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Calmodulin-Binding Proteins from Brain and Other Tissues

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1. The calmodulin contents of rabbit brain, lung, kidney and liver, of bovine aorta and uterus, and of chicken gizzard have been determined. 2. The calmodulin in all of these tissues has been shown to be present in the form of very stable complexes with several other proteins. 3. A calmodulin-binding protein of mol.wt. 22000 has been purified in high yield from bovine brain. It has been shown to interact with calmodulin and rabbit skeletal-muscle troponin C in a Ca^{2+}-dependent manner. 4. The 22000-mol.wt. protein inhibits the activation of bovine brain phosphodiesterase by calmodulin, but has very little affect on the activation of myosin light-chain kinase. 5. Calmodulin-binding proteins of mol.wts. 140000, 77000 and 61000 have also been partially purified from rabbit brain by affinity chromatography and have been shown to interact in a Ca^{2+}-dependent manner with calmodulin. 6. The apparent molecular weights of the calmodulin–calmodulin-binding protein complexes, determined by gel filtration in the presence of 6M-urea, have been shown to be similar for most of the mammalian tissues examined. 7. By using ^{125}I-labelled calmodulin, similar complexes have been demonstrated in rabbit skeletal muscle, although they are present at much lower concentrations.

It is now well established that mammalian brain calmodulin (also known as modulator protein, troponin C-like protein, Ca^{2+}-dependent regulator and cyclic phosphodiesterase-activating protein) increases the activity of phosphodiesterase (Cheung, 1970; Kakiuchi & Yamazaki, 1970) and adenyl cylase (Brostrom *et al.*, 1975) in the presence of Ca^{2+}. Recently a similar protein has been shown to be essential for the activity of the myosin light-chain kinase of skeletal muscle (Yagi *et al.*, 1978; Barylko *et al.*, 1978; Nairn & Perry, 1979) and smooth muscle (Dabrowska *et al.*, 1978). Calmodulin-type proteins have been shown to be present in all mammalian tissues so far examined (Cheung *et al.*, 1975), in birds and reptiles (Vanaman *et al.*, 1976), as well as in invertebrates, such as the starfish and sea anemone (Waisman *et al.*, 1975) and the earth worm (Waisman *et al.*, 1978). Calmodulins isolated from all these sources are remarkably similar in physical, chemical and biological properties. These facts and the very minor differences that have been reported in the amino acid sequences of calmodulin from bovine brain (Vanaman *et al.*, 1977), bovine uterus (Grand & Perry, 1978) and rat testis (Dedman *et al.*, 1978) suggest that this protein may be one of the most highly conserved proteins so far isolated.

Head *et al.* (1977), by using polyacrylamide-gel electrophoresis, showed that the troponin C-like protein in smooth muscle was present in the form of a complex that was not dissociated in 6M-urea, provided a trace of Ca^{2+} was present. The complex was dissociated when the Ca^{2+} concentration was decreased with EGTA. More recently, Grand *et al.* (1979) have confirmed that in extracts of a number of tissues, calmodulin was present as part of a very stable complex that was only dissociated when Ca^{2+} ions were removed from the system.

It is likely that a number of different proteins bind to calmodulin *in vivo*. These probably include those enzymes that have been shown to require calmodulin for full activity, namely phosphodiesterase (Cheung, 1970), adenyl cyclase (Brostrom *et al.*, 1975), myosin light-chain kinase (Yagi *et al.*, 1978; Barylko *et al.*, 1978; Nairn & Perry, 1979), phosphorylase kinase (Cohen *et al.*, 1978) and the membrane-bound ($Ca^{2+}+Mg^{2+}$)-dependent ATPase (Gopinath & Vincenzi, 1977). Wang & Desai (1977) and Klee & Krinks (1978) have purified proteins that bind to bovine brain calmodulin, but were unable to demonstrate that they possessed any detectable enzyme activity. None of the calmodulin-binding proteins so far described have been shown to be present in concentrations high enough to complex the large amounts of calmodulin present in many tissues. Further, of the proteins listed, only in the case of phosphorylase kinase (A. C. Nairn & R. J. A. Grand, unpublished work) is there evidence of the formation of a Ca^{2+}-dependent complex with calmodulin in the presence of high urea concentrations.

In the present paper we describe a method for the purification of a calmodulin-binding protein from bovine brain in concentrations of about 1g/kg of tissue. This protein has been shown to inhibit the activation of phosphodiesterase by calmodulin, and to interact with calmodulin and troponin C in a Ca^{2+}-dependent manner in the presence of 6M-urea. A

number of other proteins that also bind calmodulin have been shown to be present in all vertebrate tissues examined. Three of these proteins have been partially purified by affinity chromatography.

Materials and Methods

Materials

The proteins, other than those of myofibrillar origin, used as molecular-weight standards were supplied by Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K., as was the 5'-nucleotidase from *Crotalus atrox* venom (grade V). The 3-(4-hydroxy-5-[^{125}I]iodophenyl)-*N*-succinimidyl propionate was purchased from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.

Tissues

The brains, lungs, livers, kidneys, hearts and skeletal muscles of New Zealand White Rabbits and the gizzards of chickens were removed immediately after death. Bovine brain, uterus and aorta were obtained from a local slaughterhouse and chilled in ice immediately after removal. All tissues were either homogenized in 9M-urea/75mM-Tris adjusted to pH8.0 with 1M-HCl/1mM-$CaCl_2$/15mM-β-mercaptoethanol (buffer A) as soon as was possible or stored at -20°C until used.

Preparation of tissue homogenates for electrophoresis and gel filtration

Tissue (5g) was homogenized in approx. 20ml of buffer A, centrifuged for 30min at 76000*g* and the supernatant filtered through glass wool. These filtrates were used directly for gel filtration on Sepharose 6B and for polyacrylamide-gel electrophoresis.

Determination of the amounts of calmodulin in whole-tissue homogenates

Portions (50μl) of the whole tissue homogenates prepared as described above, except that the 1mM-$CaCl_2$ in buffer A was replaced with 5mM-EGTA, were run on polyacrylamide gels in 6M-urea/14mM-Tris/90mM-glycine, pH8.3. The gels were stained for 2h with Coomassie Blue dye (0.5%) and then destained for 3 days. The section of the gel (about 20mm×3mm) containing calmodulin, which was identified as the fastest migrating protein band, was cut out and homogenized in 2ml of 25% (v/v) pyridine. The homogenate was left overnight, transferred to a graduated 10ml centrifuge tube and then centrifuged at 700*g* for 5min. The absorbance of the supernatant was read at 600nm, and the amount of protein determined from a standard curve. The standard curve was constructed from the bands obtained when known amounts of bovine brain calmodulin, ranging from 1 to 30μg, were applied to gels and run under the same conditions. The protein concentration of a stock solution of bovine brain calmodulin used for the standard curve was determined by amino acid analysis.

Protein concentrations of the whole-tissue homogenates were determined by the method of Mejbaum-Katzenellenbogen & Dobryszycka (1959).

Survey of tissues for calmodulin-binding proteins

Samples of tissue homogenates (6ml) were applied to a column (3cm×120cm) of Sepharose 6B equilibrated with 6.5M-urea/50mM-Tris, adjusted to pH8.0 with 1M-HCl/1mM-$CaCl_2$/10mM-β-mercaptoethanol (buffer B) and eluted with the same buffer. Portions (100μl) from alternate 10ml fractions were run on polyacrylamide gels at pH8.3 in the presence of 1mM-Ca^{2+} or 5mM-EGTA. Calmodulin–calmodulin-binding protein complexes were detected in the fractions by the appearance of the fast-moving band of calmodulin on the gels when run in the presence of EGTA. On the gels run in the presence of Ca^{2+} a small amount of calmodulin could be detected in those fractions collected corresponding to the elution position of free calmodulin (i.e. a mol.wt. of about 17000).

The fractions of higher molecular weight containing the highest concentration of calmodulin, as detected by electrophoresis in the presence of EGTA, were taken to represent the peaks of elution of calmodulin–calmodulin-binding protein complexes. The molecular weights of the complexes were estimated by calibration of the column with haemoglobin (15100), bovine brain calmodulin (16700), rabbit fast-skeletal-muscle troponin I (20900), a complex of rabbit fast-skeletal-muscle troponin I and troponin C (total mol.wt. 38800), rabbit skeletal-muscle actin (41700), ovalbumin (43000), glutamate dehydrogenase (55400), bovine serum albumin (66300), transferrin (77000) and rabbit skeletal-muscle phosphorylase (92000). All standards were carbamoylmethylated with iodo[^{14}C]acetamide. The standard proteins were chromatographed two at a time with Dextran Blue to mark the excluded volume of the column and dinitrophenylglycine to mark the included volume. A calibration curve was constructed by plotting K_d against the logarithm of molecular weight (Mann & Fish, 1972).

Preparation of 140000-, 77000- *and* 61000-*mol.wt. calmodulin-binding proteins from brain*

A homogenate of rabbit brain (6ml), prepared as described above and to which ^{125}I-labelled calmodulin had been added (see below), was applied

to a column of Sepharose 6B equilibrated with buffer B and eluted with the same buffer as described above. Two peaks of radioactivity were detected, peaks I and II (Fig. 1), corresponding to the peaks of calmodulin–calmodulin-binding protein complexes detected by polyacrylamide-gel electrophoresis. Fractions were combined as indicated, concentrated with Aquacide (Bio-Rad Laboratories, Watford, Herts., U.K.), dialysed against buffer B and applied to separate columns (2cm×100cm) of Sephadex G-100 equilibrated with buffer B containing 10mM-EGTA and eluted with the same buffer.

When peak II was chromatographed under these conditions, the calmodulin was separated from the bulk of the protein that was due to the dissociation of the calmodulin–calmodulin-binding protein complex by the EGTA present (Fig. 2*b*). With peak I, however, the calmodulin was only partially resolved from the other proteins presumably because the complex was not completely dissociated under these conditions (Fig. 2*a*). This was evident because the distribution of radioactivity that was due to the presence of calmodulin in the eluate was not separated from the main protein peak, as was the case with the peak II material. The fractions obtained when peak I and peak II were run on Sephadex G-100 containing protein, but deficient in calmodulin, were pooled and dialysed against buffer B. This protein was applied to a Sepharose 4B–calmodulin affinity column equilibrated with the same buffer. After the column was washed until the absorbance of the effluent had

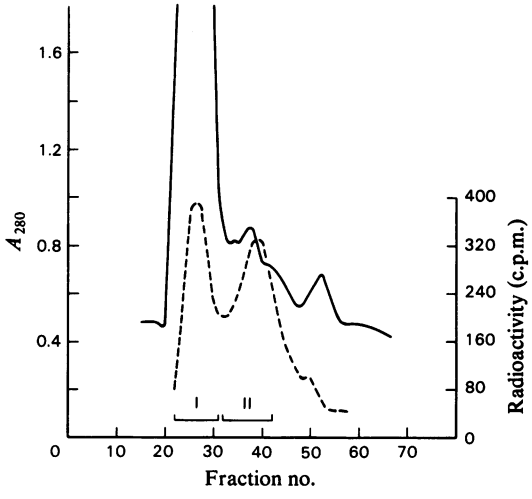

Fig. 1. *Chromatography of rabbit brain homogenate on Sepharose 6B*

Tissue homogenate (6ml), to which ^{125}I-labelled calmodulin (200μl of an approx. 5μg/ml solution) had been added, was applied to a column (3cm×120cm) of Sepharose 6B previously equilibrated and eluted with 6.5M-urea/50mM-Tris, adjusted to pH8.0 with 1M-HCl/1mM-$CaCl_2$/10mM-β-mercaptoethanol. Fractions (10ml) were collected and radioactivity determined on 200μl portions. Fractions representing peaks I and II were pooled as indicated by the bars. ——, A_{280}; ----, ^{125}I radioactivity.

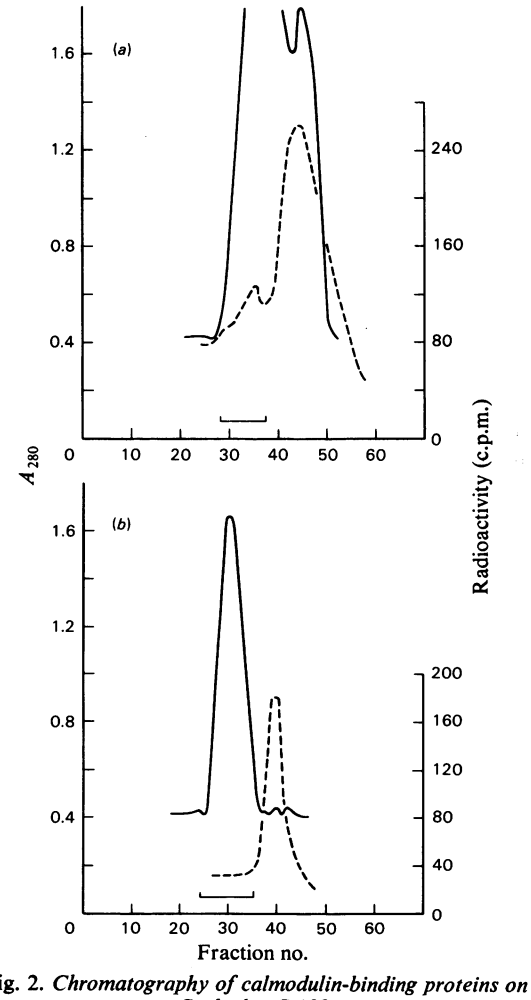

Fig. 2. *Chromatography of calmodulin-binding proteins on Sephadex G-100*

(*a*) Peak I (4ml after concentration) from Fig. 1 was applied to a column (2cm×120cm) of Sephadex G-100 equilibrated with 6.5M-urea/50mM-Tris/HCl (pH8.0)/10mM-EGTA/10mM-β-mercaptoethanol and eluted with the same buffer. Fractions (5ml) were collected and pooled as indicated. ——, A_{280}; ----, ^{125}I radioactivity. (*b*) Peak II (4ml after concentration) from Fig. 1 was applied to a column (2cm×120cm) of Sephadex G-100 equilibrated with 6.5M-urea/50mM-Tris/HCl (pH8.0)/10mM-EGTA/10mM-β-mercaptoethanol and eluted with the same buffer. Fractions (5ml) were collected and pooled as indicated. ——, A_{280}; ----, ^{125}I radioactivity.

returned to the baseline value the binding proteins were eluted with buffer B containing 10mM-EGTA.

Preparation of the 22000-mol.wt. calmodulin-binding protein from bovine brain

Bovine brain (50g) was homogenized in 500ml of buffer A, centrifuged at 11000*g* for 30min and the supernatant was filtered through glass wool. The filtrate was dialysed against buffer A and then applied to a column (3cm×12cm) of DEAE-cellulose equilibrated with the same buffer. After washing the column a gradient of NaCl (0–0.35M) was applied in the same buffer. Portions (20μl) from fractions were assayed for calmodulin-binding protein (see below).

Most of the calmodulin-binding protein passed through the DEAE-cellulose column in the void volume, although a small amount was retained and eluted with the salt gradient.

The fractions of unretarded material were combined and the pH was adjusted to 5.1 with 1M-citric acid. The solution was dialysed against 7M-urea/32.5mM-sodium citrate/25mM-citric acid (pH5.1)/1mM-$CaCl_2$/7.5mM-β-mercaptoethanol (buffer C) and centrifuged at 11000*g* for 30min. The supernatant was applied to a column (3cm×10cm) of CM-cellulose equilibrated with buffer C and the column was then eluted with an NaCl gradient (0–0.4M) in the same buffer (Fig. 3). Portions (20μl) from the fractions were assayed for calmodulin-binding proteins that were found to be present in the material eluted with the void volume and in the second peak of bound protein at a conductivity of 6–8mmho. The fractions were pooled as indicated in Fig. 3, dialysed exhaustively against 3mM-HCl, freeze-dried and shown to be a single component by polyacrylamide-gel electrophoresis (see below).

Preparation of other proteins

Rabbit skeletal-muscle troponin C and troponin I were prepared by the method of Perry & Cole (1974) from troponin isolated by the method of Ebashi *et al.* (1971). Bovine brain calmodulin was prepared either by the method of Watterson *et al.* (1976) or by the organic-solvent method of Grand *et al.* (1979).

Preparation of enzymes

Adenosine 3′:5′-cyclic monophosphate phosphodiesterase that was deficient in calmodulin was prepared from bovine brain by a slight modification of the method of Wang & Desai (1977) in that the fraction obtained after chromatography on DEAE-cellulose was subjected to gel filtration on Ultrogel A34 rather than Sephadex G-200. The partially purified enzyme was stored frozen in 10% (w/v) sucrose/50mM-Tris/40mM-HCl (pH7.6)/1mM-$CaCl_2$ until required.

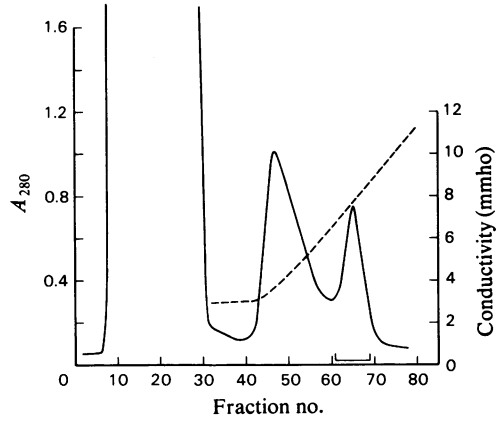

Fig. 3. *Chromatography on CM-cellulose of the partially purified bovine brain calmodulin-binding protein 22K*
The column (3cm×10cm) was equilibrated and developed with 7M-urea/32.5mM-sodium citrate/25mM-citric acid (pH5.1)/1mM-$CaCl_2$/7.5mM-β-mercaptoethanol. Bound protein was eluted with a gradient of 0–0.4M-NaCl and 10ml fractions were collected. The fractions under the bar were pooled.

Myosin light-chain kinase was prepared from rabbit skeletal muscle by the method of Pires & Perry (1977).

Preparation of calmodulin–Sepharose 4B affinity column

Bovine brain calmodulin (30mg) was coupled to 25g of wet packed Sepharose 4B by the method described by Syska *et al.* (1974) for the preparation of a troponin C–Sepharose affinity column.

Radioactive labelling of proteins

Bovine brain calmodulin was labelled with ^{125}I by the method of Bolton & Hunter (1973). The Bolton & Hunter reagent [50μl containing 80μCi of 3-(4-hydroxy-5-[^{125}I]iodophenyl)-*N*-succinimidyl propionate] was evaporated to dryness in a small test tube with a stream of N_2. Calmodulin (20μl of a solution containing 1mg/ml of 0.1M-sodium borate, pH8.5) was added and the solution was left at 0°C for 30min. The protein was diluted with 0.5ml of 0.1M-sodium borate buffer, pH8.5, and desalted on a column (1cm×10cm) of Sephadex G-50 eluted with the same buffer. The fraction containing the iodinated protein was stored frozen until required.

The 22000-mol.wt. calmodulin-binding protein and proteins used as standards for determining molecular weights were radioactively labelled at their cysteine residues with iodo[^{14}C]acetamide by the method of Wilkinson *et al.* (1972).

Detection of calmodulin-binding proteins in complexes with calmodulin

^{125}I-labelled calmodulin (5μl containing approx. 0.05μg of calmodulin and 30000d.p.m.) was added to portions (20μl) taken from the fractions obtained by the chromatographic separations described above for the preparation of the 22000-mol.wt. calmodulin-binding protein. This mixture was subjected to electrophoresis on 8% polyacrylamide gels in the presence of 6M-urea and 5mM-$CaCl_2$. After electrophoresis the gels were stained briefly, sealed in polythene bags and left in contact with Kodak Blue Brand X-ray plates for 16h.

In those fractions that contained calmodulin-binding protein no fast-moving radioactive band was visible on the X-ray plate, whereas in the fractions containing no calmodulin-binding protein a fast-moving radioactive band that was due to ^{125}I-labelled calmodulin was visible. Certain of the calmodulin-binding protein–^{125}I-labelled calmodulin complexes remained at the origin on the polyacrylamide gels, whereas others migrated into the buffer tank toward the cathode.

Phosphodiesterase assay

Bovine brain phosphodiesterase activity was assayed by a modification of the procedure of Teo & Wang (1973) as described by Watterson *et al.* (1976). Assays were carried out in a total volume of 0.5ml and contained 10μl of a solution of phosphodiesterase in 40mM-Tris (adjusted to pH8.0 with 1M-HCl)/1mM-$CaCl_2$/0.5mM-$MnCl_2$/2mM-cyclic AMP and containing 400ng of bovine brain calmodulin. After incubation for 5min at 30°C the reaction was stopped by boiling for 2min. *Crotalus atrox* venom 5′-nucleotidase (grade V, 10μl of a 0.25mg/ml solution) was added and after incubation for 10min at 30°C P_i was determined by the method of Sanui (1974).

Enough calmodulin (400ng) was added to give almost maximal activation of the phosphodiesterase. Increasing amounts of calmodulin-binding protein or rabbit skeletal-muscle troponin I were added to the assays.

Myosin light-chain kinase assays

Rabbit skeletal-muscle myosin light-chain kinase assays were performed as described by Nairn & Perry (1979).

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out on 8% polyacrylamide gels in 90mM-glycine/14mM-Tris (pH8.3), either in the presence or in the absence of 6M-urea. It was also conducted in 7.5% or 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate/0.1M-Tris/0.1M-Bicine [*NN*-bis-(2-hydroxyethyl)glycine] (pH8.3).

Analytical methods

For amino acid analysis duplicate samples were hydrolysed for 24h and 72h by the method of Wilkinson *et al.* (1972).

Tryptophan was determined spectrophotometrically by the method of Crumpton & Wilkinson (1963). Cysteine was determined as carboxymethylcysteine by amino acid analysis.

The *N*-terminal amino acid of calmodulin-binding protein was determined by the method of Wilkinson (1974).

Results

Calmodulin and troponin C content of tissues

When extracts of whole tissues in high urea concentrations were electrophoresed at pH8.3 in the presence of EGTA, calmodulin and troponin C migrated as a clear band free of all other components. This enabled the total amounts of these proteins to be rapidly determined by cutting out the band and measuring the amount of dye bound to it (see the Materials and Methods section). This method gave values ranging from about 150mg of calmodulin/kg for aorta to over 1g/kg in the case of skeletal muscle, in which tissue the protein is mainly troponin C (Table 1).

Table 1. *Total amounts of calmodulin and calmodulin-like proteins in tissue*

Values marked with an asterisk represent mainly troponin C with a small amount of calmodulin. The other values probably represent calmodulin only. Results are averages of four determinations in each case.

		Calmodulin and troponin C content	
Animal	Tissue	(mg/kg)	(% of total protein)
Rabbit	Brain	682	0.62
Rabbit	Lung	293	0.21
Rabbit	Fast skeletal muscle	1013*	0.65
Rabbit	Kidney	326	0.24
Rabbit	Liver	220	0.12
Cow	Heart	398*	0.25
Cow	Aorta	151	0.16
Cow	Uterus	241	0.24
Chicken	Gizzard	408	0.27

Gel filtration of tissue homogenates containing calmodulin-binding proteins

On gel filtration of homogenates of rabbit brain, two peaks of calmodulin–calmodulin-binding protein complex corresponding to mol.wts. of about 140000 (peak I) and 60000 (peak II) respectively (Fig. 1) were obtained. These peaks were obtained if they were identified by the presence of calmodulin detected by the electrophoretic method (see the Materials and Methods section) or by the presence of radioactivity after the addition of ^{125}I-labelled calmodulin to the homogenate before gel filtration. The distribution of calmodulin was similar in the elution profiles obtained from all the mammalian tissues examined (Table 2).

If the homogenates of tissues other than striated muscle were stored at 4°C for 24 h before gel filtration, the amount of the complex of higher molecular weight, as judged by the amount of calmodulin present, decreased. After storage for 3 days none of the higher-molecular-weight complex could be detected, but there was no clear evidence of an equivalent increase in peak II material. In the chicken gizzard only the higher-molecular-weight complex was present and no lower-molecular-weight complex could be detected even after storage for several days.

Table 2. *Molecular weights of calmodulin-binding protein complexes in various tissues*

Methods of determining the molecular weights of the complexes are described in the text (see Fig. 1). Values given were obtained from single determinations, but in all cases essentially similar results were obtained from duplicate experiments.

		Molecular weights of complexes	
		Electrophoretic	Radioactive
Animal	Tissue	method	method
Rabbit	Brain	140000	140000
		59000	57000
Rabbit	Liver	120000	120000
		53000	53000
Rabbit	Kidney	130000	150000
		58000	50000
Rabbit	Lung	140000	
		59000	
Rabbit	Skeletal muscle	35000*	150000
			61000
			37000*
Rabbit	Cardiac muscle	35000*	
Cow	Uterus	140000	
		63000	
Cow	Aorta	130000	
		60000	
Chicken	Gizzard	130000	

* Complexes of troponin I.

It seems unlikely that the complexes of lower molecular weight were due to a breakdown of the higher-molecular-weight material, since no difference in the elution profile could be seen when 1 mM-phenylmethane sulphonyl fluoride, 1 mM-NaN_3, 0.1 mM-chloromethyl L-1-tosylamido-2-phenylethyl ketone and 0.1 mM-chloromethyl *N*-α-*p*-tosyl-L-lysyl ketone hydrochloride were included in all buffers used. All extraction buffers, however, contained 9 M-urea, which alone would be expected to prevent hydrolysis by endogenous proteinases.

When ^{125}I-labelled calmodulin was added to the homogenate to detect the complexes in skeletal muscle three peaks of radioactivity were detected, two of higher molecular weights similar to those present in the brain and liver homogenates. The peak of lowest molecular weight, that contained most of the radioactivity corresponded to the only complex that could be identified by the electrophoretic method in the fractions obtained by gel filtration. This complex contained troponin I and troponin C and was eluted in the same position as a sample of the troponin I–troponin C complex prepared from the proteins isolated from rabbit fast skeletal muscle. No calmodulin from the higher-molecular-weight complexes could be seen on gels, as was the case with the other mammalian tissues, presumably due to the low concentrations present.

Isolation of 140000-, 77000- *and* 61000-*mol.wt. calmodulin-binding proteins by affinity chromatography*

The gel-filtration–affinity-chromatographic procedure (see the Materials and Methods section) offered a relatively simple method for the preparation of small amounts of calmodulin-binding protein. When the peak II protein was applied to the Sepharose–calmodulin column the EGTA buffer mainly eluted material that migrated as two bands of equal intensity, of mol.wts. 77000 and 61000 (Fig. 4), designated calmodulin-binding proteins 77K and 61K respectively. In addition a minor component of mol.wt. approx. 140000 was also eluted from the affinity column when peak II material was applied. The protein eluted when peak I material was applied to the affinity column migrated on sodium dodecyl sulphate/polyacrylamide-electrophoresis mainly as a single band of mol.wt. 140000, designated calmodulin-binding protein 140K. A considerable proportion of the calmodulin-binding protein present in peak I remained at the origin of the gel (Fig. 4*a*). It was not possible to decide whether this material was aggregated calmodulin-binding protein 140K or another unidentified protein. A variety of procedures were used in attempts to make this material migrate. For example, the polyacrylamide concentration was lowered to 5% and samples were incubated with concentrations of sodium dodecyl sulphate of up to

10%. β-Mercaptoethanol and dithiothreitol were added in the presence and absence of 6M-urea, but in all cases the band pattern obtained remained essentially unchanged.

Preparation of the 22000-mol.wt. calmodulin-binding protein

In addition to calmodulin-binding proteins 140K, 77K and 61K a calmodulin complex with a protein of mol.wt. 22000 was found to be present in brain homogenates, but the complex of this protein with calmodulin was not eluted from Sepharose (see below). For this reason an independent method was developed for its preparation (see the Materials and Methods section). On DEAE-cellulose chromatography most of the calmodulin-binding proteins were either unretarded in buffer A or eluted if the conductivity was raised to approx. 4mmho. As is the case with troponin I–troponin C complexes on fractionation on DEAE-cellulose (Perry & Cole, 1974), the complexes of calmodulin-binding protein–calmodulin were also dissociated under these conditions and calmodulin was eluted at about 7–8mmho. The subsequent chromatography on CM-cellulose separated the calmodulin-binding proteins of higher molecular weights from that of mol.wt. 22000, which was eluted reasonably pure at 6.5–8.0mmho (Fig. 3). It is probable that the larger calmodulin-binding proteins pass through the CM-cellulose in the void volume.

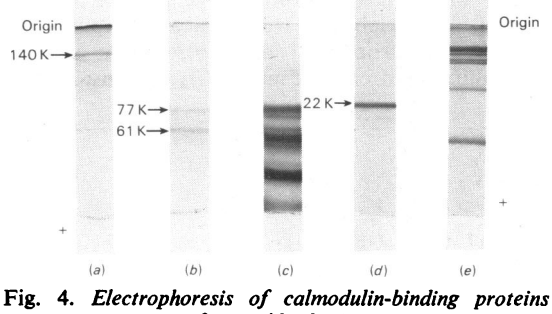

Fig. 4. *Electrophoresis of calmodulin-binding proteins from rabbit brain*

Electrophoresis carried out on 7.5% (tracks *a–c*) or 10% (tracks *d* and *e*) polyacrylamide gels in 0.1% sodium dodecyl sulphate/0.1M-Tris/0.1M-Bicine (pH8.3). (*a*) Calmodulin-binding protein 140K; (*b*) calmodulin-binding proteins 77K and 61K; (*c*) molecular weight standards (50μg in each case) transferrin (77000), glutamate dehydrogenase (55500) and chymotrypsinogen (25700); (*d*) 50μg of bovine brain calmodulin-binding protein 22K; (*e*) the molecular weight standards (50μg in each case) transferrin (77000), glutamate dehydrogenase (55500), carbonic anhydrase (29200) and haemoglobin (15100).

Fractions containing this protein, designated calmodulin-binding protein 22K, were combined, dialysed and freeze-dried. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed the protein to be reasonably pure and of mol.wt. 22000 with only minor contaminating bands (Fig. 4). The protein also migrated to the cathode as a single band on 8% polyacrylamide gels, at pH8.3 in the presence of 6M-urea. The average yield of calmodulin-binding protein 22K from bovine brain was 110mg/100g of tissue. The amino acid composition is given in Table 3. It is assumed that the *N*-terminus is blocked as no *N*-terminal amino acid could be detected by the dansyl procedure.

Interaction studies

From the studies by using ^{125}I-labelled calmodulin in the isolation procedures, it is clear that calmodulin binds to all the calmodulin-binding proteins described in the presence of Ca^{2+} and high urea concentrations. Complex formation was studied by electrophoresis on 8% polyacrylamide gels at pH8.3 in the presence and in the absence of 6M-urea. It was judged to have occurred when little or no calmodulin or troponin C migrated at its normal mobility towards the anode. When complex formation occurred with ^{125}I-labelled calmodulin no radioactive bands were observed migrating towards the anode at pH8.3 because the complexes presumably possessed a net positive

Table 3. *Amino acid analysis of calmodulin-binding protein 22K from bovine brain*

Values are means for results obtained with two preparations each analysed in quadruplicate

	Calmodulin-binding protein 22K (mol/mol)	Troponin I* (mol/mol)
Asp	14.1	17.1
Thr	9.0	3.3
Ser	20.5	8.9
Glu	14.1	33.2
Pro	14.2	6.3
Gly	30.4	7.8
Ala	16.9	15.2
Val	5.2	7.1
Met	2.7	7.4
Ile	4.3	4.9
Leu	13.8	17.9
Tyr	5.2	2.0
Phe	8.7	2.8
His	10.5	3.6
Lys	16.1	25.8
Arg	20.0	14.3
Trp	1.2	0.9
Cys	0.9	2.7

* Rabbit fast-skeletal-muscle protein (Wilkinson, 1974).

charge at that pH and therefore migrated towards the cathode.

Bovine brain calmodulin-binding protein 22K formed complexes with calmodulin and rabbit fast-skeletal-muscle troponin C only in the presence of Ca^{2+} whether 6M-urea was present or not (Fig. 5). In the presence of EGTA the complexes, which in the presence of Ca^{2+} migrate close to the origin, were dissociated and free calmodulin (Fig. 5*b*) or troponin C (Fig. 5*f*) migrated to the anode and calmodulin-binding protein (Figs. 5*d* and 5*h*) migrated to the cathode.

The stoicheiometry of the interaction was investigated by electrophoresing a known amount of the bovine brain calmodulin-binding protein 22K with increasing amounts of calmodulin from the same source on 8% polyacrylamide gels at pH 8.3 in the presence of 1mM-$CaCl_2$ and 6M-urea. At molar ratios of calmodulin to calmodulin-binding protein up to 1:1 no free calmodulin was visible on the gels, but at ratios in excess of this value free calmodulin could be seen as a fast-moving band migrating to the anode. An analogous experiment was performed in which increasing amounts of bovine brain calmodulin-binding protein 22K were added to a known amount of calmodulin. These samples were electrophoresed on 8% gels run towards the cathode. Again calmodulin-binding protein became visible only when in molar excess over the calmodulin. It is concluded that the proteins interact in the presence of Ca^{2+} at a 1:1 molar ratio.

The 140000-mol.wt. calmodulin-binding protein and the mixture of 77000- and 61000-mol.wt. calmodulin-binding proteins from rabbit brain were mixed with ^{125}I-labelled bovine brain calmodulin and electrophoresed on 8% polyacrylamide gels at pH 8.3 in the presence of 6M-urea. Under these conditions, the radioactivity labelled calmodulin was located at the origin. In the presence of EGTA, however, the complexes of calmodulin with calmodulin-binding proteins 77K and 61K were completely dissociated and the calmodulin appeared as a fast running band in the gel. The calmodulin-binding protein 140K–calmodulin complex was partially dissociated in the presence of EGTA, some of the radioactivity remaining at the origin of the gel and some moving as free calmodulin. This property of apparent partial dissociation was probably not due to modification of the binding proteins during the isolation procedure as on electrophoresis of the whole tissue homogenate under similar conditions the higher-molecular-weight calmodulin-binding protein–calmodulin complex was not totally dissociated in the presence of EGTA as judged by the distribution of the radioactivity on the gel.

Interaction of calmodulin-binding protein 22K with Sepharose 6B

As no evidence for the presence of the calmodulin-binding protein 22K in bovine brain or other tissues was obtained from the gel-filtration–affinity-chromatographic preparative procedure the possibility of an interaction between the protein and Sepharose 6B was investigated.

Bovine brain calmodulin (2mg) and bovine brain calmodulin-binding protein 22K (2mg) that had been labelled at the cysteine residue with iodo[^{14}C]-acetic acid were dissolved in 3ml of buffer B and chromatographed on a column (3cm×120cm) of Sepharose 6B equilibrated and eluted with the same buffer. Fractions (10ml) were collected and portions (100μl) from each tube were subjected to polyacrylamide-gel electrophoresis at pH 8.3 in the presence of 6M-urea and either 5mM-$CaCl_2$ or 5mM-EGTA.

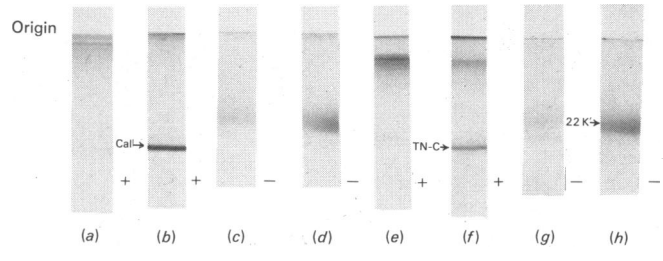

Fig. 5. *Complex formation by bovine brain calmodulin-binding protein 22K with bovine brain calmodulin and rabbit skeletal-muscle troponin C*

Electrophoresis was carried out in polyacrylamide gels in 14mM-Tris/90mM-glycine (pH 8.3)/6M-urea. Approx. 10μg of each protein was applied. 1mM-$CaCl_2$ or 5mM-EGTA was added as indicated. Gels (*a*), (*b*), (*e*) and (*f*) were run towards the anode; gels (*c*), (*d*), (*g*) and (*h*) were run towards the cathode. Abbreviations used: CBP22K, calmodulin-binding protein 22K; CAL, calmodulin; TN-C, troponin C. Samples (*a*), (*b*), (*e*) and (*f*) were also run on gels at pH 8.3 in the absence of urea (gels not shown) and similar results were obtained. (*a*) CBP22K+CAL+Ca^{2+}; (*b*) CBP22K+CAL+EGTA; (*c*) CBP22K+CAL+Ca^{2+}; (*d*) CBP22K+CAL+EGTA; (*e*) CBP22K+TN-C+Ca^{2+}; (*f*) CBP22K+TN-C+EGTA; (*g*) CBP22K+TN-C+Ca^{2+}; (*h*) CBP22K+TN-C+EGTA.

No radioactivity could be detected in any of the fractions eluted from the column. The calmodulin was readily detected on the gels, but was not present in the form of a complex as might be expected if it had been eluted with the calmodulin-binding protein. When the column was washed with 6M-guanidine hydrochloride approx. 5% of the radioactivity applied to the column was recovered. This experiment suggested that calmodulin-binding protein 22K bound to the Sepharose in such a manner that it was not readily removed by strong dissociating conditions.

Effect of bovine brain calmodulin-binding protein 22K on the activity of calmodulin-requiring enzymes

Phosphodiesterase. The addition of increasing amounts of bovine brain calmodulin-binding protein 22K to phosphodiesterase activated by calmodulin to slightly below its maximum activity produced progressive inhibition of the enzyme. About 80% inhibition was obtained with calmodulin-binding protein to calmodulin at a molar ratio of 5:1 and inhibition was almost complete with a molar ratio of 10:1 (Fig. 6). Rabbit fast-skeletal-muscle troponin I was very similar to calmodulin-binding protein 22K in its inhibitory activity on the phosphodiesterase (Fig. 6).

Myosin light-chain kinase. Myosin light-chain kinase activated by calmodulin at a molar ratio of calmodulin to kinase of 1.6:1 was not inhibited by calmodulin-binding protein 22K at 100 times the concentration of calmodulin. If the ratio of concentrations was increased to 1000, the kinase was inhibited by 10%. These results are similar to those obtained when rabbit skeletal-muscle troponin I is added to the calmodulin–myosin light-chain kinase system (A. C. Nairn, unpublished work).

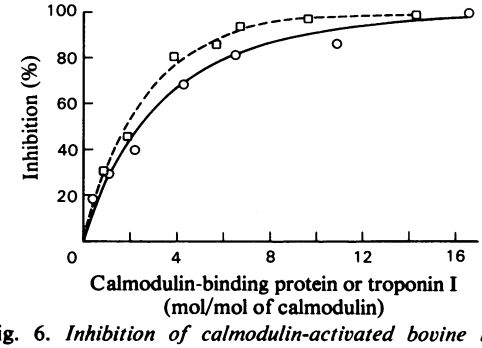

Fig. 6. *Inhibition of calmodulin-activated bovine brain phosphodiesterase by calmodulin-binding protein 22K and troponin I*

Bovine brain calmodulin-binding protein (○) or rabbit skeletal-muscle troponin I (□) was added to enzyme assays of phosphodiesterase containing 400ng of bovine brain calmodulin. For assay conditions see the Materials and Methods section.

Discussion

The values in the present paper for the calmodulin content of tissue determined by the electrophoretic method are in general higher than those reported by other workers (Lin *et al.*, 1974; Watterson *et al.*, 1976; Klee, 1977; Kato *et al.*, 1977). This is probably due to the fact that the calmodulin content was determined in conditions designed to ensure the dissociation of the calmodulin-binding protein–calmodulin complexes. The fact that on some occasions dissociation was not complete, even in the presence of urea and EGTA, suggests that the true calmodulin contents may be even slightly higher than the values listed in Table 1. The amount of Ca^{2+}-binding protein (which is mainly troponin C) in the bovine heart appears to be lower than that reported previously for troponin C in rabbit heart by using the isotope-dilution technique (Head & Perry, 1974). The reasons for this discrepancy are not clear at present.

It is apparent from this study and previous investigations from our laboratory (Head *et al.*, 1977; Grand *et al.*, 1979) that in tissue homogenates, and presumably therefore in the living tissue, all the Ca^{2+}-binding proteins, either calmodulin or troponin C, exist as complexes that are stable to high urea concentrations and are Ca^{2+}-dependent. In the present investigation we have isolated from brain four apparently different proteins that form urea-stable Ca^{2+}-dependent complexes with calmodulin and troponin C. Complexes similar to those characterized in brain and containing calmodulin-binding proteins of mol.wts. approx. 140000, 80000 and 60000 were present in most of the tissues examined. In striated muscle, however, the complexes containing these proteins were present in relatively low concentrations and the major Ca^{2+}-binding protein was troponin C. In this tissue, therefore, almost all of the urea-stable Ca^{2+}-dependent complex consisted of the troponin I–troponin C complex. The molecular weights of the three largest calmodulin-binding proteins are somewhat greater than would be expected from the apparent molecular weights of calmodulin–calmodulin-binding protein complexes deduced from the gel-filtration experiments. This discrepancy arises from the fact that even though the gel filtration of the complexes was carried out in 6M-urea (Fig. 1), the proteins were not completely denatured. Under these conditions the molecular weights obtained would be lower than the true molecular weights of the completely unfolded protein complexes (Tanford, 1968).

In addition, brain and probably most other tissues also contain a calmodulin-binding protein of mol.wt. in the range 22000–19000 that can be identified on polyacrylamide-gel electrophoresis of whole-tissue homogenates in the presence of sodium dodecyl

sulphate (R. J. A. Grand & S. V. Perry, unpublished work). This protein was not detected by the gel-filtration–affinity-chromatographic method that was used to isolate the higher-molecular-weight calmodulin-binding proteins, because of its unusual property of binding strongly to Sepharose. Other workers have reported non-specific interaction between proteins and agarose even in the presence of denaturing agents (Holroyde *et al.*, 1976).

The low-molecular-weight protein calmodulin-binding protein 22K is present in bovine brain in the largest amount of all the calmodulin-binding proteins and was isolated as a homogeneous preparation in substantial yield from this tissue. Insofar as it is generally basic in properties, inhibited the activation phosphodiesterase by calmodulin, did not inhibit the activation of myosin light-chain kinase by calmodulin and formed a urea-stable Ca^{2+}-dependent complex with troponin C, this protein is very similar to troponin I isolated from skeletal muscle. It is noteworthy, however, that apart from the preponderance of basic residues the amino acid analyses of the two proteins are quite distinct. A protein of this size and with these properties has not previously been reported to be present in brain.

As yet the three other calmodulin-binding proteins have not been isolated in quantities sufficient to enable their detailed study, although they are probably present in the tissue at concentrations in excess of 100mg/kg tissue wt. Calmodulin-binding proteins of mol.wt. 61000 have been isolated although in very low yield by Watterson & Vanaman (1976) and Klee & Krinks (1978), and of mol.wt. about 80000 by Wang & Desai (1977) and Wallace *et al.* (1978). These probably correspond to the calmodulin-binding proteins 77K and 61K described above, although the yields obtained by other workers are very much lower than those reported in the present paper.

The function of the group of calmodulin-binding proteins is far from clear, but they have one distinctive property in common; that is the ability to form urea-stable Ca^{2+}-dependent complexes with calmodulin and troponin C. This property first described for the complex between troponin I and troponin C (Perry *et al.*, 1972; Head & Perry, 1974) is, to our knowledge, unique. It suggests that there may be some common structural features between troponin I and the calmodulin-binding proteins. This hypothesis (see Perry *et al.*, 1979) is supported by the established evolutionary relationship between calmodulin and troponin C (Barker *et al.*, 1977) and the fact that sites on these two proteins that bind troponin I, and presumably the calmodulin-binding proteins, are very similar (Vanaman & Perry, 1978). Although the amino acid compositions of troponin I and the calmodulin-binding protein 22K are rather different, their pronounced basic nature and the striking similarity of certain of their properties leads one to speculate on whether these two proteins have an evolutionary relationship similar to that which exists between troponin C and calmodulin.

Note added in proof (Received 31 May 1979)

Since this paper was submitted, further work on the nature and function of the calmodulin-binding protein of mol.wt. 22000 suggests that it is identical with myelin basic protein (for review see Carnegie & Dunkley, 1975). This conclusion is based on the following evidence. (1) The sequences of 18 tryptic peptides representing approx. 80% of the protein that have been wholly or partially determined appear to be very similar to regions of bovine myelin basic protein. (2) Antibody to the protein (kindly prepared by Dr. G. K. Dhoot) specifically stains myelinated nerve. (3) The protein appears to be mainly associated with the myelin fraction of bovine brain.

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