Isolation, purification and cell-free synthesis of calmodulin from the pig anterior pituitary gland

Simon W. WALKER,* John D. WARK,† Sheila MacNEIL,‡ Homa MELLERSH,‡
Barry L. BROWN† and Stephen TOMLINSON‡

*Department of Chemical Pathology, Northern General Hospital, Sheffield S5 7AU, †Department of Human Metabolism and Clinical Biochemistry, The Medical School, Beech Hill Road, Sheffield S10 2RX, and

‡Department of Medicine, University Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, U.K.

(Received 15 July 1983/Accepted 5 October 1983)

Calmodulin was extracted and purified from pig anterior pituitary gland. The protein was characterized by its migration on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of Ca^{2+} or EGTA, its u.v. spectrum between 240 and 290 nm and the activation of calmodulin-deficient cyclic AMP phosphodiesterase. The yield was 370 mg/kg wet wt. mRNA was also extracted from the same tissue and translated in a wheat-germ cell-free translation system. Translated calmodulin was identified by its heat-stability, its co-migration with authentic anterior-pituitary calmodulin on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, its acidic isoelectric point (4.15) on flat-bed isoelectric focusing, its Ca^{2+} -dependent binding to fluphenazine–Sepharose 6B, and its co-elution from this gel with authentic unlabelled calmodulin with EGTA buffer. Calmodulin was not translated as a precursor form. In this tissue it was calculated that calmodulin accounted for 0.5–1% of the total translated protein.

There is good evidence that the secretion of anterior-pituitary hormones is dependent on the intracellular concentration of Ca^{2+} ions. Manipulations that increase cytosolic Ca^{2+} , such as incubation of pituitary cells with calcium ionophores or with high K^+ concentrations, stimulate hormone release (Schrey *et al.*, 1978; Naor *et al.*, 1980; Gershengorn, 1980; Thorner *et al.*, 1980). Furthermore, both secretion and Ca^{2+} -dependent action potentials are blocked by inhibitors of Ca^{2+} channels, such as Co^{2+} or methoxyverapamil (Schrey *et al.*, 1978; Thorner *et al.*, 1980). The way in which Ca^{2+} ions affect the secretory process is unknown, but there is growing evidence that calmodulin, the Ca^{2+} -dependent regulatory protein, may be involved (Means & Chafouleas, 1982).

Cyclic nucleotide phosphodiesterase activity has been identified in rat anterior pituitary gland and is activated by crude heat-treated anterior-pituitary extracts (Azhar & Menon, 1977); this activation is inhibited by trifluoperazine, a calmodulin antagonist (Merritt *et al.*, 1982).

In addition, a number of calmodulin antagonists, including trifluoperazine, inhibit secretion of

prolactin and thyrotropin from the pituitary in whole-cell systems (Merritt *et al.*, 1981; Fleckman *et al.*, 1981).

There is thus evidence that calmodulin may be involved in stimulus–secretion coupling in the anterior pituitary gland. However, there is little information about the synthesis of calmodulin in the anterior pituitary or, indeed, in other tissues. We therefore set out to isolate and purify calmodulin from this tissue and to extract mRNA in an attempt to demonstrate that this mRNA could direct the synthesis of calmodulin in a cell-free translation system.

Materials and methods

Materials

Anterior pituitaries and brain from freshly slaughtered pigs were obtained from the abattoir. Phenylmethanesulphonyl fluoride, pepstatin A, dithiothreitol, cyclic GMP and cyclic AMP (crystallized free acids), snake venom (*Ophiophagus hannah*), Dowex 1-X8 (200–400 mesh) anion-exchange resin, bovine heart 3',5'-cyclic nucleotide phosphodiesterase (activator-deficient), L-amino acids, spermidine, creatine kinase, phospho-

Abbreviation used: SDS, sodium dodecyl sulphate.

creatine, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Mes (4-morpholine-ethanesulphonic acid), cytochrome *c*, bovine serum albumin (fraction V), myoglobin and Coomassie Brilliant Blue R were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Purified pig brain calmodulin, proteinase K, GTP and ATP were from Boehringer (London), Lewes, E. Sussex, U.K. Epoxy-activated Sepharose 6B was from Pharmacia (G.B.), Hounslow, Middx., U.K. Acrylamide, bisacrylamide and *NNN'*-tetramethylethylenediamine were from Bio-Rad Laboratories, St. Albans, Herts., U.K. L-[³⁵S]Methionine, cyclic [8-³H]GMP and cyclic [8-³H]AMP were obtained from Amersham International, Amersham, Bucks., U.K. Oligo(dT)-cellulose T2 was from Collaborative Research, Waltham, MA, U.S.A. Freshly milled (untoasted) wheat germ was from General Mills, Minneapolis, MN, U.S.A. X-ray film (X-omat RP film), developer (Dx-80) and fixer (FX-40) were from Kodak, Manchester, U.K. Fluphenazine hydrochloride was a gift from E. R. Squibb, Moreton, Wirral, Merseyside, U.K. DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. The Ampholines and Ultradex gel were from LKB, Selsdon, South Croydon, Surrey, U.K. 2,5-Diphenyloxazole was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

All other solutions and chemicals were from BDH Chemicals, Poole, Dorset, U.K. All chemicals and reagents were A.R. grade.

Preparation of solutions

To prevent RNA digestion by ribonuclease, gloves were worn at all times when performing experiments involving RNA. The buffers used for RNA extraction were autoclaved at 104 kPa (15 lb/in²) for 10 min; plastic pipette tips and centrifuge tubes were autoclaved at 69 kPa (10 lb/in²) for 10 min; glassware was sterilized at 180°C for 1 h before use. The phenol was re-distilled before use. Scintillant fluid (modified Bray's) contained 0.4% 2,5-diphenyloxazole, 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene, 6% (w/v) naphthalene, 2% (v/v) ethylene glycol and 10% (v/v) methanol in 1,4-dioxan.

Purification of calmodulin

Calmodulin was extracted from 9.2 g of anterior-pituitary tissue obtained from freshly slaughtered pigs. The protein was purified by the method of Kakiuchi *et al.* (1981).

Preparation of partially purified pig brain calmodulin-dependent phosphodiesterase

Fresh pig brain was homogenized and the supernatant applied to DEAE-cellulose as described by

Thompson *et al.* (1979). Briefly, DEAE-cellulose was equilibrated with 5 mM-Mes (pH 6.5)/30% ethylene glycol/1 mM-dithiothreitol/5% (w/v) glucose/50 mM-NaF/20 μ M-phenylmethanesulphonyl fluoride buffer, containing 15 mM-sodium acetate. The column was washed with this buffer, and then with the same buffer, but containing 150 mM-sodium acetate. Finally, the column was eluted with 1 litre of a linear gradient of 250–1200 mM-sodium acetate, also in this buffer. Eluted fractions were assayed for phosphodiesterase in the presence and absence of a maximally stimulating concentration of calmodulin, with 20 μ M-cyclic GMP as substrate. Fractions containing calmodulin-dependent phosphodiesterase activity were pooled and stored in batches at –20°C.

Measurement of calmodulin activity

Comparison of the purified pig anterior-pituitary calmodulin with a standard preparation was carried out with activator-deficient bovine heart cyclic AMP phosphodiesterase (Sigma) as described by MacNeil *et al.* (1982). Assay of the DEAE-cellulose-column fractions for calmodulin activity during purification of this protein from the anterior pituitary (Kakiuchi *et al.*, 1981) was by the same method, except that 20 μ M-cyclic GMP was substrate at 100 μ M-Ca²⁺, and the pig brain phosphodiesterase was used.

Extraction of total nucleic acids and isolation of mRNA

Approx. 5 g of anterior pituitary glands from freshly slaughtered pigs were collected into liquid N₂, and the total nucleic acid was extracted by the method of Hall *et al.* (1979), with modifications as described by Mellersh *et al.* (1980). The nucleic acid was dissolved in sterile water to a final concentration of 80–100 A₂₅₈ units/ml (1.0 A₂₅₈ unit = approx. 50 μ g of nucleic acid).

Polyadenylated mRNA was isolated by the method of Craig *et al.* (1976) with modifications described by Mellersh *et al.* (1980).

Translation of polyadenylated mRNA from pig anterior pituitary in a wheat-germ cell-free translation system

Wheat-germ lysate was prepared by the method of Roberts & Paterson (1973). Protein synthesis was carried out for 3 h at room temperature in 25 μ l assays, in the wheat-germ system. The incubation mixture contained 50 mM-potassium acetate, 0.5 mM-magnesium acetate, 25 μ M each of 19 unlabelled L-amino acids, 29 μ Ci of L-[³⁵S]methionine, 0.25 mM-spermidine, 8 mM-phosphocreatine, 2 mM-dithiothreitol, 25 μ M-GTP, 1.0 mM-ATP, 24 mM-Hepes buffer, pH 7.0, 8 μ l of wheat-germ lysate and 0.05 A₂₅₈ unit (approx. 2.5 μ g) of mRNA.

SDS/polyacrylamide-gel electrophoresis

The purified anterior-pituitary calmodulin and the translated proteins were analysed by SDS/polyacrylamide-gel electrophoresis on 15%-acrylamide slab gels as described by Laemmli (1970).

A 50 μ l portion of the translation mixture was boiled for 1 min and the denatured protein precipitated by centrifugation at 10000 g_{av} for 5 min. The recovered supernatant was then divided into halves. One half was made up to 50 μ l to contain a final concentration of 160 mM-Tris/HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 2 mM-EGTA (EGTA sample buffer). The other half was made up identically, except that 20 mM-CaCl₂ was substituted for the EGTA (CaCl₂ sample buffer). Then 5 μ g of freeze-dried anterior-pituitary calmodulin was dissolved in 50 μ l of EGTA sample buffer, and the same amount was dissolved in 50 μ l of CaCl₂ sample buffer. The translated protein and the authentic calmodulin were then analysed on 15%-polyacrylamide slab gels, which were fixed and stained in 0.2% Coomassie Blue in 7% (v/v) acetic acid/50% (v/v) methanol for 1 h and destained overnight in 7% acetic acid/50% methanol. The gels were then dried and subjected to fluorography by the method of Laskey & Mills (1975).

Fluphenazine-Sepharose 6B affinity chromatography

A 80–160 μ l portion of translation mixture was made up to 1 ml with 10 mM-Tris/HCl (pH 7.0)/0.5 mM-CaCl₂ and applied to 3 ml of fluphenazine-Sepharose 6B affinity gel equilibrated with the same buffer. The column (6 cm $\times$ 1 cm) was eluted with 10 mM-Tris/HCl (pH 7.0)/0.5 mM-CaCl₂/0.5 M-NaCl; 1 ml fractions were collected until the ³⁵S radioactivity was less than 50 c.p.m./fraction. At this point 800 μ g of purified authentic calmodulin dissolved in 1 ml of 10 mM-Tris/HCl (pH 7.0)/0.5 mM-CaCl₂ was applied to the column. When the radioactivity eluted was again less than 50 c.p.m., the elution buffer was changed to 10 mM-Tris/HCl (pH 8.0)/10 mM-EGTA, and 0.5 ml fractions were collected. Portions (50 μ l) from each fraction were measured for radioactivity and the calmodulin content, followed by measurement of A₂₈₀.

Isoelectric focusing

A 80–160 μ l sample of the translation mixture was boiled for 1 min and the denatured protein removed by centrifugation at 10000 g_{av} for 5 min. The heat-stable proteins were then separated by flat-bed preparative isoelectric focusing over the pH range 3.5–5.0. After electrophoresis overnight, the gel was subdivided into 30 sections. Each gel section was removed into 2 ml of water; the pH of each fraction was measured and the ³⁵S radioactivity

Fig. 1. SDS/polyacrylamide-gel electrophoresis of anterior-pituitary calmodulin on 15% slab gels

Lane 1 shows the three M_r markers (A, bovine serum albumin; B, myoglobin; C, cytochrome c). The purified anterior-pituitary calmodulin was run in the presence of 2 mM-EGTA (no Ca²⁺ added) in lane 2 and in the presence of 20 mM-Ca²⁺ (no EGTA added) in lane 3.

was present in 100 μ l portions of the supernatant was determined by addition of 5 ml of scintillation fluid; the radioactivity was measured in a Packard liquid-scintillation counter.

Results

During purification the calmodulin activity, as measured in the pig brain calmodulin-dependent phosphodiesterase assay (with cyclic GMP as substrate), was eluted from DEAE-cellulose at the higher (NH₄)₂SO₄ concentration (0.3 M). In the final purification step, using fluphenazine-Sepharose 6B affinity chromatography, calmodulin was eluted as a single sharp peak of protein with EGTA-containing buffer.

Analysis of the protein by SDS/polyacrylamide-gel electrophoresis (15% slab gel) showed a characteristic Ca²⁺-dependent shift with a single band when the protein was run in buffer containing 2 mM-EGTA and a doublet when the same material was run in the presence of 20 mM-CaCl₂ (see Fig. 1).

When the purified protein was tested for activity in the calmodulin-dependent phosphodiesterase

Fig. 2. SDS/polyacrylamide-gel electrophoresis on a 15% slab gel of heat-treated proteins obtained by translation of pig anterior-pituitary mRNA in a wheat-germ cell-free translation system

Lane 1 was run in the presence of 2 mM-EGTA (no Ca^{2+} added) and lane 2 in the presence of 20 mM- Ca^{2+} (no EGTA added). The arrows indicate the position of authentic pig anterior-pituitary calmodulin run under the conditions described for lane 1 (arrow A) or lane 2 (arrow B) on the same gel. A translated protein is visible which alters its mobility in precisely the same way as the authentic calmodulin.

assay, the activation observed was parallel to that with a calmodulin standard purified from pig brain (Boehringer). The pituitary calmodulin produced half-maximal activation at 10 ng, identical with that by the standard.

The yield of calmodulin purified in this way from pig anterior pituitaries was equivalent to 370 mg/kg wet wt. of tissue.

Polyadenylated mRNA extracted and fractionated from the anterior pituitaries was approx. 1% of the total extracted nucleic acids. This polyadenylated mRNA stimulated incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable protein by 20–30-fold over the endogenous wheat-germ protein synthesis without added mRNA.

Fig. 3. Application of unheated translation mixture to fluphenazine-Sepharose gel

The translation mixture was made up to 1 ml with 10 mM-Tris/HCl (pH 7.0)/0.5 mM- CaCl_2 and applied to the column, and eluted with the same buffer until the radioactivity in 1 ml fractions was less than 50 c.p.m. At A, 800 μg of purified pig anterior-pituitary calmodulin was added, and the ^{35}S radioactivity (●) and calmodulin content, as determined by A_{280} (■), were measured in 1 ml fractions. When the baseline was stable, the elution buffer was changed at B to 10 mM-Tris/HCl (pH 8.0)/10 mM-EGTA; 0.5 ml fractions were collected and ^{35}S radioactivity and calmodulin content were measured as previously. Note the precise co-elution of authentic pig anterior-pituitary calmodulin with a peak of radiolabelled material.

Analysis of the heated translated mixture by SDS/polyacrylamide-gel electrophoresis showed that the single most prominent band of radiolabelled protein on autoradiography co-migrated with authentic calmodulin and underwent the same Ca^{2+} -dependent shift, depending on whether Ca^{2+} or EGTA was present in the sample buffer (Fig. 1). Several very faint radiolabelled bands only were seen when the endogenous control (no added mRNA) was applied to the gel, none in the calmodulin position. Similarly, when the heat-stable translated proteins were subjected to flat-bed isoelectric focusing, a prominent peak of [^{35}S]methionine radioactivity was observed to

focus at pI4.15, as described for calmodulin (Klee *et al.*, 1980).

Finally, the presence of newly synthesized [^{35}S]methionine-labelled calmodulin was demonstrated by fluphenazine-Sepharose 6B affinity chromatography. Application of unlabelled calmodulin to the column after the translation mixture had been applied did not produce any significant displacement of radiolabelled material. When the column was pulsed with elution buffer containing 10mM-EGTA, a peak of [^{35}S]methionine radioactivity co-eluted precisely with the authentic calmodulin (Fig. 3). From the radioactivity eluted in this way and the total amount of trichloroacetic acid precipitable radioactivity initially applied to the column, it was calculated that the calmodulin accounted for 0.5–1% of total translated protein.

Discussion

We have thus shown that by a relatively simple purification method (Kakiuchi *et al.*, 1981) calmodulin can be extracted from pig anterior pituitaries and purified to apparent homogeneity. The yield of calmodulin obtained by this technique was 370 mg/kg wet wt., which compares with the value quoted for other endocrine tissues (Hutton *et al.*, 1981). Interestingly, Sheaves *et al.* (1982) have described the isolation of calmodulin from bovine posterior pituitary, which appears to be a particularly rich source of the protein (740 mg/kg wet wt.).

While the present manuscript was in preparation, Hatada *et al.* (1983) described the isolation of calmodulin from rat anterior pituitary, where the concentration was 1.18 $\mu\text{g}/\text{mg}$ of cytosol protein.

Examination of the extracted purified protein by SDS/polyacrylamide-gel electrophoresis showed a single band in EGTA-containing buffer with a characteristic shift in mobility to an apparently lower M_r in Ca^{2+} -containing buffer. This shift in mobility occurred either side of the myoglobin marker (M_r 17000). This appearance, with the additional faint band of slightly higher M_r in Ca^{2+} -containing sample buffer, is very similar to that described by Molla *et al.* (1981) for octopus calmodulin.

The extracted protein was biologically active in a calmodulin-deficient cyclic AMP phosphodiesterase assay and behaved identically with the standard preparation. The value for half-maximal activation at 10 ng is similar to that measured for other purified calmodulin preparations (Sharma & Wang, 1979).

The heat-stability of calmodulin was utilized in the analysis of the translated proteins. Separation of the heat-treated translated proteins by SDS/polyacrylamide-gel electrophoresis showed that the single most prominent heat-stable trans-

lated protein was calmodulin, as judged by its identical mobility compared with authentic calmodulin. Interestingly, although this band showed the Ca^{2+} -dependent shift in mobility, only one radioactive band appeared under conditions in which Ca^{2+} was present in the sample buffer; this may simply reflect the fact that the higher- M_r component of the doublet is considerably fainter in Coomassie Blue-stained gels and therefore less easily detected by autoradiography. There was no evidence for a high- M_r precursor of calmodulin, which is consistent with other studies where mRNA has been extracted from, for example, spinach leaf (Van Eldik *et al.*, 1980), the electroplax of the electric eel (Munjaal *et al.*, 1980) and bovine lymphocytes (Kecskemethy & Schafer, 1982).

Calmodulin binds to fluphenazine-Sepharose 6B in a Ca^{2+} -dependent manner and can be specifically eluted with EGTA-containing buffers. The failure of the authentic calmodulin to displace bound labelled calmodulin from the gel presumably reflects the high capacity of fluphenazine-Sepharose 6B for calmodulin binding (Kakiuchi *et al.*, 1981). Nevertheless, a peak of radiolabelled synthesized protein co-eluted precisely with the authentic protein when the fluphenazine-Sepharose 6B was eluted with EGTA-containing buffer. Unheated translation mixture was used in this experiment in an attempt to obtain an approximate value for the contribution of calmodulin to the total synthesized protein. The yield of synthesized calmodulin was 0.5–1.0% of total translated protein, which must be a low estimate if losses are taken into account. Nevertheless, this is much less than in the electroplax of the electric eel, where about 10% of the total proteins synthesized by mRNA was calmodulin (Munjaal *et al.*, 1980).

These studies show that the amount of calmodulin is relatively high in the anterior pituitary and that mRNA coding for the protein is also present. Calmodulin is synthesized as the authentic protein, without a precursor form.

We are grateful to the Wellcome Trust for financial support. Much of this work was carried out when S. W. W. was in receipt of a Wellcome Clinical Research Fellowship. S. T. is a Wellcome Trust Senior Lecturer.

References

- Azhar, S. & Menon, K. M. J. (1977) *Eur. J. Biochem.* **73**, 73–82
- Craig, R. K., Brown, P. A., Harrison, O. S., McIlreavy, D. & Campbell, P. N. (1976) *Biochem. J.* **160**, 57–74
- Fleckman, A., Erlichman, J., Schubart, U. K. & Fleischer, N. (1981) *Endocrinology* **108**, 2072–2077
- Gershengorn, M. C. (1980) *J. Biol. Chem.* **255**, 1801–1803

- Hall, L., Craig, R. K. & Campbell, P. N. (1979) *Nature (London)* **277**, 54–56
- Hatada, Y., Munemura, M., Fukunaga, K., Yamamoto, H., Maeyama, M. & Eishichi, M. (1983) *J. Neurochem.* **40**, 1082–1089
- Hutton, J. C., Penn, E. J., Jackson, P. & Hales, C. N. (1981) *Biochem. J.* **193**, 875–885
- Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakou, M. & Kosaki, G. (1981) *FEBS Lett.* **126**, 203–207
- Kecskemethy, W. & Schafer, K. P. (1982) *Eur. J. Biochem.* **126**, 573–582
- Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- MacNeil, S., Walker, S. W., Brown, B. L. & Tomlinson, S. (1982) *Biosci. Rep.* **2**, 891–897
- Means, A. R. & Chafouleas, J. G. (1982) *Annu. Rev. Physiol.* **44**, 667–682
- Mellersh, H., Tomlinson, S. & Pollock, A. (1980) *Biochem. J.* **185**, 601–607
- Merritt, J. E., Tomlinson, S. & Brown, B. L. (1981) *FEBS Lett.* **135**, 107–110
- Merritt, J. E., Walker, S. W., Wojcikiewicz, R. J. H., MacNeil, S., Brown, B. L. & Tomlinson, S. (1982) *Biochem. Soc. Trans.* **10**, 397
- Molla, A., Kilhoffer, M.-C., Ferraz, C., Andemard, E., Walsh, M. P. & Demaille, J. G. (1981) *J. Biol. Chem.* **256**, 15–18
- Munjaal, R. P., Dedman, J. R. & Means, A. R. (1980) *Ann. N.Y. Acad. Sci.* **356**, 110–117
- Naor, Z., Leifer, A. M. & Catt, K. J. (1980) *Endocrinology* **107**, 1438–1445
- Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2330–2334
- Schrey, M. P., Brown, B. L. & Ekins, R. P. (1978) *Mol. Cell. Endocrinol.* **11**, 249–264
- Sharma, R. K. & Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 187–188
- Sheaves, R. M., Bowman, D. & Hope, D. B. (1982) *J. Neurochem.* **39**, 909–914
- Thompson, W. J., Epstein, P. M. & Strada, S. J. (1979) *Biochemistry* **18**, 5228–5237
- Thorner, M. O., Hackett, J. T., Murad, F. & MacLeod, R. M. (1980) *Neuroendocrinology* **31**, 390–402
- Van Eldik, L. J., Grossman, A. R., Iverson, D. B. & Watterson, D. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1912–1916