, 18.4 kDa and 17 kDa CaBPs

It is well known that calmodulin and some other Ca^{2+} -binding proteins, including the 21 kDa CaBP, exhibit a Ca^{2+} -dependent mobility shift in sodium dodecyl sulphate/polyacrylamide gels (Walsh *et al.*, 1984). Fig. 4(a) illustrates that the 18.4 kDa CaBP (lanes 5 and 6) exhibits a Ca^{2+} -dependent mobility shift, in common with calmodulin (lanes 1 and 2) and the 21 kDa CaBP (lanes 3 and 4). In each case the Ca^{2+} -bound species migrated more rapidly than the Ca^{2+} -free form. On the basis of the mobilities of M_r -marker proteins electrophoresed simultaneously, the 18.4 kDa CaBP was determined to have M_r 18000 in the presence of Ca^{2+} and M_r 18400 in the absence of Ca^{2+} . In contrast, the 17 kDa CaBP (lanes 7 and 8 in Fig. 4a) did not exhibit a clear Ca^{2+} -dependent mobility shift. The same proteins separated in an identical gel were subjected to transblotting and ^{45}Ca autoradiography as described in the Materials and methods section (Fig. 4b). Binding of $^{45}\text{Ca}^{2+}$ directly to calmodulin (lanes 1 and 2), the 21 kDa CaBP (lanes 3 and 4) and the 18.4 kDa CaBP (lanes 5 and 6) was observed. However, no $^{45}\text{Ca}^{2+}$ binding to the 17 kDa CaBP was detected (lanes 7 and 8). These observations suggest that only high-affinity Ca^{2+} -binding proteins may be detected by Ca^{2+} -dependent electrophoretic-mobility shifts and the transblotting/ ^{45}Ca -autoradiographic technique. The 21 kDa and 18.4 kDa CaBPs therefore appear to be high-affinity Ca^{2+} -binding proteins, whereas the 17 kDa CaBP appears to be a lower-affinity Ca^{2+} -binding protein. Indeed, high-affinity Ca^{2+} binding to the 21 kDa CaBP has been proven directly by equilibrium dialysis (see above). Amido Black staining of the nitrocellulose membrane shown in Fig. 4(b) revealed that all proteins were efficiently transblotted (results not shown).

Functional studies of novel brain Ca^{2+} -binding proteins

The 17 kDa, 18.4 kDa and 21 kDa CaBPs were tested as possible Ca^{2+} -dependent activators of two calmodulin-

Table 1. Comparison of amino acid compositions of Ca²⁺-binding proteins

Key to references: ^aWatterson *et al.* (1980); ^bCollins *et al.* (1977); ^cWalsh *et al.* (1984); ^dpresent study; ^eManalan & Klee (1984); ^fWaisman *et al.* (1983); ^gAitken *et al.* (1984); ^hIsobe & Okuyama (1981); ⁱIsobe & Okuyama (1978); ^jBerchtold *et al.* (1982). Abbreviation: N.D., not determined. + indicates that tryptophan was detected spectrophotometrically.

Amino acid	Composition (mol of residue/mol)										
	Calmodulin ^a	Troponin C ^b	21 kDa CaBP ^c	18.4 kDa CaBP ^c	17 kDa CaBP ^d	12 kDa CaBP ^d	16 kDa CaBP ^d	Calceineurin B ^e	S-100α ^h	S-100β ^f	Parvalbumin ^j
Lys	8*	9	16	12	13	12	16	15	9	8	16
His	1	1	3	5	4	2	4	2	2	5	2
Arg	6	7	9	7	5	7	6	6	0	1	1
Asp	23	22	21	20	16	22	22	28	13	9	15
Thr	12	6	8	8	6	8	10	3	4	3	5
Ser	4	7	17	12	10	13	18	11	5	5	11
Glu	27	31	27	28	21	21	30	22	15	19	10
Pro	2	1	6	6	8	4	11	3	0	0	0
Gly	11	13	19	17	21	12	19	14	6	4	9
Ala	11	13	12	10	14	8	13	5	8	5	11
Cys	0	1	3	2	2	N.D.	4	0	1	2	0
Val	7	7	8	9	9	7	5	13	8	7	5
Met	9	10	5	3	2	6	4	6	2	3	2
Ile	8	10	8	7	9	8	6	11	1	4	6
Leu	9	9	13	15	12	14	22	14	11	8	10
Tyr	2	2	5	2	4	5	6	3	2	1	0
Phe	8	10	11	7	4	10	16	12	5	7	9
Trp	0	0	2	0	1	+	1	0	1	0	0

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

Fig. 4. Ca^{2+} -dependent electrophoretic mobility shifts and $^{45}\text{Ca}^{2+}$ binding to calmodulin and 21 kDa, 18.4 kDa and 17 kDa Ca^{2+} -binding proteins

Purified Ca^{2+} -binding proteins were subjected to 0.1%-sodium dodecyl sulphate/15–20%-polyacrylamide-gradient slab-gel electrophoresis and either: stained with Coomassie Blue (a) or transblotted on to a nitrocellulose membrane and subjected to ^{45}Ca autoradiography as described in the Materials and methods section (b). Key to lanes: 1, calmodulin (7.5 μg) + 10 mM- CaCl_2 ; 2, calmodulin (7.5 μg) + 10 mM-EGTA; 3, 21 kDa CaBP (4 μg) + 10 mM- CaCl_2 ; 4, 21 kDa CaBP (4 μg) + 10 mM-EGTA; 5, 18.4 kDa CaBP (2 μg) + 10 mM- CaCl_2 ; 6, 18.4 kDa CaBP (2 μg) + 10 mM-EGTA; 7, 17 kDa CaBP (5 μg) + 10 mM- CaCl_2 ; 8, 17 kDa CaBP (5 μg) + 10 mM-EGTA.

dependent enzymes: chicken gizzard myosin light-chain kinase and bovine brain cyclic nucleotide phosphodiesterase. None of the three CaBPs could substitute for calmodulin in activating myosin light-chain kinase (results not shown). A low basal myosin light-chain kinase activity was observed in the absence of added calmodulin or other Ca^{2+} -binding proteins, and can be attributed to a trace of contaminating calmodulin normally found in myosin preparations. The fact that this basal activity was not inhibited by any of the three novel Ca^{2+} -binding proteins indicates that they do not compete with calmodulin for binding to the myosin light-chain kinase.

Similarly, none of the three CaBPs could substitute for calmodulin in the activation of bovine brain cyclic nucleotide phosphodiesterase. In each case the activity seen in the presence of Ca^{2+} and any of these three Ca^{2+} -binding proteins was indistinguishable from the basal activity observed in the absence of Ca^{2+} .

Finally, in the light of observations (Albert *et al.*, 1984) that calmodulin and other Ca^{2+} -binding proteins (S-100 and troponin C) can inhibit the activity of the Ca^{2+} -phospholipid-dependent protein kinase (protein kinase C), we investigated the effects of the 18.4 kDa, 17 kDa and 21 kDa CaBPs on protein kinase C of bovine brain (Fig. 5). In agreement with the previous observations by Albert *et al.* (1984), calmodulin caused a partial inhibition of protein kinase C: specifically a 23% decrease in the initial rate of histone phosphorylation. Neither the 18.4 kDa CaBP nor the 21 kDa CaBP had any significant inhibitory effect. However, the 17 kDa CaBP proved to be a potent inhibitor of protein kinase C (Fig. 5).

DISCUSSION

Ca^{2+} -dependent hydrophobic-interaction chromatography has proved to be a very useful tool in the purification of several brain Ca^{2+} -binding proteins, including calmodulin (Gopalakrishna & Anderson, 1982), calelectrins (Südhof *et al.*, 1984), the Ca^{2+} -phospholipid-dependent protein kinase (protein kinase C) and a novel 21 kDa Ca^{2+} -binding protein (Walsh *et al.*, 1984). Applying this technique to the large-scale purification of the novel 21 kDa CaBP, we have identified for the first time a further three Ca^{2+} -binding proteins, of M_r 17000, 18400 and 26000. These proteins are distinct from Ca^{2+} -binding proteins previously described on the basis of amino acid compositions and other properties. Furthermore, the novel calcioproteins of M_r 17000 and 18400 do not appear to be derived from the 21 kDa CaBP by proteolysis during isolation, for the following reasons. Firstly, the 18.4 kDa CaBP has higher contents of histidine, glutamic acid, leucine and valine than the 21 kDa CaBP. Similarly, the 17 kDa CaBP has higher contents of histidine, proline, glycine, alanine, valine, isoleucine and tyrosine than the 21 kDa CaBP. The 17 kDa CaBP has higher contents of histidine, proline, glycine, alanine and isoleucine than the 18.4 kDa CaBP. Secondly, it is very unlikely that limited proteolysis of the 21 kDa or 18.4 kDa CaBP would yield a peptide fragment now exhibiting strong inhibition of protein kinase C activity, as does the 17 kDa CaBP. Thirdly, semi-quantitative comparison of the data in Fig. 4 suggests that the 18.4 kDa CaBP either binds more Ca^{2+} than the 21 kDa CaBP or binds Ca^{2+} more tightly, both

Fig. 5. Effects of 21 kDa, 18.4 kDa and 17 kDa Ca^{2+} -binding proteins on the activity of protein kinase C

Bovine brain protein kinase C (80 $\mu\text{l}/\text{ml}$ of reaction mixture), prepared as previously described (Walsh *et al.*, 1984), was incubated at 30 °C with histone III-S (0.2 mg/ml) in 20 mM-Tris/HCl (pH 7.5)/5 mM- MgCl_2 /phosphatidylserine (40 $\mu\text{g}/\text{ml}$)/1,3-diolein (0.8 $\mu\text{g}/\text{ml}$)/0.5 mM- CaCl_2 in the absence of added Ca^{2+} -binding protein (○) or in the presence, at 50 $\mu\text{g}/\text{ml}$, of 21 kDa (△), 18.4 kDa (□) or 17 kDa (●) Ca^{2+} -binding proteins. Reactions were initiated by addition of [γ - ^{32}P]ATP (approx. 50000 c.p.m./nmol) to a final concentration of 10 μM . The reaction volume was 1.5 ml. Samples (0.2 ml) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [^{32}P]phosphate as described by Walsh *et al.* (1983).

of which appear inconsistent with the former being derived from the latter by proteolysis.

Hydroxyapatite-column chromatography, as applied previously in the small-scale purification of the 21 kDa CaBP (Walsh *et al.*, 1984), was found to be an inadequate final step in the large-scale preparation of this calcuprotein. Further purification was achieved therefore by ion-exchange chromatography on a column of DEAE-Sephacel. This resulted in the purification of 18.4 kDa and 17 kDa CaBPs in addition to the 21 kDa CaBP.

The data presented in this paper indicate that the 21 kDa CaBP originally reported by Walsh *et al.* (1984) is indeed a high-affinity Ca^{2+} -binding protein, as demonstrated by equilibrium dialysis and the transblotting/ ^{45}Ca -autoradiographic technique. This calcuprotein exhibits a u.v.-absorption spectrum that has characteristics in common with Ca^{2+} -binding proteins of the calmodulin superfamily. The Ca^{2+} -binding properties of this protein (1 mol of Ca^{2+} bound/mol of protein with apparent K_d approx. 1 μM in the presence of physiological [Mg^{2+}] and ionic strength) suggest a possible physiological

role in the Ca^{2+} -mediated regulation of cellular function in the brain. The 21 kDa CaBP does not activate either myosin light-chain kinase or cyclic nucleotide phosphodiesterase, both calmodulin-dependent enzymes, nor does it influence the activity of brain protein kinase C. In the light of the mechanisms of action of calmodulin and other related Ca^{2+} -binding proteins, it is reasonable to suggest that the 21 kDa CaBP regulates an enzymic activity in a Ca^{2+} -dependent manner by forming a complex with its target enzyme only in the presence of Ca^{2+} . The 21 kDa-CaBP-target-enzyme complex could then represent either the activated or the inactivated state of the enzyme.

Transblotting/ ^{45}Ca autoradiography and Ca^{2+} -dependent electrophoretic-mobility-shift experiments suggest that the novel 18.4 kDa CaBP is a high-affinity Ca^{2+} -binding protein, whereas the 17 kDa CaBP is one of low affinity. Confirmation of this conclusion will require direct quantification of Ca^{2+} -binding by, e.g., equilibrium dialysis. Like the 21 kDa CaBP, neither of these novel calcuproteins had any effect on the activity of the calmodulin-activated enzymes, myosin light-chain kinase or cyclic nucleotide phosphodiesterase. Similarly, the 18.4 kDa CaBP did not affect protein kinase C activity. However, the 17 kDa CaBP was found to be a potent inhibitor of protein kinase C. Indeed, this Ca^{2+} -binding protein was a far more potent inhibitor of protein kinase C than are calmodulin, troponin C or S-100 protein, which have been found to be capable of inhibiting protein kinase C (Albert *et al.*, 1984; McDonald & Walsh, 1985). This observation suggests a possible physiological role for the 17 kDa CaBP in the regulation of protein kinase C activity. Appropriate extracellular signals triggering membrane-bound polyphosphoinositide hydrolysis lead to the generation of diacylglycerol and inositol 1,4,5-trisphosphate intracellularly. Diacylglycerol can activate protein kinase C directly at low concentrations of free Ca^{2+} , and inositol 1,4,5-trisphosphate serves to mobilize intracellular Ca^{2+} , which also favours protein kinase C activation. As is the case in analogous systems, e.g. hormonal (catecholamine) regulation of cyclic AMP-dependent protein kinase, the appearance of second messengers and activation of their target enzymes (often protein kinases) is a transient phenomenon. Effective mechanisms must exist within the cell for removal of the second messenger and/or inactivation of its target enzyme(s). If the 17 kDa CaBP is indeed a relatively low-affinity Ca^{2+} -binding protein, it may become saturated with Ca^{2+} only after a relatively large increase in cytosolic [Ca^{2+}]. In such a state it may now inhibit the protein kinase C, which would have been activated earlier by diacylglycerol and lower concentrations of Ca^{2+} . Such a regulatory mechanism would permit inactivation of protein kinase C without removal of the second messengers responsible for its activation. In light of the importance of protein kinase C in cellular regulation (Nishizuka, 1984; Berridge, 1984; Berridge & Irvine, 1984), it would not be surprising if its activity were regulated in diverse ways.

Schwantke & LePeuch (1984) have described the identification and partial purification from rat brain of a protein of native M_r approx. 20000 that acts as an inhibitor of protein kinase C. It is not known, however, whether or not this inhibitor is a Ca^{2+} -binding protein, so that its relationship to the 17 kDa CaBP described in the present paper is unclear.

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