Two low-molecular-weight Ca^{2+} -binding proteins isolated from squid optic lobe by phenothiazine–Sephacryl affinity chromatography

James F. HEAD, Susan SPIELBERG and Benjamin KAMINER

Department of Physiology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118,
and Marine Biological Laboratory, Woods Hole, MA 02543, U.S.A.

(Received 15 October 1982/Accepted 23 November 1982)

We have isolated two Ca^{2+} -binding proteins from squid optic lobes, each of which is also able to bind phenothiazines in a Ca^{2+} -dependent manner. These proteins have each been purified and partly characterized. One of the proteins corresponds to calmodulin, in that it has a similar amino acid content to bovine brain calmodulin, including a single residue of trimethyl-lysine, it co-migrates with bovine calmodulin both on alkaline-urea- and on sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis, and will activate calmodulin-dependent phosphodiesterase. The second protein has the same subunit molecular weight as calmodulin, as determined by SDS/polyacrylamide-gel electrophoresis, M_r 17000, but migrates more slowly than this protein on alkaline-urea-gel electrophoresis. It has an amino acid composition distinct from calmodulin, containing no trimethyl-lysine, its CNBr fragments migrate on alkaline gels in a pattern distinct from those of calmodulin and it shows little ability to activate phosphodiesterase. The u.v.-absorption spectra of the proteins indicate the absence of tryptophan and the presence of a high phenylalanine/tyrosine ratio in each. Both proteins also bind 3–4 calcium ions/mol at 0.1 mM-free Ca^{2+} and each binds chlorpromazine in a Ca^{2+} -dependent manner.

The antipsychotic phenothiazines have been shown by Levin & Weiss (1976, 1977) to bind to high-affinity Ca^{2+} -dependent sites on the intracellular Ca^{2+} -binding activator protein calmodulin and to block the interaction of calmodulin with its target enzymes (e.g., Weiss *et al.*, 1980). On the basis of these observations, and of the reported low levels of Ca^{2+} -dependent binding of phenothiazines to other proteins (Levin & Weiss, 1978), an increasing number of studies have used these drugs as specific probes of calmodulin function. The Ca^{2+} -dependent calmodulin–phenothiazine interaction has also been utilized to develop affinity-chromatographic procedures for the isolation of calmodulin from various sources on immobilized phenothiazine matrices (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979).

As part of studies on Ca^{2+} -binding proteins in the nervous system of the squid we have used one of these affinity-chromatographic procedures to isolate Ca^{2+} -dependent phenothiazine-binding proteins from squid optic lobe. Two such proteins have been isolated and purified, only one of which corresponds

to calmodulin, the other being structurally and functionally distinct.

Materials and methods

Materials

Fluphenazine was given by Squibb and Sons Inc. (Princeton, NJ, U.S.A.) and trifluoperazine was given by Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A. [^3H]Chlorpromazine was purchased from New England Nuclear (Boston, MA, U.S.A.). Other chemicals and reagents were laboratory reagent grade obtained through local suppliers. Live squid were obtained through the Marine Biological Laboratories (Woods Hole).

Protein preparations

Squid optic lobes were dissected from freshly killed animals and immediately immersed in liquid N_2 . The samples remained in liquid N_2 or at -80°C in a freezer until used in the preparation.

Preparation of phenothiazine-binding proteins

All procedures were carried out at 4°C . About 25 g of optic lobe was thawed and homogenized in

Abbreviation used: SDS, sodium dodecyl sulphate.

10 vol. of 50 mM-imidazole/200 mM-NaCl/5 mM-EGTA/15 mM-2-mercaptoethanol (pH 7.2) using a glass-pestle homogenizer. The homogenate was centrifuged at 100 000 *g* for 30 min to obtain a clear supernatant. Sufficient 1 M-CaCl₂ was added to give a free Ca²⁺ concentration of approx. 1 mM and the supernatant was then applied to a column (2 cm × 10 cm) of fluphenazine-Sepharose, prepared by the method of Charbonneau & Cormier (1979), equilibrated in 50 mM-imidazole/200 mM-NaCl/1 mM-CaCl₂/15 mM-2-mercaptoethanol (pH 7.2). Unbound material was washed from the column with 500 ml of the same buffer. The column was then eluted with 100 ml of the EGTA-containing extraction buffer. Fractions (5 ml) of the eluate were collected and the protein content of each tube was monitored by alkaline-urea/polyacrylamide-gel electrophoresis.

The protein-containing fractions were pooled, dialysed for 12 h against 100 vol. of 20 mM-Tris/HCl/5 mM-EGTA/15 mM-2-mercaptoethanol (pH 7.8), and applied to a column (2 cm × 10 cm) of DEAE-cellulose (DE-52; Whatman Inc., Clifton, NJ, U.S.A.) equilibrated in the same buffer. The column was washed with 500 ml of buffer and eluted with a linear 0–0.4 M-NaCl gradient in 500 ml of buffer. Fractions (5 ml) were collected and the protein content was monitored by alkaline-urea/polyacrylamide- and SDS/polyacrylamide-gel electrophoresis.

Bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase was prepared as described by Head *et al.* (1979).

Bovine brain calmodulin was prepared as described by Head *et al.* (1982).

Gel electrophoresis

Alkaline-urea/polyacrylamide-gel electrophoresis was performed on 8% (w/v) polyacrylamide gels containing 6 M-urea by using a continuous buffer system of 25 mM-Tris/80 mM-glycine (pH 8.6) as described by Head & Perry (1974).

CNBr fragments of proteins were electrophoresed on the same gel system, but with 16% acrylamide, essentially as described by Head *et al.* (1977).

SDS/polyacrylamide-gel electrophoresis was performed on 10% (w/v) polyacrylamide gels by the method of Weber & Osborn (1969).

U.v.-absorption spectroscopy

Protein samples were dialysed at 4°C for 24 h against 200 vol. of 100 mM-NaCl/20 mM-Tris/HCl/15 mM-2-mercaptoethanol (pH 7.8) and subsequently clarified by centrifugation at 100 000 *g* for 30 min. Spectra were measured with a Beckman model 24 dual-beam spectrophotometer with matched 1 cm-light-path quartz cuvettes. The

spectra of protein solutions were measured from 310 to 240 nm with dialysis fluid as the reference solution.

CNBr cleavage

Freeze-dried protein samples were dissolved to 1 mg/ml in 70% (v/v) formic acid. CNBr, as a 1 g/ml solution in acetonitrile, was added to give a final concentration of 0.1 g of CNBr/ml. The sample was incubated for 24 h at room temperature and subsequently dried under a stream of N₂. The dried residue was redissolved in a minimum of water and sufficient aq. 1 M-NH₃ added to solubilize any undissolved material. The sample was again dried under a stream of N₂ before preparation for alkaline-urea/polyacrylamide-gel electrophoresis.

Chlorpromazine binding

Protein samples (approx. 0.5 mg/ml) were dialysed for 24 h against 500 vol. of 10 μM-[³H]-chlorpromazine (sp. radioactivity 1 Ci/mol)/200 mM-NaCl/5 mM-Tris/HCl and either 0.1 mM-CaCl₂ or 5 mM-EGTA (pH 7.6). After dialysis the protein concentration of the dialysed sample was determined by the method of Lowry *et al.* (1951). The radioactivity of portions of the dialysed sample and the dialysis fluid were determined by liquid-scintillation counting.

Ca²⁺ binding

Ca²⁺ binding at 0.1 mM-Ca²⁺ was determined by equilibrium dialysis. Protein samples (approx. 0.5 mg/ml) were first dialysed for 12 h against 10 mM-imidazole/100 mM-NaCl/1 mM-EGTA (pH 7.0), then for 12 h against the same buffer but containing 0.1 mM-EGTA and, finally, for 48 h against 400 vol. of 10 mM-imidazole/100 mM-NaCl/0.1 mM-⁴⁵CaCl₂ (sp. radioactivity 33 mCi/mol). After this period radioactivity determinations were made on portions of the dialysed sample and the dialysis fluid and the protein concentration of the dialysed sample was measured.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951). For crude protein mixtures measurements were standardized against a solution of bovine serum albumin the concentration of which was determined by total nitrogen estimation by the procedure of Strauch (1965). For purified peak I and peak II proteins, determinations were made with respect to samples of these proteins standardized by total nitrogen measurement. The nitrogen content of proteins was assumed to be 16%.

Amino acid analysis

Amino acid analysis was performed on samples hydrolysed in 6 M-HCl for 24 h at 110°C. Analyses

were performed on a Beckman 119CL analyser with the 126 data system. A column (0.6 cm × 24 cm) of Beckman W3 resin was used with the Beckman buffer system for collagen.

Phosphodiesterase assay

Phosphodiesterase activity was determined by the method of Boudreau & Drummond (1975).

Results

Affinity chromatography of whole-tissue extracts of squid optic lobes on fluphenazine-Sepharose gives two major Ca²⁺-dependent binding proteins as

identified by alkaline-urea/polyacrylamide-gel electrophoresis (Fig. 1). The mobility of one protein on this gel system corresponded to that of bovine brain calmodulin, the other protein migrated approx. 20% more slowly. The slower migrating component represented approx. 39% of the total protein eluting from the affinity column, as determined by densitometric gel scanning, the faster component represented approx. 58%. Small amounts of other more slowly migrating proteins were also consistently observed on these gels. However, these represented less than 5% of the total protein and were separated from the major components during subsequent fractionation. Approx. 10 mg of phenothiazine binding proteins were obtained from 25 g (wet wt.) of optic lobe.

The two major squid proteins were separated from each other and from the minor components by ion-exchange chromatography on DEAE-cellulose at pH 7.8, eluting with a linear 0–0.4 M-NaCl gradient; the two major proteins eluted at 0.25 M- (peak I) and 0.3 M-NaCl (peak II) respectively. The purified peak I and peak II proteins each migrated as single bands on alkaline-urea/polyacrylamide- and SDS/polyacrylamide-gel electrophoresis (Fig. 1). The apparent subunit molecular weight of each protein, as determined from its mobility on SDS/polyacrylamide gels, was 17000.

Amino acid analyses of the proteins showed that, although both had a generally similar amino acid

Fig. 1. Polyacrylamide-gel electrophoresis of squid fluphenazine-binding proteins

(a)–(d), Alkaline-urea/polyacrylamide gels [8% (w/v) acrylamide]. Samples include 5 mM-EGTA. (e)–(h), SDS/polyacrylamide gels [10% (w/v) acrylamide]. (a) 10 µg of bovine brain calmodulin; (b) 20 µg of squid optic-lobe proteins eluted from fluphenazine column by 5 mM-EGTA; (c) 10 µg of peak II protein from DEAE-cellulose chromatography; (d) 10 µg of peak I protein from DEAE-cellulose chromatography; (e) 5 µg of bovine brain calmodulin; (f) molecular-weight standards (68 000-, 45 000-, 35 000-, 26 000-, 18 000 and 14 000-*M_r* proteins); (g) 5 µg of squid optic-lobe fluphenazine-binding protein purified from DEAE-cellulose (peak II); (h) 5 µg of squid optic-lobe fluphenazine-binding protein purified from DEAE-cellulose (peak I).

Table 1. Amino acid analysis of phenothiazine-binding proteins

	Content (residues/mol)		
	Squid brain		Bovine brain calmodulin†
	I*	II*	
Asp	19.5	22.8	23
Thr	7.8	11.6	12
Ser	7.7	4.9	4
Glu	26.5	27.3	27
Pro	5.1	2.9	2
Gly	12.3	11.8	11
Ala	11.8	10.5	11
Val	7.4	7.6	7
Met	10.7	8.1	9
Ile	10.1	8.0	8
Leu	7.5	9.4	9
Tyr	2.0	1.1	2
Phe	7.4	9.0	8
Tml‡	0	0.7	1
Lys	10.1	7.4	7
His	>0.1	1.2	1
Arg	7.1	5.7	6

* Residues/17 000-mol.wt. protein.

† From Watterson *et al.* (1980).

‡ Trimethyl-lysine.

Fig. 2. Polyacrylamide-gel electrophoresis of CNBr fragments of calmodulin- and squid phenothiazine-binding proteins

Samples containing 100 μ g of CNBr fragment were dissolved in 100 μ l of 6 M-urea and electrophoresed on alkaline-urea gels [16% (w/v) acrylamide] as described in the Materials and methods section. (a) Squid peak I protein; (b) squid peak II protein; (c) bovine brain calmodulin.

Fig. 3. Activation of bovine brain calmodulin-dependent phosphodiesterase

Bovine brain phosphodiesterase was assayed in the presence of 5 μ M-cyclic AMP/0.1 mM- CaCl_2 and increasing amounts of bovine brain calmodulin (Δ), squid peak II protein (O) and squid peak I protein ($\bullet$). Activity is expressed as relative activation of the enzyme. Zero % represents basal enzymic activity (0.6 nmol/mg per min) and 100% represents maximum enzymic activity in the presence of bovine brain calmodulin (5.6 nmol/mg per min).

content, there were distinct differences, including the presence of a single residue of trimethyl-lysine in peak II but not in peak I (see Table 1). Alkaline-urea/polyacrylamide-gels of the CNBr fragments of the two proteins further confirmed that the proteins were structurally distinct (Fig. 2).

Although the CNBr fragments of peak II differed from those of peak I, they showed a striking similarity to those of bovine brain calmodulin (Fig. 2). The identity of the peak II protein with calmodulin was also suggested by its ability to activate calmodulin-dependent phosphodiesterase in a manner similar to that of bovine brain calmodulin (Fig. 3). The peak I protein had little effect on the activity of the enzyme over the same range of protein concentrations.

Fig. 4. U.v.-absorption spectra of peak I and peak II proteins

Spectra were determined by using protein samples at a concentration of 1 mg/ml in 20 mM-Tris/HCl/100 mM-NaCl/1 mM-EGTA (pH 7.8). (a) Peak I; (b) peak II.

Fig. 5. Binding of [³H]chlorpromazine to squid phenothiazine-binding proteins

Binding was determined by equilibrium dialysis of the proteins against 10 μ M-[³H]chlorpromazine/200 mM-NaCl/5 mM-Tris/HCl and either 0.1 mM-CaCl₂ or 5 mM-EGTA, pH 7.6 (see the Materials and methods section).

The u.v.-absorption spectra of the two proteins are shown in Fig. 4. Each spectrum shows the presence of a high phenylalanine/tyrosine content and the absence of tryptophan. The phenylalanine/tyrosine ratio appears to be higher in the peak II protein than in that of peak I, in accordance with the results of amino acid analysis.

Since it was possible that proteins other than calmodulin could adhere to fluphenazine-Sepharose by binding to calmodulin, rather than directly to the fluphenazine, we assessed the ability of each of the purified proteins to interact with phenothiazines by determining their binding with [³H]chlorpromazine (see the Materials and methods section). At 10 μ M-chlorpromazine and in the presence of 0.1 mM-CaCl₂ the peak I protein bound approx. 1 mol of chlorpromazine/mol and the peak II protein bound approx. 2 mol/mol. Binding was significantly reduced in each case in the absence of free Ca²⁺ (Fig. 5).

The Ca²⁺ dependence of the interaction of the two proteins with phenothiazines suggests that both are able to bind Ca²⁺ in the range of free Ca²⁺ concentrations used in these studies. This is confirmed by the determination that at 0.1 mM-free Ca²⁺ (and under the conditions described in the Materials and methods section) the peak I protein bound 3.6 ($\pm$ 0.6) mol of Ca²⁺/mol and the peak II protein bound 3.7 ($\pm$ 0.6) mol of Ca²⁺/mol. The latter value is consistent with the four high-affinity Ca²⁺-binding

sites known to be present on calmodulins from other species.

Discussion

We have purified and partly characterized two proteins from squid optic lobe that are each able to bind Ca²⁺ and phenothiazines. Our studies indicate that small amounts of other such binding proteins may also be present in this tissue. However, it remains to be determined whether these minor components bind directly to the phenothiazine or indirectly by binding to the major proteins.

The similarity in the apparent subunit molecular weights of the squid optic lobe peak I and peak II proteins means that they are not readily distinguished by SDS/polyacrylamide-gel electrophoresis. The presence of both proteins in a given preparation might therefore be undetected unless an alternative gel system, such as alkaline-urea/polyacrylamide gels, is used to resolve them. From all of the properties determined to date, including the presence of a single residue of trimethyl-lysine, the ability to activate phosphodiesterase and the CNBr peptide map, it seems likely that the squid peak II protein corresponds to calmodulin, as previously identified in squid axoplasm by Head & Kaminer (1980). The amino acid analysis of the squid peak I protein shows no trimethyl-lysine and indicates that this protein is slightly more basic than calmodulin, a fact also suggested by the slower mobility of the protein on alkaline-gel electrophoresis and its earlier elution from DEAE-cellulose. Gels of the CNBr fragments of the peak I protein also show it to be distinct from calmodulin.

Calmodulin has been shown in a number of studies to act as a Ca²⁺-sensitive switching mechanism able to activate a large number of intracellular enzyme systems (for review, see Means *et al.*, 1982). Since it is apparently ubiquitous in eukaryotes, the presence of calmodulin in the optic lobe of the squid is not unexpected. However, the presence of a second Ca²⁺-binding protein, having a similar molecular weight and present in comparable amounts to calmodulin, offers the possibility of a second mediator for Ca²⁺-regulated events in this tissue. A low-molecular-weight (M_r 13 000–15 000) Ca²⁺-binding protein has previously been identified in squid optic lobes by Alema *et al.* (1973). However, it is not possible to determine from available data the relationship, if any, of this protein to either of the proteins described in the present study. A '0-1' protein described by Guiditta *et al.* (1977) in the optic lobes of the octopus has since been shown to correspond to octopus calmodulin (Seamon & Moore, 1980).

Marshak *et al.* (1981) and Endo *et al.* (1981) have shown that phenothiazine-Sepharose columns are

able to bind S-100b from bovine brain in addition to calmodulin. It seems unlikely from our characterization to date that the squid optic lobe peak I protein corresponds to a molluscan equivalent of S-100, since S-100 has a subunit molecular weight of only 10500 and apparently only one Ca^{2+} -binding site per subunit (Isobe *et al.*, 1981). However, in the absence of a known functional role for S-100 such a relationship is difficult to assess. Future studies must be aimed at a more detailed characterization of the peak I protein and a determination of its structural and functional relationship with other such Ca^{2+} -binding proteins.

The observation in the present study and in those of Marshak *et al.* (1981) and Endo *et al.* (1981) that proteins other than calmodulin are able to bind phenothiazines in a Ca^{2+} -dependent manner should encourage caution in the use of these drugs both as diagnostic tools for defining calmodulin involvement in a given system, and as affinity ligands for the specific binding of solely calmodulin.

We thank George Crombie for performing amino acid analyses. This work was funded by a grant from the National Science Foundation PCM 8013009.

References

- Alema, S., Calissano, P., Rusca, G. & Guiditta, A. (1973) *J. Neurochem.* **20**, 681–689
- Boudreau, R. J. & Drummond, G. I. (1975) *Anal. Biochem.* **63**, 388–399
- Charbonneau, H. & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1039–1047
- Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. & Hidaka, H. (1981) *J. Biol. Chem.* **256**, 12485–12489
- Guiditta, A., Moore, B. W. & Prozzo, N. (1977) *J. Neurochem.* **29**, 235–244
- Head, J. F. & Kaminer, B. (1980) *Biol. Bull. (Woods Hole, Mass.)* **159**, 485
- Head, J. F. & Perry, S. V. (1974) *Biochem. J.* **137**, 145–154
- Head, J. F., Weeks, R. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 465–471
- Head, J. F., Mader, S. & Kaminer, B. (1979) *J. Cell Biol.* **80**, 211–218
- Head, J. F., Masure, H. R. & Kaminer, B. (1982) *FEBS Lett.* **137**, 71–74
- Isobe, T., Ishioka, N. & Okuyama, T. (1981) *Eur. J. Biochem.* **115**, 469–474
- Jamieson, G. A. & Vanaman, T. C. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1048–1055
- Levin, R. M. & Weiss, B. (1976) *Mol. Pharmacol.* **12**, 581–589
- Levin, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **13**, 690–697
- Levin, R. M. & Weiss, B. (1978) *Biochim. Biophys. Acta* **540**, 197–204
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marshak, D. R., Watterson, D. M. & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6793–6797
- Means, A. R., Tash, J. S. & Chafouleas, J. G. (1982) *Physiol. Rev.* **62**, 1–39
- Seamon, K. B. & Moore, B. W. (1980) *J. Biol. Chem.* **255**, 11644–11647
- Strauch, L. (1965) *Z. Klin. Chem. Klin. Biochem.* **3**, 165–167
- Watterson, D. M., Sharief, F. & Vanaman, T. C. (1980) *J. Biol. Chem.* **255**, 962–975
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weiss, B., Prozialeck, W., Cimino, M., Sellinger-Barnette, M. & Wallace, T. L. (1980) *Ann. N.Y. Acad. Sci.* **356**, 319–345