Purification and properties of a 23 kDa Ca^{2+} -binding protein from *Drosophila melanogaster*

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Recent reports have shown that there exists in mammalian brain a number of heat-stable Ca^{2+} -binding proteins that are distinct from calmodulin [McDonald & Walsh (1985) *Biochem. J.* **232**, 559–567]. We have attempted to characterize equivalent Ca^{2+} -binding proteins from *Drosophila*. Affigel–phenothiazine chromatography, which can be used to purify calmodulin and other Ca^{2+} -binding proteins, allowed the identification of a possible heat-stable 23 kDa Ca^{2+} -binding protein. A purification procedure for this protein has been devised. Purified 23 kDa protein shows characteristics typical of a Ca^{2+} -binding protein; there is a mobility shift on SDS/polyacrylamide gels in the presence of EGTA, and Western blotting, followed by the use of the $^{45}\text{Ca}^{2+}$ overlay technique, confirms that the 23 kDa protein does bind Ca^{2+} . $^{45}\text{Ca}^{2+}$ -binding studies indicate that this protein binds 1 mol of Ca^{2+} /mol of protein, with K_d 1.9 μM . A single band with pI 5.2 is obtained on isoelectric focusing. Analysis of Western blots of *Drosophila* tissues probed with antibodies to the Ca^{2+} -binding protein indicates that it has a widespread distribution, but is absent from muscle tissue. The antibodies also cross-react with a protein of identical molecular mass in extracts of sheep brain. The possible similarity between this *Drosophila* Ca^{2+} -binding protein and mammalian proteins is discussed, and comparison is made between this *Drosophila* protein and other Ca^{2+} -binding proteins purified from vertebrates.

INTRODUCTION

Variation in the intracellular levels of free Ca^{2+} is one way in which extracellular stimuli, such as hormones or neurotransmitters, can trigger responses in the target cells. The presence of intracellular proteins that bind Ca^{2+} can bring about the transduction of the external stimuli into physiological responses. These Ca^{2+} -binding proteins may be activated directly by Ca^{2+} , or may act as Ca^{2+} -dependent regulators of other enzymes. The most studied protein among the latter group is calmodulin, which is known to activate a number of enzymic systems, including cyclic AMP phosphodiesterase (Cheung, 1971), protein kinases (Yagi *et al.*, 1978; Payne & Soderling, 1980; Kennedy *et al.*, 1983), adenylate cyclase (Brostrom *et al.*, 1975) and a phosphoprotein phosphatase (Stewart *et al.*, 1982; Yang *et al.*, 1982). It has been proposed that there exists a family of calmodulin-like Ca^{2+} -binding proteins which possess the 'EF-hand' type of Ca^{2+} -binding site found in calmodulin (Kretsinger, 1980).

Among those proteins known to be directly activated by Ca^{2+} are the Ca^{2+} -activated proteinases, such as calpain (Imahori, 1982; Murachi, 1983), proteins involved in the Ca^{2+} -dependent fusion of membrane vesicles, such as synexin (Creutz *et al.*, 1978) and the calelectrins (Sudhof *et al.*, 1984), and proteins which may play a role in regulating the structure of the cytoskeleton, such as the gelsolins (Kwaitkowski *et al.*, 1985).

Yet another role for Ca^{2+} -binding proteins is believed to be in the buffering of intracellular free Ca^{2+} . Proteins such as calbindin (Wasserman & Taylor, 1966; Spencer *et al.*, 1976) and perhaps calretinin (Rogers, 1987) have been proposed to have such a role.

Many Ca^{2+} -binding proteins have been purified by hydrophobic-interaction chromatography (Walsh *et al.*, 1984). By this method a number of Ca^{2+} -binding proteins have been isolated from bovine brain, some of which are heat-stable (McDonald & Walsh, 1985). Although the function of these proteins is largely unknown, one (17 kDa) has been shown to inhibit protein

kinase C activity (McDonald & Walsh, 1985). In an attempt to understand how Ca^{2+} might act as a second messenger to induce specific physiological responses, we chose to investigate Ca^{2+} -binding proteins in *Drosophila*, with the hope that a combination of genetic and biochemical approaches might yield information relevant to the physiological function of these proteins.

In this report is described the purification and initial characterization of a novel 23 kDa Ca^{2+} -binding protein from *Drosophila* (DCABP-23) that is distinct from calmodulin.

MATERIALS AND METHODS

Materials

Affigel–phenothiazine was obtained from Bio-Rad. However, during the course of these experiments Bio-Rad withdrew this product from the market. Attempts to prepare phenothiazine–Sephacrose with properties similar to that produced by Bio-Rad in terms of chromatographic separation of DCABP-23 were unsuccessful. This, in part, is why a method for the purification of DCABP-23, independent of the Affigel–phenothiazine, was developed.

Sephadex G-75 (fine grade) and CNBr-activated Sepharose 4B were obtained from Pharmacia, and hydroxyapatite was from Bio-Rad. Low-molecular-mass standards for calibration of the gel-filtration columns were obtained from Pharmacia. Alkaline phosphatase-linked anti-rabbit IgG was obtained from Promega, as were the BCIP and NBT required for staining antibody conjugates. $^{45}\text{Ca}^{2+}$ was obtained from Amersham. All other chemicals were reagent grade.

Ca^{2+} binding

Radioactive Ca^{2+} binding to Western blots was performed by the method described by Maruyama *et al.* (1984). Other Ca^{2+} -binding assays were performed by a variation of this solid-phase procedure. Fractions (10 μl), containing the protein to be assayed,

were adjusted to 2 mM-EGTA and spotted on nitrocellulose filters 5 mm in diameter. The filters were then placed in 20 mM-Mops, pH 7.5, containing 5 mM-MgCl₂, and 200 mM-KCl (buffer A) with 1 mM-EGTA for 15 min. The filters were transferred to buffer A and washed for 30 min with one change of buffer, and then placed in tubes containing 1 ml of buffer A with 1 μ Ci of ⁴⁵Ca²⁺/l (2.2 μ M). After 30 min they were removed from the ⁴⁵Ca²⁺ solution and washed for 2 min in distilled water, dried, and counted for radioactivity in a liquid-scintillation counter (LKB).

For analysis of Ca²⁺ binding, the equilibrium-dialysis method described by McDonald & Walsh (1985) was used, the only variation being that 200 μ g DCABP-23 fractions were used in a volume of 400 ml. The available free Ca²⁺ is calculated by using a pK_a (Ca²⁺) value of 10.995 for EGTA (Fabiato, 1981).

Gel electrophoresis

SDS/polyacrylamide gels (15 %) were prepared and run by the method of Laemmli (1970). Gels were stained either with Coomassie Blue or with 'Stains all' as described by Campbell *et al.* (1983). Molecular-mass standards for the SDS/PAGE were obtained from Bio-Rad, and consisted of phosphorylase *b* (97 400 Da), BSA (66 200), ovalbumin (42 699), carbonic anhydrase (31 000), trypsin inhibitor (21 500) and lysozyme (14 400).

Preparation of *Drosophila* extracts

The *Drosophila melanogaster* used in these experiments were adult Canton-S, of mixed sex, and 2–5 days old. The flies were collected by rapid freezing in liquid N₂, and kept at –70 °C until required.

To prepare heat-treated crude homogenates, frozen adult flies (10 g) were ground in liquid N₂ in a mortar and pestle. The ground powder was then added to approx. 30 ml of 20 mM-Tris/HCl (pH 7.5)/1 mM-CaCl₂/1 mM-MgCl₂ and homogenized with ten strokes of a Teflon homogenizer at 900 rev./min. The homogenate was centrifuged at 50 000 *g* for 30 min, and the supernatant was cleared by pouring it over glass wool. The pellet was re-homogenized in 30 ml of the same buffer and again centrifuged at 50 000 *g* for 20 min. The supernatants were combined, and CaCl₂ was added to a final concentration of 1.5 mM. This material was then heated at 95 °C for 5 min, cooled and centrifuged at 20 000 *g* for 15 min.

Individual tissues were dissected from adult *Drosophila* in Ringer solution and kept in this solution on ice until homogenization. For Western blots the dissected tissues were either homogenized in TBS and centrifuged at 50 000 *g* for 30 min, or for the head fractions, synaptosomal fractions were prepared as previously described (Kelly, 1981). Fractions containing 30 μ g of protein were run on gels for Western blotting.

Preparation of antibodies

Antibodies were prepared by injecting 1.5 mg of the purified protein in Freund's complete adjuvant subcutaneously into a rabbit that had been pre-bled to obtain pre-immune serum. The first injection was followed 3 weeks later with a second injection of 0.4 mg of protein. The rabbit was bled on two occasions. Serum fractions were then prepared and used in Western blots directly and compared with pre-immune serum, or the antibodies were purified further by preparing a 0–50 %-(NH₄)₂SO₄ fraction of the serum and dialysing this fraction against TBS. The dialysed fraction was then passed over an Affigel-Protein A column (Bio-Rad). The IgG fraction was eluted from the column with 4 M-MgCl₂ and the MgCl₂ was removed by dialysis against TBS. The anti-DCABP-23 antibodies were further purified by repeated application of the IgG fraction to a DCABP-23 affinity column, prepared by covalently binding 2 mg of the purified

DCABP-23 to CNBr-activated Sepharose 4B (Pharmacia) as per the manufacturer's methodology. The anti-DCABP-23 antibodies were eluted from the column with 4 M-MgCl₂, dialysed against TBS, freeze-dried and stored at 4 °C.

Western blots

SDS gels were blotted to nitrocellulose filters by using the LKB Novoblot system (Khyse-Andersen, 1984). Western blots were then either treated for ⁴⁵Ca²⁺ binding as described above, autoradiographed, and then stained with Amido Black, or subjected to blocking in 1 % skimmed milk for 1 h, followed by a 1/2000 dilution of either antiserum or purified anti-DCABP-23 antibody in TBS. The filter was washed in two changes of TBS for 2 h before the alkaline phosphatase-linked anti-rabbit IgG (Promega, 1/7500 dilution in TBS) was added. After a further 1 h, the filters were again washed twice in TBS, and finally BCIP and NBT were added in the dark. The staining was usually complete within 10 min, and the filters were then washed in distilled water.

Amino acid composition

Duplicate samples of DCABP-23 were hydrolysed *in vacuo* at 110 °C in 6 M-HCl for 24 h and then analysed with a Beckman 6300 amino acid analyser.

RESULTS

Purification of DCABP-23

As a first step in the identification of novel heat-stable Ca²⁺-binding proteins from *Drosophila*, attempts were made to remove calmodulin. To achieve this, heat-treated homogenates of *Drosophila* were applied to an Affigel-phenothiazine column known to be a specific affinity matrix for calmodulin (Jamieson & Vanaman, 1979). During this chromatography, a new heat-stable Ca²⁺-binding protein was identified and named DCABP-23.

To obtain a standard method for the purification of the DCABP-23, chromatographic methods other than Affigel-phenothiazine were sought (see the Materials and methods section).

The heat-treated crude extract was first subjected to (NH₄)₂SO₄ fractionation. DCABP-23 was found to be a major component of the 50–90 %-satn. fraction (Fig. 1*b*, track A). The 50–90 %-satn.-(NH₄)₂SO₄ fraction was therefore resuspended and dialysed against 20 mM-Tris/HCl (pH 7.5)/1 mM-CaCl₂ overnight, and applied to a hydroxyapatite column equilibrated in buffer containing 1.0 mM-sodium phosphate (pH 7.5) and 200 mM-KCl. The hydroxyapatite was washed with the same buffer until the A₂₈₀ reached zero, at which point the column was developed with a gradient of 1.0–200 mM-sodium phosphate with 200 mM-KCl. The hydroxyapatite-column profile is shown in Fig. 1(*a*). A major peak of protein is eluted at about 5 mM-phosphate. When fractions from this peak are run on SDS/polyacrylamide gels, the pattern shown in Fig. 1(*b*) is obtained. The major protein constituent of the hydroxyapatite peak is DCABP-23, as defined by its cross-reaction with antibodies raised against the Affigel-phenothiazine-purified protein.

Further purification of this fraction was obtained by chromatography of the pooled peak fractions from the hydroxyapatite column on Sephadex G-75. The Sephadex column was equilibrated and run in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-CaCl₂ and 100 mM-KCl. Again a major peak of protein was observed (Fig. 2*a*), and SDS/PAGE of the peak fractions shows the presence of DCABP-23 (Fig. 2*b*). The position of the peak of DCABP-23 from the Sephadex column gives a molecular-

Fig. 1. Hydroxyapatite chromatography of DCABP-23

(a) Protein profile from the hydroxyapatite column (1.6 cm $\times$ 2.5 cm); 1.75 ml fractions were collected. (b) SDS/PAGE of peak fractions (30 μ l) from the hydroxyapatite column. For track A, a sample of the 50–90% satn.-(NH_4)₂SO₄ fraction was applied to the column. The numbers of the other tracks indicate the column fractions from which they were taken.

mass estimate of 35 kDa. Re-running of the peak fraction on the Sephadex column, but in buffer where 5 mM-EGTA was substituted for the CaCl_2 , did not alter the position of the peak. Similar molecular-mass estimates for the native form of DCABP-23 have been obtained by using h.p.l.c. gel-filtration chromatography on a TSK 3000 column (LKB).

A low-molecular-mass contaminant of 16 kDa is apparent in all preparations of DCABP-23, and all chromatographic methods that have been attempted have so far failed to resolve this contaminant from DCABP-23. However, this 16 kDa protein is a small proportion of the material eluted in the peak from

Fig. 2. Sephadex G-75 chromatography of DCABP-23

(a) Protein (□) and $^{45}\text{Ca}^{2+}$ -binding (■) profiles of fractions from the Sephadex G-75 column (1.6 cm $\times$ 48 cm); 2.0 ml fractions were collected. V_0 , 1, 2, and 3 represent the elution positions of Blue Dextran, ovalbumin (43 kDa), chymotrypsinogen A (26 kDa) and ribonuclease A (13.7 kDa) respectively. (b) SDS/PAGE of Sephadex G-75 column fractions (30 μ l). Track numbers correspond to fraction numbers (St, molecular-mass standards; see the Materials and methods section).

Sephadex G-75, and, when the protein content of the peak is determined, the estimate shows that approx. 5 mg of DCABP-23 is obtained from 30 g of flies. Densitometric scanning of overloaded SDS gels indicates that the DCABP-23 represents 90–95% of the final protein product.

Isoelectric focusing of the purified protein produced a single band with a pI of 5.2.

Is the 23 kDa protein a Ca^{2+} -binding protein?

The first indication that the DCABP-23 might be a Ca^{2+} -binding protein came from its retardation on Affigel-phenothiazine in the presence of Ca^{2+} . To confirm that the protein was indeed a Ca^{2+} -binding protein, more direct evidence was sought. Fractions from the Sephadex G-75 were assayed for their $^{45}\text{Ca}^{2+}$ -binding activity by using the nitrocellulose blotting technique (see the Materials and methods section). A peak of $^{45}\text{Ca}^{2+}$ -binding activity was observed that corresponded to the peak of protein (Fig. 2a), although the $^{45}\text{Ca}^{2+}$ -binding activity does not exactly follow the profile of the protein peak. Purified DCABP-23 has also been subjected to electrophoresis in denaturing (SDS) polyacrylamide gels, in both the presence and the absence of EGTA. In the presence of EGTA, the mobility of the protein is decreased, resulting in a molecular-mass estimate of 24 kDa (Fig. 3). This mobility shift is a property possessed by

Fig. 3. EGTA affects the mobility of DCABP-23 on SDS/PAGE

Samples of DCABP-23 were subjected to SDS/PAGE in the absence (a) or presence (b) of 2 mM-EGTA. St, molecular-mass standards (see the Materials and methods section).

Fig. 4. Direct $^{45}\text{Ca}^{2+}$ binding to DCABP-23

A sample of the peak fraction from the Sephadex G-75 column was subjected to SDS/PAGE and Western-blotted to nitrocellulose. The blot was probed with $^{45}\text{Ca}^{2+}$ followed by autoradiography (1), and then stained with Amido Black (2). St, molecular-mass standards (see the Materials and methods section).

many Ca^{2+} -binding proteins (Klee *et al.*, 1979). However, DCABP-23 subjected to SDS/PAGE does not stain blue with 'Stains all', which has been used to identify a number of other Ca^{2+} -binding proteins (Campbell *et al.*, 1983).

The most direct proof that DCABP-23 is indeed a Ca^{2+} -binding protein comes from Western blots of SDS gels of DCABP-23. When the blot is probed with $^{45}\text{Ca}^{2+}$, as described by Maruyama *et al.* (1984), the results of such an experiment are shown in Fig. 4. As shown, DCABP-23 does bind $^{45}\text{Ca}^{2+}$.

Fig. 5. Scatchard plot of Ca^{2+} binding to DCABP-23

Results are shown of equilibrium dialysis of equimolar amounts of DCABP-23 against increasing concentrations of free Ca^{2+} .

Table 1. Comparison of amino acid compositions of various Ca^{2+} -binding proteins

Key to references: ^a the present work; ^b Rogers (1987); ^c Walsh *et al.* (1984); ^d Waisman *et al.* (1983); ^e Yamakuni *et al.* (1986). + indicates that tryptophan was detected spectrophotometrically.

	Amino acid composition (mol of residue/mol)				
	DCABP-23 ^a	Calretinin ^b	21kd CaB ^c	Caligulin ^d	Calbindin ^e
Cys	1	1	3	4	4
Asx	36	23	21	22	37
Thr	6	5	8	10	12
Ser	7	13	17	18	12
Glx	15	26	27	30	39
Pro	5	4	6	11	4
Gly	14	14	19	19	16
Ala	16	12	12	13	14
Val	12	5	8	5	6
Met	2	8	5	4	6
Ile	10	5	8	6	11
Leu	15	23	13	22	40
Tyr	8	6	5	6	8
Phe	10	12	11	16	14
Lys	15	14	16	16	25
His	2	5	3	4	4
Arg	10	10	9	6	6
Trp	+	2	2	1	2

Equilibrium dialysis of DCABP-23 against concentrations of free Ca^{2+} ranging from 0.25 to 5.0 μM and containing 20 μCi of $^{45}\text{Ca}^{2+}$ were performed. A Scatchard plot of the binding of Ca^{2+} to equimolar amounts of DCABP-23 is shown in Fig. 5. The K_d for Ca^{2+} of DCABP-23 is 1.9 μM , and the saturation value for DCABP-23 binds 1 mol of Ca^{2+} /mol of protein.

Amino acid composition of DCABP-23

A comparison of the amino acid composition of DCABP-23 with that of other Ca^{2+} -binding proteins is shown in Table 1. DCABP-23 shows some overall similarities to other Ca^{2+} -binding proteins. There is a relatively high content of acidic amino acids: 28% aspartic/glutamic residues as compared with 27% for CBP-18, 29% for the bovine 21 kd CaBP and 26% for caligulin. Comparable amounts of basic residues are also present in the four proteins. Although the tryptophan content of DCABP-23 has not been determined, the u.v. absorption spectrum (Fig. 6) shows a shoulder at 290 nm, indicative of tryptophan.

Fig. 6. U.v. absorbance spectrum of DCABP-23

Samples eluted from a TSK 3000 h.p.l.c. column were analysed by using an LKB diode-array u.v. detector, and the u.v. spectrum of the peak fraction was plotted.

Fig. 7. Western plot of *Drosophila* tissues probed with anti-DCABP-23-specific antibodies

Samples of various *Drosophila* tissues were run on SDS/PAGE as described in the Materials and methods section, Western-blotted and probed with anti-DCABP-23-specific antiserum. All of the tissues that were used gave a positive signal for DCABP-23, except for muscle (results not shown). (a) Samples prepared from head fractions. (b) Other tissue samples as indicated. The arrows indicate the presence of cross-reacting proteins other than DCABP-23. The scale on the left derives from the position of the molecular-mass standards run on the same gel.

Tissue distribution of DCABP-23

To investigate the tissue distribution of DCABP-23, extracts were made from various body parts of adult *Drosophila*. These extracts were then subjected to SDS/PAGE, Western-blotted, and probed with affinity-purified anti-DCABP-23 antibodies (see the Materials and methods section). The results are shown in Fig. 7. DCABP-23 is present at high levels in the head soluble fraction, gut and ovarian tissues, which includes mature oocytes. No cross-reacting material was observed in extracts from flight

Fig. 8. Immunological comparison of DCABP-23 with mammalian proteins

Samples of DCABP-23 (tracks 1 and 3) and the equivalent of the Affigel-phenothiazine pool 4 fraction from sheep brain (tracks 2 and 4) were run on SDS/PAGE, Western-blotted and then probed with either pre-immune serum (tracks 1 and 2) or anti-DCABP-23 antiserum (tracks 3 and 4). Several non-specific bands are seen in both the pre-immune-serum- and anti-DCABP-23-antiserum-treated blots.

muscle (results not shown). The presence, in the gut extracts, of lower-molecular-mass proteins that cross-react with the anti-DCABP-23 antibodies is probably attributable to proteolytic breakdown of DCABP-23. In crude synaptosomal fractions, two larger proteins of 35 kDa and 47 kDa show weak cross-reaction with the antibodies. This may reflect a low level of antigenicity, or low levels of these two proteins in this fraction.

Antigenic similarity between DCABP-23 and a protein in mammalian brain

Homogenates from sheep brain prepared in a manner identical with that for *Drosophila* extracts were chromatographed on a phenothiazine-Sepharose column and run on an SDS/polyacrylamide gel along with purified *Drosophila* 23 kDa Ca^{2+} -binding protein. A Western blot was made from these gels, and the nitrocellulose blot was probed with anti-DCABP-23 antiserum. The sheep brain extracts were found to contain a protein of molecular mass identical with that of DCABP-23 that cross-reacted with the anti-DCABP-23 antibody (Fig. 8).

DISCUSSION

A heat-stable 23 kDa Ca^{2+} -binding protein (DCABP-23) has been identified and purified from adult *Drosophila*. DCABP-23 exhibits properties consistent with it being a Ca^{2+} -binding protein, including a mobility shift on electrophoresis in the presence of EGTA, and retardation on phenothiazine-Sepharose in the presence of Ca^{2+} . The protein also shows $^{45}\text{Ca}^{2+}$ binding on Western blot analysis. Equilibrium-dialysis studies, using $^{45}\text{Ca}^{2+}$, indicate that DCABP-23 binds Ca^{2+} with a K_d of $\sim 1.9 \mu\text{M}$ and with a stoichiometry of 1 mol of Ca^{2+} /mol of protein.

The chromatographic properties of DCABP-23 on both Sephadex G-75 and h.p.l.c. suggests that in its native form the protein has a molecular mass of 35 kDa, in contrast with the 23 kDa estimates obtained from SDS/PAGE. It is possible that this

discrepancy arises from the presence of the 16 kDa contaminating protein. If it were to bind to DCABP-23 under non-denaturing conditions to form a heterodimer, the expected molecular mass of the protein would be 39 kDa. However, the relative proportions of the two proteins argue against this.

As much as 3 mg of DCABP-23 has been purified from 10 g of flies. Assuming maximal recovery of the protein during purification, it can be estimated that the concentration of the protein, were it evenly distributed throughout the fly (approximate weight 1 mg/fly), would be in the range of 5–10 μM . Given that the stoichiometry of Ca^{2+} binding to the protein is 1 mol of Ca^{2+} /mol of protein, then this protein would have the capacity to buffer up to 10 μM intracellular Ca^{2+} , a not inconsiderable amount.

Given its extremely high concentration, it would appear unlikely that DCABP-23 has a function similar to calmodulin. It is more likely that it is involved either in buffering the free Ca^{2+} concentration, as has been suggested for calbindin (Jande *et al.*, 1981) and calretinin (Rogers, 1987), as a Ca^{2+} -activated enzyme, or as a structural component of cells. That many of the presumed Ca^{2+} -buffering proteins have the capacity to bind 4 mol of Ca^{2+} /mol of protein might argue against DCABP-23 being a Ca^{2+} -buffering protein.

Whatever the function of the protein, it is present at high levels in neural tissue, gut and ovary, and it is absent from muscle. This would indicate that it is not a troponin-like protein, nor is it a sarcoplasmic Ca^{2+} -binding protein ('SCP'). This latter class of proteins have been found in large quantities in invertebrates (Cox *et al.*, 1976; Kohler *et al.*, 1978).

The presence of a cross-reacting species of protein in sheep brain extracts suggests that DCABP-23 has a mammalian analogue. A high-affinity Ca^{2+} -binding protein of 21 kDa has been reported in bovine brain by McDonald & Walsh (1985). This protein has been purified by using phenyl-Sepharose, binding to phenyl-Sepharose in the presence of Ca^{2+} , and eluting in the presence of EGTA. Attempts have been made to try to chromatograph DCABP-23 on phenyl-Sepharose with no success. It is still possible, however, that this 21 kDa mammalian protein is analogous to DCABP-23.

A separate, heat-stable, 24 kDa Ca^{2+} -binding protein from mammalian brain has been reported by Waisman *et al.* (1983) and named 'caligulin'. This protein has a K_d for Ca^{2+} of 0.2 μM and is reported to be a monomer, although, like DCABP-23, it too is eluted from gel-filtration columns with a higher molecular mass (33 kDa). It has been suggested that this protein might be identical with the bovine brain 18 kDa Ca^{2+} -binding protein purified by Manalan & Klee (1984).

The present study identifies DCABP-23 as a high-affinity Ca^{2+} -binding protein, and, although its function is unclear, as is its relationship with known mammalian proteins, it should be possible to define and mutate the gene encoding DCABP-23. A study of the phenotype of such mutants might then be useful in defining the physiological role of DCABP-23, as well as any possible mammalian analogue. In this way it is hoped that the role *in vivo* of these Ca^{2+} -binding proteins may be identified.

I thank Ms. M. Delbridge for technical assistance, Ms. R. Condon, of the Latrobe University Centre for Protein Chemistry, for performing the amino acid analysis, and Dr. Marie Phillips for critically reading the manuscript. This work was supported by a grant from the NH/MRC.

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